Open

CME

Fecal Gluten Peptides Reveal Limitations of Serological Tests and Food Questionnaires for Monitoring Gluten-Free Diet in Celiac Disease Patients

Isabel Comino, PhD[1], Fernando Fernández-Bañares, MD, PhD[2], María Esteve, MD, PhD[2], Luís Ortigosa, MD, PhD[3], Gemma Castillejo, MD, PhD[4], Blanca Fambuena, MS[5], Carmen Ribes-Koninckx, MD, PhD[6], Carlos Sierra, MD, PhD[7], Alfonso Rodríguez-Herrera, MD, PhD[8], José Carlos Salazar, MD[9], Ángel Caunedo, MD[10], J.M. Marugán-Miguelsanz, MD, PhD[11], José Antonio Garrote, MD, PhD[12], Santiago Vivas, MD, PhD[13], Oreste lo Iacono, MD, PhD[14], Alejandro Nuñez, BSc[13], Luis Vaquero, MD, PhD[13], Ana María Vegas, MD[12], Laura Crespo, MD[12], Luis Fernández-Salazar, MD, PhD[11], Eduardo Arranz, MD, PhD[11], Victoria Alejandra Jiménez-García, MD[10], Marco Antonio Montes-Cano, MD, PhD[15], Beatriz Espín, MD, PhD[9], Ana Galera, MD[8], Justo Valverde, MD[8], Francisco José Girón, MD[7], Miguel Bolonio, MSc[6], Antonio Millán, MD, PhD[5], Francesc Martínez Cerezo[4], César Guajardo, MD[3], José Ramón Alberto, MD[3], Mercé Rosinach, MD, PhD[2], Verónica Segura, BSc[1], Francisco León, MD, PhD[16], Jorge Marinich, PhD[17], Alba Muñoz-Suano, PhD[17], Manuel Romero-Gómez, MD, PhD[5], Ángel Cebolla, PhD[17] and Carolina Sousa, PhD[1]

OBJECTIVES: **Treatment for celiac disease (CD) is a lifelong strict gluten-free diet (GFD). Patients should be followed-up with dietary interviews and serology as CD markers to ensure adherence to the diet. However, none of these methods offer an accurate measure of dietary compliance. Our aim was to evaluate the measurement of gluten immunogenic peptides (GIP) in stools as a marker of GFD adherence in CD patients and compare it with traditional methods of GFD monitoring.**

METHODS: **We performed a prospective, nonrandomized, multicenter study including 188 CD patients on GFD and 84 healthy controls. Subjects were given a dietary questionnaire and fecal GIP quantified by enzyme-linked immunosorbent assay (ELISA). Serological anti-tissue transglutaminase (anti-tTG) IgA and anti-deamidated gliadin peptide (anti-DGP) IgA antibodies were measured simultaneously.**

RESULTS: **Of the 188 celiac patients, 56 (29.8%) had detectable GIP levels in stools. There was significant association between age and GIP in stools that revealed increasing dietary transgressions with advancing age (39.2% in subjects ≥13 years old) and with gender in certain age groups (60% in men ≥13 years old). No association was found between fecal GIP and dietary questionnaire or anti-tTG antibodies. However, association was detected between GIP and anti-DGP antibodies, although 46 of the 53 GIP stool-positive patients were negative for anti-DGP.**

CONCLUSIONS: **Detection of gluten peptides in stools reveals limitations of traditional methods for monitoring GFD in celiac patients. The GIP ELISA enables direct and quantitative assessment of gluten exposure early after ingestion and could aid in the diagnosis and clinical management of nonresponsive CD and refractory CD. Trial registration number NCT02711397.**

Am J Gastroenterol 2016; 111:1456–1465; doi:10.1038/ajg.2016.439; published online 20 September 2016

[1]Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville, Spain; [2]Department of Gastroenterology, Hospital Universitari Mutua Terrassa, and CIBERehd, Terrassa, Barcelona, Spain; [3]Pediatric Gastroenterology, Hospital Universitario Nuestra Señora de La Candelaria, Tenerife, Spain; [4]Pediatric Gastroenterology, Hospital Universitari de Sant Joan de Reus, IISPV, URV, Reus, Spain; [5]Unit for the Clinical Management of Digestive Diseases and CIBERehd and Gastroenterology and Nutrition Unit, Hospital Universitario Virgen de Valme, Seville, Spain; [6]Pediatric Gastroenterology, Hepatology and Nutrition Unit, Hospital Universitario y Politécnico La Fe, Celiac Disease and Digestive Inmunopatology Unit, Instituto de Investigación Sanitaria La Fe, Valencia, Spain; [7]Pediatric Gastroenterology and Nutrition Unit, Hospital Materno-Infantil, Malaga, Spain; [8]Gastroenterology and Nutrition Unit, Instituto Hispalense de Pediatría, Seville, Spain; [9]Servicio de Gastroenterología Pediátrica, Hospital Universitario Virgen del Rocío, Seville, Spain; [10]Hospital Universitario Virgen Macarena, Seville, Spain; [11]Mucosal Immunology Laboratory, Instituto de Biología y Genética Molecular (IBGM), University of Valladolid, CSIC and Gastroenterology Unit, Hospital Clínico Universitario de Valladolid, Valladolid, Spain; [12]Clinical Analysis and Pediatrics, Hospital Universitario Río Hortega, Valladolid, Spain; [13]Servicio de Aparato Digestivo, Hospital Universitario de Leon, Leon, Spain; [14]Sección de Aparato Digestivo, Hospital del Tajo, Madrid, Spain; [15]Servicio de Inmunología, CIBER de Epidemiología y Salud Pública, Hospital Universitario Virgen del Rocío/IBiS/CSIC/Universidad de Sevilla, Seville, Spain; [16]Celimmune, Bethesda, Maryland, USA; [17]Biomedal SL, Seville, Spain. **Correspondence:** Carolina Sousa, PhD, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, C/Profesor García González, 2, ES-41012 Seville, Spain. E-mail: csoumar@us.es

Received 17 March 2016; accepted 24 June 2016

INTRODUCTION

Celiac disease (CD) is an immune-mediated systemic disorder elicited by the ingestion of gluten in genetically susceptible individuals. It has a prevalence of 1–3% in the general Western population, including the United States (1), and it is characterized by the presence of a varied array of gluten-dependent clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy (2–4).

The treatment of CD is a lifelong strict gluten-free diet (GFD). The goal of this treatment is to relieve symptoms, achieve mucosal healing, and avoid the complications associated with untreated CD (5). However, although adhering to a GFD might seem simple, completely avoiding gluten in the gluten-rich Western diet is challenging and can considerably affect the patient's quality of life (4). Estimated compliance rates vary considerably (17–80%), depending on the patient's age or the age at diagnosis, among other factors (6–10).

The adherence to the GFD can be assessed through a dietary interview performed by a registered dietitian, or patient self-reports, and by a small bowel biopsy follow-up showing mucosal healing or CD serological screening tests showing decreasing levels of antibodies. However, none of these methods offer an accurate measure of dietary compliance. Patient self-reports are considered unreliable because individuals tend to inaccurately report their level of adherence, whether intentionally or unintentionally. Although the normalization of the small intestinal architecture on multiple biopsies is a definitive evidence of a correct dietary treatment CD, there is no consensus on the relevance of the follow-up biopsies, especially in asymptomatic patients in whom clinical improvement is seen (8,11). Many physicians therefore rely on follow-up serologies to monitor compliance with the diet. Unfortunately, data clearly show that serology at follow-up has a poor correlation with mucosal healing and therefore relying solely on serology may underestimate the activity of CD (12–16).

We recently described a novel method to monitor the adherence to the GFD by detection of immunodominant gluten peptides in human feces using the anti-α-gliadin G12 antibody (17–19). Gluten peptides, in particular peptides related to the immunotoxic-α–gliadin-33-mer peptide, are resistant to gastrointestinal digestion that ensures that a significant amount of the ingested gluten is excreted in feces. Consequently, recovery of detectable amounts of the immunotoxic fraction in feces indicates that gluten has passed through the digestive tract and, therefore, that gluten has been consumed (19).

Our aim in this study has been to display the clinical usefulness of this new method of measuring fecal gluten immunogenic peptides (GIP) as a marker of adherence to GFD. We prospectively examined the compliance to the GFD of both celiac children and adults in a multicenter clinical trial. Furthermore, the response rate to GFD was evaluated by dietary questionnaire, celiac serology, and clinical response. Correlations between fecal GIP and traditional methods to monitoring the GFD were investigated.

METHODS

Study design and participants

This was a prospective, nonrandomized, partially blinded, multicenter study including CD patients on a GFD and healthy controls recruited between April 2012 and June 2014 at 13 Spanish hospitals. The study was approved by the ethics committee of each institution involved and written informed consent was obtained from all participants ≥12 years old, or from the parents or legal guardians in the case of children <12 years old.

The study group consisted of celiac patients following a GFD for at least 1 year before the inclusion in the study and they were required to have an HLA-DQ2 or HLA-DQ8 haplotype and an histologically abnormal duodenal biopsy (grade Marsh IIIB or IIIC) at the time of diagnosis, supported by positive serum anti-endomysium IgA antibodies and/or anti-tissue transglutaminase (anti-tTG) IgA antibodies. The control group comprised healthy asymptomatic subjects in whom CD had been ruled out and who were not suspected of having any other gastrointestinal condition.

Exclusion criteria for all study patients included history of kidney or liver disease, and history of severe psychiatric disease or seizure disorder. Patients in the study group who were unable to give informed consent or who were voluntarily following a GFD or a diet containing low gluten without medical prescription in the months before the inclusion in the study were also excluded.

All subjects meeting the inclusion criteria for the study were given a dietary questionnaire to complete at home and then invited for a follow-up visit in which stool and blood samples were collected. Family history of CD and data on the date of CD diagnosis, duration of the GFD, and clinical outcome were also collected for the study group.

All authors had access to the study data, and reviewed and approved the final manuscript.

Feces and blood collection

Subjects were instructed to collect 2–4 g of stool sample in a sealed container after recording their food intake for 4 days. Specimens were dropped off within 24 h of collection and were kept at −20 °C at all times until processing.

Blood samples were collected in two 3 ml vacutainer tubes with EDTA-K3 anticoagulant and centrifuged at 2,000 *g* within 30 min of collection to obtain plasma. Processed sera were stored at −80 °C until analysis. All samples were identified and labeled with a randomized numeric-code.

Quantification of GIP in stool samples

The concentration of GIP in stools was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using the iVYDAL *In Vitro* Diagnostics iVYLISA GIP-S kit (Biomedal S.L., Seville, Spain) following the manufacturer's guidelines. Briefly, stool samples were incubated for 60 min at 50 °C with gentle agitation in 9 ml of Universal Gluten Extraction Solution (UGES; Biomedal S.L.) per g of stool to release the GIP from the stool matrix. After extraction, samples were diluted 1:10 and incubated for 60 min in the provided microtiter plate coated with G12 together with

the standards and the assay's positive and negative controls. Wells were then washed and samples incubated with horseradish peroxidase-conjugated G12 antibody for another 60 min. Subsequently, plates were washed again and incubated with the horseradish peroxidase substrate. Color development was stopped with sulfuric acid and absorbance measured at 450 nm using microplate reader UVM340 (Asys Hitech GmbH, Eugendorf, Austria). The results were expressed as µg GIP per g feces. Each sample was run in duplicate and at least two different aliquots of each sample were tested on different days.

Validation of the detection method and determination of cutoff values

Validation of the G12 sandwich ELISA was performed by determining the analytical sensitivity and the diagnostic sensitivity and specificity. The analytical specificity, defined as the ability of the method to exclusively detect the analyte (20) (in this case GIP), was not evaluated in this study because it has previously been estimated using samples from cereals and other food samples (17,18,21–24).

Assessment of the analytical sensitivity was performed by determining the limit of detection, which is the lowest detectable concentration of an analyte, and the limit of quantification (LOQ), which is the lowest quantifiable concentration of the analyte. The limit of detection was calculated by running 10 replicates of one negative sample and set at the mean+3×s.d. and the LOQ was calculated experimentally using spiked samples ranging between 0.078 and 100 µg GIP per g sample. For diagnostic purposes, all samples with values below the LOQ were considered negative and all those above the LOQ were considered positive.

For calculating the diagnostic sensitivity, defined as the proportion of subjects who are positive for the condition of study and who are identified as positive by the diagnostic method (25), the samples from 73 healthy subjects known to be on a gluten-containing diet (positive controls) were used. The percentage of those with positive GIP values in stools was calculated.

Similarly, the diagnostic specificity, defined as the proportion of subjects negative for the study condition and who are identified as negative by the diagnostic method (25), was calculated using stool samples obtained from 11 healthy infants who had never ingested gluten. Of the 11 patients, 9 were younger than 6 months and were fed only GF formula and the other 2 patients were 7 and 8 months old, and consumed, besides infant formula milk, age-related complement of fruit, vegetables, rice, and maize, but in no case did their diet include any product suspected to contain gluten. Breastfed babies were excluded to avoid the possibility of gluten ingested by the mother being excreted into the breast milk and transferred to the baby. The percentage of babies with negative GIP values was then calculated to obtain the specificity of the G12 sandwich ELISA.

Determination of anti-tTG and anti-gliadin antibodies

The levels of anti-tTG IgA and anti-deamidated gliadin peptide (anti-DGP) IgA antibodies (or anti-DGP IgG in total IgA-deficient patients) were determined by ELISA using the EliA Celikey IgA and EliA Gliadin IgA/IgG kits, respectively, according to the manufacturer's protocol (Phadia, Freiburg, Germany). Measurements were performed in duplicates and the results expressed as EliA U/ml. Sera were considered positive when >10 U/ml as indicated in the manufacturer's specifications.

Dietary questionnaire

To assess gluten exposure, a structured food questionnaire of 27 items was administered to both study patients and controls to record the foods consumed during the 4 days before the collection of the stool and blood specimens. The food items were classified into eight predefined groups: dairy (milk and cheese); complex carbohydrates (bread, cereals, pasta, rice, potato, legumes, and nuts); meats (red meat, fish, cold cuts, and eggs); fruits (whole or juiced); vegetables; fats (vegetable oils, butter, and cream); sweetened beverages (sodas, bottled juices, and energy drinks); and other (baked goods, candy, snacks, etc.). Images of standard portion sizes were included as a guideline for portion quantification. Subjects were asked to record the amount and type of food consumed, brand, time of meal, and if it was labeled as gluten-free. They were also asked to note if they were aware of having consumed any gluten-containing foods.

Statistical analysis

Data analysis was performed with SPSS 23.0 for Windows (SPSS, Armonk, NY). Values obtained by ELISA for each sample were expressed as mean±s.d. Frequency distributions were calculated for all groups and expressed as relative percentages.

The χ^2 or Fisher's exact test were used to assess the strength of association between categorical variables and Kruskal–Wallis or Mann–Whitney nonparametric tests were used to compare differences between groups. A statistical probability of $P<0.05$ was considered significant for all analyses.

RESULTS

Characteristic of the participants

A total of 340 subjects were recruited and 272 (153 females and 119 males) were included in the final study after excluding 68 patients who dropped out. The demographic characteristics of the individuals enrolled in the study are outlined in **Table 1**.

There were 188 GFD-treated celiac patients (age range 1–72 years) and 84 healthy controls (age range 0–66 years) who met inclusion criteria for the study. The control group comprised: (i) positive controls (n=73), healthy children and adults on an unrestricted gluten-containing diet; and (ii) negative controls (n=11), healthy infants between 0 and 8 months of age who did not include in their diet any product suspected to contain gluten; breastfed babies were excluded to avoid the possibility of gluten ingested by the mother being excreted into the breast milk and transferred to the baby.

Sensitivity and specificity of the analytical method

To assess the validity of the G12 sandwich ELISA method in detecting GFD transgressions, both the analytical sensitivity and

Table 1. Characteristics of patients enrolled in the study

Characteristics	Patients, *n*	(%)
Total	272	
Sex		
Male	119	43.8
Female	153	56.2
Mean age at recruitment (21.7)		
Patients enrolled		
Healthy controls	84	30.9
Celiac patients	188	69.1
Celiac patients		
Total	188	
Mean age at recruitment		
0–3 Years	35	18.6
4–12 Years	79	42.0
≥13 Years	74	39.4
Time on GFD		
1–<2 Years	96	51.1
2–5 Years	54	28.7
≥6 Years	38	20.2
Healthy controls		
Total	84	
Positive controls		
Total	73	
Mean age at recruitment		
0–3 Years	6	8.2
4–12 Years	6	8.2
≥13 Years	61	83.6
Negative controls		
Total	11	
Mean age at recruitment		
0–8 Months	11	100.0

GFD, gluten-free diet.

the diagnostic sensitivity and specificity of the test were determined.

The limit of detection and LOQ that define the analytical sensitivity were found to be 0.06 (mean of 10 negative sample replicates+3 s.d.) and 0.156 µg GIP per g feces, respectively. Of the 73 healthy subjects known to be on a gluten-containing diet (positive controls), 72 had GIP levels above the LOQ and as such were correctly identified as being positive by the immunoassay, whereas all the healthy infants on a GFD formula (negative controls) had GIP levels below the LOQ and thus were correctly considered negative, yielding a diagnostic sensitivity and specificity of 98.5% and 100%, respectively. The results obtained were classified into three ranges depending on the GIP concentration and repeatability of value in different aliquots of the same sample: (i) negative values in GIP were below the LOQ; (ii) weak positive values were close to the LOQ (between 0.16 and 0.30 µg GIP per g feces), and in repeated analysis of different aliquots of the same stool, one of them could yield negative results and; (iii) strong positive values with >0.30 µg GIP per g feces always were positive in all the tested aliquots of the same sample.

Evaluation of GFD adherence by fecal GIP content in celiac patients

Stool samples from celiac patients on a GFD and healthy controls were tested for the presence of GIP by ELISA using the iVYLISA GIP-S kit. Of the 188 celiac patients examined, 56 (29.8%) were found to have detectable amounts of GIP in stools. On the contrary, all 73 positive controls on a gluten-containing diet except 1 (98.5%) had quantifiable amounts of GIP in stools (94.1% strong positive, 4.4% weak positive). The mean GIP concentrations obtained with this method were higher in the positive controls than in the celiac patients who had all been following a GFD for >1 year ($P<0.001$, **Figure 1**). As expected, none of the negative controls had measurable levels of GIP (**Figure 1c**).

In order to assess whether age, sex, duration of GFD, or family history of CD had an effect on GFD compliance, we analyzed the association of each of these parameters with the presence of GIP in stools. To evaluate the effect of age, we categorized the celiac patients according to their ability to make independent food choices into the three following groups: (i) 0–3 year olds ($n=35$), children who presumably have little to no ability to control what they eat; (ii) 4–12 year olds ($n=79$), children with moderate autonomy and thus with risk of making dietary transgressions eating at home or when dining out; (iii) ≥13 year olds ($n=74$), teenagers and adults with a high degree of autonomy to make food choices. We found a positive significant association between age and GIP content in stools that revealed increasing dietary transgressions with advancing age ($P=0.025$, **Figure 2a**). The majority (85.7%) of celiac children between 0 and 3 years of age had stool samples negative for GIP, with only 14.3% showing levels above the LOQ (5.7% strong positive and 8.6% weak positive). The proportion of celiac patients with stool samples positive for GIP increased to 27.8% in children between 4 and 12 years of age, half of whom were weak positive. Among those ≥13 years old, the proportion rose up to 39.2% with strong and weak positive. Although GIP-positive stools were more common in the older children and adults, no significant difference was seen in GIP concentrations across the different age groups ($P=0.337$, **Figure 2b**).

When further stratified by gender, adherence to the GFD was found to be closely related to the patient's gender in certain age groups. Although the percentage of celiac patients positive for fecal GIP increased with age in both men and women, there was no significant difference between males and females <13 years old ($P=0.782$ for 0–3-year age group and $P=0.834$ for 4–12-year age group). In contrast, more men in the ≥13-year-old group had positive GIP stools compared with women in the same age group

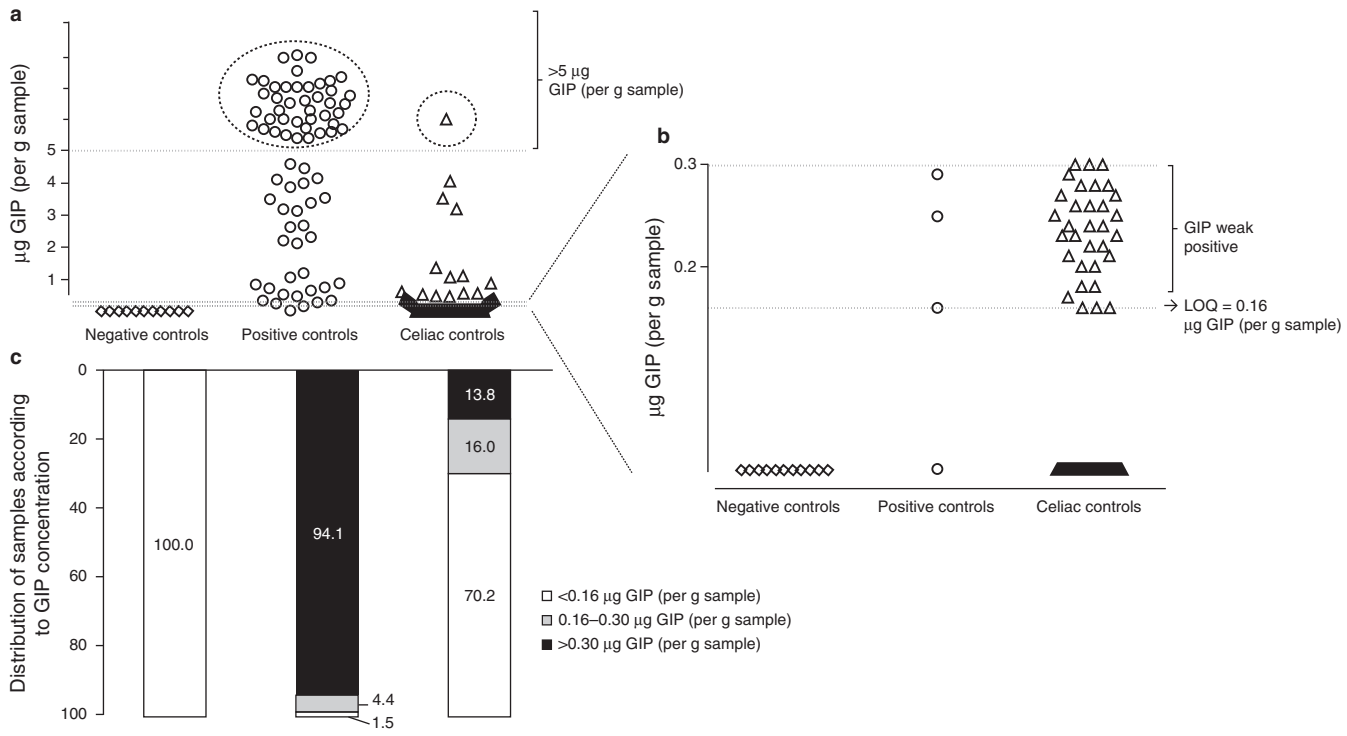


Figure 1. Concentration of GIP in stools of healthy controls and celiac patients on a GFD. (**a**) Levels of fecal GIP in positive controls, negative controls, and GFD-treated celiac patients. (**b**) Levels of fecal GIP for GFD-treated celiac patients and controls with weak positive and negative values (<0.3 µg per g sample) represented in a log base 10 scale. (**c**) Percentage distribution of controls and GFD-treated celiac patient according to GIP concentration. Each point in dot-plots represents the mean of two replicates of each sample. Celiac patients (n=188) were children and adults on GFD for ≥1 year and healthy controls (n=84) were classified into positive controls (children and adults on a gluten-containing diet, n=73); and negative controls (babies between 0 and 8 months of age who were only fed GFD formula milk, n=11). GFD, gluten-free diet; GIP, gluten immunogenic peptides.

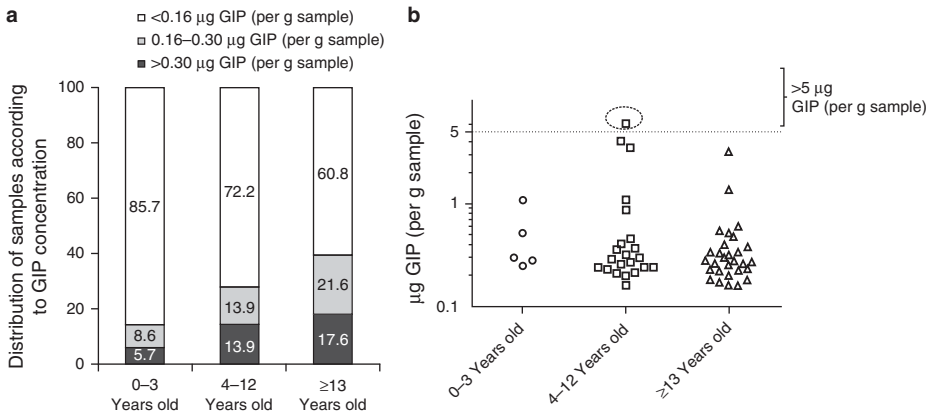


Figure 2. GFD adherence according to patient age. (**a**) Percentage distribution of celiac patients according to GIP concentration and age. (**b**) GIP concentration of celiac patients with GIP-positive stool samples (>0.16 µg GIP per g sample) represented in a log base 10 scale. Each point represents the mean of two replicates for each sample. GFD, gluten-free diet; GIP, gluten immunogenic peptides.

(60% vs. 31.5%, P=0.034), indicating higher number of dietary transgressions among men than in their female peers. In this oldest age group, 30.0% of men as opposed to 13.0% of women had strong positive GIP values in stools, and 30% vs. 18.5% had weak positive levels.

The association between the percentage of GIP-positive stools in celiac patients and the duration of the GFD is shown in **Figure 3**. Although no overall significant differences were observed, the patients who had been on the GFD for a longer period of time showed higher rates of noncompliance as evidenced by the higher

percentages of GIP-positive stools. Patients on the diet ≥6 years were more likely to have GIP-positive stools than patients on the diet for 2–5 years or only 1 year (36.8%, 33.3%, and 25%, respectively). No significant association was found between GIP levels in celiac patients and history of CD in their first- or second-degree relatives (data not shown).

Assessment of GFD adherence by dietary questionnaire and association with fecal GIP

Celiac patients and positive controls were asked to fill out a structured dietary questionnaire to assess their GFD compliance during the 4 days before the collection of the stool and plasma samples. Questionnaires were considered complete if they included information on type of food, brand, and portion size for at least 80% of the food items consumed on the first 3 days of the study. Food data from day 4 of the query was excluded, as foods consumed on the day before the sample collection would have had insufficient time to pass through the digestive tract given an ≈4-h gastric/small bowel (26) plus 7.2–86.4-h colonic transit time (19,27).

Of the 188 celiac patients who filled out dietary questionnaires, 50 (26.6%) were incomplete and thus were excluded from data analysis. For the remaining 138 patients, questionnaires were assessed for the consumption of gluten-containing foods with special emphasis on food products containing wheat, rye, barley, and oats. As shown in **Table 2**, among the 138 celiac patients who were assessed for GFD compliance by both fecal GIP and food questionnaire, 39 (28.3%) were considered noncompliant by fecal GIP analysis, whereas only 25 (18.1%) were noncompliant according to the food questionnaire, with only 9 (6.5%) of the total celiac patients being noncompliant by both methods. Of the 99 patients deemed to be gluten free by fecal GIP analysis, 82 (82.8%) were also believed to be compliant by questionnaire analysis. Conversely, of the 39 celiac patients considered to have consumed gluten by fecal GIP analysis, 27 (69.2%) did not declare any gluten consumption in the questionnaire. Analysis by Fisher's exact test failed to detect any association between the two methods but approached significance (P=0.055).

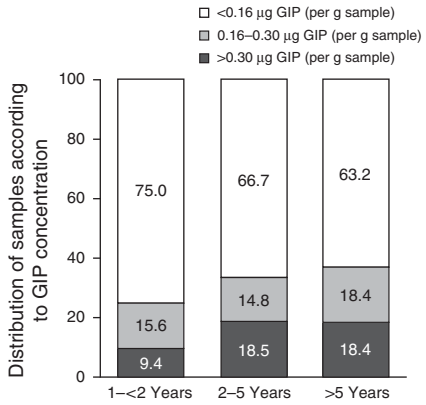


Figure 3. Percentage distribution of celiac patients according to GIP content in stools and duration of GFD. GFD, gluten-free diet; GIP, gluten immunogenic peptides.

Correlations between fecal GIP and serum antibodies

As shown in **Figure 4**, positivity for both anti-tTG IgA and GIP in stools was found in 14 patients. However, anti-tTG IgA was negative in 40 of the 56 patients with GIP-positive stools. Therefore, there was no significant association between fecal GIP and anti-tTG IgA (P=0.230). In contrast, we found an association between GIP and anti-DGP IgA levels (P=0.044). Elevated anti-DGP IgA titers were found in 11 patients, 6 of whom were also positive for GIP in stool. Negative anti-DGP IgA levels were found in 160, and of these, 114 had undetectable levels of GIP in stools as well.

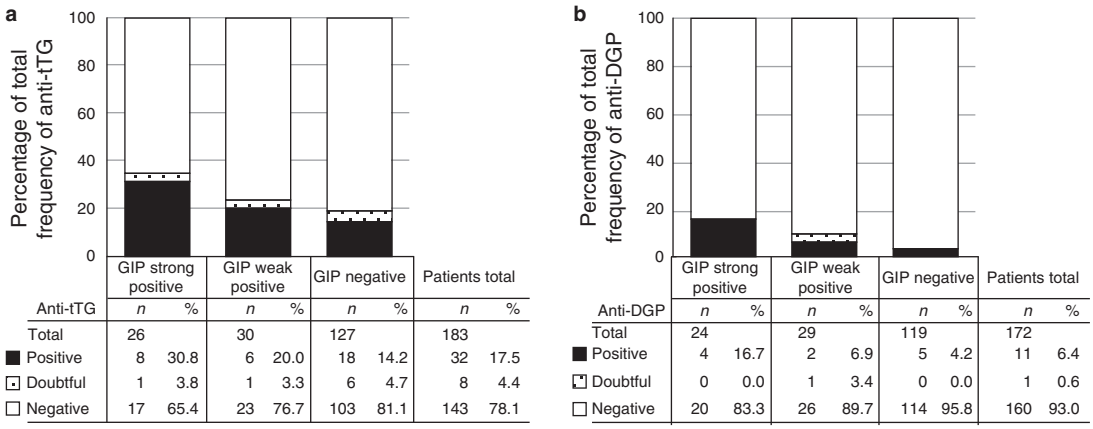
Association between fecal GIP content and clinical outcome

Clinical data were available for 182 of the 188 participating celiac patients. From them, only 9 (4.9%) reported persistent symptoms despite being on a GFD for ≥12 months (median 2.6 years range 1–5). Of these, 7 were males and 2 females with a total median age of 22.4 years (range 8–46). The most frequent symptoms or analytical abnormalities were iron deficiency anemia (n=4), diarrhea (n=3), abdominal pain (n=3), weight loss (n=1), dermatitis herpetiformis (n=1), short stature (n=1), and constipation (n=1). All 9 symptomatic patients had negative celiac serological markers at the time of stool sample collection except 2 (22.2%) who were positive for anti-tTG IgA and another two who were for anti-DGP IgA antibodies; no patients were positive for both anti-tTG and anti-DGP.

Table 2. Evaluation of GFD adherence by fecal GIP and dietary questionnaire

	GIP positive		GIP negative		Total	
Dietary questionnaire	***n***	**%**	***n***	**%**	***n***	**%**
	39		99		138	
GFD noncompliant	9	23.1	16	16.2	25	18.1
GFD compliant	27	69.2	82	82.8	109	79.0
Inconclusive[a]	3	7.7	1	1.0	4	2.9

GFD, gluten-free diet; GIP, gluten immunogenic peptides.
[a]Inconclusive, dietary questionnaire containing foods that could not be objectively assessed for gluten content.



Anti-tTG	GIP strong positive *n*	%	GIP weak positive *n*	%	GIP negative *n*	%	Patients total *n*	%
Total	26		30		127		183	
■ Positive	8	30.8	6	20.0	18	14.2	32	17.5
⊡ Doubtful	1	3.8	1	3.3	6	4.7	8	4.4
□ Negative	17	65.4	23	76.7	103	81.1	143	78.1

Anti-DGP	GIP strong positive *n*	%	GIP weak positive *n*	%	GIP negative *n*	%	Patients total *n*	%
Total	24		29		119		172	
■ Positive	4	16.7	2	6.9	5	4.2	11	6.4
⊡ Doubtful	0	0.0	1	3.4	0	0.0	1	0.6
□ Negative	20	83.3	26	89.7	114	95.8	160	93.0

Figure 4. Evaluation of GFD adherence in celiac patients according to celiac disease serologies and association with fecal GIP content. (**a**) Correlation between anti-tTG-IgA and fecal GIP. (**b**) Correlation between anti-DGP-IgA and fecal GIP. GIP content was divided into three groups according to GIP titers by enzyme-linked immunosorbent assay (ELISA): positive (>0.30 μg GIP per g feces), weak positive (0.16 and 0.30 μg GIP per g feces), and negative (<0.16 μg GIP per g feces). Levels of anti-tTG-IgA and anti-DGP-IgA are expressed as EliA U/ml and classified as positive (>10 EliA U/ml), indeterminate (7–10 EliA U/ml), and negative (<7 EliA U/ml). Anti-DGP, anti-deamidated gliadin peptide antibody; Anti-tTG, anti-tissue transglutaminase antibody; GFD, gluten-free diet; GIP, gluten immunogenic peptides.

Analysis of the association between presence of GIP in stools and clinical outcome revealed a significant association between GIP and symptoms typical of gluten consumption (P=0.019). Of the 9 patients with persistent symptoms, 3 (one-third) had undetectable levels of GIP in stools by GIP ELISA but 6 were positive, indicating that gluten exposure could be responsible for the symptoms in two-thirds of the celiac patients not responding to the diet. In addition, 27.2% (47 out of 173) of the asymptomatic celiac patients had detectable levels of GIP in stools, 44.6% of them (21 cases) being strong positive. Interestingly, 5 (out of 6) of the symptomatic and 20 (out of 47) of the asymptomatic GIP stool-positive patients reported gluten consumption in the food questionnaire.

DISCUSSION

This is the first multicenter study to assess dietary compliance to a GFD in celiac patients based on the quantification of GIP in stools. GIPs are excreted in feces only when gluten is ingested, thus detection in stools of celiac patients on a GFD implies that there has been gluten exposure and that a dietary transgression has been made. Our results showed that ~30% of the analyzed celiac patients on a GFD for at least a year had detectable amounts of GIP in their stools, suggesting that almost one-third of the celiac patients on a GFD were not compliant. In contrast, noncompliance was detected in ~18% of the patients when assessing adherence either by dietary questionnaire or by determination of anti-tTG antibodies in serum alone.

Prior studies using indirect methods based on dietary self-reports, food interviews, or follow-up serologies have estimated that between 17 and 80% of celiac patients are not compliant with the GFD (6–10). The variation found in the rate of adherence between the different studies may be due to differences in study design, the method used for evaluation of compliance, and/or differences in the characteristics of the study population. In the current study, using a direct and quantitative method of gluten detection in feces we found that the number of celiac patients noncompliant with the diet ranged from 15% in children ≤3 years old to almost 40% in teenagers and adults. This association between lack of GFD compliance and advancing age is likely due to the patient's increasing autonomy and ability to make dietary choices that would increase the risk of dietary transgressions. Children ≤3 years old, on the other hand, are highly dependent on their parents to be fed and would have strict control over the diet. Moreover, we found that among subjects ≥13 years old, dietary transgressions were significantly more frequent in men than in their female peers, as indicated by the positive levels of GIP found in the stools of up to 60% of men compared with ~30% of women within the same age group. The higher proportion of noncompliant male patients compared with females could be attributed to milder symptoms found in men or to stricter self-control over the diet in women. In addition, we also observed a tendency for more patients to be noncompliant the longer they had been on the diet. Altogether, these data show how increasing control over the diet could yield an increase in dietary adherence, as demonstrated by the fourfold greater adherence seen in children ≤3 years old who have strong parental control over their diet but no social pressure as compared with the adherence of 13 year olds and older adult males who are under little parental control but are subject to strong social influences.

There is considerable controversy over whether the evaluation of adherence to a GFD during clinical follow-up should be based on dietary questionnaires, symptom improvement, reduction of CD-related antibodies, histological recovery, or a combination of these parameters. Small bowel biopsy for the assessment of mucosal inflammation and villous atrophy is the "gold standard" for CD diagnosis. Therefore, mucosal healing would be the ideal parameter to monitor GFD adherence and for clinical management. However, because of its invasiveness, relative risk, and cost (especially

in asymptomatic patients), small bowel biopsy is not a practical method for monitoring disease activity and assessing dietary compliance in celiac patients (6).

Dietary questionnaires, although considered to be helpful in evaluating diet compliance (16), are subjective and rely on the patient's knowledge of the GFD and honesty when completing the questionnaire to accurately determine compliance (28,29). In this study, although ~18% (25 out of 138) of the celiac patients studied were considered not compliant according to the food questionnaire, only 9 out of 25 patients had also detectable levels of GIP in stools. Interestingly, 70% of the patients who showed positive levels of GIP in stools did not declare any gluten consumption in the food questionnaire. This could be because of the patients purposely not recording the gluten consumption in the questionnaire or inadvertent gluten ingestion that the questionnaire, as opposed to the GIP ELISA, would not be able to detect.

Some clinical researchers, in the absence of other practical alternatives, have recommended that serology should be performed annually to monitor adherence (8,30). However, recent studies have shown serology should not be considered a surrogate marker of intestinal recovery and ought to be used as a measure of mucosal healing only if supported by a small bowel biopsy showing improvement of the intestinal damage (15,30–33). In the present work, patients with strong positive values for GIP in stools were 2 and 4 times more likely to have positive anti-tTG and anti-DGP IgA antibodies, respectively, than patients with undetectable levels of GIP. However, of the patients with GIP levels >30 μg per g of feces, 65% had negative anti-tTG-IgA antibody titers and 83% had negative anti-DGP-IgA antibodies that would be consistent with prior reports on the low sensitivity of the serology for monitoring response to the diet.

Although seemingly intuitive, clinical response is not an optimal method for monitoring adherence to the GFD as a large number of celiac patients are asymptomatic or minimally symptomatic at presentation and in these cases it would not be feasible to use clinical response as an indicator of mucosal healing and GFD compliance (31). A controlled study examining the effects of gluten challenge found that symptoms were absent in 22% of celiac patients despite the presence of significant villous atrophy in the small bowel biopsy (34). Although the patients who reported symptoms despite being on a GFD for at least 12 months were few in this study (4.9%), we found a high correlation between the presence of symptoms and GIP with almost 67% of the symptomatic patients having detectable levels of GIP in stools, and this would indicate that gluten consumption, whether voluntary or inadvertent, could be responsible for the symptoms in these patients. These results are consistent with previous studies showing that even after adoption of a GFD, 4–30% of patients report persistent symptoms (5), and that gluten exposure is the most frequent cause of not responding to the diet, with only 10% being considered to have refractory CD (28).

Refractory CD is characterized by the persistence of symptoms and villous atrophy despite adherence to a strict GFD for >12 months. The diagnosis requires exclusion of other diseases that can cause similar symptoms and villous atrophy as well as the confirmation of a strict GFD. Detection of GIP in stools using the immunoassay described in this study would be a valuable tool in the differential diagnosis of refractory CD. In this study we found that 67% of the celiac patients with persistent symptoms despite being on a GFD had detectable levels of GIP in their stools and therefore could be having refractory CD if no other causes for the persistent symptoms are found. As both the presence of symptoms and GIP in stools may indicate potential dietary infringement, their joint use could have a high positive predictive value for monitoring the dietary compliance of celiac patients.

Similar findings to the ones observed in this study using GIP quantification in stools have recently been published by our group measuring GIP in urine with immonochromatographic strips (35). In this prior study we found that GIP is detectable in urine 6–48 h after gluten ingestion, whereas it has been shown to remain detectable in stools for up to 4 days (19). Urine analysis could be used in conjunction with the fecal test for early detection of dietary infringements and the monitoring of GFD compliance in CD.

The inability to directly measure GFD adherence is an unsolved problem for both clinicians and researchers and for which the analysis of GIP is a possible solution. Clinically, GIP analysis would allow celiac patients to detect unintentional gluten contamination and prevent the complications associated with untreated CD. In addition, during a gluten challenge it could be used to verify gluten consumption and avoid the underdiagnosis of CD. Clinical research for the development of novel therapies in CD could also benefit from fecal GIP analysis to ensure gluten exposure in control subjects of clinical trials and permit evaluation of drug efficacy.

In conclusion, we have observed a low GFD compliance rate among patients on an established GFD using GIP analysis that is lower than that shown with traditional dietary questionnaire or serological methods. The method of fecal GIP analysis proposed in this work is an accurate method that enables a direct and quantitative assessment of gluten exposure early after ingestion. Because of its high sensitivity and noninvasive nature it could become the method of choice for monitoring adherence to the GFD and a way for improving the diagnosis and clinical management of nonresponsive CD and refractory CD.

ACKNOWLEDGMENTS

We are grateful to Carolina Argüelles for her valuable suggestions in the writing of the manuscript and for helpful discussions.

CONFLICT OF INTEREST

Guarantor of the article: Carolina Sousa, PhD.

Specific author contributions: Study concept and design: I.C., F.F.-B., M.E., L.O., G.C., B.F., C.R.K., C.S., A.R.-H., J.C.S., Á.C., J.M.M.M., J.A.G., S.V., O.L.I., A.N., L.V., A.M.V., L.C., L.F.-S., E.A., V.A.J.G., M.A.M.-C., B.E., A.G., J.V., F.J.G., M.B., A.M., C.G., J.R.A., M.R., M.R.-G., V.S., F.L., J.M., A.M.S., Á.C. and C.S.; acquisition of data: I.C., V.S., F.L., A.M.S., Á.C. and C.S.; analysis and interpretation of data: I.C., V.S., F.L., A.M.S., Á.C. and C.S.; technical and material support: I.C., V.S., F.L., A.M.S., Á.C. and C.S.; drafting of

the manuscript: I.C., Á.C. and C.S.; critical revision of the manuscript and important intellectual content: I.C., F.F.-B., M.E., L.O., G.C., B.F., C.R.K., C.S., A.R.-H., J.C.S., A.C., J.M.M.M., J.A.G., S.V., O.L.I., A.N., L.V., A.M.V., L.C., L.F.-S., E.A., V.A.J.G., M.A.M.-C., B.E., A.G., J.V., F.J.G., M.B., A.M., C.G., J.R.A., M.R., M.R.-G., V.S., F.L., J.M., A.M.S., Á.C. and C.S.

Financial support: This work was supported by grants from Ministerio de Ciencia e Innovación and FEDER funds (DELIAC, IPT-2011-0952-900000), and Corporación Tecnológica de Andalucía (SINGLUCHECK, 1737/0118). We also thank the generous volunteer subjects who enrolled in the study.

Potential competing interests: Á.C. and F.L. own stock in Biomedal SL. Other authors declare no conflict of interest.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ The treatment of celiac disease (CD) is a lifelong strict gluten-free diet.
- ✓ The goal of gluten-free diet is to relieve symptoms, achieve mucosal healing, and avoid the complications associated with untreated CD.
- ✓ The adherence to the gluten-free diet varies considerably (17–80%).
- ✓ The adherence to the gluten-free diet can be assessed by indirect methods based on dietary self-reports, food interviews, or follow-up serologies. None of these methods offer an accurate measure of dietary compliance.

WHAT IS NEW HERE

- ✓ This is the first trial to assess dietary compliance to a gluten-free diet in celiac patients based on the quantification of gluten immunogenic peptides in stools.
- ✓ Fecal gluten immunogenic peptide analysis is an accurate and noninvasive method that enables a direct and quantitative assessment of gluten exposure early after ingestion.
- ✓ Gluten immunogenic peptide analysis reveals a low diet compliance rate among patients on a gluten-free diet and limitations of dietary questionnaire or serological methods for monitoring diet.
- ✓ Assessment of gluten in stool is a way for improving the diagnosis and clinical management of nonresponsive CD and refractory CD.

REFERENCES

1. Catassi C, Gatti S, Fasano A. The new epidemiology of celiac disease. J Pediatr Gastroenterol Nutr 2014;59:S7–9.
2. Sollid LM, Jabri B. Triggers and drivers of autoimmunity: lessons from coeliac disease. Nat Rev Immunol 2013;13:294–302.
3. Lundin KE, Qiao SW, Snir O *et al.* Coeliac disease - from genetic and immunological studies to clinical applications. Scand J Gastroenterol 2015;50:708–17.
4. Vriezinga SL, Schweizer JJ, Koning F *et al.* Coeliac disease and gluten-related disorders in childhood. Nat Rev Gastroenterol Hepatol 2015;12:527–36.
5. Ludvigsson JF, Bai JC, Biagi F *et al.* Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. Gut 2014;63:1210–28.
6. Ciacci C, Cirillo M, Cavallaro R *et al.* Long-term follow-up of celiac adults on gluten-free diet: prevalence and correlates of intestinal damage. Digestion 2002;66:178–85.
7. Högberg L, Grodzinsky E, Stenhammar L. Better dietary compliance in patients with coeliac disease diagnosed in early childhood. Scand J Gastroenterol 2003;38:751–4.
8. Pietzak MM. Follow-up of patients with celiac disease: achieving compliance with treatment. Gastroenterology 2005;128:135–41.
9. Herman ML, Rubio-Tapia A, Lahr BD *et al.* Patients with celiac disease are not followed up adequately. Clin Gastroenterol Hepatol 2012;10:893–9.
10. Galli G, Esposito G, Lahner E *et al.* Histological recovery and gluten-free diet adherence: a prospective 1-year follow-up study of adult patients with coeliac disease. Aliment Pharmacol Ther 2014;40:639–47.
11. Husby S, Koletzko S, Korponay-Szabó IR *et al.* European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Guidelines for the diagnosis of coeliac disease. J Pediatr Gastroenterol Nutr 2012;54:136–60.
12. Dickey W, Hughes DF, McMillan SA. Disappearance of endomysial antibodies in treated celiac disease does not indicate histological recovery. Am J Gastroenterol 2000;95:712–4.
13. Kaukinen K, Sulkanen S, Mäki M *et al.* IgA-class transglutaminase antibodies in evaluating the efficacy of gluten-free diet in coeliac disease. Eur J Gastroenterol Hepatol 2002;14:311–5.
14. Kaukinen K, Peräaho M, Lindfors K *et al.* Persistent small bowel mucosal villous atrophy without symptoms in coeliac disease. Aliment Pharmacol Ther 2007;25:1237–45.
15. Vahedi K, Mascart F, Yves JM *et al.* Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. Am J Gastroenterol 2003;98:1079–87.
16. Bannister EG, Cameron DJ, Ng J *et al.* Can celiac serology alone be used as a marker of duodenal mucosal recovery in children with celiac disease on a gluten-free diet? Am J Gastroenterol 2014;109:1478–83.
17. Morón B, Cebolla A, Manyani H *et al.* Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. Am J Clin Nutr 2008;87:405–14.
18. Morón B, Bethune MT, Comino I *et al.* Toward the assessment of food toxicity for celiac patients: characterization of monoclonal antibodies to a main immunogenic gluten peptide. PLoS One 2008;3:e2294.
19. Comino I, Real A, Vivas S *et al.* Monitoring of gluten-free diet compliance in celiac patients by assessment of gliadin 33-mer equivalent epitopes in feces. Am J Clin Nutr 2012;95:670–7.
20. Armbruster D, Terry P. Limit of blank, limit of detection and limit of quantitation. Clin Biochemist Rev 2008;29:S49–52.
21. Comino I, Real A, de Lorenzo L *et al.* Diversity in oat potential immunogenicity: basis for the selection of oat varieties with no toxicity in coeliac disease. Gut 2011;60:915–22.
22. Comino I, Real A, Gil-Humanes J *et al.* Significant differences in coeliac immunotoxicity of barley varieties. Mol Nutr Food Res 2012;56:1697–707.
23. Comino I, Real A, Moreno ML *et al.* Immunological determination of gliadin 33-mer equivalent peptides in beers as a specific and practical analytical method to assess safety for celiac patients. J Sci Food Agric 2013;93:933–43.
24. Real A, Comino I, Moreno ML *et al.* Identification and *in vitro* reactivity of celiac immunoactive peptides in an apparent gluten-free beer. PLoS One 2014;9:e100917.
25. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microb Rev 2010;23:550–76.
26. Urgesi R, Cianci R, Bizzotto A *et al.* Evaluation of gastric and small bowel transit times in coeliac disease with the small bowel PillCam®: a single centre study in a non gluten-free Diet adult italian population with coeliac disease. Eur Rev Medical Pharmacol Sci 2013;17:1167–73.
27. Wagener S, Shankar KR, Turnock RR *et al.* Colonic transit time-what is normal? J Pediatr Surg 2004;39:166–9.
28. Leffler DA, Dennis M, Hyett B *et al.* Etiologies and predictors of diagnosis in nonresponsive celiac disease. Clin Gastroenterol Hepatol 2007;5:445–50.
29. Hall NJ, Rubin GP, Charnock A. Intentional and inadvertent non-adherence in adult coeliac disease. A cross-sectional survey. Appetite 2013;68:56–62.
30. Haines ML, Anderson RP, Gibson PR. Systematic review: the evidence base for long-term management of coeliac disease. Aliment Pharmacol Ther 2008;28:1042–66.
31. Sharkey LM, Corbett G, Currie E *et al.* Optimising delivery of care in coeliac disease -comparison of the benefits of repeat biopsy and serological follow-up. Aliment Pharmacol Ther 2013;38:1278–91.

32. Dickey W, Hughes DF, McMillan SA. Patients with serum IgA endomysial antibodies and intact duodenal villi: clinical characteristics and management options. Scand J Gastroenterol 2005;40:1240–3.
33. Hopper AD, Hadjivassiliou M, Hurlstone DP *et al.* What is the role of serologic testing in celiac disease? A prospective, biopsy-confirmed study with economic analysis. Clin Gastroenterol Hepatol 2008;6:314–20.
34. Lähdeaho ML, Mäki M, Laurila K *et al.* Small-bowel mucosal changes and antibody responses after low- and moderate-dose gluten challenge in celiac disease. BMC Gastroenterol 2011;11:129.
35. Moreno ML, Cebolla Á, Muñoz-Suano A *et al.* Detection of gluten immunogenic peptides in the urine of patients with coeliac disease reveals transgressions in the gluten-free diet and incomplete mucosal healing. Gut 2015; doi:10.1136/gutjnl-2015-310148.