Chapter

Current Trends in the GFD Follow-Up

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Abstract

A poor adherence to a gluten-free diet (GFD) have a negative impact on people with celiac disease (CD). However, committing to a gluten-free lifelong carries social and economic burden and, a high degree of knowledge, motivation and a continuous effort. It is essential that the patient understands its disease, how to perform a GFD and the consequences that entail if the patient is not followed in the long term. However, a large percentage of patients does not still achieve a complete mucosal healing, likely due to a poor adherence to the GFD. We describe the current tools for the control of adherence to a GFD, with a special focus on the detection of gluten immunogenic peptides (GIP) in feces and urine, as GIP detection allows direct evidence that the gluten that has been ingested. GIP are becoming useful biomarkers for this aim. Here, we summarize the current information about the main applications and limitations of the use of the GIP determinations in the follow up of celiac disease.

Keywords: celiac disease, gluten immunogenic peptides (GIP), gluten-free diet (GFD) follow-up, POCT gluten contamination, gluten-free products

1. Introduction

Celiac disease (CD), also known as gluten-sensitive enteropathy or celiac sprue, is a common immune-mediated inflammatory disease that primarily affects the small intestine caused by an autoimmune response to dietary gluten and related proteins in genetically predisposed individuals, with the human leukocyte antigen, HLA-DQ2, and/or HLA-DQ8 haplotypes. It is estimated that approximately 0.5 to 2 percent of the population around the world is affected by this condition [1–5].

Hence, pathogenesis of CD depends on genetic and environmental factors. The main environmental factor is the gluten intake [6]. Digestion of gluten in the gastrointestinal tract generates immunoactive peptides, of which the 33-mer of alfa-gliadin (p57–68) has become a reference for its resistant to digestion and specific activity [7]. For simplification of the huge variability of peptides that are generated, the gluten digested fractions that could stimulate T cell in most celiac patients, they are referred as gluten immunogenic peptides (GIP) [7–9].

GIP that are encountered in the CD patients gut lumen, cross to the lamina propria using either the transcellular or paracellular path, leading to the activation of both adaptive and innate immune responses. This finally results in a structural change in

the small intestinal mucosa, intraepithelial lymphocyte infiltration and in a defective digestion and malabsorption of nutrients, amongst others [6, 10].

CD clinical manifestations are highly variable, as it could range from the classical gastrointestinal symptoms (e.g., malabsorption, diarrhea, steatorrhea, weight loss, bloating, flatulence, abdominal pain), to extraintestinal symptoms (e.g., dermatitis herpetiformis, arthritis, neurological symptoms, anemia, osteopenia, osteoporosis, tooth enamel defects, aphthous stomatitis, hypertransaminasemia) or with no symptoms at all [1, 6, 11]. Moreover, in the worst cases, when the disease remains undetected or not treated properly, it is associated with an increased risk of bone fracture or intestinal lymphoma [10, 12].

Currently, the unique available treatment for CD is to adhere to a strict lifelong GFD. Once the dietary treatment is established, CD associated symptoms, and risks of long-term complications, decrease, as the histology of the small bowel architecture is restored. Different studies have shown that the 95% of the children achieved a complete mucosal healing after two years of a GDF follow-up, whereas in adults a 34% and 66% accomplished duodenal mucosal recovery after two and five years, respectively [12, 13].

2. Is a complete removal of gluten from the diet achievable?

GFD should be mainly based on natural foods without gluten: fruits, vegetables, legumes, gluten-free pseudocereals (rice, corn, millet, sorghum, buckwheat, amaranth and quinoa), tubers, meat, fish, nuts and dairy products. This food selection can be supplemented with certified gluten-free products, whose purpose is to replace foods traditionally made with gluten, such as bread, pasta, pastries, etc. [14, 15].

Despite the GFD efficacy, a significant number of CD patients does not report a good adherence to the treatment [6]. Several studies based on serological tests, dietetic questionnaires and GIP detection in stool and urine, revealed that up to 45% of the children, 64% of the teenagers and 69% of adults commit diet transgressions whilst following the GFD [13, 16]. It has been estimated that the mean exposure to gluten in many patients may exceed 100 mg/day, which may be sufficient to produce persistent symptoms, enteropathy, and long-term complications [17, 18].

Effectively, going on a strict GFD is an arduous task, unimaginable elements such as lipsticks or plasticine also might be composed of gluten. The adherence to GF-life implies on the one hand, a deep understanding of the condition and gluten ubiquity by the patients and their closest social circles. On the other hand, GFD implicates sensory, nutritional, motivational, social, and economic difficulties [6, 19–21].

Naturally, gluten immunoactive peptides can be mainly found in wheat, rye and barley, which are widely used to make food products such as bread, beer, pasta, cakes, pastries and biscuits. Despite the low nutritional and biological value of this protein mixtures, it is, after sugar, the most used additive in industry [22, 23]. Its multiple properties, thermostability, the fact that it can act as a binding and extending agent, it can hold moisture and improve flavors and textures (it can be used as thickener, emulsifier, or gelling agent) make of it an excellent additive. Thus, less obvious gluten sources include processed foods (e.g., snacks or reconstituted meat and seafood), medications and cosmetic products [23]. Equally important is to be aware of cross-contaminations which can easily occur by contacting other foods that contain gluten or by using the same utensils to cook or manipulate one and the other without sanitizing them properly [14].

In sensorial and nutritional terms, GF food is not preferred versus their gluten containing versions. Industry efforts to make tastier and with nicer textures make those products higher in carbohydrates and lipids, mainly saturated fat, thus resulting in high-calorie foods which give high glycaemic index (GIP) [24, 25]. Moreover, because of the development of new formulas, a shorter shelf-life, the need of special packaging due to a higher microbial and fungal contamination risk, cleanings for GF manufacturing lines and accreditations for labelling as GF food amongst others, makes this food 200–500% more expensive than their gluten-containing counterparts [19, 24]. What for instance for a Spanish coeliac citizen translates into an increasement of 1000€ in its shopping card per year [26]. Some countries as Italy, Canada or the USA offer a gluten-free tax or subsidies deduction for those who are on a GFD for proven medical reasons [27].

CD is also associated with increased risk of suffering some psychiatric conditions as depression, anxiety, eating disorders, autistic spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). It is argued that some of them are developed by a specific biological mechanism by a "gut-brain" relationship, and others are developed indirectly for the social and motivational implications involved [28]. After starting the GFD, as CD related symptoms improve, the incidence of anxiety and depression in celiac patients decreases. However, after certain period, the psychological conditions increase again, probably due to the difficulties to match the professional and social life with the requirements of the diet. Isolation, shame, fear of becoming contaminated by gluten and worries about being a bother are common amongst CD patients [29].

From all the above, the achieved adherence is strongly associated with cognitive, emotional, and socio-cultural issues, membership of an advocacy group and regular dietetic follow-up [20]. Therefore, it is important that the coeliac has psychological, nutritional, caregivers and social support and on hand tools to adapt and continue with excellent adherence to the gluten-free diet.

3. How much gluten is harmful in celiac disease without significant clinical consequences?

The difficulty of absolute adhesion to the GFD makes patients to frequently ask doctors how much gluten they may tolerate.

Over the last years several studies have tried to answer to this very demanded question. Although the responses obtained differ, probably because methodology differences amongst studies, some studies support that small daily amounts of <50 mg could be tolerated by most celiac patients. However, for some of the patients amounts as little as 10 mg per day could lead to an immunological and histological response [30–32].

Those studies mentioned above were done involving a gluten challenge, meaning a certain amount of gluten was administered daily. The frequency to which those voluntary and/or involuntary gluten exposures occurs may be even more relevant to cause persistent histological damage than occasional high gluten intake [31, 33].

There is unfortunately not a simple answer to the question of this section that can be given to all patients. Ultimately, different gluten intake patterns may lead to negative impact on celiac patients. Studying patients on a case-by-case basis could be a very time-consuming trial and inaccurate. To facilitate this task, both sides, healthcare

providers and patients, should be provided with the correct tools and appropriate monitoring methodology to find the tolerable threshold for each patient. Otherwise, a single response would be offered: following GFD as strict as possible.

4. Current tools and strategies to measure the adherence to the GFD

In case of children and teenagers, ESPGHAN guidelines recommend follow-up visits after CD diagnosis. The first one should be programmed 3-–6 months after. Then, subsequent visits should be taken every 6 months until normalization of TGA levels, and every 12–24 months thereafter, unless there are concerns, complications or symptoms do persist and a sooner review is needed [3]. Likewise, there are similar universal agreement for adults [1, 2, 4, 5].

The aim of this monitorization is to evaluate the adherence to the treatment (GFD) and to detect any complication, which can be done either directly asking for the diet followed or fecal/urine GIP determinations, or indirectly, observing the clinical evolution from diagnosis (symptoms persistence, nutritional status and new clinic manifestations or associated complications) [16, 34, 35]. For this purpose, the following tools are used in clinical practice:

4.1 Clinical assessment

Once the GFD has been established, it is analyzed if symptoms and risk of complications have decreased, and quality of life is enhanced [2]. Despite a decrease in symptoms is associated with a response to the GFD, it has some weak points. On the one hand, several studies have stated that is an unreliable marker regarding recovery from intestinal atrophy [32, 36–39]. On the other hand, there are patients who are asymptomatic or express insignificant symptomatology, for whom the clinical assessment cannot be used as treatment monitoring indicator [36].

Additionally, other causes may motivate GI symptoms that could be easily confused with CD symptoms [37]. Therefore, clinical symptoms should not be considered as reliable method to evaluate the adherence to the GFD.

4.2 Dietetic review

Regular or periodical visits to an experienced dietitian in CD are recommended by different world-known guides [5, 35]. Relying on professional guidance is considered as key driver to accomplish the GFD. In order to evaluate the compliance grade, professionals are informed from patients' self-reports, for which the Standardized Dietician Evaluation [SDE] is commonly used, and on CD specialized dietetic questionnaires [16], such as, the Celiac Dietary Adherence Test [CDAT] or the Biagi Score [38, 40, 41].

Despite they have been advocated for being cost-effective and not invasive, they show some limitations. Firstly, questionnaires need to be translated and validated in all languages and cultures and do not register gluten real consumption. Secondly, self-reports are imprecise and subjective, as they depend on the patient's knowledge about the GFD and its fear to be judged [11, 42]. All these limitations resulted in the poor sensitivity of the dietary questionnaires to predict either villus atrophy or poor adherence in adult patients [39, 43].

Owing to those limitations, scientific research advocates more for the use of these tools to provide education for the avoidance of future inadvertent gluten exposures [36].

4.3 Endoscopy with duodenal biopsies

Duodenal biopsies provided by expert personnel or image analysis by advance technologies give a direct idea of the state of the GI mucosa. Either Marsh-Oberhuber classification or changes in villous height: crypt depth ratio (Vh:Cd) are used for its evaluation [44].

Despite current guidelines suggest follow-up biopsies every 1–2 years when symptoms persist, its use is frequently debated [1, 5, 35, 45]. On the one hand, endoscopies are invasive, expensive and need of experienced professionals to be done and interpreted and/or alternatively, specialized equipment to control biopsies and Vh:Cd determinations. On the other hand, despite an apparent strict compliance with GFD, mucosal damage could persist for years in certain adults [36, 39]. Therefore, the use of endoscopy in the follow-up tends to be more reduced.

4.4 Antibody-type serological biomarkers

Anti-gliadin antibody (anti-AGA), anti-endomysial antibody (anti-EMA), anti-tissue transglutaminase (anti-tTG)- TG2, TG6, TG3 and anti-deamidated gliadin peptide (anti-DGP), are the specific serologic antibody biomarkers in CD [46, 47].

Whereas their presence and their level above certain concentrations (e.g., anti-TG2 ≥ 10 times above the upper limit of baseline level), are very useful in the diagnosis of CD with a high sensitivity and specificity, they are not convenient biomarkers to check during the follow-up because of the low sensitivity [3, 39, 43]. Negative results are achieved with a reduction of gluten consumption, but frequent low quantities of gluten can also reduce the antibody level. Most of the individuals who do not portray a strict adherence to the GFD but do reduce the level of gluten consumption could also lead to a normalization of serology, without achieving a mucosal healing [1, 39, 47].

Other reasons that need also to bear in mind when using the serological biomarkers in the follow-up are:

- Cross-reaction with antibodies of enteric infections, other autoimmune diseases or chronic conditions may happen leading to false positive results [45].

- Once the GFD is prescribed, it usually takes ≥6–24 months to negativize. Some of them never reach full normalization of the serology. The timing of normalization can significantly vary amongst individuals [35].

- Patients with general or specific immunodeficiencies (in IgA or IgG) would lead to false negative results [45].

4.5 Novel biomarkers

The methods mentioned above have some weaknesses regarding GFD follow-up. Therefore, a general accepted tool for the follow-up of CD is still pending to be

available. The following biomarkers are providing new information and advantages over the traditional tools [46, 48]:

4.5.1 Interleukin-2 (IL-2)

To be diagnosed with CD through conventional strategies it is mandatory that the patients are ingesting enough gluten in their diet [1, 3]. However, thanks to the increase in popular awareness of celiac disease and other gluten or wheat related conditions, many of the people who suspect that gluten is causing damage to them, reduce or suppress gluten in the diet before they are diagnosed. Consequently, the people on GFD are asked to go through a gluten challenge, which consists of long gluten exposure periods, for example, children are asked for three-months gluten challenge, to provoke intestinal damage and increasement of CD specific antibodies [1, 3]. Due to physical discomfort caused by this challenge in many people, it raises a lot of rejection and/or abandonment. Therefore, interest in new diagnostic techniques for the population on GFD is growing [48, 49].

Despite those new CD diagnosing techniques still need significant daily gluten consumption, those are based on bigger intakes in shorter periods of time, counted in days. These provocations are not centred in altering the gastrointestinal mucosal state or CD specific antibodies by itself, but the initiation of an unleashing of messengers, molecules, cells of the innate and adaptive immunity that can serve as biomarkers in the diagnosis of CD in people with GFD [48, 49].

Serum IL-2 is one of the most consistently upregulated cytokines in celiac patients, peaking 4 hours after consumption of gluten containing foods and becoming undetectable for most of the patients by 6 days after initial gluten exposure. Likewise, it is correlated with timing and severity of symptoms [35, 46, 50].

However, interleukins are a type of cytokine, molecules released by the immune system that are used for signaling amongst cells, not only in CD but many other conditions. T CD4+ and CD8+ activated cells are the mayor sources of IL-2. IL-2 regulates the activities of white blood cells responsible for immunity [51]. Therefore, the use of IL-2 in the follow-up by its own it is controversial, it would be more clarifying to look at a panel of biomarkers that are up- or down-regulated during gluten exposure in celiac patients.

4.5.2 Gluten-specific CD4 T cells

Gluten-specific CD4 T cells which have a central role on CD pathogenesis, are released into the blood 6 days after the start of a three-day gluten challenge. Those gluten-specific T cells can be detected by gIFN ELISpot, IP-10 ELISA or visualized by flow cytometry [48].

4.5.3 Urinary and fecal gluten immunogenic peptides

GIPs generated after glutens gastrointestinal partial hydrolysis, contain sequences that are immunoactive in CD patients. The presence in stools or urine of gluten peptides is a direct proof of previous gluten consumption. Those GIP can be detected by immunomethods developed from food analytical products,

GIP can be detected either in urine and stool after 2–15 hours and 12–120 hours of gluten intakes, respectively, by using immunoassays in LFIA and ELISA platforms. As low as 50 mg gluten intakes could be detected [52, 53]. The fact that GIPs gave an

objective precise approach for determining voluntary or involuntary gluten consumption has made of them to be increasingly used in clinical trials of non-dietary therapies of CD, and studies with healthy and celiac children and adults [39, 43, 54–57]. Despite the undeniable direct association and effectiveness of the novel biomarkers to predict adherence to GFD, they are still not broadly implemented in the field.

4.5.4 Comparison of the current tools for GFD follow-up

The table showed below (**Table 1**) summarizes the most used current tools for GFD follow-up, presenting the advantages and disadvantages of each technique.

The curation of celiac patient could be considered when it achieves a complete mucosal healing, however, the risk of gluten exposure to deteriorate unremittingly the intestinal mucosa is always a threat. This complete healing process can only be accomplished by a full adherence to a GFD. The timing that would take for a complete recovery could vary patient to patient.

GFD assessment tool	Advantages	Disadvantages
Clinical assessment	1. Cheap. 2. Not invasive.	1. It cannot be used with asymptomatic patients, which may represent about two thirds of the CD population. 2. The symptoms caused must be differentiated from the ones that might have other origins. 3. Unreliable regarding recovery from intestinal atrophy.
Dietetic review	1. No requirement of instrumentation. 2. Not invasive. 3. Availability of standardized questionnaires.	1. Must be translated and validated in all languages and cultures. 2. Time consuming 3. Imprecise and subjective, as they are subject to the responses of each patient 4. Poor sensitivity to predict either villus atrophy and adherence
Endoscopy with duodenal biopsies	1. Determination of the gluten intake consequences through checking GI mucosal state.	1. Expensive 2. Invasive 3. Need either of experienced professionals or form specialized equipment 4. The reversibility of the damage can vary in time from patient to patient
Antibody type serological biomarkers	1. Cost effective. 2. Positive results might indicate continuous exposure to normal gluten containing diets.	1. Semi-invasive 2. Negative values in most treated patients with gluten exposure. Low sensitivity. 3. Cannot be used with immunocompromised patients 4. False positives might be obtained due to crossreactions with other antibodies 5. The timing of normalization can significantly vary amongst individuals 6. There is no linear correlation between serology values and recovery of the intestinal mucosa

GFD assessment tool	Advantages	Disadvantages
Interleukin-2	Low evidence for the utility in the follow-up.	
Gluten-specific CD4 T cells		1. Expensive instrument. 2. Required highly skilled technicians.
Gluten immunogenic peptides	1. Cost effective 2. Non-invasive. 3. Direct indicator that a gluten intake has been committed. 4. May help to identify the source of gluten exposure. 5. May estimate the amount of gluten consumption	1. Window of detection per sample is limited to hoursdays. 2. May require multiple samples (at least two) to increase accuracy and reliability.

Table 1.
Advantages and disadvantages of the existing current tools for GFD adherence assessment.

5. Can GIP determinations be the "gold standard" for exploring adherence?

In contrast to the rest of the presented methods, GIPs determination is the only tool that directly evidences the gluten intake whilst the rest of them try to determine the consequences.

5.1 What are the peptides included in the term?

Any gluten peptide that has immunoactivity with CD patients' T cells can be considered a GIP [58].

Since GIP have been detected in feces and urine, they probably could be located along the gastrointestinal tract and/or blood [59].

GIP present in stool and urine are derived from the digestion process. When gluten is ingested, it is partially digested to different size oligopeptides by digestive enzymes [60]. However, there are certain sequences that could be resistant to gastrointestinal digestion by the hydrolytic enzymes from human, such as, the immunodominant 33-mer alpha gliadin peptide, which has demonstrated to be resistant to gastric, pancreatic and intestinal brush-border membrane proteases [7]. Gluten is a protein rich in proline (P) and glutamine (Q) amino acids, what gives it its hydrophobic quality and at the same time it makes certain of those P and Q rich sequences hard to digest. Some of those indigestible sequences have the capacity of triggering an immune response in CD patients [60, 61].

During the last decades, considerable efforts have been made to map coeliac immunogenic motifs, a work that from time to time, is updated to add newly found gluten immunogenic sequences to the hundreds that have already been described as such [62]. Some immunogenic gluten epitopes may be tolerated at different level depending on the CD patient, as each person may have a different sensitivity towards the different epitopes [30, 32]. It has been demonstrated T cells have more affinity by the peptides presented by the HLA-DQ2 complexes than the ones presented by HLA-DQ8 ones. Therefore, the immune system response between individuals who have one or the other molecule would also be different [63]. However, it must be stated that not all gluten peptides are involved in the development of CD, as some may not contain immunogenic sequences. The immunogenicity of each GIP for T cell activation could be variable

depending on the specificity and repetitions of immunogenic T cell epitopes [63, 64]. This amount can easily vary depending on the peptide's gluten source. Hence, the 33-mer of the alpha-2-gladin is recognized for being the most immunodominant gluten peptide and used to be the referent GIP in analytical determinations. It contains three overlapping T-cell epitopes, namely PFPQPQLPY (DQ2.5-glia-α1a, one copy), PYPQPQLPY (DQ2.5-glia-α1b, two copies) and PQPQLPYPQ (DQ2.5-glia-α2, three copies) [61]. The deamidation of certain glutamine residues by the TG2 enhances the immunogenicity. TG2 has preference for QxP sites, where x, can be any amino acid [65].

5.2 Methods to determine GIP in human specimen

GIP have been detected in stool by ELISA and LFIA [43]. LC–MS, SPR, ELISA and LFIA [43, 59, 66, 67] have shown to determine urine GIP. Each method shows a different level of sensitivity and simplicity of execution.

The described methods for SPR, ELISA and LFIA to detect GIP in human stool and urine are immunoassays based on the G12 and/or A1 antibodies.

The study made by Palanski *et al*., [59] with LC–MS described for the first time the kind of gluten derived peptides that could be found in urine after gluten intake. The smallest peptide had 1,33 KDa and the largest was 4,28 KDa, for non-CD people after the intake of 18 g of gluten. 10/16 of these peptides showed at least 1 epitope for A1, 6/10 for G12 and 6/16 for both (**Table 2**). GIPs in stool have not been described so far.

Molecular size (KDa)	Sequence	Nº of epitopes for G12	Nº of epitopes for A1	Nº of volunteers that was encountered
4.28	SQQPEQTISQ**QP**QQPFPQQPH**QPQQPY**PQQQPYGSSL	**2**	**1**	6/8
3.46	PQQPPFSQQQQQQQQQQQPPFSQQQQPVL	0	0	3/8
3.41	PyrQQQQPPFSQQPPISQQQQPPFSQQQQPQF	0	0	1/8
3.33	TQ**QPQ**QPFPQQ**PQQPFP**QTQ**QPQQPFP**Q	**3**	**2**	3/8
3.15	FL**QP**QQPFPQQ**PQQPY**PQ**QPQQPF**PQ	**3**	**1**	1/8
3.10	TQ**QP**QQPFPQQ**P**QQPFPQQ**PQQPFP**Q	**3**	**2**	3/8
2.76	LGQQQPFPP**QQPYPQP**QPFPSQQP	0	**1**	5/8
2.51	SQ**QP**QQPFPQQPH**QPQQPY**PQ	**2**	**1**	5/8
2.35	P[I/L]**QP**QQPFPQQ**P**QQPFPQPQc	**2**	**2**	2/8
2.35	PyrQTFPHQPQQQVPQPQQPQQP	0	0	5/8
2.29	GQQQPFPPQQPYPQPQPFPS	0	**1**	8/8
2.21	GQQQPFPPQQPYPQPQPFP	0	**1**	7/8
1.89	QPFPPQQPYPQPQPFP	0	**1**	2/8
1.46	SCHVMQQQCCQ	0	0	5/8
1.39	CHVMQQQCCQd	0	0	1/8
1.33	SCHVMQQQCC	0	0	6/8

Table 2.
Modified from Palanski et al., *[59]. Gluten derived peptide sequences found in urine that show epitopes for G12, in bold and for A1 in red.*

Both A1 and G12 antibodies do not only detect gluten derived epitopes present in the α-gliadin 33-mer, but detect most immunogenic peptides [61, 68]. Furthermore, the G12 monoclonal antibody, has shown to capture most of the immunoactivity of digested gluten from different sources with an immunoaffinity resin [58, 68, 69],

Products for measuring GIP in feces and urine are currently on the market, adapted for both professional and domestic use [43]. Those ones for home use are based on the LFIA, whose mechanism of use and interpretation of the results are simple and already well known by the general population due to the familiarity of this type of test during the COVID-19 pandemic. Kits for professional use were designed in ELISA and LFIA formats, methods routinely used in clinical laboratories with the potential to provide quantitative data of GIP concentration. Facilitating user-adapted detection, allows GIP to be used as a biomarker of GFD adherence by both, professionals who perform the patient's follow-up and by the patient itself. In this way, they can detect failures and improve adherence to their treatment. Those kits are commercialized by the names of GlutenDetect Urine and Stool, LFIA for domestic use, iVYCHECK GIP Stool and iVYCHECK GIP Urine, LFIA for professional use and iVYLISA GIP Stool, ELISA for professional use.

The LFIA test for detecting GIP in urine has a LLoQ of 2.5 ng GIP/mL, for stool in stool is 0.3 μg GIP/g feces, where the LLoQ was established as the rate in which the 95% of the samples to that concentration get a positive result, using as a measurand the 33-mer (**Figure 1**). Those LFIA are semiqualitative tests, generally providing a binary result "positive" or "negative" that can be easily interpreted. However, they have been also conveniently used for semi-quantitative determination with a lateral flow reader.

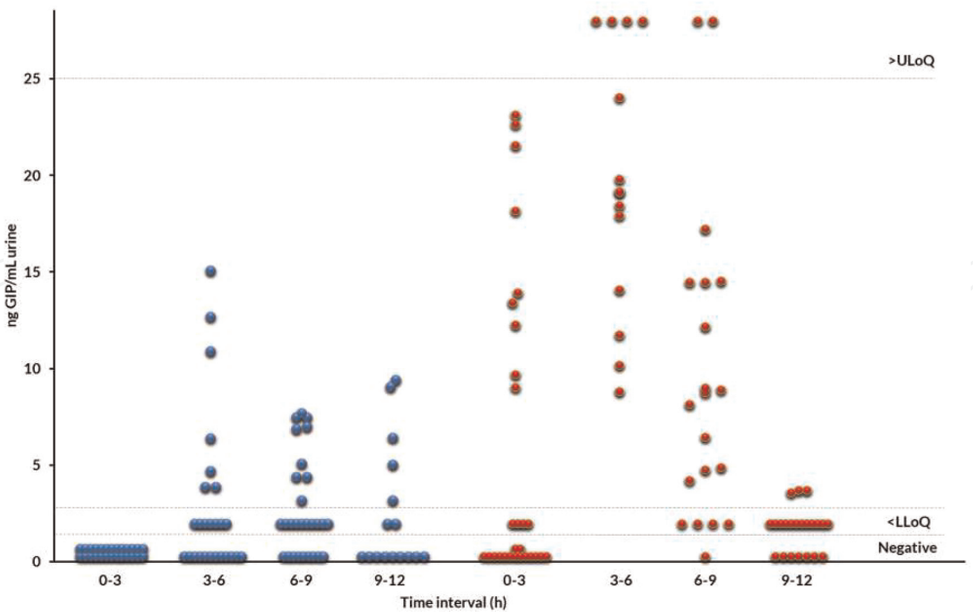

Figure 1.
Urinations collected after 2 g of encapsuled [52] and 8 g of free gluten intake, represented with blue and red spots respectively, during a 12 hours' time lapse, presented in 3 hours intervals (0–3 h, 3–6 h, 6–9 h and 9–12 h) for GIP detection and quantification. The urinations come from 21 healthy individuals for 2 g intake and from 15 healthy individuals for 8 g intake. For the dynamic range allowed, there were some urines that were positive de visu *but undetectable for the reader, those urines are the ones on the <LLoQ range; the ones on the >ULoQ are the ones detectable by the reader but whose concentration was undistinguishable. The negative urines are given the concentration below 2 ng GIP/mL.*

The ELISA test for detecting GIP in stool has a LLoQ of 0,3 μg α-gliadin 33mer/g feces. The ELISA test is a semi-quantitative method. Therefore, it allows to differentiate those stools for their GIP content within a dynamic range.

The reproducibility, repeatability and the diagnostic features of those tests are summarized in the table below (**Table 3**):

	iVYCHECK GIP Urine	**iVYCHECK GIP Stool**	**iVYLISA GIP Stool**
Diagnostic sensitivity *	90.18% (95%IC: 84.22–96.14%)	94.60% (95%IC: 86.00–100%)	97.10% (95%IC: 90.20–100%)
Diagnostic specificity *	98.28% (95%IC: 95.48–100%)	100% (95%IC: 98.80–100%)	83.30% (95%IC: 63.30–100%)
Positive predictive value *	98.06% (95%IC: 94.91–100%)	100% (95%IC: 98.60–100%)	91.90% (95%IC: 81.80–100%)
Negative predictive value *	91.20% (95%IC: 85.83–96.57%)	95.20% (95%IC: 87.60–100%)	93.75% (95%IC: 78.80–100%)
Reproducibility	97.00% (95%IC: 95.00–98.00%)	98.00% (95%IC: 96.00–99.00%)	CV ≤ 22%
Repeatability	98.00% (95%IC: 94.00–100%)	98.00% (95%IC: 94.00–100%)	CV ≤ 17%

** Associated with people without restrictions on gluten consumption in the diet. Intake not controlled.*

Table 3.
Features of the urine and stool GIP detecting kits in the market for professional use.

5.3 How [when] to collect samples for GIP determinations

The timing for the sampling is an important issue to maximize outcomes. The understanding of the GIP excretion dynamics helps us to select the most convenient time window in which this involuntary ingestion/transgression occurred [52, 53, 70] and even models have been made to estimate the relative amount of this transgression [17].

The studies by Coto *et al.,* [52, 53] with healthy volunteers, and Burger *et al*., with celiac patients [70] had allowed us to know some key issues about the dynamics of GIP excretion (related to single-dose intakes of gluten) in feces and urine (**Table 4**):

	Stool		**Urine**
	LFIA	**ELISA**	**LFIA**
Minimum gluten intake amount that has been detected (single dose)	50 mg	50 mg	50 mg
Excretion window for 50 mg	12–84 h	0–84 h	3–12 h
Excretion window for 2 g	0–132 h	12–204 h	1–15 h
Peak of GIP (time after gluten intake)	24–48 h	24–48 h	6–9 h

Table 4.
Summary of the performance of GIP detecting test according to GIP excretion dynamics.

In feces, GIP excretion is delayed for at least 1 day and wash out in 2–7 days, whilst in urine the excretion peak occurs earlier, and it is narrower than for stool. In feces, as expected, a higher consumption of gluten was correlated to a higher concentration of

GIP in the sample, and to a longer detection period after single gluten intake. In urine the excretion of gluten over time behaves in a similar way regardless of the consumption, with a higher variability on the GIP concentration than in feces (**Figure 1**) [39, 52, 53].

Regarding those differences, it could be assumed that feces are presented as a more convenient sample for a dietary practice evaluation, whereas urine would facilitate the identification of a punctual transgression and would require multiple samples to assess routine diet. As a counterpoint, it is convenient to bear in mind that patients and laboratory professionals are often reluctant to collect and use stool samples, and that for the optimal use of urine, the time relationship between the expected gluten exposure and sample collection must be considered.

In addition, visualizing these generalized behaviors, recommendation of use based on time and the amount of gluten ingested/GIP detected have been generated to help for a better understanding and interpretation of the results obtained [52, 53].

Likewise, it is necessary to understand that each individual works as a different bioreactor and that, although certain behavior patterns can be established, not everyone will do it in the same way. However, there will be certain factors that can be controlled to reduce this inter-individual variability, such as fluid intake or time of the day to collect the samples [52].

6. What would be a practical strategy for assessing adherence by using GIP determinations?

The presence of GIP, either in a urine or stool sample, is a direct indicative that a gluten intake has been committed in the previous hours or days to the sample collection, respectively [52, 53, 70].

The frequency (daily or occasional), the amount of immunoactive gluten to which a celiac is exposed, and the individual sensitivity to GIP have a direct impact on the recovery of the gastrointestinal mucosa.

The dynamic of GIP excretion, average harmful gluten exposure (0.1–0.5 g daily gluten intake for celiac population), distribution of daily meals, analytical sensitivity of the immunoassays, individual variability in metabolisms and habits, studies of correlation of GIP multitesting results with villus atrophy, practical issues and statistical analysis of the results, have been considered to propose protocols of the assessment GFD adherence tests and interpretation of the results with the two kind of samples [30, 33, 52, 53, 57].

Urine GIP: Determination of the presence/absence of GIP in three different urine samples a week, collected with an interval of two days, and at least one of the three having been collected on a weekend. The work led by Ruiz-Carnicer *et al*., where 77 celiac patients who had been on at least a two-year GFD participated, showed that urine tests had a diagnostic sensitivity of 94.4% regarding villous atrophy when the three urine samples collected during the same week had GIP presence. Two out of three of those urines were collected at the weekend, Saturday, and Sunday, and the third one on the day of the medical visit. With this protocol, negative predictive values for intestinal mucosa recovery of 3/3 negative urine GIP reached 97% in this study [39, 52].

Fecal GIP: Determinations of GIP presence/absence in two different stool samples a week, collected with an interval of three to four days, and at least one being representative of the consumptions during the weekend [70–73].

Thus, the studies carried out have made it possible to establish different degrees of adherence to treatment according to the number of samples in which GIP have been found, making three classifications: "excellent adherent," "good adherent" or "poor adherent" [39].

ELISA tests allowed to establish a GIP concentration value for the stool samples, and it has been stipulated that the finding of values higher than 0.6 μg GIP/g feces point to poor adherents which may increase risk of villus atrophy [71, 72].

If it is understood that humans are animals of habits and customs, the determination of GIP in three urines or two stools in the same week, is a practical and objective procedure to perform assessment of the celiac patient's adherence to the treatment. GIP detection allows to distinguish the degree of compliance of the patient to the prescribed diet, and predict its probability to cure or remission, probably even before long-term damage. Furthermore, several studies have showed that the repeated presence of GIP correlated higher with the duodenal mucosal damage than the traditional tools for monitoring adherence to the GFD such as serology, symptomatology, or dietary questionnaires [39].

In conclusion, GIP determinations, following a clinical validated protocol, appear to be a cost-effective, non-invasive, objective and straight forward strategy to assess GFD adherence. In addition, it may allow to predict with some accuracy when the gluten ingestion has been committed, which may enable to identify the source of gluten contamination. That information would serve to prevent future repetition of gluten exposure, improving the chances for a full GFD adherence and complete intestinal mucosa recovery. The GIP presence in human excretions is the direct evidence that the cause of the toxicity in CD, the gluten peptides, has been circulating in the patient body. At this point, does it makes sense to investigate alternative endpoints to proof deficiencies in the dietary treatment of the celiac disease?

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Conflict of interest

Ángel Cebolla is the founder and current CEO of Biomedal S.L., and it is the co-inventor of the patent "Detecting gluten peptides in human fluids" No. WO/2016/005643.

Irati Mendia is employee at Biomedal S.L.

Appendices and Nomenclature

ADHD	attention deficit hyperactivity disorder
ASD	autistic spectrum disorder
anti-AGA	Anti-gliadin antibody
anti-EMA	anti-endomysial antibody
anti-tTG	anti-tissue transglutaminase

CDAT	Celiac Dietary Adherence Test
CD	Celiac Disease
ELISA	Enzyme linked immunosorbent assay
ESPGHAN	The European Society for Pediatric Gastroenterology Hepatology and Nutrition
GF	Gluten Free
GFD	Gluten Free Diet
GI	Gastrointestinal
GIP	Gluten Immunogenic Peptides
IL-2	Interleukin-2
LFIA	Lateral flow immunoassay
LC-MS	Liquid chromatography–mass spectrometry
LLoQ	Lower limit of quantification
ULoQ	Upper limit of quantification
POCT	Point-of-care testing
SPR	Surface plasmon resonance
TG2	anti-tissue transglutaminase type 2
Vh:Cd	Villous height: crypt depth ratio

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