REVISTA ESPAÑOLA DE
ENFERMEDADES DIGESTIVAS
The Spanish Journal of Gastroenterology

Title:
Detection and quantification of gluten immunogenic peptides in feces of infants and their relationship with diet

Authors:
María Roca , Ester Donat , Etna Masip, Paula Crespo Escobar, Victoria Fornes-Ferrer, Begoña Polo, Carmen Ribes-Koninckx

DOI: 10.17235/reed.2018.5549/2018
Link: PubMed (Epub ahead of print)

Please cite this article as:
Roca María, Donat Ester, Masip Etna, Crespo Escobar Paula, Fornes-Ferrer Victoria, Polo Begoña, Ribes-Koninckx Carmen. Detection and quantification of gluten immunogenic peptides in feces of infants and their relationship with diet. Rev Esp Enferm Dig 2018. doi: 10.17235/reed.2018.5549/2018.

REVISTA ESPAÑOLA DE
ENFERMEDADES DIGESTIVAS
The Spanish Journal of Gastroenterology

OR 5549

Detection and quantification of gluten immunogenic peptides in feces of infants and their relationship with diet

María Roca[1], Ester Donat[1,2], Etna Masip[1,2], Paula Crespo-Escobar[1], Victoria Fornes-Ferrer[3], Begoña Polo[1,2] and Carmen Ribes-Koninckx[1,2]

[1]Celiac Disease and Digestive Immunopathology Unit. Medical Research Institute La Fe. Valencia, Spain. [2]Pediatric Gastrohepatology Unit. University Hospital La Fe. Valencia, Spain. [3]Biostatistics Unit. Medical Research Institute La Fe. Valencia, Spain

Received: 26/2/2018
Accepted: 8/8/2018

Correspondence: María Roca Llorens. Unidad de Enfermedad Celíaca e Inmunopatología Digestiva. Instituto de Investigación Sanitaria La Fe. Torre A, 6.ª planta. Av. Fernando Abril Martorell, 106. 46026 Valencia, Spain. e-mail: maria_roca@iislafe.es

Authors´ contributions: Guarantor of the article: María Roca. Specific author contributions: MR and CRK have contributed to the conception or design of the study. MR, ED, EM, PC, VF, BP and CRK have contributed to the acquisition, analysis or interpretation of data for the study. MR, ED, EM, PC and CRK have contributed to drafting the study or critical revision for important intellectual content. All authors read and approved the final version of the manuscript.

Acknowledgements: We would like to thank all the families who participated in the study. We are grateful to the Asociación de Celiacos de la Comunidad Valenciana for their collaboration.

REVISTA ESPAÑOLA DE
ENFERMEDADES DIGESTIVAS
The Spanish Journal of Gastroenterology

Declaration of funding interests: This study was partially supported by a grant from the Asociación de Celiacos y Sensibles al Gluten de la Comunidad de Madrid.

ABSTRACT

Background: there are no effective methods to easily control the correct adherence to a gluten-free diet (GFD) in celiac disease (CD) patients.

Aim: to assess the sensitivity and specificity of a rapid immunochromatographic (IC) test that detects gluten immunogenic peptides (GIP) in feces, compared to an enzyme-linked immunosorbent assay (ELISA) method.

Methods: fecal samples from healthy infants were analyzed by a rapid IC test and ELISA, both methods are based on the anti-gliadin 33-mer monoclonal antibody. Group 1 included infants aged from 6 to 24 months, with an unrestricted consumption of gluten containing cereals. Group 2 (negative controls) was comprised of infants aged from 0 to 6 months, either breastfed or formula fed who had never ingested gluten.

Results: In group 1 (n = 34), all infants had positive values by ELISA, the mean was 13.13 µgGIP/g (range 0.56-46.79). The IC test was negative in 5/20 cases and there was a significant correlation (p=0.006) between the mean daily gluten intake and GIP in feces. In group 2 (n = 20), all the samples were negative by both methods. Moreover, the Kappa Fleiss concordance index (Kappa = 0.79 IC95% [0.616, 0.965]) indicated a moderate concordance between both methods.

Conclusions: according to our results, both methods are highly specific. However, the ELISA test had a higher sensitivity. Although we found a significant correlation between the amount of gluten consumed and GIP recovery in feces, further studies are needed to clarify the impact of individual confounding factors in GIP recovery.

Key words: Celiac disease. Gluten-free diet. Gluten immunogenic peptides.

INTRODUCTION

Although new treatments besides dietary restrictions are being extensively investigated, celiac disease (CD) patients still need to comply with a lifelong gluten free diet (GFD) in order to achieve and maintain a histological recovery and avoid complications. However, despite the importance of a strict adherence to a GFD, there are no efficient tools to assess dietary compliance. Neither voluntary transgressions nor involuntary gluten consumption due to food contamination or mislabeling can be accurately detected, especially if this only happens occasionally, Moreover, these dietary deviations will only lead to clinical symptoms or overt disorders in exceptional cases. Serum anti-tissue transglutaminase antibodies (a-tTG) or anti-deamidated gliadin peptide antibodies (a-DGP) can be in the normal range, especially after years of compliance when only an occasional gluten exposure occurs. Only repeated and severe noncompliance that eventually leads to a histological lesion will cause an increase in a-tTG levels. Although DGP antibodies seems to perform better than a-tTG for this purpose, a recent study by Monzani reported a sensitivity of 60% for anti-DGP IgA and 76% for DGP IgA+IgG; a-tTG IgA had a sensitivity of 24% in the same pediatric population (1). On the other hand, dietary interviews, which are frequently considered as the gold standard for GFD monitoring, are unreliable as voluntary transgressions will be concealed by the patients and any non-voluntary actions will not be identified (2).

Several markers have been suggested as potential indicators of dietary transgressions such as permeability tests, fecal calprotectin, REG I, plasma total alkylresorcinols (3-6) intestinal-fatty acid binding protein (I-FABP] or citrulline. However, these tests are unspecific (7-8) markers that reflect enterocyte damage (9) and are not specific for CD. Furthermore, positive results of these tests will only be obtained when associated with intestinal histological damage. Thus, available tools are unable to detect occasional gluten exposure that may hamper total gut mucosa recovery in the celiac patient (10-16). In this respect, a diet with zero gluten intake is impossible to achieve due to that fact that gluten is a common component in the food industry. Therefore, there is a need for accurate, non-invasive tools to detect minimal gluten intake as a preliminary step to further investigate the potential harmful long-term effects of cumulative minimal quantities of gluten consumption that are unavoidable in real life.

A new promising approach has been recently reported showing that gluten ingestion can be revealed by the detection of gluten immunogenic peptides (GIP) in human feces and urine. GIP were detected using a competitive ELISA with an antigliadin 33-mer monoclonal antibody (17-19). The hypothesis of this study is that both ELISA and a new rapid test are highly efficient methods for GIP detection in feces and therefore would be useful tools for diet monitoring in CD patients. The aim of the present study was to assess the true sensitivity and specificity of this ELISA method by testing it in infants that received exclusively breast milk or formula feeding as compared to infants with a controlled gluten intake. Furthermore, the association between this method and a new rapid immunochromatographic visual test (iVYCheck®GIP) was evaluated for use at the patients "bedside".

MATERIAL

Subjects

Healthy children aged 0 to 2 years, pertaining to families of the hospital staff or relatives of patients at the Pediatric Department of La Fe Hospital were prospectively recruited to the study, between February 2015 and June 2016. Children were classified in 2 different groups. Group 1 included infants aged 6 to 24 months, who consumed unrestricted gluten containing cereals, the daily gluten intake was calculated from a dietary questionnaire. Group 2 (negative controls) was comprised of infants aged 0 to 6 months, who were fed exclusively with breast milk (BM) and/or infant formula (IF) and had never ingested gluten. In breastfed infants, the mothers' diet was considered, paying special attention to dietary restrictions and gluten consumption.

All recruited children met the following inclusion criteria: age 0 to 2 years, gestational age > 37 weeks, appropriate birth weight and with no known underlying chronic inflammatory disease. The exclusion criteria were the following: vaccination in the month prior to enrollment, hospital admissions 2 months prior to enrollment, any intake of steroidal or non-steroidal anti-inflammatory drugs, antibiotics or any other drug during the 2 weeks prior to recruitment or a history of signs or symptoms of infection or gastrointestinal disease (diarrhea, vomiting, hematochezia and fever).

The study was approved by the Ethical Committee of Universitary and Politècnic La Fe Hospital. Written, informed consent was obtained from the parents of all the children who participated in the study prior to their enrollment.

Fecal sampling

Parents were provided with a specific plastic screw-capped container and were instructed to collect a small amount of feces within 4 hours after defecation. Samples were kept in the fridge at home and brought to the laboratory no later than 7 days after collection and were then stored at 20 °C until analysis. For group 1, feces were collected on the 3rd day of the dietary survey.

Dietary questionnaire

Parents were asked to complete a specifically developed food record (FR) covering the three days prior to the stool sample collection. They documented all gluten-containing food, as well as brands and portion sizes that were consumed by the infants. Moreover, in order to ensure that the subjects ate gluten at least one week prior to stool collection and not only during the three previous days, the parents had to fill in a general questionnaire about gluten consumption during the previous week. The FRs and the gluten content of the consumed products were assessed by a specialist dietitian.

The amount of gluten was calculated by multiplying the grams of gluten containing vegetable proteins of each reported product by 0.8, according to a generally accepted method (20). First, the dietitian identified all the brands of different types of gluten-containing products available. Thereafter, the information of the exact amount of gluten-containing proteins from each foodstuff was obtained from different sources such as food labels, specific food composition tables, WHO-FAO website, company official websites or information provided directly by the company.

METHODS

Samples were defrosted and homogenized before analysis by two different methods as follows:

Sandwich ELISA for GIP analysis

A commercial sandwich enzyme-linked immunosorbent (ELISA) assay kit (iVYLISA ®GIP-S, Biomedal SL, Spain) based on the G12 monoclonal antibody was used to analyze GIP in feces. Before analysis, a strict protocol was followed for peptide extraction (2). The measuring range for this method is 0.156-5.000 µg GIP/g feces. Fecal samples were analyzed according to the manufacturer´s instructions. Each sample was analyzed in duplicate and at least two different aliquots of each sample were tested.

IC test for detection of GIP

Fecal samples were also analyzed using a rapid IC test (iVYCheck ®GIP Stool, Biomedal, Spain) based on the G12 monoclonal antibody, according to the manufacturers´ instructions. The limit of detection is 0.3 µg GIP/g feces. Each sample was analyzed in duplicate and at least two different aliquots of each sample were tested. The appearance of a red band corresponds to a positive result, the intensity was graded as follows: strong intensity of the signal (+++); low intensity of the signal (+/++) and negative result (-). The appearance of a blue line indicated that the test was valid. Any opposing results of the same sample between both methods were reanalyzed.

Statistics

Variables were summarized by the mean (SD) and median (1^{st}, 3^{rd} quartile) in the case of quantitative variables and by absolute (N) and relative (%) frequencies in the case of categorical variables. The Kappa Fleiss concordance index was calculated between both methods. The Pearson's correlation was also calculated to test the association between the mean daily gluten intake and the concentration of GIP in feces. Analysis was performed using the R software (version 3.3.2) and *irr* package (version 0.84). A p-value lower than 0.05 was considered as statistically significant.

RESULTS

A total of 54 healthy infants (27 female) were recruited to the study. Group 1 included 20 infants aged 6 to 24 months, who consumed gluten containing cereals. The negative

control group comprised 34 infants aged 0 to 6 months, who had never ingested gluten. Of these, 11 received IF, 8 had mixed feeding and 15 were exclusively breastfed (BF). All lactating mothers were on a normal gluten containing diet, except for one CD mother who was on a GFD.

In group 1, the daily gluten intake calculated from the dietary questionnaire ranged from 0.5g to 10.5g/day. According to the ELISA analysis, all infants had GIP values of > 0.156µg/g feces, the mean was 13.13 µgGIP/g feces (range 0.56-46.79). In addition, a significant correlation ($r = 0.59$, $p = 0.006$) was found in this group between the mean daily gluten intake and the concentration of GIP in feces (Fig. 1).

5 of 20 samples were negative according to the IC test (Table 1). These pertain to infants with different levels of gluten consumption and do not correspond to the 5 children with the lowest gluten intake (Table 1). However, GIPs were detected by IC strips in all infants who consumed more than 5.6 g gluten/day. Moreover, there was a statistically significant association between GIP in feces that quantified by ELISA and the intensity of the results observed via the IC Strips, IC95% [1.04, 1.22] p-value = 0.005. Thus, higher ELISA values corresponded to a greater intensity in the IC strip response (Fig. 2).

In group 2 (controls), all infants had values of < 0.156µgGIP/g feces according to the ELISA analysis. The IC test results were also negative, independently of the feeding pattern such as exclusively BF, mixed feeding or exclusively IF feeding.

There was a 100% sensitivity and specificity for the sandwich ELISA and a sensitivity and specificity of 75% and 100%, respectively for the IC strips. The Kappa Fleiss concordance index (Kappa = 0.79 IC95% [0.616, 0.965]) indicated a moderate concordance between both methods ($p < 0.001$).

DISCUSSION

A rapid IC test to detect GIP in feces compared to the sandwich ELISA method was assessed in this study. For that purpose, children with different types of feeding and gluten consumption were recruited. To our knowledge, this is the first study to assess infants with exclusive BF, mixed feeding and exclusive IF feeding as negative controls by both GIP testing methods. All samples from these infants had negative values by

ELISA and the IC test. Our results showed that both methods are highly specific (100%), although the ELISA test had a higher sensitivity than IC strips (100% and 75%, respectively). Previous studies (2) have shown a diagnostic sensitivity and specificity of 98.5% and 100%, respectively for the G12 sandwich ELISA method.

Overall, there was a significant correlation between the amount of gluten consumed and GIP recovery in feces by ELISA. However, a wide inter-individual variation was observed. The study by Comino et al. used different methods for GIP detection in feces, competitive ELISA, immunochromatographic test and western blot. This study also found a positive correlation between the amount of gluten consumed and the amount detected, even though a large variation between individuals was also observed. The results from both studies support the idea that absorption of gliadin peptides along the gastrointestinal tract may vary among individuals (18), this is probably related to different individual gastrointestinal conditions such as the microbiome or the gastrointestinal transit time impact on the final GIP concentration in feces. A potential benefit of the correlation between the amount of gluten consumed and the recovery of GIP in feces would be the detection of dietary deviations and the possibility of a quantitative estimation of transgressions.

We detected GIP by a sandwich ELISA in all 20 samples of the children in group 1, these had a minimum daily gluten consumption of 500 mg. This is in line with the study by Comino et al (18), which showed that a competitive ELISA could detect the ingestion of 50 mg to 30g of gluten from processed bread in feces.

According to our results, 5 of these 20 samples were negative by IC strips, although they do not correspond to the 5 children who consumed less gluten, nor to the samples in which a lower amount of GIP was detected by ELISA. However, all samples with results above 4.66 µg GIP/g feces by ELISA were GIP-positive by IC strips. This disagreement could be due to a lower sensitivity of the chromatographic method as compared to the ELISA. Likewise, a heterogeneous distribution of GIP in feces, despite a careful sample homogenization, could also contribute to some of the observed discrepancies. A limitation of our study is that although parents were given very precise instructions about feces collection, storage and transport, we cannot completely rule out some deviations from the recommended protocol.

In group 2 (negative controls), all infants had negative values by ELISA and the IC test, independently of exclusive BF, mixed feeding or exclusive IF feeding. These babies do not consume gluten, although small traces of gluten excreted through breast milk could reach the digestive tract. However, previous studies from our group (21) as well as from other authors (22-23) have shown that the gluten concentration in breast milk is negligible and peptides in breast milk may also be degraded via the enteromammary circulation. In addition, the 100% specificity observed confirms that there was no cross reactivity with other dietary / intraluminal antigens.

Dietary questionnaires and serological CD markers are commonly used for the control of the adherence to the GFD of CD patients. However, these tools are not able to detect small involuntary and/or occasional dietary transgressions (2). The detection of GIP in feces via ELISA or IC strips, would be useful as a non-invasive method for the control of GFD. Although, each method should have a different use. The ELISA test is able to quantify the amount of GIP detected in feces, although it requires an infrastructure and qualified technician and should be performed in a laboratory. The IC strip is a simple visual qualitative method, although not as sensitive as the ELISA, would be aimed at self-control by the patient for suspected involuntary transgressions. Self-management of dietary compliance could be achieved by this rapid easy to use test. The improvement of dietary control means that complications related to dietary transgressions, could be avoided. This would result in a positive impact on quality of life of CD patients. Moreover, it could be a decisive tool to empower a patients´ self-management of the disease. However, the method should be optimized to improve the low negative predictive value of IC.

In conclusion, both methods are highly specific according to our results. However, the ELISA test has a higher sensitivity. Although we found a significant correlation between the amount of gluten consumed and GIP recovery in feces, further studies are needed to clarify the impact of individual confounding factors in GIP recovery.

REFERENCES

1. Monzani A, Rapa A, Fonio P, et al. Use of deamidated gliadin peptide antibodies to monitor diet compliance in childhood celiac disease. J Pediatr Gastroenterol Nutr

2011;53(1):55-60. DOI: 10.1097/MPG.0b013e3182145511

2. Comino I, Fernández-Bañares F, Esteve M, et al. Fecal Gluten Peptides Reveal Limitations of Serological Tests and Food Questionnaires for Monitoring Gluten-Free Diet in Celiac Disease Patients. Am J Gastroenterol 2016;111(10):1456-65. DOI: 10.1038/ajg.2016.439

3. Duerksen DR, Wilhelm-Boyles C, Parry DM. Intestinal permeability in long-term follow-up of patients with celiac disease on a gluten-free diet. Dig Dis Sci 2005;50(4):785-90. DOI: 10.1007/s10620-005-2574-0

4. Ertekin V, Selimoğlu MA, Turgut A, et al. Fecal calprotectin concentration in celiac disease. J Clin Gastroenterol 2010;44(8):544-6. DOI: 10.1097/MCG.0b013e3181cadbc0

5. Planas R, Pujol-Autonell I, Ruiz E, et al. Regenerating gene Iα is a biomarker for diagnosis and monitoring of celiac disease: a preliminary study. Transl Res 2011;158(3):140-5. DOI: 10.1016/j.trsl.2011.04.004

6. Lind MV, Madsen ML, Rumessen JJ, et al. Plasma alkylresorcinols reflect gluten intake and distinguish between gluten-rich and gluten-poor diets in a population at risk of metabolic syndrome. J Nutr 2016;146(10):1991-8. DOI: 10.3945/jn.116.236398

7. Capone P, Rispo A, Imperatore N, et al. Fecal calprotectin in coeliac disease. World J Gastroenterol 2014;20(2):611-2. DOI: 10.3748/wjg.v20.i2.611

8. Duerksen DR, Wilhelm-Boyles C, Veitch R, et al. A comparison of antibody testing, permeability testing, and zonulin levels with small-bowel biopsy in celiac disease patients on a gluten-free diet. Dig Dis Sci 2010;55(44):1026-31. DOI: 10.1007/s10620-009-0813-5

9. Adriaanse M, Leffler DA. Serum markers in the clinical management of celiac disease. Dig Dis 2015;33(2):236-43. DOI: 10.1159/000371405

10. Kaukinen K, Peräaho M, Lindfors K, et al. Persistent small bowel mucosal villous atrophy without symptoms in coeliac disease. Aliment Pharmacol Ther 2007;25(10):1237-45. DOI: 10.1111/j.1365-2036.2007.03311.x

11. 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(3):311-5. DOI: 10.1097/00042737-200203000-00017

12. Tursi A, Brandimarte G, Giorgetti GM. Lack of usefulness of anti-transglutaminase antibodies in assessing histologic recovery after gluten-free diet in celiac disease. J Clin Gastroenterol 2003;37(5):387-91. DOI: 10.1097/00004836-200311000-00007

13. Tursi A, Brandimarte G, Giorgetti, GM, et al. Endoscopic and histological findings in the duodenum of adults with celiac disease before and after changing to a gluten-free diet: A 2-year prospective study. Endoscopy 2006;38(7):702-7. DOI: 10.1055/s-2006-925178

14. Biagi F, Campanella J, Martucci S, et al. A milligram of gluten a day keeps the mucosal recovery away: A case report. Nutr Rev 2004;62(9):360-3. DOI: 10.1111/j.1753-4887.2004.tb00062.x

15. Rashtak S, Ettore MW, Homburger HA, et al. Comparative usefulness of deamidated gliadin antibodies in the diagnosis of celiac disease. Clin Gastroenterol Hepatol 2008;6(4):426-32. DOI: 10.1016/j.cgh.2007.12.030

16. Vallejo-Díez S, Bernardo D, Moreno ML, et al. Detection of specific IgA antibodies against a novel deamidated 8-Mer gliadin peptide in blood plasma samples from celiac patients. PLoS ONE 2013;8(11):e80982. DOI: 10.1371/journal.pone.0080982

17. 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(5):e2294. DOI: 10.1371/journal.pone.0002294

18. 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(3):670-7. DOI: 10.3945/ajcn.111.026708

19. 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 2017;66(2):250-7. DOI: 10.1136/gutjnl-2015-310148

20. Overbeek FM, Uil-Dieterman IG, Mol IW, et al. The daily gluten intake in relatives of patients with coeliac disease compared with that of the general Dutch population. Eur J Gastroenterol Hepatol 1997;9(11):1097-9. DOI: 10.1097/00042737-199711000-00013

21. Peter Koehler, editor. Analysis of gluten in human milk samples in coeliac and non-coeliac mothers. In: Proceedings of the 26th Meeting of the Working Group on Prolamin Analysis and Toxicity: Leuven: Verlag Wissenschaftliche Scripten; 2013. pp. 39-44

22. Chirdo FG, Rumbo M, Anon MC, et al. Presence of high levels of non-degraded gliadin in breast milk from healthy mothers. Scand J Gastroenterol 1998;33(11):1186-92. DOI: 10.1080/00365529850172557

23. Troncone R, Scarcella A, Donatiello A, et al. Passage of gliadin into human breast milk. Acta Paediatr Scand 1987;76(3):453-6. DOI: 10.1111/j.1651-2227.1987.tb10498.x

Table 1. Characteristics of infants in group 1 and the GIP analysis results

	Age (months)	Sex	BF	Gluten ingested per day (grams)	ELISA (µg GIP/g feces)	IC strips
1	6	m	n	0.5	0.56	-
2	7	f	y	1.32	1.02	+
3	6	f	n	1.32	1.63	++
4	13	f	n	5.6	2.13	-
5	18	f	n	1.14	2.31	+
6	13	m	n	3.24	2.78	+
7	6	m	y	1.32	2.96	++
8	7	m	y	1.9	2.81	-
9	6	f	n	1.26	3.38	+
10	12	f	n	4.225	4.45	-
11	20	f	y	3.7	4.48	++
12	22	f	y	2.98	4.66	-
13	13	m	y	6.5	20.00	+++
14	14	f	n	6.365	20.5	+
15	7	f	y	1	21.50	++
16	14	m	n	2.2	21.5	+++
17	13	f	n	4.27	22.21	+
18	7	f	n	5.5	37.50	+++
19	7	m	y	2.28	46.79	+++
20	16	m	n	10.05	39.50	++

m: male; f: female; BF: breast feeding.

Results of GIP analysis by ELISA are expressed as µg GIP/g feces. The results of the IC strips were graded according to the intensity of the response: strong intensity of the signal (+++); low intensity of the signal (+/++) and negative result (-).

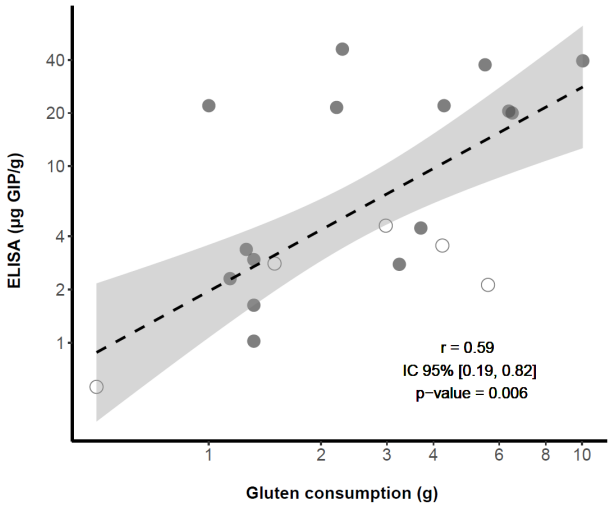

Fig. 1. Scatterplot of the mean daily gluten intake and the concentration of GIP in feces by ELISA in group 1. White dots represent infants with no response detected by the IC strips.

REVISTA ESPAÑOLA DE
ENFERMEDADES DIGESTIVAS
The Spanish Journal of Gastroenterology

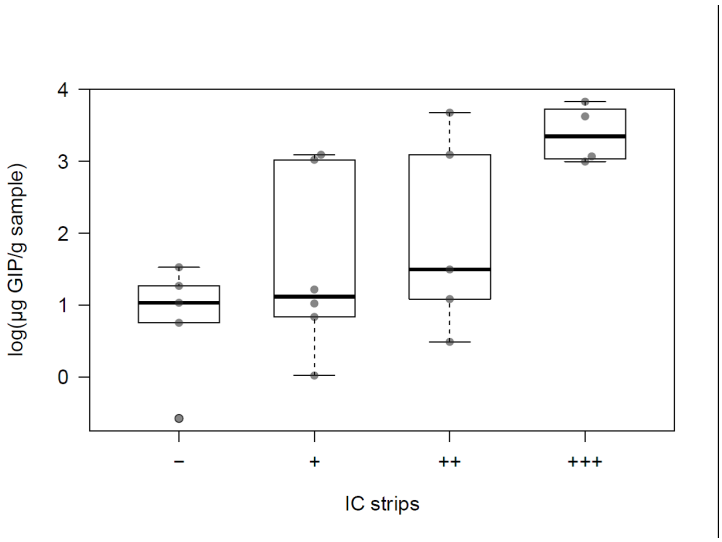

Fig. 2. Boxplot of the IC strips results and the concentration of GIP in feces by ELISA in group 1.