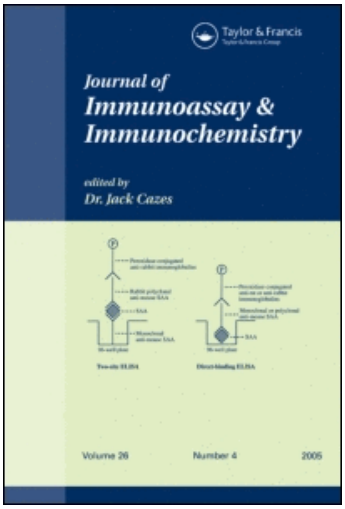

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Zeno Földes-Papp[a]; Ulrike Demel[a]; Desiree Berry[b]; Wolfgang Domej[a]; Dietmar Fuchs[c]; Gernot Peter Tilz[a]
[a] Clinical Immunology, Jean Dausset Laboratory, Graz University M.S. and Hospital, Graz, LKH, Austria [b] Sutherland Centre of Immunology, Protein Laboratory, University of Sydney, Sydney, NSW, Australia [c] Institute of Medical Biochemistry, University of Innsbruck, Innsbruck, Austria

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TISSUE TRANSGLUTAMINASE ANTIBODY DETERMINATION IN CELIAC DISEASE. ANALYSIS OF DIAGNOSTIC SPECIFICITY OF ANTI-HUMAN IgA-TYPE ASSAYS

Zeno Földes-Papp,[1] Ulrike Demel,[1] Desiree Berry,[2] Wolfgang Domej,[1] Dietmar Fuchs,[3] and Gernot Peter Tilz[1,*]

[1]Clinical Immunology and Jean Dausset Laboratory, Graz University M.S. and Hospital, Auenbruggerplatz 8, A-8036 Graz, LKH, Austria
[2]Sutherland Centre of Immunology, Protein Laboratory, University of Sydney, Sydney NSW 2229, Australia
[3]Institute of Medical Biochemistry, University of Innsbruck, Fritz Pregel Strasse 3, A-6020 Innsbruck, Austria

ABSTRACT

Biopsy is onerous and, for this reason, immunodiagnostics in sera of celiac disease patients are an "additional diagnostic standard." The objective of the study was to investigate the variability in diagnostic specificity of ELISAs for the

*Corresponding author. E-mail: gernot.tilz@uni-graz.at

detection of IgA anti-tissue transglutaminase antibodies in serum of celiac disease patients who underwent biopsy. All patients were included in the study on the basis that they had a small intestinal biopsy. We studied 18 patients with histological proven celiac disease (7 male, 11 female, mean age $\pm$ SD: 35 ± 19 years) from Graz, Austria. Healthy control subjects were also entered into the study. The determinations of the anti-tissue transglutaminase antibodies were simultaneously performed together with the endomysium and gliadin antibody markers. We analysed the 216 serum values according to Cochran's non-parametric Q-test. The complexity to the analysis reflects the complexity of the diagnostic situation with the patients. No real differences were found in the reactions of the anti-human IgA-type anti-tissue transglutaminase ELISAs. Based on these results, an association was established between the outcomes of anti-human IgA-type ELISAs for the specific antigen and patients with histologically proven celiac disease, treated for celiac disease after histology was carried out and the diagnosis was made, and healthy controls. The detection of IgA anti-tissue transglutaminase antibodies in serum is a promising alternative to the indirect immunofluorescence determination of IgA-type endomysium antibodies. One ELISA for the specific antigen showed some advantage with respect to its extended scale of detection. Immunopathology of celiac disease can be based on the results of the appropriate IgA anti-tissue transglutaminase ELISAs under uncomplicated gastrointestinal conditions.

Key Words: Immunodiagnostics; Tissue transglutaminase antibodies (tTG); Diagnostic specificity; ELISAs; Immunopathology; Celiac disease; Cochran's Q-test

INTRODUCTION

Celiac disease is classified on the basis of characteristic small intestinal histology.(1) Serological tests developed in the last decade provide a non-invasive diagnostic means.(2) IgA-type anti-endomysium antibodies, and IgA-type and IgG-type anti-gliadin autoantibodies in serum immunologically characterize celiac disease. An increased serum titre of anti-endomysium autoantibodies (EMA) is considered indicative of celiac disease

(3–8) and the presence of two of three positive serological tests (IgA-type anti-endomysium and/or IgA and IgG-type anti-gliadin) are supportive of the diagnosis.(9)

EMA's are mainly of the IgA-type.(10) The specificity and sensitivity of IgA EMA lie in the range of 91–100% and 74–100%.(11–18) IgA EMA does not detect all gluten-sensitive individuals. The positive and negative predictive values are between 79 and 100% and 95 and 100%.(12–14,18) An increased titre of EMAs is dependent, to a great extent, upon severity of the mucosal damage, age, and genetic factors.(19–22) However, the assay sensitivity is influenced by the diagnostic way, namely patients are biopsied on the basis of clinical symptoms or seropositive patients are biopsied in order to confirm the diagnosis.(2) Very recently, a new celiac disease subgroup was described with anti-endomysium and anti-transglutaminase antibodies of the IgG-type in the absence of selective IgA deficiency.(3) The determination of IgA EMA is being replaced by the determination of antibodies to the specific antigen.

Tissue transglutaminase is the endomysial autoantigen in celiac disease.(23) The preferred substrate of this enzyme is gliadin, a component of wheat gluten, initiating mucosal damage by an immunological process in individuals with a certain genetic predisposition.

A serum antibody status for the diagnostic support of celiac disease is now routine, but to our knowledge, this is the first clinical study to determine whether differences in the diagnostic specificity between appropriate IgA anti-transglutaminase ELISAs really exists. In all, 81 sera were examined from patients with histologically proven celiac disease, treated for celiac disease after histology was carried out and the diagnosis was made, and from healthy controls. Further, we compared the diagnostic specificity of the IgA anti-transglutaminase determination with the determination of anti-endomysium and anti-gliadin antibodies. In this pilot study, the IgA anti-transglutaminase ELISAs were the superior test.

EXPERIMENTAL

Design

We investigated, here, the variability in diagnostic specificity of the IgA tTG determinations by four ELISAs (items n) of p comparable individuals (dependent trials). Because the patients were diagnosed by proven celiac disease or they were treated for celiac disease after histology was done

and the diagnosis was histologically confirmed, we tested according to Cochran's non-parametric Q-test.(24) Our detailed mathematical analysis is given in the Appendix.

Patients and Materials

The procedures followed were in accord with the ethical standards of the Helsinki Declaration of 1975 as revised in 1983. All patients were included in the study on the basis that they had a small intestinal biopsy. We studied 18 patients (7 male, 11 female, mean age $\pm$ SD: 35 ± 19 years) from Graz, Austria. Nine patients were treated for celiac disease by diet after histology was performed and the diagnosis made. Nine healthy control subjects were also entered into the study. The patients were otherwise unselected. All 81 sera were routine samples in the investigation of celiac disease. The sera were collected over a period of nine months and stored at -20°C.

ELISAs

IgA anti-tissue transglutaminase antibodies (IgA tTG) were determined by commercially available ELISAs from Sweden (Pharmacia), Germany (Immunodiagnostics), Italy (Eurospital), and the United Kingdom (Binding Site). Each kit contained tTG antigen and anti-human IgA antibody, for example, from the sheep (monoclonal; Eurospital) or rabbit (polyclonal; Banding Site), labeled with a peroxidase (horseradish peroxidase) for quantifying the amount of captured IgA anti-tissue transglutaminase autoantibodies. The chromogenic substrate was 3,3′,5,5′-tetramethylbenzidine. However, one kit (Sweden, Pharmacia) had an advantage in the scaling of the signal (signal resolution in the indicator response of the secondary detection antibody). In the case of the kits from Italy (Eurospital) and from Germany (Immunodiagnostics), the samples were difficult to handle because of prolonged incubation steps (Eurospital: 1 h for sera at room temperature, 1 h for conjugate at room temperature, 25 min for substrate at room temperature; Immundiagnostics: 1 h for sera at 4°C, 1 h for conjugate at room temperature, 25 min for substrate at room temperature). Reference sera (IgA tTG calibrators), positive and negative controls, cut-off points, calibration curves, and intra-assay precision, as well as inter-assay precision, were provided by the specified suppliers. All analytical values of assay quality control were remeasured by us and confirmed. IgG anti-gliadin (IgG AGA), IgA anti-gliadin (IgA AGA), and IgA anti-endomysium autoantibody (IgA EMA) titres were determined by standard

assays, i.e., indirect immunofluorescence for EMA and ELISAs for AGA. All assays were done in duplicate measurements. The means of the determinations given in Tables 1–3 were expressed as either plus level or negative level of the outcome. The plus sign represented the high level (increased antibody value) and the minus sign the low level (non-increased antibody level).

RESULTS

The comparative study was made concerning the specificity of the immunodiagnostic methods for celiac disease. The diagnostic performance was assessed of class A anti-transglutaminase antibodies, class A anti-endomysium antibodies, and class A and class G anti-gliadin antibodies. All 18 patients with histologically proven celiac disease, nine healthy control subjects, and the bulk data are given in Table 1. The means of 216 serum values of antibodies are shown, whereby a plus or 1 sign represents the high level (increased titre level) and a minus or 0 sign the low level (non-increased titre level).

Four items (observations), n, were considered, namely the IgA tTG determinations by four ELISAs of p comparable individuals of dependent trials. Differences between the + and − levels of detected IgA tTG (quantitative variables) were then tested whether there is any solid evidence of their existence. To understand the analysis technique being used, it is important to state that Cochran's Q-test is an appropriate nonparametric test for examining change in a dichotomous variable across more than two observations such as in the situation at hand. The equivalent test for the case of two observations (as opposed to more than two) is McNemar's test, with which most reader's are more familiar. In other words, McNemar's test is a special case of Cochran's Q-test for

$$x_j = \begin{pmatrix} x_{1j} \\ x_{2j} \end{pmatrix}.$$

However, we do not present here any matrix calculations. The complexity to the type of analysis reflects the complexity of the diagnostic situation with the patients. This experimental design indicated what could legitimately be concluded about current clinical hypotheses from the patients entered into the study. We obtained answers that are as little affected by experimental error as possible. For example, we could perform the analyses even with a minimum of information about the patients' samples.

No positive IgA-tTG ELISA reactions were found in healthy controls. In assessing patients who had histologically proven celiac disease, but

Table 1. IgA-Type Anti-Tissue Transglutaminase Autoantibody Activities in Blood from Patients with Histologically Proven Celiac Disease, Treated for Celiac Disease After the Diagnosis was Histologically Confirmed, and from Healthy Controls. An Increased Value Is Indicated by +/1. A Non-increased Value Is Indicated by −/0. Elisa 1 Was from Sweden (Pharmacia), Elisa 2 from Germany (Immunodiagnostics), Elisa 3 from Italy (Eurospital), Elisa 4 from United Kingdom (Binding Site)

p Trials		*n* Items			
Conditions	Patient's Nr	tTG Elisa 1	tTG Elisa 2	tTG Elisa 3	tTG Elisa 4
Celiac disease, with	11498/00	+/1	+/1	+/1	+/1
IgA anti-gliadin +	11421/00	+/1	+/1	+/1	+/1
and IgA anti-endomysium +	2857/00	+/1	+/1	+/1	+/1
or IgG anti-gliadin +	2051/00	+/1	+/1	+/1	+/1
	14612/00	+/1	+/1	+/1	+/1
	16154/99	+/1	+/1	+/1	+/1
Celiac disease, with IgA anti-gliadin + and IgA anti-endomysium− and IgG anti-gliadin−	1582/00	−/0	+/1	+/1	−/0
Celiac disease, with IgA anti-gliadin− and IgA anti-endomysium + and IgG anti-gliadin +	13012/00	+/1	+/1	+/1	+/1

Celiac disease, with IgA anti-gliadin− and IgA anti-endomysium− and IgG anti-gliadin + ($p=9$)	1494/00	−/0	−/0	+/1	−/0
Patients treated for celiac disease, with IgA anti-gliadin + or IgA anti-endomysium + or IgG anti-gliadin + ($p=9$)	11699/00	−/0	−/0	−/0	−/0
	11664/00	−/0	−/0	−/0	−/0
	11655/00	−/0	−/0	−/0	−/0
	10970/00	−/0	−/0	−/0	−/0
	5272/00	−/0	−/0	−/0	−/0
	4835/00	−/0	−/0	−/0	−/0
	4762/00	−/0	−/0	−/0	−/0
	2809/00	−/0	−/0	−/0	+/1
	1985/00	−/0	−/0	+/1	−/0
Healthy controls ($p=9$)	11044/00	−/0	−/0	−/0	−/0
	4850/00	−/0	−/0	−/0	−/0
	4667/00	−/0	−/0	−/0	−/0
	6039/00	−/0	−/0	−/0	−/0
	1479/00	−/0	−/0	−/0	−/0
	1704/00	−/0	−/0	−/0	−/0
	2132/00	−/0	−/0	−/0	−/0
	14992/00	−/0	−/0	−/0	−/0
	15051/00	−/0	−/0	−/0	−/0

Table 2. Differentiation of the Antibody Status for Patients with IgA Anti-Endomysium Autoantibodies and Healthy Control. An Increased Value of IgA-Type Anti-Tissue Transglutaminase Autoantibody Is Indicated by +/1. A Non-increased Value Is Indicated by −/0. For (qualitative) Comparison We Included the Antibody Status of the Healthy Control Subjects. ELISA 1 Was from Sweden (Pharmacia), ELISA 2 from Germany (Immunodiagnostics), ELISA 3 from Italy (Eurospital), ELISA 4 from United Kingdom (Binding Site)

p Trials				*n* Items			
Conditions			Patient's Nr	tTG ELISA 1	tTG ELISA 2	tTG ELISA 3	tTG ELISA 4
IgA Anti-endomysium	Patients with IgA Anti-gliadin	IgG Anti-gliadin					
+	+	−	11498/00	+/1	+/1	+/1	+/1
+	+	+	11421/00	+/1	+/1	+/1	+/1
+	+	−	2857/00	+/1	+/1	+/1	+/1
+	+	+	2051/00	+/1	+/1	+/1	+/1
+	+	+	14612/00	+/1	+/1	+/1	+/1
+	+	+	16154/99	+/1	+/1	+/1	+/1
+	−	+	13012/00	+/1	+/1	+/1	+/1
+	−	+	11699/00	−/0	−/0	−/0	−/0
+	+	−	11655/00	−/0	−/0	−/0	−/0
	(*p* = 9)						

Healthy controls with IgA Anti-endomysium	IgA Anti-gliadin	IgG Anti-gliadin					
−	−	+	11044/00	−/0	−/0	−/0	−/0
−	−	−	4850/00	−/0	−/0	−/0	−/0
−	−	+	4667/00	−/0	−/0	−/0	−/0
−	−	−	6039/00	−/0	−/0	−/0	−/0
−	−	+	1479/00	−/0	−/0	−/0	−/0
−	−	−	1704/00	−/0	−/0	−/0	−/0
−	−	+	2132/00	−/0	−/0	−/0	−/0
−	+	−	14992/00	−/0	−/0	−/0	−/0
−	−	−	15051/00	−/0	−/0	−/0	−/0
	($p=9$)						

Table 3. Differentiation of the Serological Status (Antibody Status) for Patients with IgA Anti-Gliadin Autoantibodies and Healthy Control. An Increased Titer of IgA-Type Anti-Tissue Transglutaminase Autoantibody Is Indicated by +/1. A Non-increased Titer Is Indicated by −/0. For (Qualitative) Comparison We Included the Antibody Status of the Healthy Control Subjects. ELISA 1 Was from Sweden (Pharmacia), ELISA 2 from Germany (Immunodiagnostics), ELISA 3 from Italy (Eurospital), ELISA 4 from United Kingdom (Binding Site)

p Trials				*n* Items			
Conditions			Patient's Nr	tTG ELISA 1	tTG ELISA 2	tTG ELISA 3	tTG ELISA 4
IgA Anti-gliadin	Patients with IgA Anti-endomysium	IgG Anti-gliadin					
+	+	−	11498/00	+/1	+/1	+/1	+/1
+	+	+	11421/00	+/1	+/1	+/1	+/1
+	+	−	2857/00	+/1	+/1	+/1	+/1
+	+	+	2051/00	+/1	+/1	+/1	+/1
+	+	+	14612/00	+/1	+/1	+/1	+/1
+	+	+	16154/99	+/1	+/1	+/1	+/1
+	−	−	1582/00	−/0	+/1	+/1	−/0
+	−	+	11664/00	−/0	−/0	−/0	−/0
+	+	−	11655/00	−/0	−/0	−/0	−/0
+	−	+	4762/00	−/0	−/0	−/0	−/0
	($p=10$)						

	Healthy controls with						
IgA Anti-gliadin	IgA Anti-endomysium	IgG Anti-gliadin					
−	−	+	11044/00	−/0	−/0	−/0	−/0
−	−	−	4850/00	−/0	−/0	−/0	−/0
−	−	+	4667/00	−/0	−/0	−/0	−/0
−	−	−	6039/00	−/0	−/0	−/0	−/0
−	−	+	1479/00	−/0	−/0	−/0	−/0
−	−	−	1704/00	−/0	−/0	−/0	−/0
−	−	+	2132/00	−/0	−/0	−/0	−/0
+	−	−	14992/00	−/0	−/0	−/0	−/0
−	−	−	15051/00	−/0	−/0	−/0	−/0
	($p=9$)						

were untreated and positive for IgA AGA and/or IgA EMA and/or IgG AGA (dependent $p = 9$ trials, Table 1), the analysis of the data yielded the same probability of positive reactions of the four ELISAs for the specific antigen in each trial within the confidence interval 0.999 ($\hat{Q} = 20.4 < \chi^2_{0.001;8} = 26.1$).

We notice, for example, that the reactions of the kits had differences which occurred between the trials at the confidence interval of 0.990 ($\hat{Q} = 20.4 > \chi^2_{0.01;8} = 20.1$), but there was no evidence indicating any real difference between the four IgA-tTG ELISAs at the 0.001 level of significance. We now considered the nine patients with treated celiac disease (dependent $p = 9$ trials, Table 1) and positive values for IgA AGA or IgA EMA or IgG AGA. They were tested against the four IgA-tTG ELISAs. The experimental data of Table 1 indicated that the true mean difference between the trials was also zero ($\hat{Q} = 7.0 < \chi^2_{0.001;8} = 26.1$) within the confidence interval 0.999 ($1 - \alpha$). Taken together, based on these results, the association was also proven between the outcomes of anti-human IgA-type ELISAs for the specific antigen and histological confirmed celiac disease before and after treatment by diet.

Further, we differentiated the antibody status (gliadin, endomysium, tTG) for every patient and control separately (Tables 2 and 3). The serial data of patients with IgA EMA (Table 2, dependent $p = 9$ trials) were compared with all of the four ELISAs assaying the specific antigen. The evaluation gave significant mean differences of the outcomes between the trials ($\hat{Q} = 32.0 > \chi^2_{0.001;8} = 26.1$). For the serial data of patients with IgA AGA (Table 3, dependent $p = 10$ trials), significant differences were also obtained within the confidence interval 0.999 ($\hat{Q} = 32.4 > \chi^2_{0.001;9} = 27.9$).

DISCUSSION

In this clinical study, we evaluated four appropriate ELISAs for antigen tissue-transglutaminase before and after treatment of histologically proven celiac disease. The four items, n, namely IgA tTG determinations in serum by the four ELISAs, were considered under p conditions of comparable individuals in dependent trials. The effects of experimental error could be greatly reduced by the experimental design and analysis. In particular, the analysis showed the general location and spread of the observations of the body of data. The primary outcome of this study is that we could not find any significant difference in the reactions of the four anti-human IgA-type anti-tissue transglutaminase ELISAs within the confidence interval 0.999 (Table 1). Based on these findings, the association between the outcomes of anti-human IgA-type ELISAs for the specific antigen and

histological confirmed celiac disease before and after treatment by diet has also been proven (Table 1). The patients improved symptomatically following treatment with a gluten-free diet. The patients failed to attend for a repeat of biopsies. The differences in the differentiation of the antibody status for patients with IgA anti-endomysium antibodies (Table 2) and IgA anti-gliadin antibodies (Table 3), compared with IgA anti-transglutaminase antibodies, were due to a response of patients to a gluten-free diet after histology was done. In patients with no IgA EMA, a positive reaction was found only in one case with the kit from Germany and the kit from Italy (patient 1582/00), and in two cases with the Italian kit (patients 1582/00 and 1494/00). One ELISA for the specific antigen (Sweden, Pharmacia) showed an advantage regarding its extended scale of signal resolution (signal detection). Our findings support the view that immunopathology of celiac disease can be based on the results of the appropriate IgA-tTG ELISAs under uncomplicated gastrointestinal conditions. In particular, the detection of IgA anti-tissue transglutaminase antibodies in serum is well suited for monitoring the success in disease treatment under uncomplicated gastrointestinal conditions.

Celiac disease is proven by histology showing the villous atrophy of the small intestine. Biopsy is onerous and, for this reason, immunodiagnostics in sera are an "additional diagnostic standard." Gliadin and endomysium antibodies have widely been used with moderate overall sensitivity. Anti-transglutaminase antibody studies in celiac disease have already been done before (for review see reference 2). Despite the fact that there is the need for a more specific and sensitive assay, no standardization and no reference protocols, as well as materials, are presently available.(17) Therefore, non-commercial test protocols have been worked out very recently,(17) but data on the comparative evaluation of IgA anti-tissue transglutaminase ELISAs with clinical implications are not given.

CONCLUSIONS

The experimental design and analysis of the clinical study indicated what could legitimately be concluded about the specificity of immunodiagnostics from the patients entered into the study. The results of serial data of four IgA anti-transglutaminase ELISAs demonstrated the potential of the anti-human IgA-type ELISA assay to replace indirect immunofluorescence for IgA anti-endomysium antibodies by the determination of antibodies to the specific antigen, namely tissue-transglutaminase. Quantification of IgA tTG levels by the appropriate ELISAs is useful,

especially in cases of celiac disease with uncomplicated gastrointestinal conditions.

APPENDIX: MATHEMATICAL ANALYSIS

The mathematical analysis was performed from the tables of signs (Tables 1–3) with $n \cdot p \geq 30$ signs. Instead of a + and − notation, we used the notation 1 (increased antibody value) and 0 (non-increased antibody value) for the quantitative evaluations. Differences between the 1 (+) and 0 (−) levels of detected IgA tTG (quantitative variables) were analyzed in the following way: The ith observation (item, object) was characterized by p dependent trials (patients, conditions). We wrote for the j term of the trial

$$x_j = \begin{pmatrix} x_{1j} \\ \vdots \\ x_{nj} \end{pmatrix},$$

and, for the ith observation (item)

$$x_i = (x_{i1}, x_{i2}, \ldots, x_{ip}).$$

We omitted those x_j and x_i which did not contribute to the differentiation between the null hypothesis H_0 and some alternative hypothesis H_A. The $n \times p$ matrix $\boldsymbol{X}$ associated with the experimental data (table of signs) was then

$$\boldsymbol{X}_{n\times p} = \begin{pmatrix} x_{11} & \cdots & x_{1p} \\ \vdots & \ddots & \vdots \\ x_{n1} & \cdots & x_{np} \end{pmatrix}$$

Now, we obtained, from the experimental data, the following four matrices

$$\boldsymbol{T} = \boldsymbol{X}^T \cdot 1 \quad \text{and} \quad \boldsymbol{T}^2, \quad \text{with } j = 1, 2, \ldots, p$$

as well as

$$\boldsymbol{L} = \boldsymbol{X} \cdot 1 \quad \text{and} \quad \boldsymbol{L}^2, \quad \text{with } i = 1, 2, \ldots, n,$$

where 1 is a unit vector with p or n 1's. ^{T} denotes the transpose of $\boldsymbol{X}$. Then, Cochran's Q-criterion appropriate to test the null hypothesis H_0 was calculated for $\lambda = p - 1$ dependent trials

$$\hat{Q} = \frac{\lambda \cdot [p \cdot (\boldsymbol{T}^2) \cdot 1 - \boldsymbol{T}^T \cdot 1]}{p \cdot \boldsymbol{T}^T \cdot 1 - (\boldsymbol{L}^2)^T \cdot 1}.$$

Values of $\hat{Q} < \chi^2_{\alpha;\lambda}$ with $\alpha < 0.001$ were considered for keeping the null hypothesis $\hat{Q} < \chi^2_{\alpha;\lambda} \rightarrow H_0$ that the true mean difference was zero against some alternative hypothesis. The quantitative analysis was necessary in order to compare directly the IgAtTG ELISA raw data under the different conditions of celiac disease patients or patients treated for celiac disease and healthy control subjects.

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