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Radioimmunological detection of anti-transglutaminase autoantibodies in human saliva: a useful test to monitor coeliac disease follow-up

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SUMMARY

Background
Serum radioimmunoassay (RIA) tissue transglutaminase autoantibodies (tTG-Abs) proved to be a sensitive test also during coeliac disease (CD) follow-up. We demonstrated that RIA tTG-Abs could be detected in human saliva.

Aim
To evaluate salivary RIA tTG-Abs in coeliac children on gluten-free diet (GFD).

Methods
Saliva and serum samples from 109 coeliac children were evaluated at diagnosis (group 1: 71 females, median age 9.4 years) and 58 of them on GFD: 36 after 3–6 months (group 2a), 34 at 9 months or more (group 2b). Two gender- and age-matched control groups: 89 gastroenterological patients (group 3) and 49 healthy subjects (group 4) participated in the study. Saliva and serum tTG-Abs were detected by RIA and compared with serum tTG-Abs ELISA and IgA anti-endomysium antibodies (EMA).

Results
Salivary RIA tTG-Abs were found in 94.5%, 66.7% and 50.0% of groups 1, 2a and 2b CD patients and in 98.2%, 72.2% and 50.0% of corresponding serum samples, respectively. tTG-Abs decreased with GFD progression and a correlation was found between saliva and serum titres ($r = 0.75$, $P = 0.0001$). During the CD follow-up, salivary and serum RIA sensitivities were comparable, and higher with respect to EMA and ELISA.

Conclusions
This study demonstrates that it is possible to detect salivary tTG-Abs with high sensitivity not only at CD diagnosis, but also during GFD.

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INTRODUCTION

Coeliac disease (CD) is an autoimmune enteropathy caused by the ingestion of gluten in genetically susceptible individuals and characterized by villous atrophy and crypts' hyperplasia of small bowel mucosa. CD may appear in a classical presentation, with gastrointestinal complaints and growth failure, or with extra intestinal manifestations and 'atypical forms'[1] characterized by symptoms such as anaemia[2] and short stature[3] or with a silent form. The high prevalence of the disease in the general population, 1:266 worldwide subjects in Europe,[4] largely in an asymptomatic form, and the complications of a long-lasting CD, make the early diagnosis of this disease, possibly in childhood, crucial. The detection of CD-related serum autoantibodies, in particular those directed against tissue transglutaminase (tTG-Abs), gives a valid contribution to the identification of candidates for the intestinal biopsy.[5–8] Nevertheless, the utility of the serum tTG-Abs in monitoring the diet-adherence of coeliac subjects is debated, particularly for adult CD patients.[9, 10]

Serum tTG-Abs detected with a fluid-phase radioimmunoprecipitation method (RIA) proved to be a sensitive test, not only at diagnosis but also during follow-up, with the result that this is the most reliable in detecting the non-adherence to the diet.[11, 12] It is well known that a good compliance to the diet is particularly important in children and in adolescents, permitting at the same time to obtain optimal growth[13, 14] and to avoid complications including autoimmune pathologies.[15, 16] Recently, we demonstrated that RIA tTG-Abs could be detected in human saliva, a body fluid that can easily be obtained by non-invasive techniques and bypassing the unpleasant blood sample collection is particularly helpful in children for CD-screening purposes.[17] The aim of this study was to evaluate the salivary RIA tTG-Abs assay capacity to monitor CD follow-up. For this purpose, we compared results obtained from testing a large cohort of coeliac children (at diagnosis and during the follow-up) and control sera in the following methods: RIA tTG on saliva, RIA tTG on serum, commercial ELISA tTG on serum and indirect immunofluorescence (IF) method anti-endomysium antibodies (EMA) on serum.

MATERIAL AND METHODS

Subjects

Patients with CD and control subjects were enrolled sequentially from November 2001 to June 2006 from the Paediatric Department, CD referral centre of 'Sapienza' University of Rome. Paired saliva and serum samples were collected from 109 non-immunoglobulin A (IgA) deficient coeliac children at disease diagnosis (group 1: 38 males; median age 9.4 years, all receiving a gluten-containing diet); 58 out of 109 of these patients were followed-up once or twice during a gluten-free diet (GFD): 38 from 3 to 6 months (group 2a: 21 females, median age 9.5 years), and 34 on a GFD from 9 months or more (group 2b: 23 females, median age 11.9 years). As control groups, we tested 89 gastroenterological controls who underwent upper endoscopy for failure to thrive, gastro-oesophageal reflux disease, vomiting or abdominal pain (group 3: 57 females, median 10.1 years) and 49 healthy controls (group 4: 21 females, median age 9 years). Groups 1 and 3 patients underwent routine endoscopy and biopsy. For the histological examination, one specimen was taken from the duodenal bulb and four specimens were taken from the second and third duodenal portions. All these samples were subsequently evaluated by a single pathologist experienced in CD scoring according to Marsh modified classification.[18] All group 1 patients showed subtotal/total villous atrophy (type 3b/3c) of the duodenal mucosa, whereas group 3 subjects showed a normal duodenal mucosa. All parents/guardians gave informed consent for the participation of their child in the study. The Ethics Committee at the Paediatric Department, 'Sapienza' University of Rome, approved the study.

Collection and treatment of saliva and serum samples

The investigated subjects were not allowed to eat in the morning of serum and saliva collection, which occurred between 8 and 11 AM. Unstimulated whole saliva samples of all subjects participating in the study were obtained by direct spitting into a sterile plastic tube in a time span not exceeding 10 min, collected in ice and subsequently spun within 2 h at 8 000 *g* for 10 min at 4 °C. The saliva supernatant, used for the tTG antibody detection, was aliquoted and stored at

−80 °C until analysis. Serum samples were aliquoted and stored at −20 °C until analysis.

Saliva tTG-Abs RIA

Salivary tTG-Abs were detected using a previously described method,[17] with some modifications. Briefly, radiolabelled [^{35}S]-methionine tTG was incubated overnight at 4 °C with 150 μL of saliva sample. Twenty-five microlitres of goat anti-human IgA-Agarose (Sigma, St Louis, MO, USA) were subsequently added and the solution was incubated for 3 h at 4 °C in a rotating platform. After exhaustive washings, SDS was added to each tube to resuspend the pellet that was then transferred into a scintillation vial. This last step was repeated once again. Each vial was counted in a β-counter after the adding of scintillation liquid solution (Pakard, Meriden, CT, USA). Salivary autoantibody levels were expressed as an Ab-index calculated as follows: (sample cpm − negative standard sample cpm)/(positive standard control cpm − negative standard control cpm). Saliva samples were considered tTG-Abs positive if the Ab-index was above 0.036. A receiver operator characteristic (ROC) plot was used to identify the optimal threshold value for saliva tTG-Abs presence.

Serum tTG-Abs RIA

The full-length human tTG cDNA (kindly provided by Prof. George Eisenbarth, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Science Center, Denver, CO, USA) was transcribed and translated *in vitro* in the presence of ^{35}S-methionine (PerkinElmer Life Sciences, Boston, MA, USA) using the TNT-coupled rabbit reticulocyte system (Promega, Madison, WI, USA). Presence of tTG-Abs was detected by a previously published RIA method, where an ROC analysis was used to identify the optimal threshold value for sensitivity and specificity.[19] Serum levels of tTG-Abs were calculated and expressed as in the salivary RIA. Serum samples were considered tTG-Abs positive when the Ab-index was above 0.050. An ROC plot was used to identify the optimal threshold value for saliva tTG-Abs presence.

Serum ELISA tTG-Abs

IgA tTG detection was performed by a commercial, sandwich type, enzyme immunoassay (ELISA) (INOVA Diagnostics, San Diego, CA, USA).

EMA method

EMA IgA, tested in sera diluted 1:5, were detected by an indirect IF method using sections from the distal portion of monkey oesophagus as substrate (Menarini, Firenze, Italy).[5]

Statistical analysis

Mann–Whitney nonparametric *U*-test was used to determine differences between groups. A two-tailed $P < 0.05$ was considered significant. Correlation between the results obtained with the serum tTG-Abs RIA and the saliva tTG-Abs RIA was examined with linear regression analysis. Saliva tTG-Abs RIA method sensitivity and specificity were analysed using an ROC plot.[19]

RESULTS

Salivary RIA tTG-Abs

A total of 103 out of 109 group 1 CD subjects had tTG-Abs (94.5%) with a mean Ab-index of 0.373 ± 0.338 (Figure 1). Frequency of tTG-Abs positive subjects in groups 2a and 2b patients was significantly lower compared with group 1 patients ($P < 0.0001$ and $P < 0.0001$, respectively). Salivary mean tTG-Abs titres of group 1 children were significantly higher compared with GFD groups 2a (0.136 ± 0.178) and 2b (0.054 ± 0.12) children ($P < 0.001$ and $P < 0.001$, respectively). Group 1 salivary tTG-Abs titres were significantly higher ($P < 0.001$) in comparison with gastrointestinal (group 3) (mean Ab-index -0.004 ± 0.041) and healthy controls (group 4) (-0.022 ± 0.05). Two out of 89 group 3 patients were found weakly tTG-Abs positive. The specificity of the saliva RIA method was 97.7%. A high correlation ($r = 0.75$, $P < 0.0001$) between salivary and serum tTG-Abs levels was found. Salivary RIA assay detected Abs in a significantly higher number of group 2a ($P < 0.01$) and 2b ($P = 0.01$) GFD patients in comparison with EMA method (Table 1). Figure 2 shows salivary tTGAb-index in nine coeliacs at the diagnosis and at the time of the follow-up.

Serum RIA tTG-Abs

Using previously established ROC plot thresholds, corresponding to the 99th percentile of a control

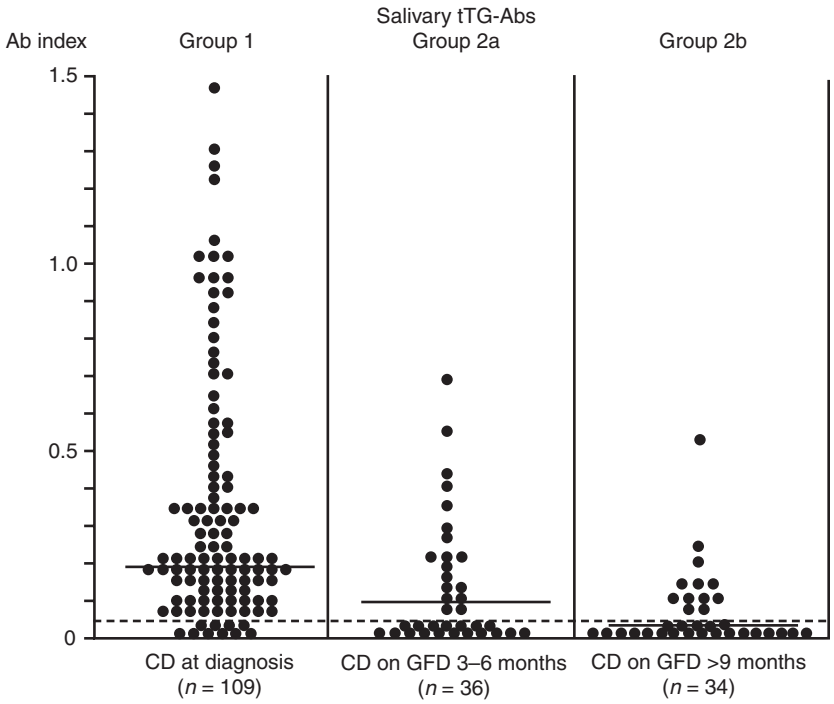

Figure 1. Distribution of salivary transglutaminase (tTG) full-length autoantibody (Abs) levels in 109 coeliac patients at disease diagnosis (group 1) and in 70 coeliac patients after different periods of gluten-free diet (GFD). The *y*-axis indicates the tTG-Abs indexes. The *x*-axis indicates the three groups of patients analysed. The horizontal dashed line represents the limit of positivity of salivary tTG-Abs method. The horizontal line drawn for each of the three groups of coeliac patients analysed represents the median of tTG-Abs values.

Table 1. Percentages of coeliac patients found positive in saliva and serum samples with the different methods

	Group 1 (n = 109) at diagnosis (%)	Group 2a (n = 36) follow-up 3–6 months (%)	Group 2b (n = 34) follow-up ≥9 months (%)
tTG-Abs RIA			
Saliva	103/109 (94.5)	24/36 (66.7)	17/34 (50.0)
Serum	107/109 (98.2)	26/36 (72.2)	17/34 (50.0)
tTG-Abs ELISA serum	106/109 (97.2)	24/36 (66.7)	14/34 (41.0)
EMA serum	101/109 (92.7)	12/36* (33.3)	4/34** (12.0)

* $P < 0.01$ vs. salivary tTG-Abs RIA and serum tTG-Abs RIA and ELISA.
** $P = 0.01$ vs. salivary tTG-Abs RIA, serum tTG-Abs RIA and serum tTG-Abs ELISA.
RIA, radioimmunologic assay; tTG, tissue transglutaminase; Abs, autoantibodies; EMA, anti-endomysium antibodies; ELISA, enzyme-linked immunosorbent assay.

population,[11] 107 out of 109 (98.2%) group 1 CD patients were positive for presence of serum tTG-Abs (Figure 3). During the GFD follow-up, both the percentage of positive subjects and the RIA tTG-Abs mean levels progressively decreased from groups 1 to 2b patients (Table 1). Mean serum tTG-Abs titres at diagnosis (0.783 ± 0.356) were significantly higher with respect to those found during the follow-up (group 2a: 0.269 ± 0.35; group 2b: 0.132 ± 0.2) ($P < 0.0001$ vs. each GFD group). No presence of tTG-Abs was detected in groups 3 and 4 subject sera. Serum tTG-Abs RIA immunoassay detected Abs in a significantly higher number of groups 2a ($P < 0.01$) and 2b ($P = 0.01$) GFD patients in comparison with EMA method (Table 1).

ELISA tTG-Abs in serum

As shown in Table 1, 106 out of 109 (97.2%) group 1 CD patients were positive for presence of ELISA tTG-Abs. During the GFD follow-up, both the percentage

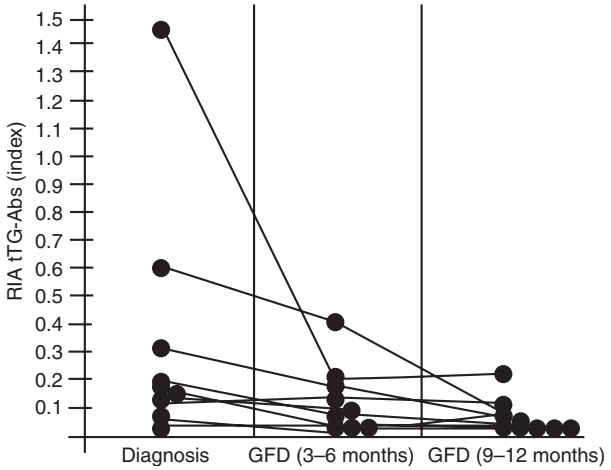

Figure 2. Levels of salivary transglutaminase (tTG) autoantibody (Abs) in nine coeliac children at diagnosis and during the follow-up on a gluten-free diet.

of positive subjects and the ELISA tTG-Abs mean levels progressively decreased from groups 1 to 2b patients (Table 1). Serum ELISA tTG-Abs were detected in a significantly higher number of groups 2a ($P < 0.01$) and 2b ($P = 0.01$) GFD patients in comparison with EMA method (Table 1). No presence of tTG-Abs was found in sera of groups 3 and 4 subjects.

EMA in serum

EMA IgA were detected in 101 out of 109 (92.7%) group 1 patients. During the GFD follow-up, the percentage of positive subjects decreased with time, from groups 1 to 2b patients (Table 1) reaching 12% in the latter group 2b of GFD patients. EMA test proved to be highly specific (100%) in control subjects (group 3) and in healthy controls (group 4).

DISCUSSION

Coeliac disease shows different clinical manifestations from a severe malabsorption syndrome to a silent, asymptomatic picture, this last form being increasingly recognized nowadays. For the serious complications of a long-lasting, nondiagnosed CD such as endocrinopathy,[15] infertility, low birth-weight infants[20] and cancer,[21] the identification of highly reliable assays is crucial, permitting not only the diagnosis, but also the possibility to monitor the compliance of the GFD. In fact, permanent withdrawal of gluten-containing food is the only effective current treatment of CD. In a previous study,[11] analysing the serum of CD patients at diagnosis and during the follow-up on a GFD, we demonstrated that, for detection of CD-related autoimmunity, a fluid-phase RIA method is more sensitive than solid-phase ELISA and IF EMA assays.

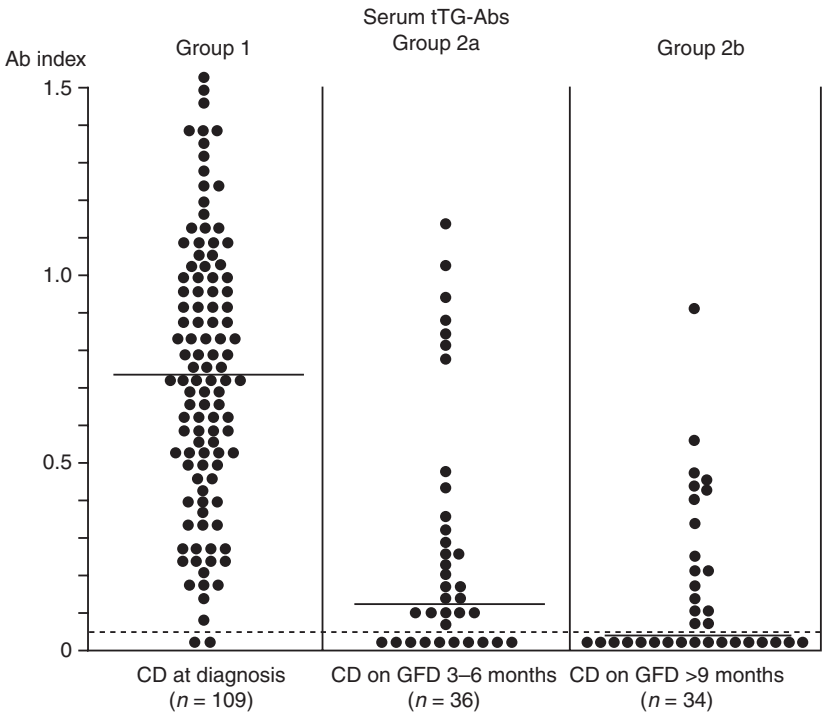

Figure 3. Distribution of serum transglutaminase (tTG) full-length autoantibody (Abs) levels in 109 coeliac patients at disease diagnosis (group 1) and in 70 coeliac patients after different periods of gluten-free diet (groups 2a and 2b). The *y*-axis indicates the tTG-Abs indexes. The *x*-axis indicates the three groups of patients analysed. The horizontal dashed line represents the limit of positivity of serum tTG-Abs method. The horizontal line drawn relative to each of the three groups of coeliac patients analysed represents the median of tTG-Abs values.

Subsequently, using a similar fluid-phase RIA format and in contrast with data obtained with a salivary ELISA immunoassay,[22] we found that it is possible to detect with high sensitivity, the tTG autoantibodies at the CD diagnosis also in human saliva.[17] In this study, we confirm and extend our previous results, demonstrating that it is possible to detect salivary tTG autoantibodies with high sensitivity also in coeliac patients on a GFD, when the autoantibody titres tend to decrease quite dramatically. At disease diagnosis, when the tTG-Abs are usually found at high titres, we found that the performance of the salivary and of the three serum assays was quite similar, whereas during the GFD follow-up when the autoantibody titres tend to decrease progressively, salivary and serum RIA tTG-Abs methods detect higher frequencies of autoantibody positive patients with respect to ELISA and IF assays (EMA). In particular, during the GFD follow-up, salivary RIA assay was able to detect tTG-Abs in almost 50% of patients, in a significantly higher percentage with respect to EMA (12.0%, $P < 0.001$). The higher performances obtained with the RIA both in serum and in saliva fluids are probably because of the use of a sensitive fluid-phase antigen–antibody reaction, which allows a better exposition of conformational tTG antigen reactive epitopes.[23] These epitope domains seem to be better preserved in fluid-phase rather than in solid-phase procedures.[11] In fact, previous findings related to measurement of antibodies present in other autoimmune diseases (e.g. autoimmune diabetes) clearly showed the superiority both in sensitivity and specificity of fluid – with respect to solid-phase assays.[24, 25]

In literature, long-lasting follow-up studies focusing on the decline of serum tTG-Abs on a GFD in CD children, are quite rare. In this study, the elimination of gluten from the diet of CD patients at diagnosis resulted in a significant decrease in the humoural immune response in all the CD patients investigated. The decrease was common to all four methods utilized. However, after 9 months or more of GFD, serum and salivary RIA tTG-Abs were still detectable (at lower levels) in 50% of CD patients. In the same group of patients, ELISA and EMA immunoassays detected 41% and 12% of antibody-positivity, respectively. This finding is not surprising, if we consider the higher sensitivity crucial of the RIA method in detecting tTG immuno-reactivity with respect to ELISA and EMA methods.[26–28] In a previous study, analysing CD patients on a GFD, we found that the serum RIA tTG-Abs assay could detect four times more Ab-positive patients compared with an ELISA immunoassay.[11] The high frequency of salivary and serum RIA tTG-Abs positive patients on a GFD suggests the high sensitivity of RIA method in monitoring the intake of small, probably unrecognized, amounts of gluten. In fact, previous findings demonstrated that EMA are not effective in revealing the assumption of a small quantity of gluten (up of 0.5 g/day or less).[29] Furthermore, it is revealing that there are recent reports showing severe CD-related histological lesions in adult patients with normal levels of ELISA tTG-Abs.[30, 31]

In conclusion, although definite diagnosis of CD still relies on the intestinal biopsy findings, our data confirm that it is possible to monitor, at diagnosis and during the follow-up, the course of CD by salivary human-tTG radiobinding assay and that the detection of tTG-Abs in saliva represents a reliable, non-invasive, easy to perform, powerful tool to follow the correct diet of coeliac subjects, by-passing the unpleasant blood sample collection. There are no technical aspects limiting the applicability of this new method, neither in saliva collection, nor in the assay procedure that employs instruments commonly used in the clinical research laboratory. Presence of salivary tTG-Abs during the coeliac follow-up may reveal a nonstrict adherence to the diet, with possible nutritional[32] and immunological implications.[16]

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