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A proposed reference change value for an IgA anti-tissue transglutaminase immunoassay to improve interpretation of serial results in celiac patients

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ABSTRACT

Background: Celiac disease (CD) is an autoimmune disorder caused by an inappropriate immunological response to gluten ingestion in genetically susceptible individuals. IgA anti-tissue transglutaminase (tTG) antibodies have been widely employed as a specific biochemical marker for CD. Recent studies have also shown its usefulness in evaluating patient compliance with a gluten-free diet.
Methods: A group of 28 subjects with CD was selected for the study. Each fulfilled the requirement of a gluten-free diet for more than one year. IgA anti-tTG determination was performed every two months for half a year. These data were used to estimate the biological variation (BV) of IgA anti-tTG in celiac patients and to calculate the reference change value (RCV).
Results: The within-subject biological variation (CV_I) and between-subject biological variation (CV_G) were 19.2% and 75.6%, respectively, and the index of individuality was 0.25. The RCV calculated using these data together with our analytical imprecision (5.7%) was 55.5% for a 95% level of significance.
Conclusions: We have determined for the first time the BV and the RCV for IgA anti-tTG in a celiac population. This value and the probability curve generated from our data could be a valuable tool for monitoring patients' adherence to dietary treatment.

1. Introduction

Celiac disease (CD) is an autoimmune disorder characterized by an inappropriate immunological response to ingested gluten (from wheat, barley, rye or oats) in genetically susceptible individuals [1]. The disease is typically characterized by malabsorption that results from inflammatory injury of the mucosa of the small bowel. CD is now recognized as a common disorder diagnosed at all ages and affecting 0.5–1.0% of all adults. Complete and lifelong adherence to a gluten-free diet (GFD) is the only accepted treatment that allows one to lead a normal life and helps in the recovery of the intestinal mucosa [2,3].

Since the identification of tissue transglutaminase (tTG) as the major target antigen recognized by anti-endomysial antibodies in 1997 [4], several commercial immunoassays detecting the presence of IgA/IgG anti-tTG antibodies have been developed. The second generation assays, which use highly purified or recombinant human tTG antigens, have been reported to have excellent sensitivities and specificities [5]. Therefore, IgA anti-tTG determination has been widely employed as a biochemical marker of CD in screening and diagnosis [6].

Histological evaluation of duodenal biopsy remains the gold standard for a definitive diagnosis of CD but it is not practical for monitoring treatment efficacy. The quality of life measurements have also been shown to be responsive to treatment, but their sensitivity to patients' compliance with dietary gluten-withdrawal seems to be limited [7]. Recently, the use of serial determinations of certain CD-related antibodies, including IgA anti-tTG, at different points in time was demonstrated to be a valuable tool for monitoring disease activity and patients' compliance with the GFD [8].

Traditionally, a single cut-off is used for the clinical interpretation of IgA anti-tTG results and all values above or below it are given a positive or negative consideration, respectively. However, this IgA anti-tTG interpretation may not be a valid approach when it is employed as a complementary tool for monitoring CD if this test shows a marked individuality

Abbreviations: CD, celiac disease; anti-tTG, anti-tissue transglutaminase; GFD, gluten-free diet; RCV, reference change value; BV, biological variation; CV_A, analytical imprecision; CV_I, within-subject biological variation; CV_G, between-subject biological variation.

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[9]. After implementation of the GFD, most patients experience a significant reduction of IgA anti-tTG values which reach a "negative" concentration one year later and which normally exhibit fluctuations inside this negative range or near the cut-off point [10]. This situation makes it difficult to differentiate between normal physiological variation and diet transgression due to short gluten exposure.

The most widely accepted approach to establish a criterion for dynamic assessment of a biochemical quantity and to define when the difference between two consecutive results indicates a change in the patient's health status is the so-called reference change value (RCV), a concept described in the early eighties [11]. RCV encompasses both biological and analytical variation and defines how large the difference between two consecutive determinations is statistically significant [12].

The aims of this study were to estimate the biological variability of IgA anti-tTG in patients with steady CD and to calculate the RCV for IgA anti-tTG in order to provide an additional tool for monitoring GFD adherence in celiac patients.

2. Material and methods

2.1. Study population

Forty volunteers (10 men and 30 women) from Toledo, Spain, with previously diagnosed CD were initially enrolled to perform serial testing. All of them needed to satisfy two criteria to be included: having a GFD for at least one year and carrying an adequate fulfillment of the GFD supported by clinical and analytical data. The selection process was carried out in collaboration with the Gastroenterology Unit in our hospital. A written consent was signed by every patient enrolled in the study.

All subjects were evaluated analytically four times for six months at a regular interval of two months. These analyses consisted of a biochemical and hematological profile which included IgA anti-tTG test and total IgA determination. After that, ten patients were discarded due to: gluten transgression in their diet, not keeping the planned follow-ups, missing some of the scheduled visits, or showing a continuous decrease in anti-tTG values, reflecting that CD was still in an improvement phase after the GFD establishment. Two patients were also discarded for having all their anti-tTG values under 0.5 U/mL, since change concentrations inside this range were considered to be irrelevant in celiac state. Therefore, data from 28 of the 40 original patients were used for the RCV calculation.

2.2. Anti-tTG antibody assay

The samples were collected in the morning from fasting patients in a sitting position. Serum samples were centrifuged at 3500 *g* for 10 min and processed in the next two hours.

The IgA anti-tissue transglutaminase determination (EliA™ Celikey™ IgA, Thermo Scientific-Phadia, Freiburg, Germany) based on an enzyme fluoroimmunoassay was performed on an automated InmunoCap 250 analyzer (Phadia AB, Uppsala, Sweden). This assay employs human recombinant tTG antigen produced in eukaryotic baculovirus/insect cell systems. Results are expressed in arbitrary units by milliliter (U/mL) based on a six-point standard curve. A pooled serum from patients whose IgA anti-tTG concentration was at the same range as the selected celiac patients (mean = 3.4 U/mL, range 2.8–3.8 U/mL) was processed twenty-five times as internal control to establish our laboratory between-run imprecision. In addition, our laboratory participated in an external quality assessment program from the Institute for Quality Assurance (Euroimmun, Lübeck, Germany), which provided a correct result for CD interpretation according to our IgA anti-tTG results in all the evaluations performed.

Total IgA determination was also carried out in all samples to discard IgA deficiency in any patient. This assay was conducted by an immunonephelometric technique on an Immage 800 (Beckman Coulter, Brea, CA, USA). Patients were considered partial IgA-deficient if their IgA concentration was equal or lower to 82 mg/dL according to our population levels.

2.3. Statistical analysis

All samples from volunteers were collected under standardized conditions in our single institution and hence we assumed minimal pre-analytical variation. IgA anti-tTG results obtained for each individual were analyzed using the Shapiro–Wilk test to check normal distribution. Outliers were considered using Tukey method and data were discarded if results were lower than $Q_1 - 1.5 \cdot (Q_3 - Q_1)$ or greater than $Q_3 + 1.5 \cdot (Q_3 - Q_1)$, where Q_1 is the first quartile and Q_3 corresponds to the third quartile. No outliers were detected in the IgA anti-tTG determinations for each patient, and in addition, no values were found which exceeded ± 3 standard deviations from the grand mean for all patients. Thus, all results were considered for the biological variation (BV) calculation.

The analytical coefficient of variation (CV_A) was calculated from the between-run data of our pooled serum. Of the twenty-five pooled samples processed, three data were excluded as outliers according to the Tukey method.

An analysis of variance (ANOVA) was employed to estimate the components of BV. This procedure provided the total within-subject variance, including both within-subject and analytical variances (S^2_{I+A}), and the total variance, including the three sources of variation: between-subject, within-subject and analytical variances (S^2_{G+I+A}).

In our study, like most of the previous works in the BV field, within-subject and between-subject biological variations were expressed as coefficients of variation termed CV_I and CV_G, respectively. CV_I was computed as $100 \cdot (S^2_{I+A} - S^2_A) / M$ and CV_G was calculated as $100 \cdot (S^2_{G+I+A} - S^2_{I+A}) / M$, where M is the IgA anti-tTG mean value for all data.

The index of individuality (II), which indicates whether significant changes in a patient's health status can be detected by comparing it with population-based reference values ($II > 1.4$), or whether each patient should be evaluated against himself or herself ($II < 0.6$), was also calculated as CV_I / CV_G.

The RCV was calculated using the formula of Harris and Fraser: $RCV = Z \cdot 2^{1/2} \cdot (CV_A^2 + CV_I^2)^{1/2}$, where Z is a constant equal to 1.96 to define a 95% confidence interval for a two-tailed distribution.

The number of samples taken from a patient required to achieve a certain percentage of closeness to the true homeostatic setting point was calculated from the following formula, based on a simple standard error of the mean: $n = [Z \cdot (CV_A^2 + CV_I^2)^{1/2} / D]^2$, where D is the percentage deviation allowed from the mentioned true homeostatic setting point.

The programs SPSS 15.0 and Microsoft Excel were used to perform statistical analyses and create graphs.

3. Results

The mean age of the 28 finally-selected subjects (7 men and 21 women) was 33.8 years (range 15–70 years). No patients presented an IgA deficiency and all had their total IgA concentration inside our population range.

Four participants did not achieve a full fall in their IgA anti-tTG levels since they had some determinations above the cut-off point recommended by the manufacturer (7 U/mL). The obtained IgA anti-tTG values ranged between 0.5 and 8.2 U/mL (Fig. 1A) and the mean for the overall group of subjects was 2.7 U/mL. IgA anti-tTG results displayed a normal distribution for all patients except for three of them according to the Shapiro–Wilk test. Consequently, normality was assumed and no mathematical transformations were applied to data. The graphic representation of the CV(%) against the individual

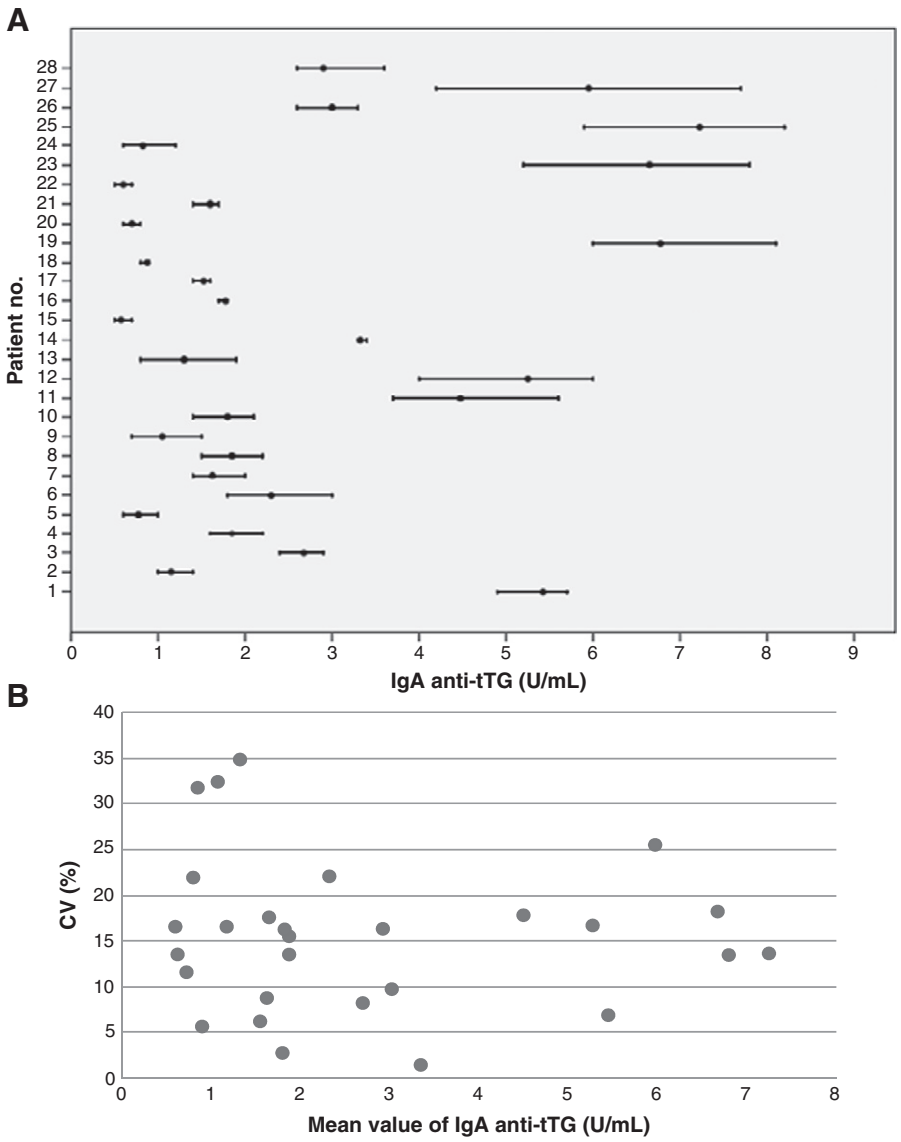


Fig. 1. Mean (dots) and range (bars) of IgA anti-tTG concentrations for the 28 celiac patients regularly monitored every two months for half a year that were selected for the calculation of biological variability (A). Graphic representation of the coefficient of variation (CV, %) estimated for each patient's samples against the corresponding mean value of IgA anti-tTG (B).

IgA anti-tTG mean showed no presence of trend or bias in calculated imprecision in relation to the IgA anti-tTG value (Fig. 1B).

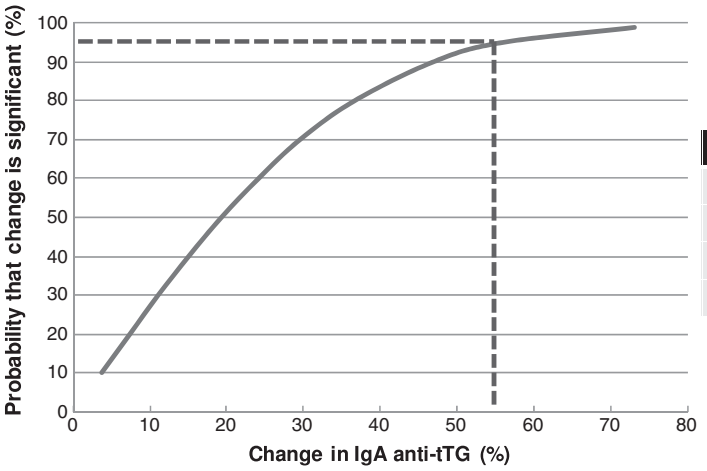
As previously described, no outliers were detected, so all results were included in the ANOVA. The CV_I and CV_G found were 19.2% and 75.6%, respectively. The CV_A estimated employing our pooled serum from patients was 5.7%. Using this data and the corresponding bi-directional value of Z for a 95% level of significance (1.96), the RCV for IgA anti-tTG was 55.5%. The index of individuality was 0.25, which indicated that our IgA anti-tTG immunoassay showed a marked individuality (II < 0.6).

The values of CV_I and CV_A were also employed for assessing the number of specimens required. In this case, obtaining four samples from each subject allowed us to achieve about 20% of closeness to the true homeostatic setting point with a 95% level of confidence. The deviation percentage could be reduced by increasing the number of samples. Therefore, seven samples would be required to obtain an estimation of the homeostatic setting point within 15% of the true value, while sixteen specimens would be needed to decrease this deviation percentage below 10%.

A graphic, which associates the change between two consecutive results to the probability that the change is significant, was generated using a modification of the RCV formula that makes the Z-score (and thus the probability) the unknown: $Z = \text{change} / (2)^{1/2} \cdot (CV_A^2 + CV_I^2)^{1/2}$ (Fig. 2). This curve could be used as a tool to estimate the probability of being significant for any change between two consecutive IgA anti-tTG determinations from the same patient. In addition, this equation was employed to create a table of information which relates the most usual probability thresholds to the percentage of change (Table annexed to Fig. 2).

4. Discussion

Life-long avoidance of gluten exposure is the only accepted treatment in celiac patients and it is essential to minimize the risk of complications associated with CD. Several options have been developed to check the GFD effectiveness and to monitor patient compliance with dietary restrictions, such as evaluation by experienced nutritionists, clinical examination, self-reported compliance, small bowel biopsy and biochemical tests [1]. However, many of them have shown important limitations in their routine applicability or unsatisfactory reliability of their results. Some authors have proposed the specific CD-related antibodies as serum markers for CD activity, although their usefulness for



P	%
80	36.5
90	46.7
95	55.5
99	73.1

Fig. 2. Curve generated from the RCV equation relating the percentage of change between two consecutive results of IgA anti-tTG to the probability that this change is significant. The dashed line marks the percentage variation between two IgA anti-tTG determinations needed to reach a 95% statistical significance level. The table on the right indicates the percentage of change (%) needed for statistical significance at different probability thresholds (*P*).

assessing dietary compliance has been considered controversial [13,14]. This issue is possibly due to significant differences, both in the antibodies employed and in the experimental design, which can be found in most of the published studies.

More recently, it has been proved that serum concentration of IgA anti-tTG is useful in monitoring CD evolution [8] and that its decrease is inversely correlated with the degree of patient compliance with the GFD [10]. In this context, we have determined the biological variability of an IgA anti-tTG immunoassay for the first time and we have also applied these data to calculate the RCV, which could be used to interpret the serial results obtained in the follow-up of celiac patients.

As previously mentioned, a reliable serological testing in celiac adults could be carried out after one year or more from the GFD implementation [7,8], and thus we considered this temporary cut-off point in the selection of our study population. After this time, IgA anti-tTG values remain stable or even decrease in strictly compliant patients, while it is likely to increase in individuals who are partially adherent to the GFD, as was reported in a 4-year follow-up study [10]. Consequently, given that levels of strict compliance with the GFD vary widely and may be as low as 36% [15], there will be a significant proportion of patients in all celiac populations with incomplete negativization of IgA anti-tTG concentrations, or at least near the diagnosis cut-off point, making their monitoring difficult. This issue is in concordance with the IgA anti-tTG range observed in some of the 28 patients selected for our study.

Detecting gluten transgression is crucial to avoid an enhancement of the long-term complication risk and a deterioration of quality of life conditions [2,16]. The lack of compliance with the GFD often displays some non-classical and unspecific celiac symptoms, especially if it is caused by unnoticed small amounts of gluten. Thus, the IgA anti-tTG RCV could be an interesting additional option when these symptoms are reported by patients.

The use of a "compliance" cut-off instead of the classical IgA anti-tTG "screening" cut-off has been previously discussed by Nachmann and collaborators [10]. They calculated a new "compliance" cut-off value from the ROC curves using dietary and clinical data to categorize GFD adherence. Despite the specificity and sensitivity of antibody tests improved employing this cut-off, they considered the use of serial results for follow-up purposes to be more reliable, rather than a dichotomous positive–negative interpretation based on a cut-off value. Precisely, RCV is the best strategy to check significant differences between serial quantitative measurements.

Other immunological assays that are gaining importance in CD assessment are those based on antibodies against deamidated gliadin peptides (anti-DGP), which have a similar performance to IgA anti-tTG [17]. Although there is a current discussion of whether anti-DGP test efficiency and throughput are better or not than anti-tTG ones in celiac adults [18], some authors have observed higher sensitivity at comparable good specificity for anti-DGP in the evaluation and monitoring of CD in children [19,20]. Therefore, we propose anti-DGP assay as a likely suitable candidate for further RCV studies in the follow-up assessment of celiac children.

It is quite important in RCV calculation to establish the clinical decision-making context, i.e. if RCV will be used for the evaluation of a change in any direction or for the assessment of an increase or decrease, since the value of Z-score depends on it [21]. As commented before, we believe that the main application of our results would be to detect a significant rise in IgA anti-tTG levels as a result of gluten exposure. In contrast, we have employed a two-tailed Z-score. This can be explained by our intention that the RCV proposed in this study would also be useful for monitoring an IgA anti-tTG fall after establishing further measures in dietary treatment, for example, in patients suspected of not having a strict adherence to the GFD.

Another problematic issue was the selection of subjects. RCV is normally calculated from healthy people according to the Harris and Fraser's original proposal [22]. Nevertheless, it is obvious that this is not possible for biochemical quantities whose result is zero or below the detection limit of the test in healthy individuals, such as IgA anti-tTG immunoassays. Therefore, there is no other way of estimating the biological variation of this test except with a group of patients with CD. As previously carried out by Katzmann and colleagues in individuals with monoclonal gammopathies [23], we have used serial data from clinically stable celiac adults who had a good fulfillment of the GFD according to the available clinical and analytical data. Furthermore, we performed an additional second process of exclusion of patients with high probability of gluten transgression during the study, in order to minimize the effect caused by other sources of variation in the calculation of CV_I and CV_G.

It is well-established that when a quantity has a strong individuality, the conventional population-based reference intervals may not provide useful information for monitoring health status [9,22]. The index of individuality obtained for our IgA anti-tTG immunoassay was clearly lower than 0.6, indicating that the best approach to detect changes with this test, when used as a monitoring tool, is to evaluate each patient by comparing with his or her previous result using the proposed RCV.

Although RCV is the most appropriate and accepted strategy to evaluate serial results, some disadvantages and limitations have also been discussed [24,25]. Recently, many of them were appropriately answered

by Fraser and colleagues [26]. In accord with them, we have made an effort to be rigorous in several of these critical points, like selection of reference individuals, standardization of pre-analytical conditions, fixed frequency of test determination, rationale decision about the Z-score employed, as well as being cautious about our conclusions. We have also overcome the problem of employing commercial control materials for the estimation of inter-day imprecision due to the lack of interchangeability with serum samples for some quantities [27]. In this study, a pooled serum was used to calculate our analytical imprecision and its concentration was coherent with the range of values observed in celiac patients. It is noteworthy that our CV_A was less than half the CV_I, which indicates that our IgA anti-tTG determination is adequate and it satisfies the desirable analytical quality specifications [28].

It would be possible to extend our results to other laboratories and IgA anti-tTG assays since CV_I is constant over time, geography and methodology [21]. Moreover, CV_I was demonstrated to be similar in health and most chronic stable diseases [29] and hence, our RCV could be used even if other pathologies were present in celiac patients. Note that to put into practice our RCV, the laboratory CV_A for its IgA anti-tTG test must be similar to the one determined in this study. In addition, we recommend the calculation of the RCV in other celiac populations and the employment of different immunoassays to check the validity of our results.

In any case, like other RCV proposals [23,30–33], our intention is not to replace clinical judgment in the follow-up of celiac patients. RCV must be used as a complementary tool that provides additional information about a change in the serial determination of a biochemical quantity. In particular, IgA anti-tTG RCV could be valuable in helping clinicians decide if there has been a transgression of gluten in the diet. It may also provide insight into additional behavior measures to achieve a better compliance of the GFD.

In conclusion, for the first time, our study provides a RCV for IgA anti-tTG test, which is the most commonly used determination in CD monitoring. This value, obtained by employing well-established methods for calculating biological variation, may be helpful for assessing the probability that celiac patients with a GFD fulfillment have ingested unappreciable traces of gluten, which could compromise their long-term quality of life.

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