<0.05 for all comparisons). Peak concentrations of Ntx were found during the second 2400–0800 sampling [1323.3 (134.4) nmol/mmol] and trough concentrations during the 2000–2400 time period [812.1 (98.8) nmol/mmol; P <0.01]. Ntx in both samples collected at 2400–0800 were higher than in the samples collected at 1200–1600, 1600–2000, and 2000–2400 (P <0.05 for all comparisons).

Considering the marked diurnal variations in PICP and ICTP, it may be difficult to interpret the finding of no rhymicity in PINP concentrations. There are no available data in adults or in children for comparison of the finding. Perhaps differences in assay sensitivity and metabolism of the propeptides may play a role, necessitating a larger study population than the present to detect possible variations in PINP. Our observation of no diurnal variation in PIIINP concentrations, however, is in accord with a previous report in children (1).

Using an immunoassay, a study of a group of healthy girls, 10–14 years of age, revealed nocturnal peak concentrations of DPD at 0300 and trough concentrations at 1300 (6). The findings are in accord with observations of adults based on immunoassay (7) and HPLC (8) for assessment of total DPD. Furthermore, our results obtained by an immunoassay that specifically measures the free fraction of DPD are consistent with the findings of Robins et al. (4). One study using HPLC, however, found a statistically

significant circadian variation only in total, not in free, DPD (9), thus supporting suggestions that in adults, diurnal variations in free DPD may be less marked than in total DPD. Whether this may reflect that the ratio between free and total DPD may vary between children and adults is unclear. Furthermore, the present finding of diurnal variations in Ntx corrected for creatinine is in accord with studies in adults (10). However, when Ntx was not corrected for creatinine in adults, a circadian variation could not be detected. To what extent diurnal variations in DPD and Ntx in children may depend on variations in creatinine needs further study.

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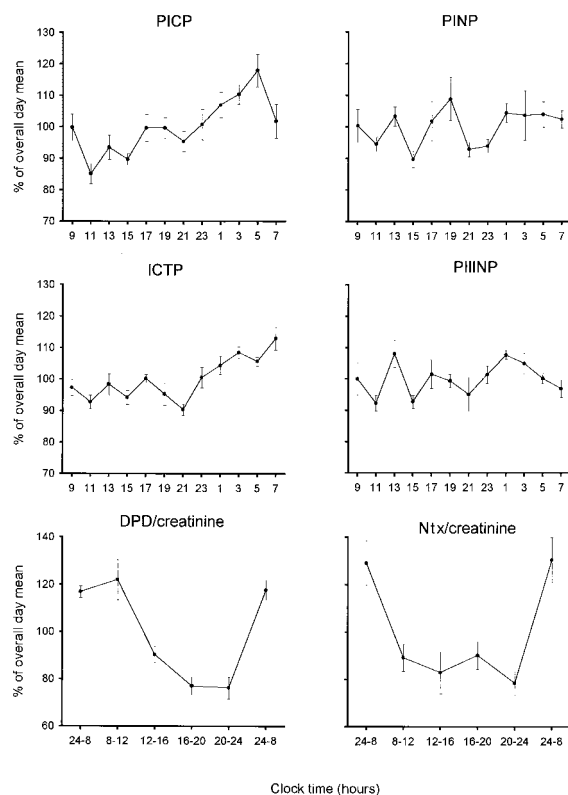
Fig. 1. Mean ± SE 24-h profiles of serum PICP, PINP, ICTP, and PIIINP and 32-h profiles of urine DPD and Ntx (standardized for creatinine) in seven healthy children expressed as percentages of the overall day mean.

Diagnostic Accuracies for Celiac Disease of Four Tissue Transglutaminase Autoantibody Tests Using Human Antigen, *Silvia Martini,[1] Giulio Mengozzi,[2*] Giuseppe Aimo,[2] Roberto Pagni,[2] and Carla Sategna-Guidetti[1]* ([1] Dipartimento di Medicina Interna, Università di Torino, 10126 Torino, Italy; [2] Unità Operativa Autonoma Laboratorio Analisi Chimico-Cliniche, Azienda Ospedaliera San Giovanni Battista, Corso Bramante, 88, 10126 Torino, Italy; * author for correspondence: fax 39-011-676052, e-mail gmengozzi@molinette.piemonte.it)

Celiac disease (CD), a genetic, immunologically mediated small bowel enteropathy that causes malabsorption, is one of the more common disorders in Western countries and is frequently underdiagnosed because of its protean presentations (1). Early diagnosis and treatment with a gluten-free diet may reduce the risk for nutritional (2),

bone *(3)*, and obstetric complications *(4)*, as well as malignancies *(5, 6)* and increased mortality rates *(7, 8)*. Histologic demonstration of a flat small intestinal mucosa, together with clinical improvement when patients are on a gluten-free diet, continues to be the gold standard for its diagnosis. The serologic detection of anti-endomysium antibodies (EmAs) is used to support the diagnosis and to screen populations at risk; the sensitivity and specificity of these tests are 70–95% and 100%, respectively *(9)*.

The identification of tissue transglutaminase (tTG) as the main antigen of EmAs *(10)* allows a new diagnostic approach to CD. A large number of ELISA methods, mainly based on commercially available guinea pig tTG, have been produced; however, these methods have a lower diagnostic accuracy than EmAs *(11–13)*. Assuming that possible interspecies differences could be responsible for such discrepancies, the use of human tTG antigen was recently suggested *(14)*.

The first aim of this study was to clarify the diagnostic accuracy of four commercially available anti-human tTG-IgA ELISA methods in both controls and EmA-positive CD patients. The second aim was to compare anti-tTG results obtained by these different methods and their feasibility in practice.

The study population consisted of 34 untreated, newly diagnosed, and ambulatory EmA-positive CD patients (26 women and 8 men; median age, 36 years; range 23–65 years) seen consecutively at the University Department of Internal Medicine between May and September 2000. The diagnosis was made in all cases by the typical histologic appearance of small intestinal mucosa, which recovered after gluten withdrawal. We also studied 91 controls (51 women and 40 men; median age, 38 years; range 21–75 years), including 34 healthy volunteers without a familial history of CD (healthy controls) and 57 diseased controls, including 30 patients with inflammatory bowel disease (15 with Crohn disease; 15 with ulcerative colitis) and 27 patients with other diseases: 8 with malignancies (1 each with prostate cancer, pleural mesothelioma, hepatocarcinoma, non-Hodgkin lymphoma, Hodgkin disease, and 3 with gastric cancer), 4 with autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, thyroiditis), 7 with chronic liver diseases (6 with postviral cirrhosis and 1 alcoholic cirrhosis), and 8 with heart failure (2 of them with associated severe arterial hypertension). CD was excluded in all either by both clinical history and IgA-EmA negativity or by duodenal biopsy, the latter being performed in patients who underwent upper endoscopy for other problems.

The study was performed according to the principles of the Declaration of Helsinki and oral informed consent was obtained from each participant.

Three to five biopsy specimens were obtained from the aboral part of the duodenum with standard forceps during upper endoscopy. The mucosal pattern was graded according to Marsh's classification *(15)*. Detection of serum antibodies against endomysial structures was performed by immunofluorescence using commercially available slides of monkey esophagus (Alfa Biotech):

positive samples showed the characteristically thin fluorescent honeycomb-like network around the smooth muscle fibers *(16)*. Sera were tested at 1:10 initial dilution with the inclusion of positive and negative controls in every batch of tests. All slides were read blindly by at least two of the authors with a Leitz Orthoplan fluorescence microscope (magnification, ×25 and ×40).

Both qualitative and quantitative assessments were performed without knowledge of the diagnoses, using four different commercially available sandwich-type ELISA packages: (*a*) DRG (DRG Diagnostics; distributed by Pantec S.r.L.), requiring a 1:250 predilution of sera, two 1-h incubations, and three washing cycles before use, after the first incubation and before reading [measuring range, 0–1000 arbitrary units/mL (AU/mL)]; (*b*) EU-tTG® IgA (Eurospital S.p.A.), including ready-for-use microplates and suggesting a 1:26 predilution of sera with two 1-h incubations separated by a three-time washing cycle to be repeated before reading (measuring range, 0–30 AU/mL); (*c*) Immunodiagnostik (Immunodiagnostik; distributed by Li StarFISH s.a.s.), requiring a 1:250 predilution of sera, two 1-h incubations and three steps of five washing cycle before use, after the first incubation, and before reading (measuring range, 0–40 AU/mL); (*d*) Celikey™ (Pharmacia & Upjohn), in which sera are prediluted 1:100, microplates must be washed once before use, and two 30-min incubations with two washing steps are carried out (measuring range, 0–100 AU/mL).

All incubations were performed at room temperature, except for the first step in the DRG and Immunodiagnostik assays, which were carried out at 4 °C, as suggested by the manufacturers. Measurements were done in the same laboratory room and by a single operator; sera were thawed only once before determinations. The same samples were tested in parallel runs during two consecutive days with two ELISA methods per day. Three different batches with the same reagent lots were used for each tested assay over a 2-week period. Intraassay CVs were <5% for all studied reagent sets.

Titers were expressed as AU, calculated according to a calibration curve provided by the manufacturer in the Celikey and DRG assays, and as a percentage of one reference calibrator in EU-tTG and in Immunodiagnostik. For each reagent set, two cutoff thresholds were indicated by the manufacturers (Fig. 1) with borderline values included in a "gray zone" between them.

The one-way ANOVA and unpaired Student *t*-test or the Kruskal–Wallis and Wilcoxon Mann–Whitney *U*-tests were applied according to either gaussian or non-gaussian distribution of data, respectively. The Pearson coefficient was used for correlation studies. ROC-curve analysis was done both to estimate the performance of each assay and to calculate sensitivity and specificity at various cutoff thresholds.

Table 1 shows the overall prevalence of tTG antibodies defined as negative, borderline, or positive according to the manufacturer's indications, as well as the sensitivity, specificity, and accuracy of each assay based on the qualitative analysis.
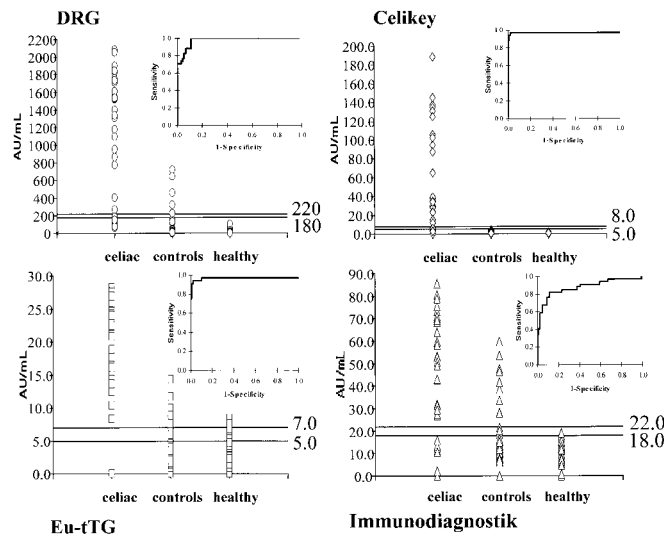
Fig. 1. Distribution of individual IgA anti-tTG concentrations measured with the four tested assays (DRG results were arbitrarily truncated at 2000 AU/mL for clarity).

The study population was subdivided into CD patients, diseased controls, and healthy volunteers. *Bold lines* indicate low and high limits of reference values suggested by the suppliers. ROC curves are shown for each of the studied anti-human tTG ELISA tests; areas under the curve (and their 95% confidence intervals) were 0.978 (0.958–0.998), 0.969 (0.912–1.026), 0.965 (0.908–1.022), and 0.881 (0.801–0.961) for the DRG, Celikey, Eu-tTG, and Immunodiagnostik assays, respectively.

Median (range) anti-tTG concentrations were as follows (in AU/mL): DRG, 1529 (61.0–3713), 11.2 (2.7–720), and 7.1 (0–104) for CD patients, diseased controls, and healthy controls, respectively ($P <0.0001$ for differences among the three groups); Celikey, 35 (0–188), 0.5 (0.1–3.8), and 0.7 (0.2–2.3), respectively ($P <0.0001$); Eu-tTG, 22.4 (0–28.2), 3.2 (0.9–14.3), and 3.9 (0.9–8.4), respectively ($P <0.0001$); Immunodiagnostik, 51 (2.4–86), 13.1 (6.1–60), and 11.6 (0.8–18.9), respectively ($P <0.0001$; Fig. 1). Pairwise comparison between CD and either diseased or healthy controls always yielded a P value <0.0001, whereas the difference between the two control groups was statistically significant only with DRG and Immunodiagnostik assays ($P = 0.01$ and $P = 0.001$, respectively).

The results of the four ELISAs coincided in 68% of all examined participants; in particular, 26 of 34 CD patients were positive and 1 negative with all assays; 35 of 57 controls were negative with all four reagent sets, 4 of whom had a positive titer with all but the Celikey; and 23 of 34 healthy volunteers had negative results with the four ELISAs. Correlations among titers of different methods were >0.9 for each pairwise comparison, as assessed by linear regression analysis. We saw no significant correlation between antibody concentrations and the histologic score of mucosal lesions with any of the examined assays.

We used ROC curves and areas under the curve to compare the diagnostic accuracies of the four assays (Fig. 1). Furthermore, cutoff thresholds providing the best combination of sensitivity and specificity were derived: 58.9 AU/mL for DRG (sensitivity and specificity of 100% and 89%, respectively); 9.9 AU/mL for Eu-tTG (94% and 98%); 2.3 AU/mL for Celikey (97% and 98%); and 22 AU/mL for Immunodiagnostik (82% and 89%).

As a result of the development of several ELISAs to detect IgA anti-tTG, many laboratories replaced EmA testing in their routine CD panel. Notwithstanding, however, a good agreement between tTG and EmAs, with reported anti-tTG sensitivities of 85–95% and specificities of 65–90% for the detection of EmA-positive sera, failed to meet expectations *(11–13)*. One reason is probably related to the use of guinea pig antigen. Since the introduction of recombinant human tTG antigen either in ELISA or radioligand assays *(14, 17, 18)*, there have been few reports concerning both the clinical performance of these methods and their agreement in the detection of IgA anti-tTG as compared with EmAs. To our knowledge, this is the first study in which different commercially available methods using human antigen are examined.

Some ELISAs appear more cumbersome and difficult to implement. Possible advantages of ELISAs over EmAs are mainly the lower costs, better reproducibility, availability of automated assays, and adoption of recombinant tech-

Table 1. Prevalence of IgA anti-tTG autoantibodies detected by tested ELISAs (−, ±, +: number of negative, borderline, and positive values, respectively, based on the qualitative analysis) according to manufacturer indications.[a]

	DRG			Eu-tTG			Celikey			ImmunoDiagnostik		
	−	±	+	−	±	+	−	±	+	−	±	+
Celiac disease, n = 34	8	0	26	1	0	33	5	2	27	6	0	28
Controls												
Healthy, n = 34	34	0	0	24	7	3	34	0	0	31	3	0
Diseased, n = 57	52	0	5	42	5	10	57	0	0	43	4	10
Sensitivity, % (95% CI)												
Borderline values as positive	77 (69–84)			97 (94–100)			85 (79–92)			82 (76–89)		
Borderline values as negative	77 (69–84)			97 (94–100)			79 (72–87)			82 (76–89)		
Specificity, % (95% CI)												
Borderline values as positive	95 (91–99)			73 (65–80)			100 (100–100)			81 (75–88)		
Borderline values as negative	95 (91–99)			86 (80–92)			100 (100–100)			89 (84–95)		

[a] Sensitivity, specificity, and 95% confidence intervals of each assay were calculated assuming borderline values either as positive or as negative.

nologies; on the other hand, there is still a rather wide discrepancy among methods, probably related to differences in the extraction and preparation of the antigen. One CD patient in our study had negative results with all four assays with IgA anti-tTG concentrations even lower than those of controls in two of the tested ELISAs. This might be tentatively accounted for by the occurrence of autoantibodies directed against an antigen different from tTG, yet leading to EmA positivity *(19)*.

Moreover, for each package a gray zone within the reference limits renders borderline values difficult to interpret: they should be considered according to the clinical setting. In this study, we tested only samples drawn from EmA-positive CD patients; performances of anti-tTG ELISAs need further evaluation in a prospective cohort of patients referred to a malabsorption clinic, including those with EmA-negative results and a larger number of controls affected by diverse pathologies. This might avoid the need to use borderline values. At present, we are unable to recommend the complete replacement of EmAs with recombinant human anti-tTG ELISAs for CD screening purposes, although the combination of the two methods can be proposed, especially when faced with borderline values that fall in the gray zone.

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Determination of Malondialdehyde as Dithiobarbituric Acid Adduct in Biological Samples by HPLC with Fluorescence Detection: Comparison with Ultraviolet-Visible Spectrophotometry,

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Living cells are constantly exposed to reactive oxygen species, some of which are capable of initiating lipid peroxidation by abstraction of an allylic proton from a polyunsaturated fatty acid. This process, by multiple stages leading to the formation of lipid hydroperoxides, is a known contributor to the development of atherosclerosis *(1)*.

The thiobarbituric acid (TBA) test is an easy and quick assay for the assessment of lipid peroxidation in which malondialdehyde (MDA) is derivatized. The rationale and methodology have been discussed in detail elsewhere *(2, 3)* and have rightfully been criticized for low specificity and artifact formation because only a fraction of the MDA measured was generated in vivo *(1, 3)*. Furthermore, the TBA derivatization procedure itself leads to the formation of several MDA-unrelated ultraviolet (UV)-absorbing and fluorescent species, the latter as shown in Fig. 1, A–C. Despite this fact, the method remains one of the most useful and commonly used measurements of oxidative damage because of its simplicity. In recent years, several HPLC-based TBA assays have evolved with increased specificity *(4–7)*, but nevertheless the spectrophotometric methods remain commonly used. In the present report, MDA was quantified in plasma, erythrocytes, and liver homogenates from 3-month-old guinea pigs by either our "modern" TBA test based on HPLC with fluorescence detection, which quantifies only the genuine MDA(TBA)$_2$ adduct, or by the original less specific and less sensitive spectrophotometric method, which measures the total absorbance of several species. The purpose was to evaluate the use of the original TBA test by today's standards by assessing the overestimation and sensitivity in the various applications that are routinely performed in several laboratories.

All compounds were of the highest quality available. Specifically, butylated hydroxytoluene (BHT), tetrame-