Jeff Terryberry*, Jani Tuomi, Subo Perampalam, Russ Peloquin, Eric Brouwer, Detlef Schuppan[a] and Stefano Guandalini[a]

Diagnostic accuracy of a fully automated multiplex celiac disease antibody panel for serum and plasma

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Abstract

Background: An automated multiplex platform using capillary blood can promote greater throughput and more comprehensive studies in celiac disease (CD). Diagnostic accuracy should be improved using likelihood ratios for the post-test probability of ruling-in disease.
Methods: The Ig_plex™ Celiac Disease Panel on the sqidlite™ automated platform measured IgA and IgG antibodies to tTG and DGP in n = 224 CD serum or plasma samples. Diagnostic accuracy metrics were applied to the combined multiplex test results for several CD populations and compared to conventional single antibody ELISA tests.
Results: With multiple positive antibody results, the post-test probability for ruling-in untreated and treated CD increased to over 90%. The number of samples positive for more than one antibody also increased in untreated CD to ≥90%. Measurement of all four CD antibodies generate cut-off dependent accuracy profiles that can monitor response to treatment with the gluten-free diet (GFD). Higher positive tTG and DGP antibodies are seen more frequently in confirmed CD without (81%–94%) than with GFD treatment (44%–64%). In CD lacking biopsy confirmation, overall agreement of plasma to serum was ≥98% for all antibodies, and 100% for venous to capillary plasma.
Conclusions: The Ig_plex Celiac Disease Panel increases the likelihood of confirming CD based on the post-test probability of disease results for multi-reactive markers. Specific positivity profiles and cut-off intervals can be used to monitor GFD treatment and likely disease progression. Using serum, venous and capillary plasma yield comparable and accurate results.

Keywords: celiac disease; diagnostic accuracy; gluten; likelihood ratio; multiplexing.

Introduction

Celiac disease (CD), wheat allergy (WA) and non-celiac wheat (gluten) sensitivity (NCWS, NCGS) represent different wheat-induced inflammatory disorders, for practical reasons here termed gluten-related disorders (GRD). GRDs, with an estimated global prevalence of up to 5%, have received increasing attention as a clinically relevant spectrum of diseases in recent evaluations of nomenclature and classification [1–4]. Epidemiological studies estimate a worldwide prevalence of CD of approximately 1%, with an additional 1% of CD cases undiagnosed, indicating a need for improvements in screening for CD in the general population [5, 6]. In addition, the need to rule out CD before labeling a patient with NCGS is necessary prior to initiating a gluten-free diet (GFD). Further, a portion of patients labeled with NCGS may not be reacting to gluten, but to fermentable oligo-, di-, mono-saccharides and polyols or wheat amylase trypsin inhibitors, showing only a placebo/nocebo effect. It is thus evident that before administering a GFD, accurate diagnostic criteria and markers are needed to characterize people with CD or other GRD as this leads to different nutritional and treatment strategies [2, 7–11].

Gluten-containing cereal prolamins, such as gliadin in wheat, secalin in rye and hordein in barley, have a high number of proteolysis-resistant sequences, resulting in the persistence of larger peptides in the gut. These peptides

[a]Detlef Schuppan and Stefano Guandalini contributed equally to the manuscript.

***Corresponding author: Jeff Terryberry,** SQI Diagnostics Systems Inc., 36 Meteor Dr. Toronto, ON M9W 1A4, Canada, Phone: +416-674-9500, E-mail: jterryberry@sqidiagnostics.com
Jani Tuomi: Microdrop LLC., Houston, TX, USA
Subo Perampalam, Russ Peloquin and Eric Brouwer: SQI Diagnostics Systems Inc., Toronto, ON, Canada
Detlef Schuppan: Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA; and Institute of Translational Immunology, University Medical Center, Johannes-Gutenberg University, Mainz, Germany
Stefano Guandalini: Department of Pediatrics, Section of Gastroenterology, Hepatology and Nutrition, University of Chicago Celiac Disease Center-Comer Children's Hospital, Chicago, IL, USA

can then trigger an innate immune response or activate small-bowel mucosal CD4+ T cells in patients who carry the necessary genetic predisposition for CD, the antigen presenting molecules HLA-DQ2 and/or -DQ8. Intestinal tissue transglutaminase (tTG), a ubiquitous enzyme that is released from intestinal cells during mechanical or cellular stress, has been identified as the autoantigen of CD, against which the patients' IgA and IgG antibodies, previously described as endomysial autoantibodies, are directed. Importantly, tTG crosslinks and/or deamidates the partly digested gluten peptides in the intestinal lamina propria and increases their immunogenic potential [12, 13]. The importance of antibody detection to the diagnosis of CD is well recognized. Currently, tTG ELISA tests are used as assays to diagnose CD, and tTG-IgA is widely recommended as the most reliable screening test. However, the clinical performance of sensitivity and specificity may vary depending on the quality of the tTG antigen and the antibody isotype detected [11, 14, 15].

Selective deamidation of gliadins by tTG also increases circulating antibody levels to gliadin peptides in celiac patients. Such serum deamidated gliadin peptide (DGP) antibodies have been proven to be additional indicators of CD. Testing for DGP IgA and IgG antibodies, along with the IgA and IgG tTG antibodies, is recommended in children ≤2 years old, and in patients with serum IgA levels <0.2 g/L where tTG IgA and DGP IgA are likely to yield false-negative results [15–17].

Diagnostic accuracy studies using likelihood ratios (LR), post-test probability of disease and positive predictive value (PPV) have received increased focus as a means to supplement the clinical interpretation of multiple diagnostic test strategies. Multiplexing the known celiac antigen immunoassays increases the diagnostic accuracy of CD detection with multiple LR probability estimates. However, previous studies have only looked at various combinations of two or three CD-related antibodies and reported on associated PPV or LR values [18–23].

The current study comprehensively integrates, for the first time, the diagnostic accuracy values of LR and other metrics for all four CD-related antibodies simultaneously and demonstrates the increased post-test probability of having celiac based on multiple validated Ig_plex test results. Further, this study explores the Ig_plex test's ability to monitor response to treatment and serologically differentiate CD with and without biopsy-confirmation. In addition, this study compares multiple sample matrices to support the use of plasma, including finger-stick capillary-derived plasma, to increase the accessibility of patient sampling. Previously, finger-stick capillary blood samples have only been used in various point-of-care assays. Capillary blood-derived plasma has now been expanded for use in the automated Ig_plex Celiac Disease Panel multiplexing assay to detect and monitor CD [24–27].

Materials and methods

Subject population

Samples from a total of 224 well-characterized subjects (Table 1) were evaluated according to published criteria and Standards for Reporting of Diagnostic Accuracy Studies guidelines. Subject demographics, gastrointestinal symptoms, diagnostic criteria, treatment duration and laboratory testing methods were recorded [1, 9, 28–30]. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans with subject consent obtained for use of samples.

Subject samples were obtained from academic collaborators and commercial sources with defined eligibility criteria. Clinically defined sera of patients with biopsy-confirmed (Marsh III) active or treated CD were collected from the Department of Pediatric Gastroenterology and Nutrition at the Massachusetts General Hospital (MGH) for Children in Boston and the Celiac Center at Beth Israel

Table 1: Classification and demographics of n = 224 subject samples evaluated for diagnostic accuracy.

Group	Subject classification		Sample matrix/pairing	Number of samples	Gender		Age group		Criteria/definition [1, 6–9, 28]
					M	F	Pediatric	Adult	
1	Confirmed CD with	GFD (≥1 month)	Serum	52	11	41	7	45	Marsh III
2	Marsh III biopsy criteria	Not yet GFD treated	Serum	31	6	25	5	26	Marsh III
3	CD without confirmation by Marsh III biopsy		Serum	20	8	12	5	15	Classical
4	(includes potential and classical CD)		Plasma	40	13	27	3	37	Classical (n = 37) potential (n = 3)
5	Wheat allergy		Plasma	20	14	6	12	8	Oral food challenge and skin prick
6	Normal		Plasma	61	39	22	6	55	Healthy donor

CD, celiac disease; GFD, gluten-free diet.

Deaconess Medical Center (BIDMC), Boston, MA. Samples were collected between 2011 and 2014, aliquoted and frozen at −80 °C. Fresh freezer aliquots experiencing no more than two freeze/thaw cycles were used in the study.

The Oslo definition of classical CD describes the presence of a gluten-induced enteropathy presenting with diarrhea, malnutrition or a malabsorption syndrome (indicated by weight loss, steatorrhea and edema secondary to hypoalbuminemia). The term potential CD is used for individuals with normal small intestinal mucosa who are at increased risk of developing CD as indicated by positive CD serology [1, 8].

In this study, serum and plasma (EDTA) samples from CD patients that did not meet the Marsh III histology criteria for biopsy-confirmed CD, included mostly classical and several potential CD [1, 28]. The uniformly-defined classical CD samples and three cases of potential CD were labeled as CD without biopsy confirmation and collected from commercial serum banking enrollment programs including Access Biologics (Vista, CA, USA), SeraCare (Milford, MA, USA), in.vent Diagnostica (Berlin, Germany) and Cleveland Clinic (CC, Cleveland, OH, USA) and stored under the same conditions as the confirmed CD samples. In addition, prospective sample collections of CD samples were initiated (May–Oct 2018) to obtain additional (n = 49) paired serum-plasma samples and paired venous-capillary plasma samples (Table 2) from BioIVT (Westbury, NY, USA) and DxBiosamples (San Diego, CA, USA). Capillary samples were obtained by finger-prick using the Microvette 100 EDTA plasma collection tube (Thermo Fisher Scientific Inc., Portage, MI, USA). WA subjects were obtained from AbBaltis (Sittingbourne, UK) and BioIVT. WA subjects were defined as individuals who had both positive results at wheat allergen extract testing either by serum specific IgE or skin prick tests and had shown clinical reaction at the food challenge test [6, 7]. Individual normal human plasma samples were collected from BioIVT.

Testing methods

Automated Ig_plex™ Celiac Disease Panel: The Ig_plex Celiac Disease Panel (SQI Diagnostics, Toronto, ON, Canada) quantitatively measures the presence of both IgA and IgG autoantibodies to tTG and DGP antigens for a single sample in a multiplex assay format on the fully automated sqidlite platform, and offers the ability to add additional antigens or allergens to the printed microarray to increase the differential diagnostic potential of the panel. Dual-layer multiplexing at the capture and detector level facilitates both antigen screening and autoantibody isotyping. Antigen microarrays were non-contact printed by piezoelectric dispensing on covalent-binding epoxy-silane derivatized glass 96-well plates. Recombinant tTG (baculovirus/Sf9 insect cell expressed) and DGP peptides representing immunodominant epitopes from α- and γ-gliadins linked to a protein carrier are printed in multiple replicate spots to ensure precise within-well measurements. The plate is loaded with standard, controls and samples with a primary incubation of 75 min at 500 rpm shaker speed. Antibody binding is detected after washing (BioTek, Winooski, VT, USA) the plate by secondary anti-IgA and anti-IgG antibodies labeled with fluorescent dyes which are incubated for another 45 min, washed and scanned (Sensovation AG, Radolfzell, Germany).

Sqidlite™ automated microarray system: The Ig_plex Celiac Disease Panel was run on the sqidlite automated microarray. The sqidlite system is a multiplex immunoassay platform that automates the assay process for diluting and running samples, standards, controls, assay plate loading, incubating, washing and drying for each individual sample. Once the assay's immunochemical reactions have completed, the sqidlite system performs a three-color fluorescent scan of each well in the microarray, analyzes the data and generates a report containing results for all assay markers. The sqidlite system also includes internal quality checks in the data analysis.

Manual ELISA kits: For method comparison studies, FDA-approved ELISA kits were used for detecting IgA and IgG antibodies to tTG and DGP. ELISA testing conducted at SQI Diagnostics employed the following assays from Inova Diagnostics, Inc. (San Diego, CA, USA): Quanta Lite tTG IgG Cat# 708755 Lot# 042287; Quanta Lite tTG IgA Cat# 708760 Lot# 043755; Quanta Lite DGP IgG Cat# 704520 Lot# 042636; Quanta Lite DGP IgA Cat# 704525 Lot# 043751. The assays were performed as specified in the kit's package inserts.

Analytical precision

Analytical precision was measured using % coefficient of variation (%CV) with acceptable levels of variation being ≤15% CV. Precision acceptance criteria were based on FDA and WHO guidelines for immunoassays and bioanalytical methods [31, 32]. Intra-assay (within-run) precision was determined with two positive control (PC)

Table 2: Classification and demographics of n = 49 subject samples evaluated for matrix equivalence studies.

Group	Subject classification	Sample matrix/pairing	Number of samples	Gender		Age group		Criteria/definition [1, 6–9, 28]
				M	F	Pediatric	Adult	
7	CD without confirmation by Marsh III biopsy	Serum/plasma matched samples	34	8	26	1	33	Classical
8		Venous/capillary plasma matched samples	13 (subset of group 7)	4	9	1	12	Classical
9	Wheat allergy	Serum/plasma matched samples	5	5	0	4	1	Oral food challenge and skin prick
10	Normal	Serum/venous plasma/ capillary plasma matched samples	10	6	4	1	9	Healthy donor

CD, celiac disease.

subject samples per analyte run in replicates of 16 on each of five plates. The cumulative total of the 16 replicates across the five plates (n = 80 repeat measurements) was used to determine the inter-assay (between-run) precision. Inter-assay precision included variables for days (n = 3), operators (n = 2), lots of reagents (n = 2), sqidlite platforms (n = 3) and assay sites (n = 2).

Diagnostic accuracy

The cut-off value indicates the criterion interval that predicts a positive condition. Screening cut-off values were determined in the pooled CD population by receiver operator curve (ROC) analysis to maximize sensitivity, specificity, Youden index and positive LR+. Interval cut-off values were calculated at maximum specificity. Method comparison studies for the Ig_plex Celiac Disease Panel reported % overall agreement. Indeterminate values (IND) within ± 2 standard deviations of the cut-off were removed from the analyses of n = 49 matrix equivalence samples and n = 70 positive and negative samples tested in both the ELISA and Ig_plex assays. Matrix equivalence studies reported % overall agreement and correlation coefficient.

The following definitions and computations of Bayesian diagnostic parameters were used to compare the different CD populations in the microarray based on standard 2 × 2 concordance table analysis with A = true positives, B = false positives, C = false negatives and D = true negatives:

- TPR is the true positive rate or sensitivity = A/(A + C).
- TNR is the true negative rate or specificity = D/(B + D).
- PPV is the positive predictive value or precision = A/(A + B).
- Accuracy (overall agreement) is the proportion correctly classified = (A + D)/(A + B + C + D).
- Youden index is the sensitivity + specificity − 1.
- LR+ is the positive likelihood ratio, or the ratio of TPR (sensitivity) to FPR (1 − specificity). (A/(A + C))/(B/(B + D)).
- Post-test probability (p) is the adjusted PPV based on the known prevalence of CD (pretest probability = 1%).

Calculations and ROC analyses were conducted using MedCalc (version 16.4.3) and NCSS12 (version 12.0.10).

Ig_plex Celiac Disease Panel multiple test likelihood ratios

To investigate the clinical utility of multiplexing, combined specificity measures and multiple LR+ calculations were determined based on combinations of single test clinical performance indices. Computing post-test odds after a series of diagnostic tests is more informative than using the single test sensitivity/specificity method. When using multiple tests, the probabilities are converted to odds ratios (OR) then back to probabilities: odds = probability/(1 − probability). For multiplexed tests, post-test odds = pretest odds × LR1 × LR2 × LR3… × LRn for discreet positive tests. Post-test odds are then converted back to a probability by the following: probability = odds/(1 + odds) [33].

Results

Analytical performance of the Ig_plex Celiac Disease Panel

The analytical precision results of the Ig_plex Celiac Disease Panel are reported in Table 3. Intra-assay precision of each of the five plates was ≤15% CV for all PC plasma samples and antibodies tested. Inter-assay precision across all five plates was ≤15% CV for each PC and antibody.

Five plates were run over 3 days by two operators, on three sqidlite platforms across two testing sites with PC

Table 3: Ig_plex Celiac Disease Panel precision performance.

Sample	Plate	tTG IgA intra-assay (n = 16)		tTG IgG intra-assay (n = 16)		DGP IgA intra-assay (n = 16)		DGP IgG intra-assay (n = 16)	
		Avg U/mL	%CV	Avg U/mL	%CV	Avg U/mL	%CV	Avg U/mL	%CV
PC1	1	109.1	6.4	53.5	7.4	80.0	10.8	95.1	9.7
	2	110.1	8.9	67.0	8.2	80.1	7.3	102.4	8.1
	3	126.0	13.5	57.1	7.0	82.1	10.9	113.5	6.9
	4	100.9	4.8	58.9	9.8	79.0	8.0	98.4	6.7
	5	125.8	9.7	66.2	9.2	79.4	9.6	96.7	14.8
Inter-assay (n = 80)		114.4	12.8	60.6	12.0	80.1	9.3	101.2	11.4
PC2	1	54.0	10.2	24.9	6.0	34.1	8.3	29.2	10.1
	2	56.6	6.4	30.3	10.6	37.2	8.4	31.7	9.0
	3	56.4	10.9	29.3	5.8	38.3	11.8	36.3	11.9
	4	52.9	6.6	28.7	7.6	36.2	8.0	30.8	8.5
	5	59.2	8.5	29.5	8.0	36.3	11.2	27.7	9.1
Inter-assay (n = 80)		55.8	9.4	28.5	10.3	36.4	10.3	31.1	13.6

CV, coefficient of variation; DGP, deamidated gliadin peptide; PC, positive control; tTG, tissue transglutaminase.

assayed in replicates of 16 on each plate for intra-assay precision %CV. A total of 80 results across the five plates were obtained for inter-assay precision %CV.

Ig_plex Celiac Disease Panel cut-off determination

To establish the screening cut-off value for the assay, clinically defined CD samples (n = 143) were compared to the normal samples (n = 61). The cut-off values that maximized sensitivity, specificity, Youden index and positive LR were selected (Table 4). The screening cut-off was then used for method comparison and matrix equivalence accuracy studies. In addition, 20 WA samples were tested as a related disease control group and compared to the CD population. None of the WA samples demonstrated antibodies to tTG or DGP IgG and IgA isotypes.

Method comparison to ELISA

Agreement (accuracy) of the Ig_plex Celiac Disease Panel to FDA-approved ELISA kits was conducted by standard 2×2 concordance table analysis and reported in Table 5. The method comparison study for the Ig_plex Celiac Disease Panel used the screening cut-offs to evaluate n = 70 CD subjects.

Matrix equivalence

To extend the sample matrix use of the Ig_plex Celiac Disease Panel, matrix equivalence studies were sequentially performed using matched normal and CD samples to measure agreement between 1) serum and venous plasma and 2) venous and capillary plasma.

Serum to plasma equivalence

To validate the use of plasma as the sample matrix, equivalence testing was conducted on matched serum and plasma samples drawn consecutively from 34 CD subjects without biopsy confirmation, five WA subjects and 10 healthy donors (Table 2). Agreement analysis of observed results between both matrices is summarized in Table 6.

Venous to capillary plasma equivalence

To verify that capillary EDTA plasma was also a reliable matrix for the Ig_plex Celiac Disease Panel, paired venous and finger-prick blood (Microvette 100 EDTA) draws (n = 23, Table 2) were commissioned at commercial enrollment sites. Agreement (n = 21 + 2 IND) and correlation analyses were then conducted on the pooled results (Table 7, Figure 1).

Table 4: Clinical performance features for selecting screening cut-off values for CD serum and plasma samples.

Antibody	Cutoff (U/mL)	TPR (Sens.)	±95% CI	TNR (Spec.)	±95% CI	Youden Index	Max LR+
tTG IgA	≥5.6	0.96	0.91–0.98	0.98	0.91–0.99	0.94	58.4
tTG IgG	≥6.4	0.89	0.83–0.94	0.98	0.91–0.99	0.87	54.2
DGP IgA	≥14.7	0.82	0.70–0.91	0.93	0.84–0.98	0.75	12.5
DGP IgG	≥10.5	0.83	0.72–0.92	0.90	0.79–0.97	0.73	8.4

CI, confidence interval; DGP, deamidated gliadin peptide; LR, likelihood ratio; TNR, true negative rate; TPR, true positive rate; tTG, tissue transglutaminase.

Table 5: Agreement of Ig_plex Celiac Disease Panel multiplexed results to single ELISA results.

Agreement	tTG IgA	tTG IgG	DGP IgA	DGP IgG
Positive agreement	98% (46/47)	100% (29/29)	97% (37/38)	88% (32/36)
Negative agreement	100% (23/23)	82% (32/39)	87% (27/31)	91% (29/32)
Overall agreement	99% (69/70)	90% (61/68)	93% (64/69)	90% (61/68)

DGP, deamidated gliadin peptide; tTG, tissue transglutaminase.

Table 6: Serum to plasma agreement analysis results for Ig_Plex Celiac Disease Panel.

Agreement	tTG IgG	DGP IgG	tTG IgA	DGP IgA
Positive agreement	100% (8/8)	100% (12/12)	91% (10/11)	100% (10/10)
Negative agreement	100% (41/41)	97% (36/37)	100% (38/38)	100% (39/39)
Overall agreement	100% (49/49)	98% (48/49)	98% (48/49)	100% (49/49)

DGP, deamidated gliadin peptide; tTG, tissue transglutaminase.

Table 7: Venous to capillary (finger prick) plasma agreement analysis results for Ig_Plex Celiac Disease Panel.

Agreement	tTG IgG	DGP IgG	tTG IgA	DGP IgA
Positive agreement	100% (2/2)	100% (5/5)	100% (3/3)	100% (3/3)
Negative agreement	100% (19/19)	100% (16/16)	100% (18/18)	100% (18/18)
Overall agreement	100% (21/21)	100% (21/21)	100% (21/21)	100% (21/21)

DGP, deamidated gliadin peptide; tTG, tissue transglutaminase.

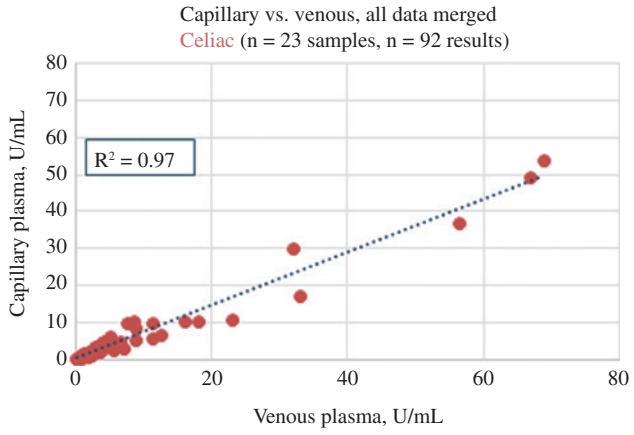

Figure 1: Combined normal and celiac capillary vs. venous plasma sample correlation, R^2.

Multiple likelihood ratios of the Ig_plex Celiac Disease Panel

Multiple LR+ calculations can aid in ruling-in the diagnosis of CD when screening suspected cases or the general population [18, 20]. Increased clinical utility of the Ig_plex Celiac Disease Panel's multiplexing capabilities is further exemplified using the multiple LR+ approach [33]. To calculate the multiple LR+ from several positive test results, the pretest OR is first calculated with CD prevalence = 0.01. Using the LR+ values obtained from the screening cut-off, LR+ combinations in multi-reactive samples yielded a much higher post-test probability of having CD, especially if one of the positive tests were for tTG (IgA or IgG). Samples positive for both DGP IgA and IgG antibodies also increase the probability of disease in all test combinations, even in the absence of tTG positivity (Table 8).

Diagnostic accuracy of the Ig_plex Celiac Disease Panel

Diagnostic accuracy evaluation was performed on subjects stratified as Marsh III biopsy-confirmed CD with and without GFD treatment and CD without Marsh III biopsy confirmation that includes potential and classical CD.

Table 8: Multiple test positivity increases post-test probability of CeD.

Antibody	LR+	Post-test probability for CeD, %								
		One test positive	Two tests positive				Three tests positive			Four tests positive
			tTG IgA tTG IgG	tTG IgA DGP IgA	tTG IgG DGP IgG	DGP IgA DGP IgG	tTG IgA DGP IgA DGP IgG	tTG IgA tTG IgG DGP IgG	tTG IgA tTG IgG DGP IgA	tTG IgG DGP IgG DGP IgA	
tTG IgA	58.4	37.0	96.9	88.0			98.4	99.6	99.7		100.0
tTG IgG	54.2	35.0	96.9		82.0			99.6	99.7	98.3	100.0
DGP IgA	12.5	11.0		88.0		51.2	98.4		99.7	98.3	100.0
DGP IgG	8.4	9.0			82.0	51.2	98.4	99.6		98.3	100.0

CD, celiac disease; DGP, deamidated gliadin peptide; LR, likelihood ratio; tTG, tissue transglutaminase.

Both serum and plasma matrices were available for the CD population without biopsy confirmation to further validate the use of plasma as a sample type.

Multiplexing and response to GFD treatment

Rank correlation analyses were conducted to examine if tTG or DGP antibody positivity in terms of measured U/mL were correlated to GFD treatment duration. Treatment time was available for n = 40 of the confirmed CD with GFD group and ranged from 1 to 184 weeks (median = 20 weeks). No significant Spearman's rank correlation coefficient was found for any antibody in terms of GFD treatment time within this group. Thus, high and low positive values were equally distributed over the entire treatment time.

Diagnostic accuracy results for the treated and untreated biopsy-confirmed CD populations are presented in Table 9. At the screening cut-off value, tTG IgA antibody detection in confirmed CD without GFD intervention sensitivity and specificity were 0.97 (95% CI = 0.83–0.99) and 0.98 (0.91–0.99), respectively. GFD treatment as a whole, did not reduce the sensitivity and specificity of tTG IgA detection. For tTG IgG antibodies, the sensitivity and specificity for confirmed CD without GFD treatment were 0.97 (0.83–0.99) and 0.98 (0.91–0.99), respectively. In this subpopulation, almost all cases (30/31) had both tTG IgA and IgG. At the screening cut-off, GFD treatment had little impact on the sensitivity or diagnostic accuracy.

DGP IgA clinical performance in Marsh III biopsy-confirmed CD without GFD treatment, gave a sensitivity of 0.97 (0.83–0.99) and specificity of 0.89 (0.83–0.94) at the screening cut-off. A decrease to 0.90 (0.80–0.96) sensitivity was observed in response to GFD treatment. At the screening cut-off, DGP IgG detection in biopsy-confirmed CD without GFD had a high sensitivity of 0.97 (0.83–0.99) and specificity of 0.98 (0.91–0.99). In response to GFD treatment, this sensitivity decreased significantly approaching a $p \leq 0.05$ level (two-sided t-test $p = 0.07$) to 0.75 (0.63–0.86).

Positive test intervals and response to GFD treatment

Based on the drop-in sensitivity for DGP IgG in response to GFD treatment, we investigated whether increasing the test interval to stratify high positives and low positives [23] would better partition GFD treatment response. ROC analyses of biopsy-confirmed CD indicated that if the screening cut-off interval reported in Table 4 was increased to the threshold interval reported in Table 10, a significant drop in sensitivity was found in response to GFD treatment for two of the analytes. This is interpreted to mean that at higher test interval, fewer high positives are seen in the confirmed CD with the GFD treatment group than in the confirmed CD without GFD treatment. For tTG IgA the decrease was 15.9%, for tTG IgG, 45.7%, for DGP IgA, the decrease was 23.5% and for DGP IgG, 35.8%. Significance was calculated with a two-sided t-test assuming equal variance in the two CD populations.

Diagnostic accuracy in CD serum and plasma

To further evaluate the use of plasma matrix to report CD results, both serum and plasma from subjects without Marsh III biopsy confirmation inclusive of potential or

Table 9: Diagnostic accuracy metrics for the biopsy-confirmed CD populations.

Antibody	Biopsy	GFD	TPR (Sens.)	TNR (Spec.)	Accuracy	PPV	NPV	LR+
tTG IgA	+	+	0.97	0.98	0.98	0.97	0.98	59.0
	+	−	0.98	0.98	0.98	0.98	0.98	59.8
tTG IgG	+	+	0.97	0.98	0.98	0.97	0.98	59.0
	+	−	0.90	0.98	0.95	0.98	0.92	55.1
DGP IgA	+	+	0.97	0.89	0.91	0.81	0.98	8.4
	+	−	0.90	0.89	0.89	0.87	0.92	7.9
DGP IgG	+	+	0.97	0.98	0.98	0.97	0.98	59.0
	+	−	0.75	0.98	0.87	0.97	0.81	44.6

DGP, deamidated gliadin peptide; GFD, gluten-free diet; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; TNR, true negative rate; TPR, true positive rate; tTG, tissue transglutaminase.

Table 10: GFD treatment response as a function of test interval.

Antibody	Confirmed CeD	Cut-off interval	Sens. TPR	95% CI		Significance, p
				Lower	Upper	
tTG IgA	No GFD	≥34.9	0.94	0.79	0.99	0.190
	+GFD	≥34.7	0.79	0.65	0.89	
tTG IgG	No GFD	≥29.6	0.81	0.63	0.93	0.002[a]
	+GFD	≥30.3	0.44	0.30	0.59	
DGP IgA	No GFD	≥32.8	0.81	0.63	0.93	0.097
	+GFD	≥32.0	0.62	0.47	0.75	
DGP IgG	No GFD	≥29.2	0.81	0.63	0.93	0.012[a]
	+GFD	≥29.1	0.52	0.38	0.66	

[a]Significant $p \leq 0.05$. CD, celiac disease; CI, confidence interval; DGP, deamidated gliadin peptide; GFD, gluten-free diet; TPR, true positive rate; tTG, tissue transglutaminase.

Table 11: Diagnostic accuracy metrics for CD without biopsy confirmation serum and plasma samples.

Antibody	Matrix	TPR (Sens.)	TNR (Spec.)	Accuracy	PPV	NPV	LR+
tTG IgA	Serum	1.00	0.98	0.99	0.95	1.00	61.0
	Plasma	0.90	0.98	0.95	0.97	0.94	54.9
tTG IgG	Serum	0.90	0.98	0.96	0.95	0.97	54.9
	Plasma	0.88	0.98	0.94	0.97	0.92	53.4
DGP IgA	Serum	0.95	0.92	0.93	0.79	0.98	11.6
	Plasma	0.90	0.87	0.88	0.82	0.93	6.9
DGP IgG	Serum	0.75	0.97	0.91	0.88	0.92	22.9
	Plasma	0.83	0.95	0.90	0.92	0.89	16.8

DGP, deamidated gliadin peptide; LR, likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; TNR, true negative rate; TPR, true positive rate; tTG, tissue transglutaminase.

classical CD were collected [1, 28]. Both serum and plasma samples from these subjects showed similar sensitivity, specificity and accuracy for all analytes (Table 11). Modest, non-significant decreases in plasma DGP IgA sensitivity and accuracy were seen compared to serum.

CD sample multi-reactivity and disease progression

At the screening cut-off, samples were found to decrease in the frequency of multiple positive test results when comparing Marsh III biopsy-confirmed CD to the CD population without Marsh III biopsy confirmation. Interestingly, the number of samples positive for all four antibodies decreased from 93.5% in biopsy-confirmed CD to 55.0% in sera from CD without confirmation and to 47.5% in CD without confirmation plasma. For biopsy-confirmed CD without GFD, 28/31 (90.3%) of samples had all four antibodies positive, 2/31 (6.5%) samples demonstrated three positive results and one subject two positive results. In the cases of CD without confirmation serum, multi-reactivity was reduced in frequency to: 11/20 (55.0%) for four positives; 6/20 (30.0%) for three positives; 2/20 (10.0%) for two positives; 1/20 (5.0%) for one positive. In CD without confirmation plasma, 19/40 (47.5%) were positive for all four antibodies; 7/40 (17.5%) for three positives; 9/40 (22.5%) for two positives; 4/40 (10.0%) for one positive and one sample no positive result.

Discussion

The precise Ig_plex Celiac Disease Panel was used to evaluate clinical performance metrics when testing all four relevant antibody CD markers in a multiplexed panel: tTG IgA, tTG IgG, DGP IgA and DGP IgG. The sensitivity, specificity, diagnostic accuracy, LR and post-test probabilities of disease were compared in CD populations with and without Marsh III biopsy confirmation and the clinical utility of using venous and capillary plasma assessed.

Sensitivity and specificity of serum tTG IgA is typically high (e.g. 94.3% and 95.7%) with a PPV of 95.7%. The Ig_plex Celiac Disease Panel demonstrated equivalent or better performance for tTG IgA as that reported for standard ELISA in the literature [15, 19, 34, 35]. In the group without biopsy confirmation, high sensitivity and specificity was preserved indicating that tTG IgA is an accurate screening marker in both serum (0.99) and plasma (0.96). Ig_plex detection of tTG IgG also showed high sensitivity (0.97) and specificity (0.98) in confirmed CD with a diagnostic accuracy of 0.98. For screening tTG IgG in plasma, the accuracy was 0.93. These values are comparable to other high sensitivity (up to 0.95) and specificity (up to 1.0) tTG IgG kits reported in the literature which typically use recombinant tTG [36].

Compared to confirmed CD without GFD treatment, both plasma DGP IgA and IgG demonstrated modest decreases in sensitivity and accuracy in CD without confirmation. However, when DGP positive results were combined with other test results like tTG IgA, the combined tests increased the post-test probability of ruling-in CD in plasma beyond that seen for tTG IgA alone. Further, 10% of CD without biopsy confirmation samples did not have measurable tTG IgA or IgG but did have DGP IgA or IgG antibodies in five of the six observed cases. This demonstrates the power of the Ig_plex Celiac Disease Panel multiplex test, that when multiple LR+ values are determined including those for DGP in the absence of tTG positivity, there is a greater probability of screening for and ruling-in CD. When three to four tests are positive, there is over 95% probability the subject will have CD. A detailed examination of multiple LR+ probabilities and the percentage of multi-reactive test results for the four positive test combinations promote the diagnosis of CD in a screening population and allows for more marker-oriented monitoring and management of patients.

Similar to the results reported here, Vermeersch et al. [20, 23] found that LR+ for CD markedly increased with double positivity and increasing antibody levels of IgA anti-tTG and IgG anti-DGP. Subjects with double positivity and high antibody levels had a high probability for having CD. The detected fraction of CD subjects with double positivity and high antibody levels was reported as 59–67%. Our results extend this observation to all

four autoantibody markers where a distinct pattern of multi-reactivity was observed in the different CD populations. In CD samples without confirmation, 47–67% of subject samples had all four antibodies positive. In the current study, antibody multi-reactivity increased in the confirmed CD group when compared to the CD without confirmation group.

Leffler et al. suggested that if more than one celiac serologic test is positive, it strengthens the argument that the subject has a true, if mild, form of CD when examining potential CD subjects [37]. Dahlbom et al. concluded that detection of IgA/IgG tTG has the potential of detecting most untreated CD patients, including those with IgA deficiency, which supports the use of multiple LR+ combinations for more than one positive test to rule-in CD [21]. Although total IgA was not herein tested, the use of the four Ig_plex antibodies reduces the occurrence of false negatives as shown by the multiple LR+ values.

Raising the cut-off for a positive result to the threshold interval above the screening cut-off significantly reduced the sensitivity of tTG IgG in the confirmed CD group with GFD treatment by 46% compared to just a 16% reduction in confirmed active CD without GFD. Thus, the degree of positivity or relative affinity for tTG IgG antibodies were reduced in response to GFD treatment in the subject samples evaluated here. When the DGP IgA cut-off was raised, sensitivity dropped 23.5% in the GFD treatment group. For DGP IgG, the sensitivity dropped significantly by 35.8% at almost 3-fold elevated cut-off when comparing confirmed active CD without GFD to confirmed CD with GFD. This threshold analysis is important in the determination of how positive test levels change with respect to treatment. Within the GFD treatment group there is no correlation of treatment time and antibody positive level. However, there is a significant relation of treatment to degree of antibody positivity when comparing to the confirmed CD without any GFD treatment group. While likely, prospective studies need to demonstrate whether tTG IgG and DGP IgG alone or in combination with the other multiplex analytes are sensitive indicators of compliance to GFD treatment.

In one study, DGP IgA (sensitivity 87.4, specificity 97.2) and the combination of DGP IgA + IgG (sensitivity 94.7, specificity 89.6) were able to monitor compliance to GFD in CD children [38]. It was concluded that both anti-DGPs showed higher sensitivity than tTG IgA in monitoring compliance with GFD but did not out-perform tTG IgA for CD screening. These findings agree in part with those reported here, with the Ig_plex Celiac Disease Panel tTG IgG test result providing additional benefit. A published guideline recommended that for monitoring adherence to GFD, serological testing of IgA tTG or IgA (or IgG) DGP antibodies should be conceived [10].

In addition, confirmed CD without GFD had high sensitivity for both DGP IgA and IgG (0.97 each). At the optimal screening cut-off point, DGP IgG sensitivity decreased to 0.75 in the CD group lacking Marsh III biopsy confirmation. Given the differential changes in antibody positivity and multi-reactivity detected by the Ig_plex Celiac Disease Panel in different CD populations, this multiplex assay could prove useful in patient management such as monitoring disease prevalence and progression in view of the iceberg paradigm of CD. Correlation of our multi-reactive antibody profiles to symptoms and biopsy results, which has been performed for tTG IgA only, awaits further study [14, 20, 39–41].

Altogether, the diagnostic efficiency of the Ig_plex Celiac Disease Panel was demonstrated in four ways: (1) plasma was as clinically accurate as serum for use as a sample matrix; (2) multi-reactive test results obtained from the Ig_plex Celiac Disease Panel increase the probability of ruling in CD when screening the general population; (3) multiplexing the known CD antibodies might facilitate effective GFD treatment monitoring and (4) multiplexing all four tTG and DGP antibodies might aid the ability to monitor the progression of CD.

As a control group, those with WA had no celiac antibody reactivity. Although this multiplex platform will not support or rule out a diagnosis of NCGS/NCWS, a condition for which reliable biomarkers for diagnosis are lacking [42], when Wheat IgE testing along with CD testing results are negative (thus also making WA less likely, as WA is most often IgE-mediated), the health care provider may want to discuss the possibility of NCGS/NCWS with the patient. Adding other known wheat extract/gluten allergens like fermentable oligo-, di-, mono-saccharides and polyols or wheat amylase trypsin inhibitors or other cereal prolamins to the microarray might potentially facilitate its use in screening for WA subtypes, NCGS/NCWS and other intolerances to grains [8, 42–47].

Furthermore, this study demonstrates that venous plasma is a diagnostically accurate sample matrix and that the use of finger-prick capillary plasma is also viable for evaluating potential CD antibodies based on agreement and correlation to venous plasma. In a screening study of 198 children using capillary blood plasma, 13.6% tested positive for tTG IgA, tTG IgG or gliadin IgA antibodies in a rapid immunochromatographic test [26]. In another study, 31 of 41 children who screened positive for tTG IgA or IgG using finger-prick capillary plasma, were confirmed positive on a serum-based endomysial antibody test [27]. If screening undiagnosed or self-diagnosed GRDs is

facilitated by using finger-prick collection methods, with multiplexed quantitative results obtained in a clinical laboratory, then a physician-guided use of GFD treatment can be performed with improved treatment and disease monitoring [3, 10, 11, 14–17, 48]. Additional prospective case finding and follow up studies in large populations using capillary plasma for our Ig_plex Celiac Disease Panel are underway.

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