



(51) International Patent Classification:

A61K 38/16 (2006.01) C07K 16/16 (2006.01)
C07K 14/415 (2006.01) G01N 33/53 (2006.01)

(21) International Application Number:

PCT/US2017/019292

(22) International Filing Date:

24 February 2017 (24.02.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/299,609 25 February 2016 (25.02.2016) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

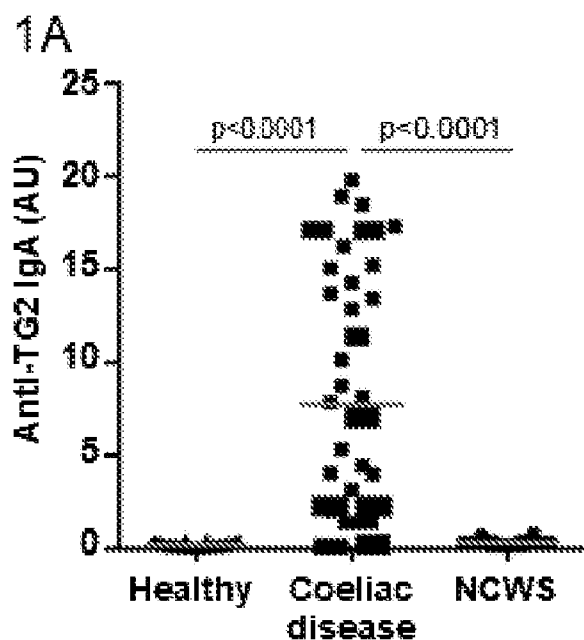
Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

(54) Title: SYSTEMIC IMMUNE ACTIVATION AND BIOMARKERS OF NONCELIAC WHEAT/GLUTEN SENSITIVITY



(57) Abstract: The present invention provides a method of using increased levels of one or more biomarkers to identify subjects having non-celiac gluten sensitivity or non-celiac wheat sensitivity. The identification would aid the physician or health professional to determine a specific treatment for the patient, for example, a diet that eliminates wheat, rye, and/or barley. In one embodiment, the biomarkers are one or more of soluble CD 14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and intestinal fatty acid -binding protein (FABP2). The present invention also provides a method of using the same markers to monitor the response to treatment for non-celiac wheat sensitivity in a subject.

**SYSTEMIC IMMUNE ACTIVATION AND BIOMARKERS OF NON-CELIAC
WHEAT/GLUTEN SENSITIVITY**

[0001] This application claims the benefit of U.S. Serial No. 62/299,609, filed February 25,
5 2016, the entire content and disclosure of which are incorporated by reference into this
application.

[0002] Throughout this application, various references or publications are cited. Disclosures of
these references or publications in their entireties are hereby incorporated by reference into this
10 application in order to more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

[0003] This invention relates in general to the mechanisms behind the onset of non-celiac gluten
sensitivity (NCGS) or non-celiac wheat sensitivity (NCWS) and relevant biomarkers therefor.

BACKGROUND OF THE INVENTION

[0004] Celiac disease is a common autoimmune disorder with genetic, environmental, and
immunologic components. It is characterized by an immune response to ingested wheat gluten
and related proteins of rye and barley that leads to inflammation, villous atrophy, and crypt
20 hyperplasia in the small intestine. Among the most common manifestations of celiac disease
are abdominal pain, diarrhea, weight loss, bone disease, and anemia. The disease is strongly
associated with genes for the specific class II human leukocyte antigens (HLA) DQ2 and DQ8
that are involved in presenting specific immunogenic peptides of gluten proteins to CD4⁺ T
cells in the small intestine. Transglutaminase 2 (TG2) appears to be an important player in the
25 disease, both as a deamidating enzyme that can enhance the immunostimulatory effect of
gluten, and as a target autoantigen in the ensuing immune response. The major B cell
responses in patients with celiac disease target native and deamidated gluten sequences, as well
as the TG2 autoantigen. Among these, the IgA anti-TG2 antibody is currently considered the
most sensitive and specific serologic marker, whereas antibodies to native gluten proteins have
30 low specificity for celiac disease and have been reported to be elevated in a number of other
conditions.

[0005] Some individuals experience a range of symptoms in response to ingestion of wheat and related cereals, yet lack the characteristic serologic, histologic, or genetic markers of celiac disease. The terms non-celiac gluten sensitivity (NCGS) or non-celiac wheat sensitivity (NCWS) are generally used to refer to this condition, which is currently understood as the collection of non-specific symptoms in response to ingestion of gluten-containing cereals, and the resolution of such symptoms upon removal of those foods from diet in individuals in whom celiac disease and IgE-mediated wheat allergy have been ruled out. The condition is associated with gastrointestinal symptoms, most commonly including bloating, abdominal pain, and diarrhea, as well as certain extra-intestinal symptoms, among which fatigue, headache, anxiety, and cognitive difficulties feature prominently. Accurate figures for the prevalence of NCWS are not available, although estimates that put the number at similar to or greater than for celiac disease (1%) are often cited. Despite the commonly used terminology for the condition, the identity of the component(s) of wheat and/or related cereals responsible for triggering the associated symptoms remains uncertain. While recent controlled trials have indicated a prominent role for gluten, non-gluten proteins and fermentable short chain carbohydrates have also been suggested by some studies to drive aberrant immune responses or to be associated with symptoms.

[0006] The potential mechanisms behind the onset of symptoms in NCWS remain unknown and no biomarkers have been identified. However, a small number of studies point to increased antibody reactivity to gluten proteins, moderately raised intraepithelial lymphocyte numbers, increased intraepithelial and lamina propria eosinophil infiltration, and enhanced expression of intestinal tight junction protein claudin 4, Toll-like receptor 2 (TLR2), or IFN- γ , suggesting intercellular junction and/or immune abnormalities in subsets of affected individuals. Human intestinal epithelial surfaces are colonized by large communities of microorganisms and are in constant contact with an abundance of highly immunogenic microbial products. Compromised intestinal epithelial integrity has been linked to extensive systemic innate and adaptive immune responses that are a consequence of microbial translocation from the lumen into circulation. Systemic immune activation in response to microbial translocation is a noted component of HIV infection and inflammatory bowel disease.

[0007] Therefore, there is a need to determine the mechanisms behind the onset of symptoms in NCWS and relevant biomarkers for accurate diagnosis of NCWS.

SUMMARY OF THE INVENTION

5 [0008] The present invention arises from studies that examined whether sensitivity to wheat in the absence of celiac disease is associated with systemic immune activation in response to translocated microbial products that may be linked to an enteropathy. The studies led to identification of a panel of biomarkers that would be useful for accurate diagnosis of NCWS.

10 [0009] Study participants included individuals who reported intestinal and/or extra-intestinal symptoms in response to the ingestion of wheat and in whom celiac disease and wheat allergy were ruled out (n=80), patients with biopsy-confirmed celiac disease (n=40), and healthy controls (n=40). A subset of individuals with sensitivity to wheat underwent six months of a self-monitored diet that excluded wheat, rye, and barley (n=20). Blood serum samples were analyzed for markers of intestinal cell damage and systemic immune response to microbial
15 products.

[0010] In comparison to healthy controls and celiac disease patients, individuals with wheat sensitivity had significantly increased serum levels of soluble CD14 (sCD14) and lipopolysaccharide (LPS)-binding protein (LBP), as well as antibody reactivity to bacterial LPS and flagellin and to wheat gluten. Circulating levels of intestinal fatty acid-binding
20 protein (FABP2), a marker of intestinal epithelial cell damage, were significantly elevated in the affected individuals and correlated with the innate and adaptive immune responses to bacterial products. There was a significant change towards normalization of the levels of FABP2 and immune activation markers following the restrictive diet. These data reveal a state of enhanced systemic immune response to microbial products in conjunction with a
25 compromised intestinal epithelium affecting a subset of individuals who experience sensitivity to wheat in the absence of celiac disease.

[0011] The results demonstrate the presence of objective markers of systemic immune activation and epithelial cell damage in individuals who report sensitivity to wheat in the absence of celiac disease. The principal component analysis (PCA) of the entire dataset demonstrated that the
30 identified panel of biomarkers was capable of clustering the healthy control, celiac disease, and NCWS subjects into three distinctly discernible groups.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A-F show markers of celiac disease and immune reactivity to wheat gluten. Serum levels of IgA antibody to TG2 (Fig. 1A), IgG antibody to deamidated gliadin (Fig. 1B), IgA antibody to deamidated gliadin (Fig. 1C), IgG antibody to native gliadin (Fig. 1D), IgA antibody to native gliadin (Fig. 1E), and IgM antibody to native gliadin (Fig. 1F) in cohorts of healthy controls, celiac disease patients, and individuals identified as having NCWS. Horizontal red lines indicate the median for each cohort.

[0013] Figures 2A-F show markers of systemic immune response to microbial components. Serum levels of LBP (Fig. 2A), sCD14 (Fig. 2B), EndoCAb IgG (Fig. 2C), EndoCAb IgM (Fig. 2D), IgG antibody to flagellin (Fig. 2EA), and IgM antibody to flagellin (Fig. 2F) in cohorts of healthy controls, celiac disease patients, and individuals with NCWS. Horizontal red lines indicate the median for each cohort.

[0014] Figures 3A-C show intestinal epithelial cell damage and correlation with systemic immune activation. Fig. 3A shows serum levels of FABP2 in cohorts of healthy controls, celiac disease patients, and individuals identified as having NCWS. Correlation of serum levels of FABP2 with LBP (Fig. 3B) and sCD14 (Fig. 3C) in individuals with NCWS. Horizontal red lines indicate the median for each cohort.

[0015] Figure 4 shows PCA score plot for the complete dataset of serologic markers (anti-TG2 IgA; anti-deamidated gliadin IgG and IgA; anti-gliadin IgG, IgA, and IgM; LBP; sCD14; EndoCAb IgG, IgA, and IgM; anti-flagellin IgG, IgA, and IgM; and FABP2) measured in healthy controls, celiac disease patients, and individuals with NCWS. Subjects are plotted in two dimensions using the first and second principal components (PC1 and PC2).

[0016] Figures 5A-E show symptoms and anti-gliadin antibody reactivity in response to restrictive diet. Figures 5A-B show composite scores for intestinal symptoms (bloating, abdominal pain, diarrhea, epigastric pain, and nausea) and extra-intestinal symptoms (fatigue, headache, anxiety, memory and/or cognitive disturbances, and numbness in arms and/or legs) before and after 6 months of a diet free of wheat, rye, and barley in a cohort of 20 NCWS patients. Figures 5C-E show levels of IgG, IgA, and IgM antibody to gliadin proteins before and 6 months after starting the diet in the NCWS cohort. Each individual is represented by a dot and the two points corresponding to the same individual are connected by a line. Each box indicates

the 25th-75th percentiles of distribution, with the horizontal line inside the box representing the median.

[0017] **Figures 6A-F** show markers of intestinal epithelial cell damage and systemic immune activation in response to the restrictive diet. Levels of LBP, sCD14, EndoCAb IgM, anti-flagellin IgG, anti-flagellin IgM, and FABP2 before and 6 months of a diet free of wheat, rye, and barley in the cohort of 20 NCWS patients are shown. Each individual is represented by a dot and the two points corresponding to the same individual are connected by a line. Each box indicates the 25th-75th percentiles of distribution, with the horizontal line inside the box representing the median.

[0018] **Figure 7** shows correlation between serum levels of LBP and sCD14 in individuals with NCWS.

[0019] **Figures 8A-B** show IgA responses to LPS and flagellin. Serum levels of EndoCAb IgA (Fig. 8A) and IgA antibody to flagellin (Fig. 8B) in cohorts of healthy controls, celiac disease patients, and individuals identified as having NCWS. Horizontal red lines indicate the median for each cohort.

[0020] **Figure 9** shows correlation between serum levels of EndoCAb IgM and anti-flagellin IgM in NCWS individuals.

[0021] **Figures 10A-B** show correlation between serum levels of FABP2 and (Fig. 10A) EndoCAb IgM and (Fig. 10B) anti-flagellin IgM in individuals with NCWS.

[0022] **Figure 11** shows correlation between serum levels of FABP2 and IgA antibody to TG2 in patients with celiac disease.

DETAILED DESCRIPTION OF THE INVENTION

[0023] In one embodiment, the present invention provides a method of identifying a subject having non-celiac wheat sensitivity, comprising the step of determining blood levels of one or more markers in said subject, said markers are soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and/or intestinal fatty acid-binding protein (FABP2), wherein increased levels of a panel of said markers in the subject as compared to control levels indicate that said subject has non-celiac wheat sensitivity. In one embodiment, the levels of said markers are determined in serum or plasma. In one embodiment, the control levels are derived from healthy subjects or

subjects having celiac disease. In one embodiment, the anti-lipopolysaccharide antibodies are IgM antibodies. In one embodiment, the anti-flagellin antibodies are IgM antibodies and/or IgG antibodies. In one embodiment, the anti-gliadin antibodies are IgG, IgA, and/or IgM antibodies.

[0024] In one embodiment, the above method would involve using a statistical algorithm (e.g. those based on a principal component discriminant statistical analysis or a similar method) of the above panel of biomarkers to predict whether said subject has non-celiac wheat sensitivity.

[0025] In one embodiment, the above method further comprises treating the identified subject with a gluten-free diet that eliminates wheat, rye, and/or barley, or any another treatment that is developed in the future. In one embodiment, the subject is treated with gluten-free diet for six months or more. In another embodiment, the subject is treated with gluten-free diet for less than six months.

[0026] Wheat is a complex substance, and one who has immune reactions or sensitivity to wheat may be reactive to one or more components of wheat. Currently, removal of the immunological trigger (wheat) is the basis of treatment once a NCWS patient is properly diagnosed. Alternative methods of treatment are being developed for wheat/gluten sensitivity. For example, approaches can be developed to target uptake of toxic wheat/gluten peptides by enhancing their enzymatic breakdown, by sequestering gluten proteins, or by restoring epithelial barrier function. One can also envision treating wheat/gluten sensitivity by hindering the activation of gluten-specific immune cells. Identification of gluten epitopes implicated in the immune response to gluten would open the possibility of devising methods for tolerizing some individuals with NCWS. Modulation of the immune system might also be possible in the future via, for example, anti-cytokine therapy.

[0027] In one embodiment, the present invention provides a method of providing a therapeutic intervention for a subject having NCWS, comprising first of identifying the subject with NCWS by determining blood levels of one or more markers in said subject, said markers are soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and/or intestinal fatty acid-binding protein (FABP2), wherein increased levels of a panel of said markers in the subject as compared to control levels indicate that said subject has non-celiac wheat sensitivity. In one embodiment, the blood levels of said markers are determined in serum or plasma. In one embodiment, the control levels are derived from healthy subjects or subjects having celiac disease. In one embodiment,

the anti-lipopolysaccharide antibodies are IgM antibodies. In one embodiment, the anti-flagellin antibodies are IgM antibodies and/or IgG antibodies. In one embodiment, the anti-gliadin antibodies are IgG, IgA, and/or IgM antibodies. The identified subject is then treated with a gluten-free diet, e.g. a diet that eliminates wheat, rye, and/or barley, or any another treatment that is devised in the future.

[0028] In another embodiment, the present invention provides a method of monitoring response to a treatment for non-celiac wheat sensitivity in a subject, comprising the step of determining blood levels of one or more markers in said subject before and after a treatment, said markers are anti-gliadin antibodies, soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, and/or intestinal fatty acid-binding protein (FABP2), wherein decreased levels of said markers in said subject after treatment indicate that the subject has responded to the treatment; otherwise the subject has not responded to the treatment. In one embodiment, the treatment comprises a diet free of one or more of wheat, rye and barley. In another embodiment, the treatment comprises any or all of currently available treatments or those developed in the future. In one embodiment, the anti-lipopolysaccharide antibodies are IgM antibodies. In one embodiment, the anti-flagellin antibodies are IgM antibodies and/or IgG antibodies. In one embodiment, the anti-gliadin antibodies are IgG, IgA, and/or IgM antibodies.

[0029] In another embodiment, the present invention provides a method of determining a treatment course for non-celiac wheat sensitivity in a subject, comprising the step of determining blood levels of one or more markers in said subject before and after a treatment, said markers are anti-gliadin antibodies (IgG, IgA, and/or IgM), soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide IgM antibodies, anti-flagellin antibodies (IgG and/or IgM), and/or intestinal fatty acid-binding protein (FABP2), wherein decreased levels of said markers in said subject after treatment indicate that the subject has responded to the treatment and should continue to be treated with the treatment. Alternatively, if the levels of the markers do not decrease after treatment, it indicates that the subject has not responded to the treatment and should be treated with an alternative therapy. In one embodiment, the treatment comprises a diet free of one or more of wheat, rye and barley. In another embodiment, the treatment comprises any or all of currently available treatments or those developed in the future.

[0030] In another embodiment, the present invention provides a multi-analyte assay kit that aids in diagnosis of non-celiac wheat sensitivity in a subject. In one embodiment, the kit is an immunoassay kit comprising reagents that detect the presence of a panel of the markers disclosed herein. This assay can be in the form of ELISA, microarray, bead-based, or any other immunologic method that detects antibody-antigen interaction. In one embodiment, the kit includes isolated antibodies (e.g. monoclonal antibodies) specifically immunoreactive with a panel of the markers disclosed herein, and means for detecting the binding of the markers to the antibodies. In one embodiment, the antibodies are attached to a solid support, such as polymeric beads, dip sticks, 96-well plate or filter material. The detecting means of the kit may include labeled secondary antibodies. The solid surface reagent in the above kit can be prepared by any known techniques for attaching protein material to solid support material. For example, these attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Optionally, the kit may further comprise a standard or control information so that the test sample can be compared with the control information standard to determine if the test amount of a marker detected in a sample is a diagnostic amount consistent with a diagnosis of non-celiac wheat sensitivity.

[0031] In one embodiment, the present invention provides a kit that aids in diagnosis of non-celiac wheat sensitivity in a subject, the kit comprises reagents that detect the presence of one or more markers in a sample from said subject, said markers are soluble CD14, lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and/or intestinal fatty acid-binding protein (FABP2). In one embodiment, the sample is a blood, serum or plasma sample. In one embodiment, the reagents are antibodies that specifically recognize one or more of the markers.

EXAMPLE 1

Immune Activation in Non-Celiac Wheat Sensitivity

[0032] In the study described herein, we investigated 1) whether systemic immune activation in response to translocated microbial products may be a feature of NCWS, 2) whether such systemic immune activation is linked to a compromised intestinal epithelium, and 3) whether

the systemic immune activation or damage to the epithelium is responsive to the elimination of wheat and related cereals from diet.

METHODS

[0033] *Patients and controls.* The study included 80 individuals with NCWS who met the criteria recently proposed by an expert group¹ and who were identified using a previously described structured symptom questionnaire^{2,3} (a modified version of the Gastrointestinal Symptom Rating Scale designed to rate symptoms commonly associated with NCWS). All NCWS subjects reported experiencing intestinal and/or extra-intestinal symptoms after ingestion of gluten-containing foods, including wheat, rye, or barley. The reported symptoms in all subjects improved or disappeared when those foods were withdrawn for a period of 6 months, and recurred when they were re-introduced for a period of up to 1 month. Individuals were excluded if they were already on a restrictive diet in the past 6 months, if they were positive for the celiac disease-specific IgA anti-endomysial and/or anti-TG2 autoantibody or for intestinal histologic findings characteristic of celiac disease, or if they were positive for wheat allergy-specific IgE serology or skin prick test. A total of 6 intestinal biopsies, including 2 from the duodenal bulb and 4 from the distal duodenum, were taken from each individual. Serum samples from all 80 NCWS subjects while on a diet that contained wheat, rye, and/or barley were available. In addition to the above specimens, serum samples were available from 20 of the above NCWS individuals both prior to and after 6 months of a self-monitored diet free of wheat, rye, and barley. These individuals were asked to complete the previously described questionnaire³ prior to initiating the diet and immediately following its completion. For this study, specific intestinal symptoms (bloating, abdominal pain, diarrhea, epigastric pain, and nausea) and extra-intestinal symptoms (fatigue, headache, anxiety, memory and cognitive disturbances, and numbness in arms or legs), selected on the basis of being the most commonly reported symptoms by patients in this population as previously found⁴, were considered for analysis. Symptoms were scored from 0 to 3 as follows: 0 = absent; 1 = occasionally present; 2 = frequently present; and 3 = always present. A total score, based on the sum of individual symptom scores, was calculated for each individual at the two time points (before and after the diet). The study also included 40 serum samples from patients with biopsy-proven active celiac disease and 40 serum samples from healthy subjects (both groups on normal non-restrictive diet), recruited as part of the same protocol that included the NCWS individuals. All cases of celiac disease were biopsy-

proven and diagnosed according to established criteria.⁵ Screening questionnaires were used to evaluate the general health of unaffected controls. Individuals who had a history of liver disease, liver function blood test results (AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase), total protein, albumin, globulin, and bilirubin) outside the normal range, or a recent infection were excluded from all cohorts in the study.

[0034] All samples were collected with written informed consent under institutional review board-approved protocols at St. Orsola-Malpighi Hospital, Bologna, Italy. Serum specimens were kept at -80 °C to maintain stability. This study was approved by the Institutional Review Board of Columbia University Medical Center.

[0035] **Assays.** Established serologic markers of celiac disease, including IgA antibody to TG2 and IgG and IgA antibodies to deamidated gliadin, were measured as previously described.^{6,7}

[0036] Serum IgG, IgA, and IgM antibodies to native gliadin were measured separately by the enzyme-linked immunosorbent assay (ELISA) as previously described,^{6,8} with the following modification: the secondary antibodies were HRP-conjugated anti-human IgG (GE Healthcare), IgA (MP Biomedicals), or IgM (MP Biomedicals). Serum IgG, IgA, and IgM antibodies to bacterial flagellin were measured separately using a similar protocol for detecting antibodies to gliadin, with the following modification: plates were coated with a 2 µg/mL solution of highly purified flagellin from *Salmonella typhimurium* (InvivoGen).

[0037] Levels of serum IgG, IgA, and IgM endotoxin-core antibodies (EndoCAb) (Hycult Biotech), lipopolysaccharide (LPS)-binding protein (LBP) (Hycult Biotech), soluble CD14 (sCD14) (R&D Systems), and intestinal fatty acid-binding protein (FABP2) (R&D Systems) were determined by ELISA, according to the manufacturers' protocols.

[0038] **Data analysis.** Group differences were analyzed by the Kruskal-Wallis one-way analysis of variance, with post-hoc testing and correction for multiple comparisons. Correlation analysis was performed using Spearman's *r*. A multivariate principal component analysis (PCA) was carried out on the entire dataset to reduce data dimensionality and to assess clustering. The effect of the restrictive diet was assessed by the Wilcoxon matched-pairs test. All **P** values were 2-sided, and differences were considered statistically significant at **P**<0.05. Statistical analyses were performed with Prism 6 (GraphPad) and Minitab 17 (Minitab) software.

RESULTS

[0039] Patients and controls. The demographic and clinical characteristics of the study cohorts are included in Table 1. Twenty-one (26%) NCWS individuals carried the HLA DQ2 and/or DQ8 genes, a rate not substantially different than in the general population. Small intestine duodenal biopsy showed a normal mucosa (Marsh 0) in 48 (60%) and mild abnormalities, represented by an increased intraepithelial lymphocyte number (Marsh 1), in 32 (40%). In contrast, all celiac disease patients in this study carried the HLA DQ2 and/or DQ8 markers and presented with Marsh 3 grade intestinal histologic findings.

Table 1. Demographic and clinical characteristics of study cohorts.

Subject group	Number of subjects	Mean age, years [SD]	Female s no. (%)	Celiac disease-associated HLA DQ2 and/or DQ8—no. (%)	Intestinal biopsy histologic grade: Marsh 0; Marsh 1; Marsh 3—no. (%)
NCWS					
Non-restrictive diet	80	34.6 [10.3]	62 (78)	21 (26)	48 (60); 32 (40); 0
Before and after restrictive diet*	20	34.0 [10.7]	19 (95)	7 (35)	9 (45); 11 (55); 0
Active celiac disease	40	34.5 [13.7]	30 (75)	40 (100)	0, 0, 40 (100)
Healthy	40	35.0 [12.8]	30 (75)	-	-

*Intestinal biopsy taken prior to dietary restriction.

[0040] Markers of celiac disease and immune reactivity to gluten. As expected, only the active celiac disease patients exhibited significantly elevated IgA antibody reactivity to TG2, as well as IgG and IgA antibody reactivity to deamidated gliadin, when compared with healthy controls ($P<0.0001$ for each comparison) (Fig. 1A-C). Celiac disease patients also displayed increased IgG and IgA ($P<0.0001$ for each), but not IgM, antibody reactivity to native gliadin when compared to healthy controls (Fig. 1D-F). In the NCWS cohort (while being on a diet that did not restrict the intake of wheat and related cereals), IgG, IgA, and IgM antibodies to native gliadin were all significantly higher than in the healthy control group ($P<0.0001$, $P<0.0001$, and $P=0.018$ respectively) (Fig. 1D-F). However, IgA reactivity to native gliadin in this NCWS cohort was lower than in the celiac disease group ($P=0.015$). There was no association between antibody reactivity to native gliadin and the presence of HLA-DQ2 and/or DQ8 genes in the NCWS group.

[0041] Systemic innate immune activation. Serum levels of both LBP and sCD14 were significantly elevated in individuals with NCWS in comparison to celiac disease patients and healthy individuals ($P<0.0001$ for each comparison) (Fig. 2A-B). There was a highly significant

correlation between serum LBP and sCD14 ($r=0.657$, $P<0.0001$) (Fig. 7). Neither LBP nor sCD14 was found to be significantly elevated in celiac disease patients when compared to healthy controls.

[0042] B cell response to microbial antigens. When compared with the healthy control and celiac disease cohorts, the NCWS group had significantly higher levels of EndoCAb IgM ($P<0.0001$ and $P=0.028$, respectively) (Fig. 2D), but not IgG or IgA (Fig. 2C; Fig. 8A). In contrast to the NCWS cohort, the celiac disease group had higher levels of EndoCAb IgA when compared with the NCWS and healthy control groups ($P=0.021$ and $P=0.032$, respectively) (Fig. 8A), but not IgG or IgM (Fig. 2C-D).

[0043] Furthermore, the levels of IgG and IgM antibodies to flagellin were significantly elevated in the NCWS cohort when compared with the healthy control group ($P=0.001$ and $P=0.009$, respectively) (Fig. 2E-F). These antibodies were not significantly elevated in the celiac disease cohort, although there was a trend towards higher IgA reactivity to flagellin when compared with healthy controls ($P=0.059$) (Fig. 8B). The increased IgM antibody response to flagellin correlated with the elevated EndoCAb IgM in the NCWS cohort ($r=0.386$, $P<0.0001$) (Fig. 9).

[0044] Systemic immune activation is associated with increased intestinal epithelial cell damage. In comparison with the healthy control group, serum concentrations of FABP2, a marker of intestinal epithelial cell damage, were significantly elevated in the NCWS cohort, as well as in the celiac disease group ($P<0.0001$ for each) (Fig. 3A). In addition, the FABP2 concentrations in the NCWS cohort correlated with levels of LBP ($r=0.360$, $P=0.001$) and sCD14 ($r=0.461$, $P<0.0001$) (Fig. 3B-C). The FABP2 concentrations in the NCWS group also correlated with EndoCAb IgM ($r=0.326$, $P=0.003$) and anti-flagellin IgM antibody reactivity ($r=0.239$, $P=0.03$) in the NCWS cohort (Fig. 10A-B). In the celiac disease cohort, FABP2 concentrations correlated with the levels of IgA antibody to TG2 ($r=0.559$, $P<0.0001$) (Fig. 11).

[0045] Multivariate analysis of dataset. PCA was used to assess similarities and differences between the subjects in the three cohorts based on the generated data and to determine whether they can be grouped. Most of the variability in the data could be explained by the first two components (54%). The score plot of the first and second components for the entire dataset demonstrated the clustering of the healthy control, celiac disease, and NCWS subjects into three discernible groups, with some outliers (Fig. 4).

[0046] Systemic immune activation and intestinal epithelial cell damage respond to dietary restriction. Levels of the above markers of immune activation and gut epithelial cell damage were also measured in 20 of the above NCWS subjects before and 6 months after initiation of a diet free of wheat, rye, and barley. All individuals reported symptom improvement at the end of 6 months, which was reflected in a significant reduction in both the intestinal and extra-intestinal composite symptom scores ($P<0.0001$ for each) (Fig. 5A-B), accompanied by a decline in IgG, IgA, and IgM anti-gliadin antibodies ($P<0.0001$, $P=0.002$, and $P=0.004$, respectively) (Fig. 5C-E). In conjunction with this, we found a statistically significant reduction in the serum levels of LBP ($P=0.0002$), sCD14 ($P=0.0006$), EndoCAb IgM ($P=0.006$), anti-flagellin IgG and IgM antibodies ($P=0.002$ and $P=0.003$, respectively), and FABP2 ($P=0.003$) after the completion of the diet (Fig. 6A-F). The magnitude of change in the measured biological markers did not correlate significantly with that for the symptom scores.

DISCUSSION

[0047] As expected, individuals with sensitivity to wheat in the absence of celiac disease did not exhibit significantly elevated antibody responses to TG2 or deamidated gliadin sequences. This indicates that in contrast to celiac disease, the observed humoral immune response to gluten in NCWS is independent of TG2 enzymatic activity and HLA-DQ2/DQ8, and is likely to target certain epitopes that are distinct from those in celiac disease. We hypothesized that the enhanced antibody response to native gliadin in NCWS individuals, particularly IgG and IgM isotypes, may be a consequence of ongoing intestinal epithelial barrier defects. If so, such defects might also give rise to an inadequate regulation of the interaction between the gut microbiota and systemic circulation, resulting in peripheral immune activation. To examine this, we measured the levels of LBP and sCD14 as indicators of the translocation of microbial products, particularly LPS, across the epithelial barrier. Translocated circulating LPS can result in the rapid secretion of LBP by gastrointestinal and hepatic epithelial cells, as well as sCD14 by CD14⁺ monocytes/macrophages.⁹ sCD14 binds LPS in the presence of LBP to activate TLR4.¹⁰ We found significantly elevated serum levels of both LBP and sCD14 in individuals with NCWS in comparison to celiac disease patients and healthy controls. The high degree of correlation between serum LBP and sCD14 suggested that these molecules are concurrently expressed in response to the stimulus in NCWS individuals.

[0048] We also quantified serum levels of antibody to LPS core oligosaccharide, or EndoCAb, which is known to modulate in response to bacterial endotoxin in circulation.¹¹ As they are involved in the neutralization of circulating endotoxin, EndoCAb immunoglobulins are typically depleted in response to an acute LPS exposure, but eventually rise due to the B cell anamnestic response.¹⁹ Individuals in the NCWS cohort exhibited increased levels of EndoCAb IgM. To demonstrate that the systemic immune response in individuals identified as having NCWS would not be limited to only LPS if driven by translocated microbial products, we also measured serum levels of antibody to flagellin, the principal substituent protein of the flagellum in Gram-positive and -negative bacteria. We found that levels of IgG and IgM antibodies to flagellin were significantly elevated in the NCWS cohort. Considering that no individuals in this study had evidence of infection, these observations are suggestive of a translocation of microbial products from the gastrointestinal tract that contributes to the observed innate and adaptive immune activation in the NCWS cohort.

[0049] Circulating bacterial components, such as LPS and flagellin, bind to their respective TLRs on various cells, including macrophages and dendritic cells, which results in signaling through the myeloid differentiation factor 88 (MyD88) adaptor protein.¹⁰ Ultimately, MyD88 signaling leads to the activation of transcription factor nuclear factor- κ B (NF- κ B) and increased expression of various pro-inflammatory cytokines that can exert deleterious systemic effects.^{9, 12} A systemic innate immune activation model would be consistent with the generally rapid onset of reported symptoms in NCWS.⁴ In addition, circulating microbial products can bind to TLRs on other cells to trigger a more localized inflammatory response. For example, LPS binds directly to TLR4 on the luminal surface of brain blood vessels, resulting in local cytokine secretion in the brain that has been shown to activate the microglia to displace inhibitory synapses.¹³ In HIV infection, where the presence of microbial translocation is linked to intestinal epithelial damage, increased systemic immune activation in response to bacterial antigens is associated with cognitive deficits.¹⁴ Such a pathway might contribute to some of the neurocognitive symptoms experienced by NCWS individuals.

[0050] Subsequently, we hypothesized that the observed systemic immune activation in response to microbial products in individuals with NCWS may be linked to increased intestinal enterocyte damage and turnover rate. FABP2 is a cytosolic protein specific to intestinal epithelial cells that is rapidly released into systemic circulation upon cellular damage.¹⁵ Alterations in circulating

FABP2 concentration, reflecting epithelial cell loss and changes in enterocyte turnover rate, are useful for identifying acute intestinal injury.¹⁵⁻¹⁸ Elevated circulating FABP2 has been shown to be associated with increasing degrees of villous atrophy in celiac disease,¹⁹ and with microbial translocation in HIV²⁰ that is in turn linked to damaged intestinal epithelial barrier integrity.²¹

5 Similar to the celiac disease patients, the NCWS individuals in this study were found to have raised circulating FABP2 levels, indicating increased intestinal epithelial cell damage. FABP2 concentrations in the NCWS cohort correlated strongly with levels of LBP and sCD14, suggesting a link between the intestinal epithelial cell damage and the acute systemic immune activation in response to translocated microbial products. The FABP2 concentrations in the
10 NCWS group also correlated with IgM antibody reactivity towards microbial antigens, though less strongly in comparison to LBP and sCD14 responses, as might be expected for a systemic antibody response. In the celiac disease cohort, FABP2 concentrations correlated with the increased IgA antibody to TG2, confirming the existence of a close relationship between the mucosal autoimmune response and the intestinal damage in this disease.

15 **[0051]** In contrast to celiac disease, however, investigations of small intestine biopsies in NCWS subjects in this and other studies have not found villous atrophy or mucosal architectural abnormalities,^{2, 4, 22, 23} even if significant inflammatory changes are seen.²³ One possible explanation for this could be that the epithelial damage associated with NCWS is in regions other than the duodenum from where biopsies are generally taken in such individuals. This would be
20 plausible because FABP2 is expressed primarily by the epithelial cells of the jejunum,^{16, 24} which may point to this region of the small intestine as a potential primary site of mucosal damage in NCWS. Different sections of the gastrointestinal tract have unique cellular, structural, and immunologic features that make them vulnerable to specific insults.²⁵ Another possibility is that the epithelial changes associated with NCWS might be more subtle in comparison to celiac
25 disease, without overt remodeling of the mucosa. For example, TNF- α -mediated enhancement of enterocyte loss has been shown to cause mucosal barrier dysfunction and physical gaps in the epithelium that require confocal and scanning electron microscopy for visualization.²⁶

[0052] On the other hand, despite the established extensive villus damage associated with celiac disease, neither LBP nor sCD14 levels were found to be significantly elevated in the celiac
30 disease group, thus standing in stark contrast to the NCWS cohort. In addition, among the immunoglobulin responses to microbial antigens, only IgA antibodies appeared to be increased

in celiac disease. These data suggest that there is an effective mechanism for the neutralization of microbial products that may cross into the lamina propria in most cases of celiac disease, possibly in part via the localized IgA response and mucosal phagocytic cells. These mechanisms are known to be essential for the immune surveillance of luminal antigens and the elimination of microbial products that cross the epithelial barrier, thereby reducing the likelihood of their translocation into the submucosa and access to blood vessels.⁹ Such mucosal immune responses may be lacking or inadequate in individuals with NCWS. Instead, what we observed were enhanced IgM responses to gliadin, LPS, and flagellin in the NCWS cohort, which clearly contrasted with the celiac disease group. In humans, IgM memory B cells are present in the peripheral blood and contribute to the expression of IgM antibodies to a diverse variety of antigens, offering a first line of defense against potential pathogens.²⁷ Exposure to unmethylated CpG sequences, which are abundant in the bacterial and viral genomes, can result in TLR9-dependent proliferation and differentiation of these B cells, independent of direct interaction with their respective antigens or T cell involvement.²⁸ Acute microbial translocation from the gut, as the data from our study suggest, would be expected to enhance the secretion of IgM antibodies in the periphery via this pathway. These IgM B cells would be additionally stimulated upon encounter with specific antigens, such as the translocated microbial components or gliadin sequences, and may contribute to the observed IgM antibody responses.^{28, 29} Recognition and agglutination of antigens by IgM and IgG antibodies can result in the activation of the classical complement pathway and Fc receptor-mediated endocytosis by macrophages,³⁰ further contributing to the ongoing systemic immune response.

[0053] The hallmark of NCWS is the onset of intestinal and/or extra-intestinal symptoms upon ingestion of gluten-containing foods, i.e., wheat, rye, and barley, and the alleviation of symptoms upon their withdrawal from diet. To determine whether the patient-reported symptom resolution upon the elimination of these foods would be associated with the amelioration of intestinal epithelial cell damage and a reduction in microbial translocation and systemic immune activation, we examined the above markers in a subset of NCWS subjects prior to and after a diet that excluded wheat and related cereals. The results indicated a significant decline in the markers of immune activation and gut epithelial cell damage, in conjunction with the improvement of symptoms. However, the magnitude of change in the measured biological markers did not correlate significantly with that for the symptom scores. This appears to be similar to

observations in celiac disease patients, where symptoms are known to be a poor predictor of disease activity and associated biomarkers.^{31, 32} A limitation of this portion of the study was the absence of a healthy control group to assess the potential impact of the dietary restriction in unaffected individuals.

5 [0054] In summary, the results of this study on individuals with sensitivity to wheat in the absence of celiac disease demonstrate 1) significantly increased serum levels of sCD14 and LBP, as well as antibody reactivity to microbial antigens, indicating systemic immune activation, 2) an elevated expression of FABP2 that correlates with the systemic immune responses to bacterial products, suggesting compromised intestinal epithelial barrier integrity and increased microbial
10 translocation, and 3) a significant change towards normalization in the levels of the immune activation markers, as well as FABP2 expression, in response to the restrictive diet, which is associated with improvement in symptoms. Our data establish the presence of objective markers of systemic immune activation and epithelial cell damage in the affected individuals. The results of the multivariate data analysis suggest that a selected panel of these may have utility for
15 identifying NCWS patients or patient subsets in the future.

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What Is Claimed Is:

1. A method of identifying a subject having non-celiac wheat sensitivity, comprising the step of determining blood levels of one or more markers in said subject, said markers are selected from the group consisting of soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and intestinal fatty acid-binding protein (FABP2), wherein increased levels of said markers in said subject as compared to control levels would support a diagnosis of non-celiac wheat sensitivity.
2. The method of claim 1, wherein said control levels are derived from healthy subjects or subjects having celiac disease.
3. The method of claim 1, wherein levels of said markers are determined in serum or plasma.
4. The method of claim 1, wherein said anti-lipopolysaccharide antibodies are IgM antibodies.
5. The method of claim 1, wherein said anti-flagellin antibodies are one or both of IgM antibodies and IgG antibodies.
6. The method of claim 1, wherein said anti-gliadin antibodies are one or more of IgA, IgM and IgG antibodies.
7. The method of claim 1, wherein the method comprises a statistical algorithm to predict whether a subject has non-celiac wheat sensitivity.
8. The method of claim 1, wherein the method further comprises the step of treating said subject with a diet free of one or more of wheat, rye and barley.
9. A method of treating a subject having non-celiac wheat sensitivity, comprising the steps of:

- i. determining blood levels of one or more markers in said subject, said markers are selected from the group consisting of soluble CD14, lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and intestinal fatty acid-binding protein (FABP2), wherein increased levels of said markers in said subject as compared to control levels indicate said subject has non-celiac wheat sensitivity; and
- ii. treating the subject identified in (i) with a diet free of one or more of wheat, rye and barley.

10. The method of claim 9, wherein said control levels are derived from healthy subjects or subjects having celiac disease.

11. The method of claim 9, wherein levels of said markers are determined in serum or plasma.

12. The method of claim 9, wherein said anti-lipopolysaccharide antibodies are IgM antibodies.

13. The method of claim 9, wherein said anti-flagellin antibodies are one or both of IgM antibodies and IgG antibodies.

14. The method of claim 9, wherein said anti-gliadin antibodies are one or more of IgA, IgM and IgG antibodies.

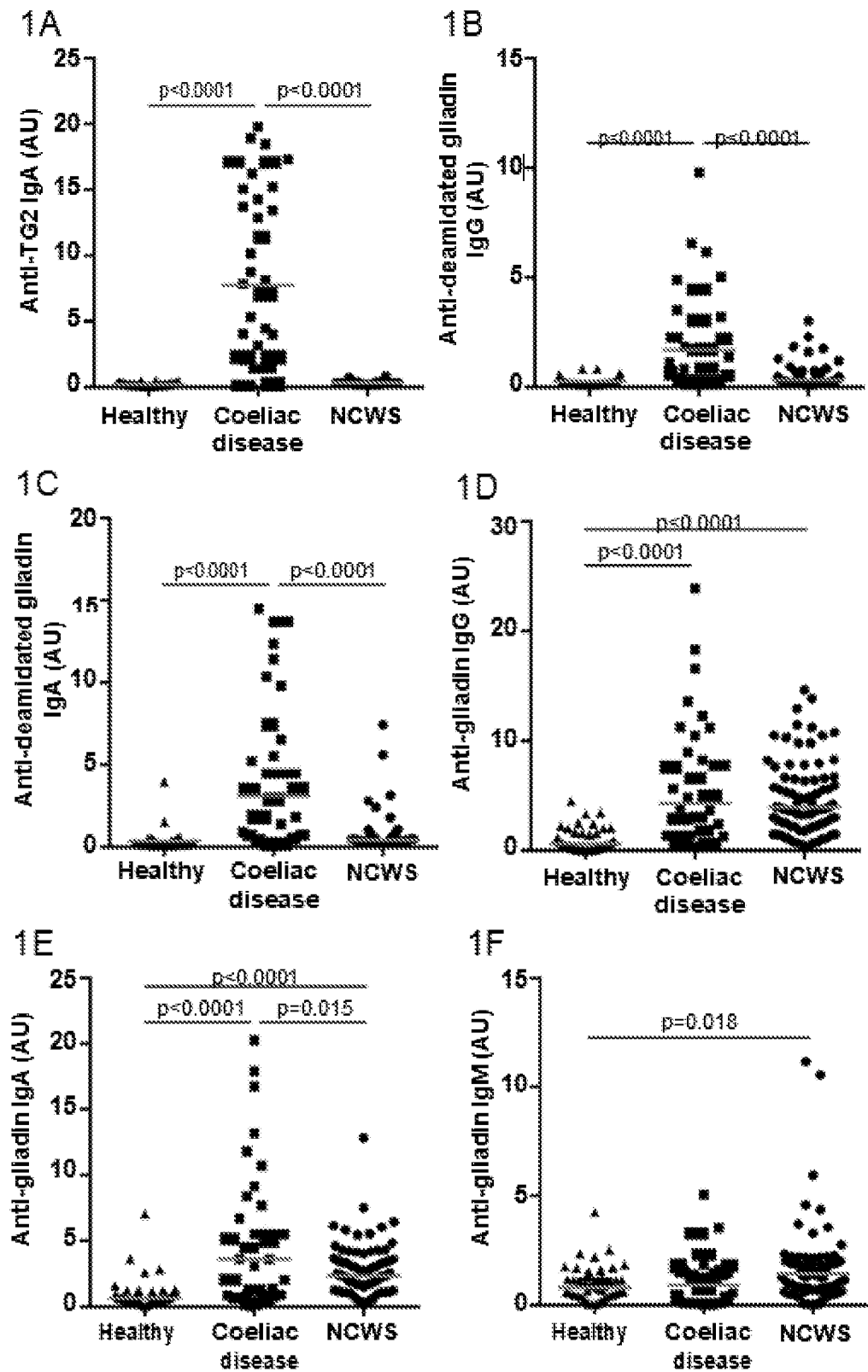
15. A method of monitoring response to a treatment for non-celiac wheat sensitivity in a subject, comprising the step of determining blood levels of one or more markers in said subject before and after said treatment, said markers are selected from the group consisting of soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and intestinal fatty acid-binding protein (FABP2), wherein decreased levels of said markers in said subject after said treatment indicate that said subject has responded to said treatment, otherwise the subject has not responded to the treatment.

16. The method of claim 15, wherein levels of said markers are determined in serum or plasma.
17. The method of claim 15, wherein said anti-lipopolysaccharide antibodies are IgM antibodies.
- 5 18. The method of claim 15, wherein said anti-flagellin antibodies are one or both of IgM antibodies and IgG antibodies.
19. The method of claim 15, wherein the anti-gliadin antibodies are one or more of IgG, IgA, and IgM antibodies.
- 10 20. The method of claim 15, wherein the treatment comprises a diet free of one or more of wheat, rye and barley.
- 15 21. A method of determining a course of treatment for non-celiac wheat sensitivity in a subject, comprising the step of determining blood levels of one or more markers in said subject before and after said treatment, said markers are selected from the group consisting of soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and intestinal fatty acid-binding protein (FABP2),
- 20 wherein decreased levels of said markers in said subject after said treatment indicate said subject should continue to be treated with said treatment, wherein when the levels of the markers do not decrease after said treatment, said subject should be treated with an alternative treatment.
- 25 22. The method of claim 21, wherein levels of said markers are determined in serum or plasma.
23. The method of claim 21, wherein said anti-lipopolysaccharide antibodies are IgM antibodies.
- 30

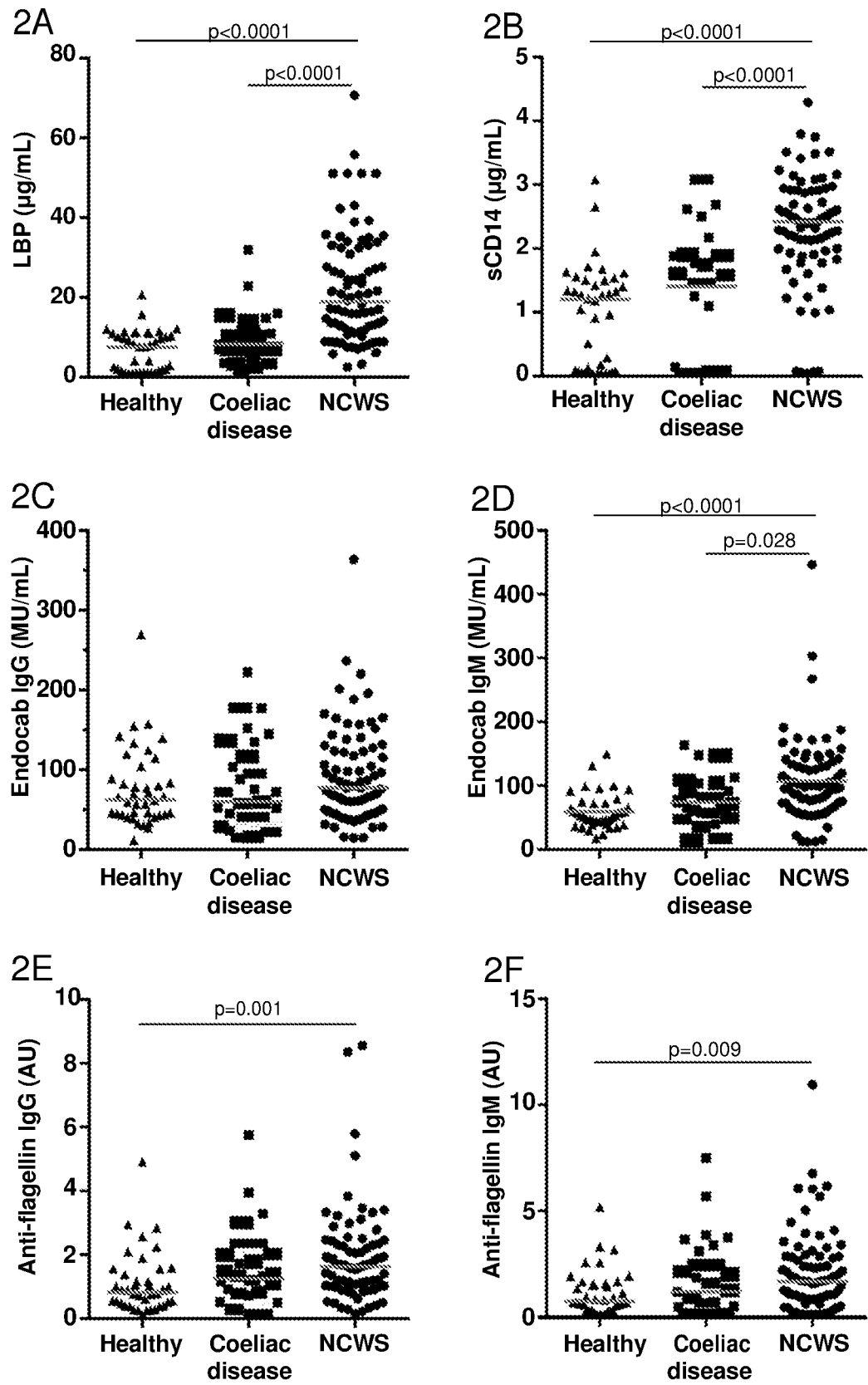
24. The method of claim 21, wherein said anti-flagellin antibodies are one or both of IgM antibodies and IgG antibodies.
25. The method of claim 21, wherein the anti-gliadin antibodies are one or more of IgG, IgA,
5 and IgM antibodies.
26. The method of claim 21, wherein the treatment comprises a diet free of one or more of wheat, rye and barley.
- 10 27. A kit that aids in diagnosis of non-celiac wheat sensitivity in a subject, comprising reagents that detect the presence of one or more markers in a sample from said subject, said markers are selected from the group consisting of soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP), anti-lipopolysaccharide antibodies, anti-flagellin antibodies, anti-gliadin antibodies, and intestinal fatty acid-binding protein (FABP2).
15
28. The kit of claim 27, wherein the sample is a blood, serum or plasma sample.

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Figures 1A-F

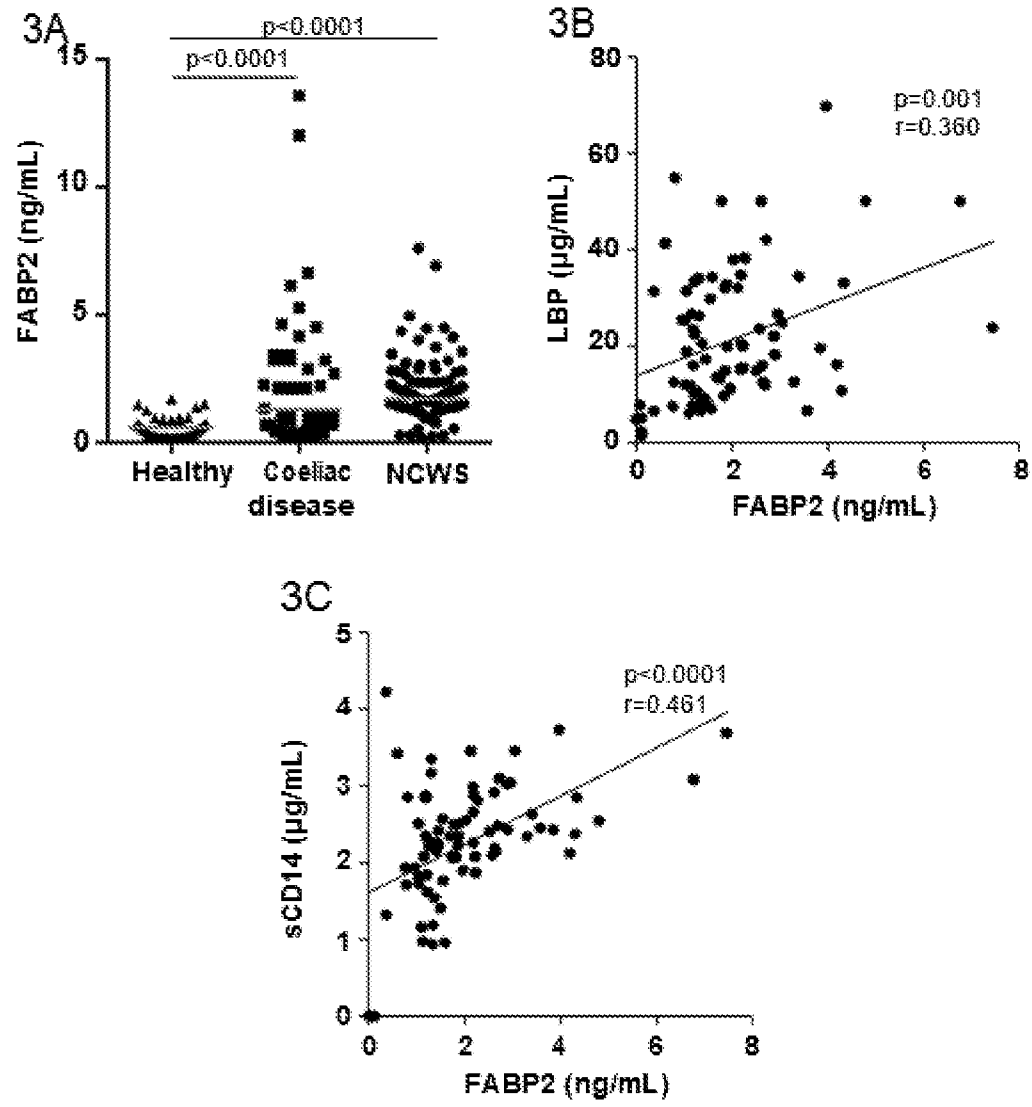


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Figures 2A-F



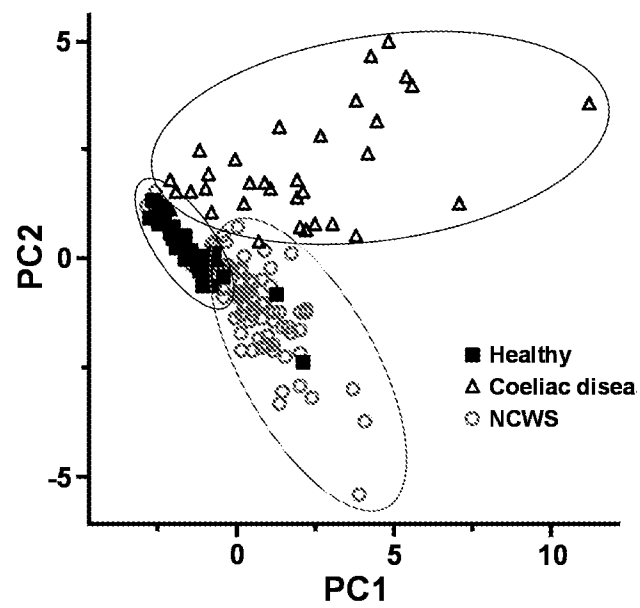
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Figures 3A-C

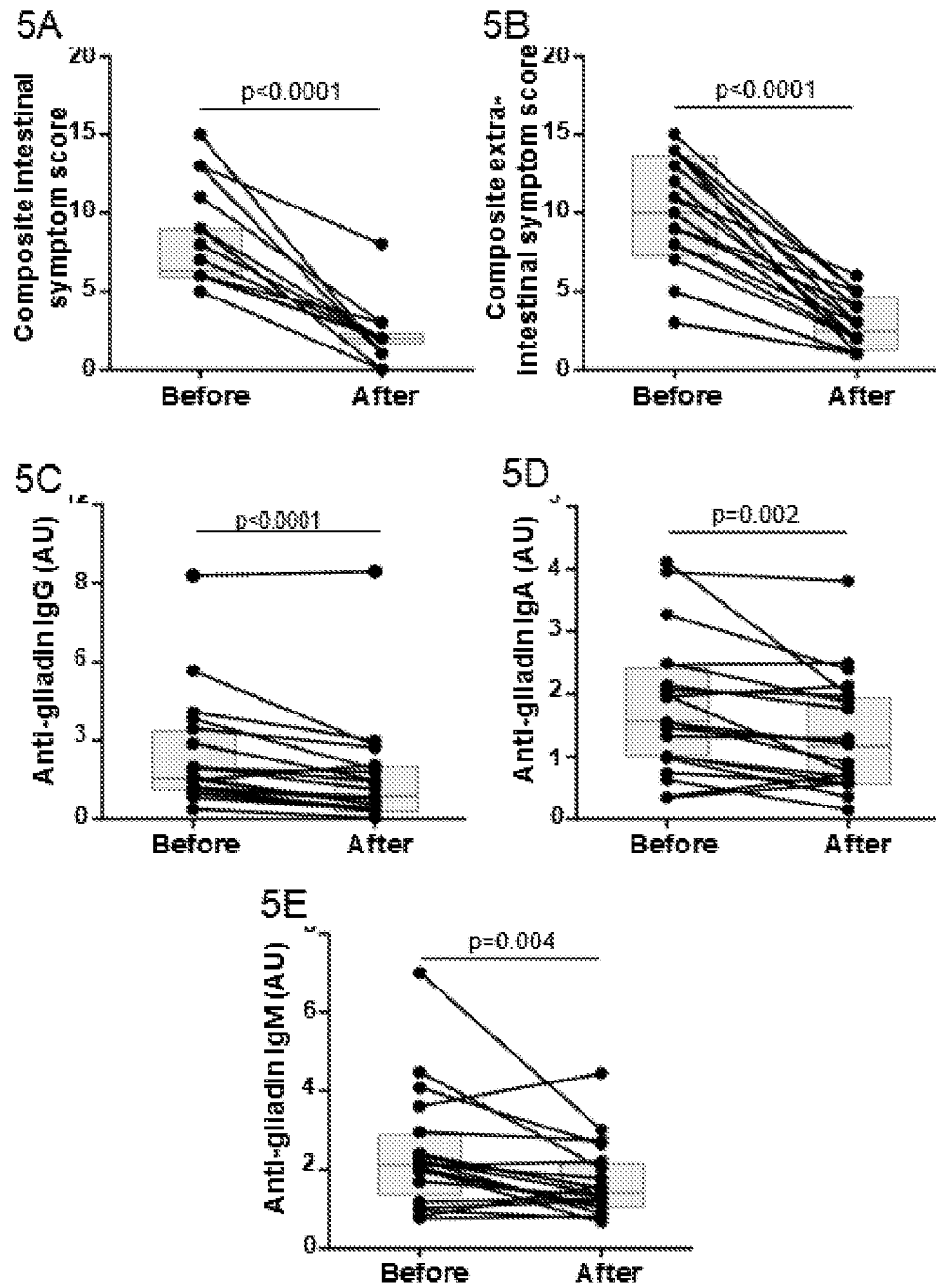


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Figure 4

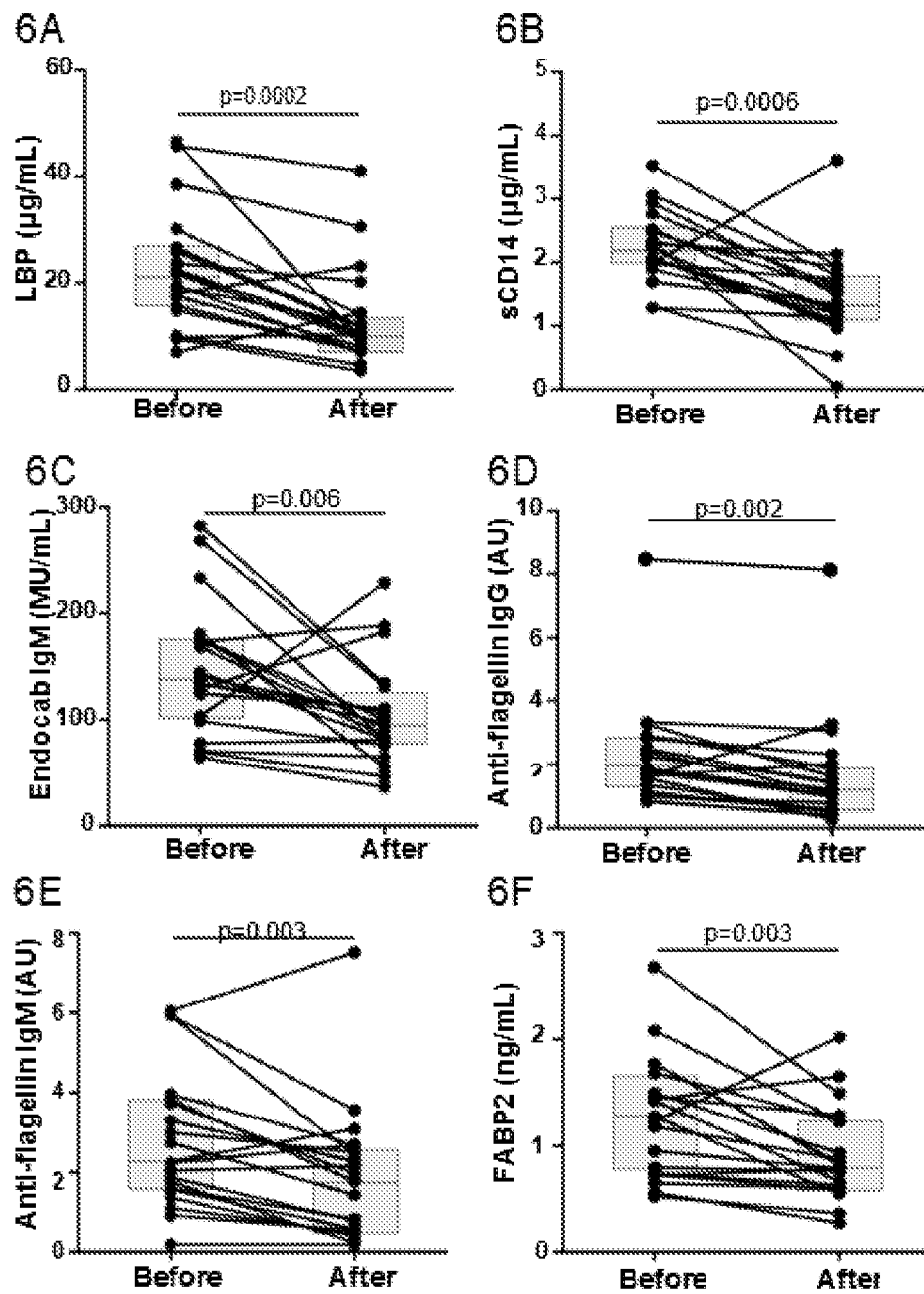


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Figures 5A-E



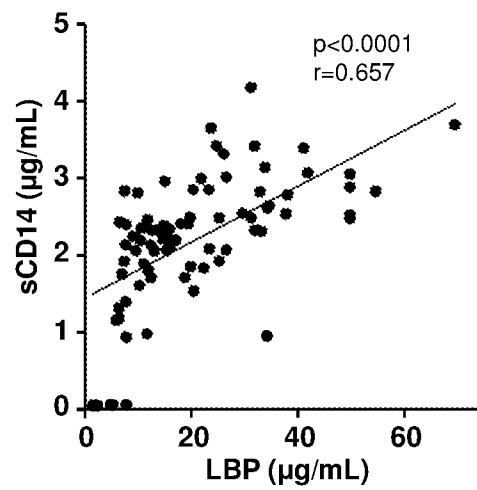
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Figures 6A-F



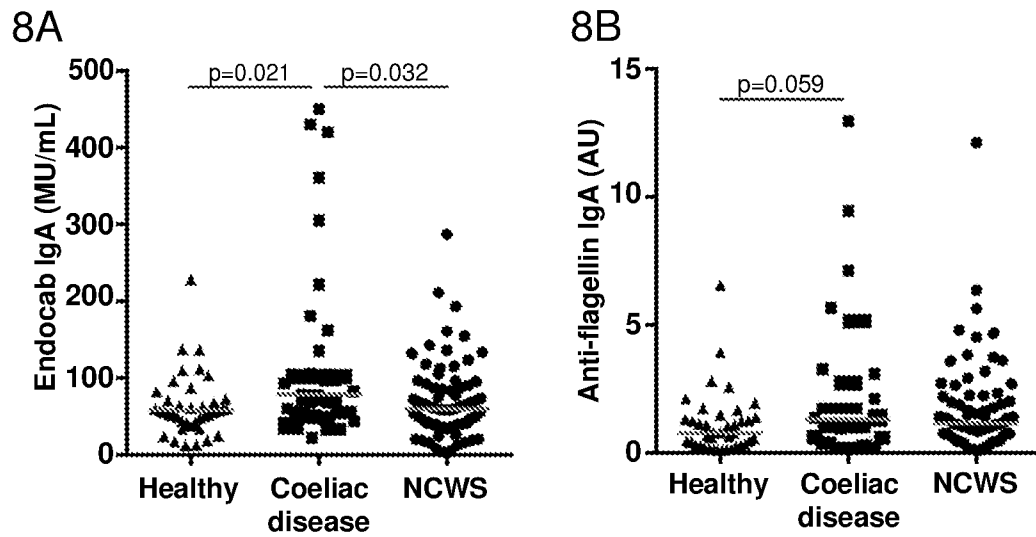
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Figure 7



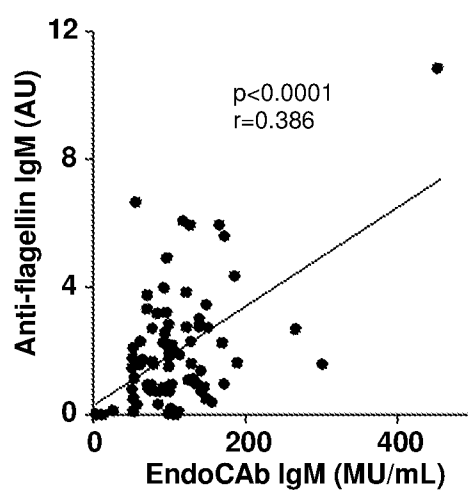
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Figures 8A-B

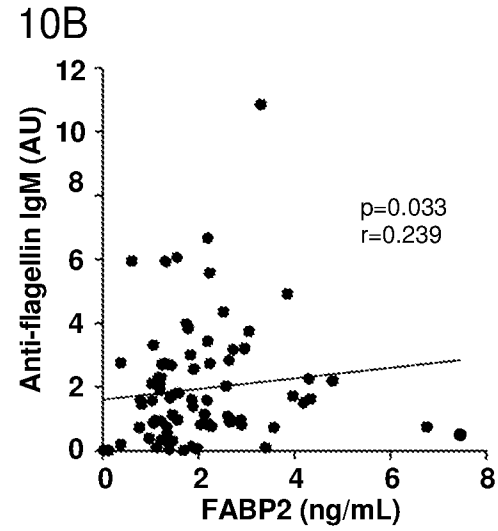
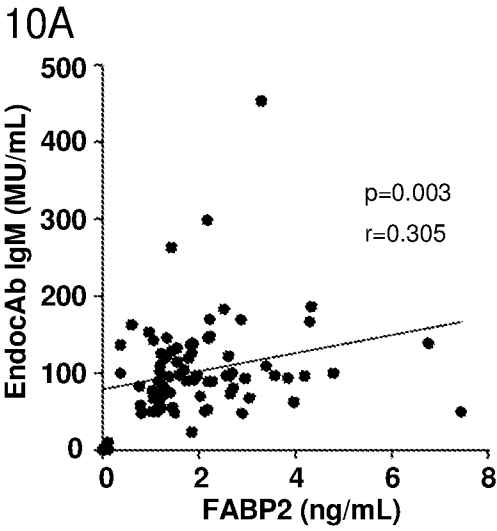


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Figure 9

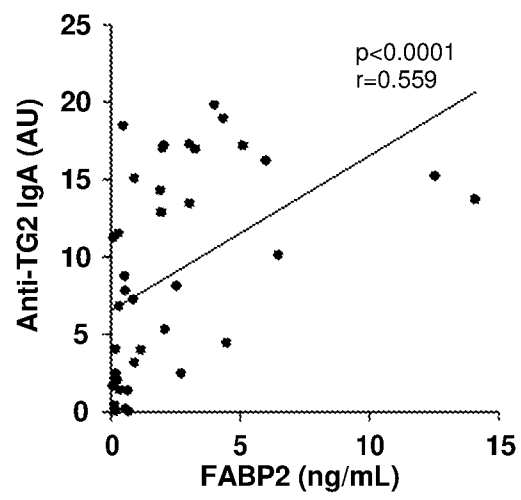


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Figures 10A-B



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Figure 11



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/019292

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/16; C07K 14/415; C07K 16/16; G01N 33/53 (2017.01)

CPC - A23L 33/105; C07K 14/415; G01N 33/6893; G01N 2800/06; G01N 2800/24 (2017.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/133.1; 424/171.1; 424/185.1; 506/9 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0190571 A1 (VOJDANI) 26 July 2012 (26.07.2012) entire document	1-3, 6-11, 14-16, 19-22, 25-28
Y		4, 5, 12, 13, 17, 18, 23, 24
Y	US 2014/0186855 A1 (IMMUNOSCIENCES LAB, INC. et al) 03 July 2014 (03.07.2014) entire document	4, 12, 17, 23
Y	US 8,409,819 B1 (BARKEN) 02 April 2013 (02.04.2013) entire document	5, 13, 18, 24
A	US 2014/0037661 A1 (BAAR et al) 06 February 2014 (06.02.2014) entire document	1-28
A	CARROCCIO et al. "Non-celiac wheat sensitivity as an allergic condition: personal experience and narrative review," American Journal of Gastroenterology, 05 November 2013 (05.11.2013), Vol. 108, No. 12, Pgs. 1845-1852. entire document	1-28
A	CATASSI et al. "Non-Celiac Gluten Sensitivity: The New Frontier of Gluten Related Disorders," Nutrients, 26 September 2013 (26.09.2013), Vol. 5, Pgs. 3839-3853. entire document	1-28
P, X	US 2016/0377629 A1 (CYREX LABORATORIES LLC) 29 December 2016 (29.12.2016) entire document	1-28

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

12 April 2017

Date of mailing of the international search report

18 MAY 2017

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