ORIGINAL RESEARCH

The Relevance of Using the C3d/Immunoglobulin G Test in Clinical Intervention

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ABSTRACT

Context • A large subset of the population is afflicted with a wide range of food-related inflammatory conditions, with at least 100 million people affected worldwide. The C3d/immunoglobulin G (IgG) test measures both the innate and adaptive responses of the immune system.

Objective • The study intended to validate the C3d/IgG test for food sensitivity for its ability to manage the symptoms of patients with intestinal and extraintestinal symptoms.

Design • The research team designed a retrospective study based on a cohort of patients treated at a medical center.

Setting • The patients were seen at Progressive Medical Center of Atlanta, an integrative medicine clinic, and patients' samples were analyzed at Dunwoody Laboratory.

Participants • The study included 30 individuals, 9 males and 21 females, ranging in age from 7-71 y who presented with symptoms associated with food sensitivity.

Intervention • The study reviewed the treatment and results of patients who were placed on an exclusion dietary regimen for treatment of possible food sensitivity. From an initial C3d/IgG test, foods causing elevated anti-C3d/IgG, with the exception of ones causing mild reactions, were identified and eliminated from each patient's diet.

Outcome Measures • At baseline and at an average of 10.7 mo on the dietary regimen, 2 C3d/IgG tests were performed on each patient's serum by the method of indirect enzyme-linked immunosorbent assay (ELISA). Both food sensitivities and chief complaints were reassessed in that second test to determine if participants' symptoms improved with food elimination. Outcomes were based on the status of the patients' primary complaints.

Results • Patients who complied with the avoidance of anti-C3d/IgG dietary antigens demonstrated a statistically significant reduction in C3d/IgG-testing sensitivity and a marked reduction in symptoms that they had reported before beginning the diet. The *P* values were .000002, .007, and .001 for changes in the severe, high, and moderate test results, respectively, between the initial and second test.

Conclusion • Overall, patients' well-being improved when C3d/IgG food sensitivity decreased as a result of an exclusion diet, demonstrating that food removal based on the C3d/IgG test could be an effective approach to patients' care. (*Altern Ther Health Med.* 2014;21(1):##-##.)

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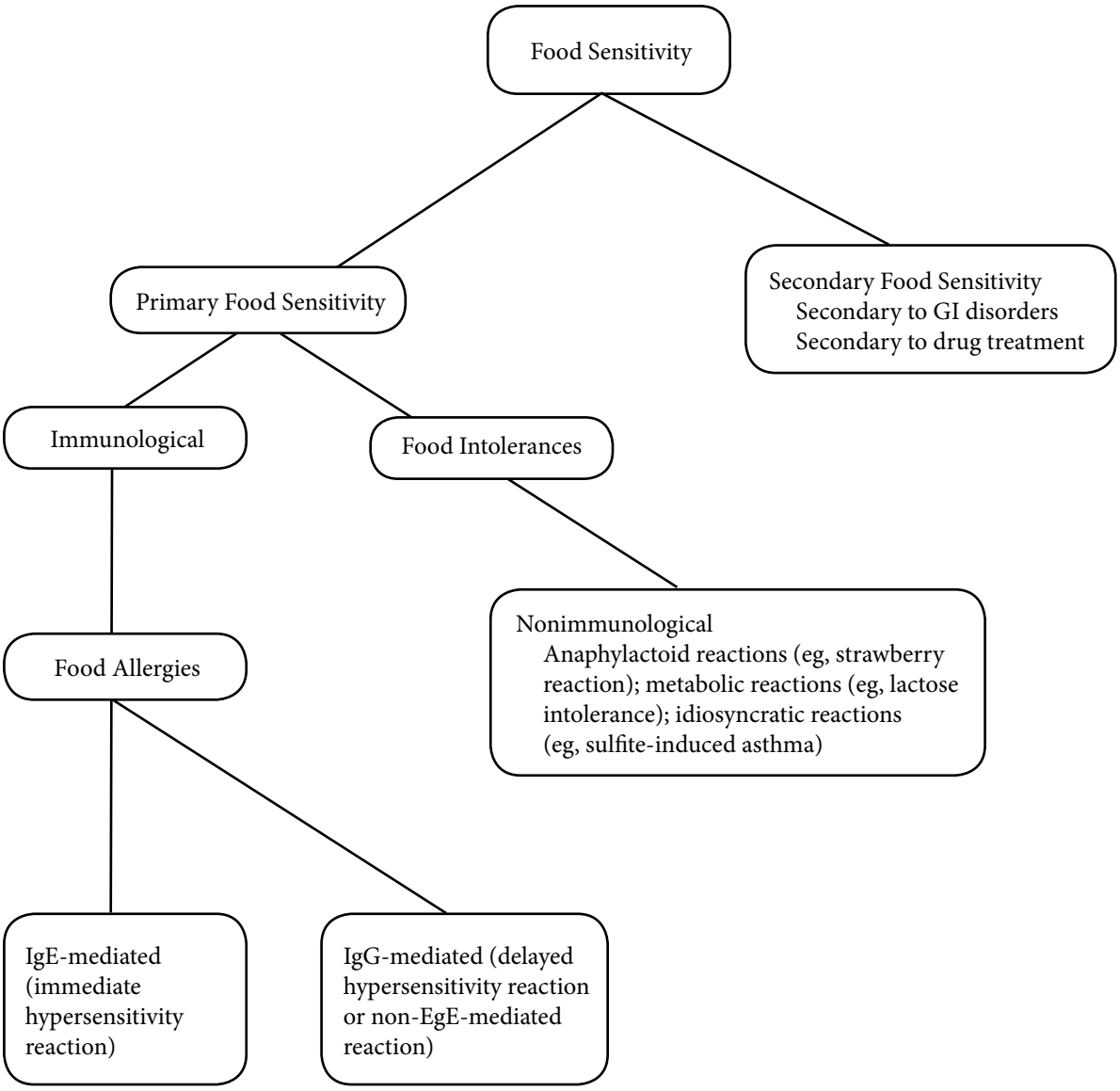
A great amount of time has been invested in studying the mechanism and effector molecules involved in food allergies. Effective pharmacotherapies have been formulated to alleviate a wide variety of symptoms, but nothing close to being curative has been developed. Food allergies involve 2 major types of reactivity, immediate (IgE-mediated) and delayed (IgG-mediated). Immunoglobulin E (IgE) mediates type 1 hypersensitivity reactions, and immunoglobulin G (IgG) mediates type 2 and type 3.[1] Immunological food sensitivities are characterized as hypersensitivity and inflammatory responses to an immune-mediated reaction from the ingestion of an offending food (Figure 1).[2]

IgG is an effector molecule of adaptive immunity, and its pleiotropic effect is demonstrated by its ability to modulate inflammation through the activation of the complement system, opsonization (binding of antigens), and the mediation

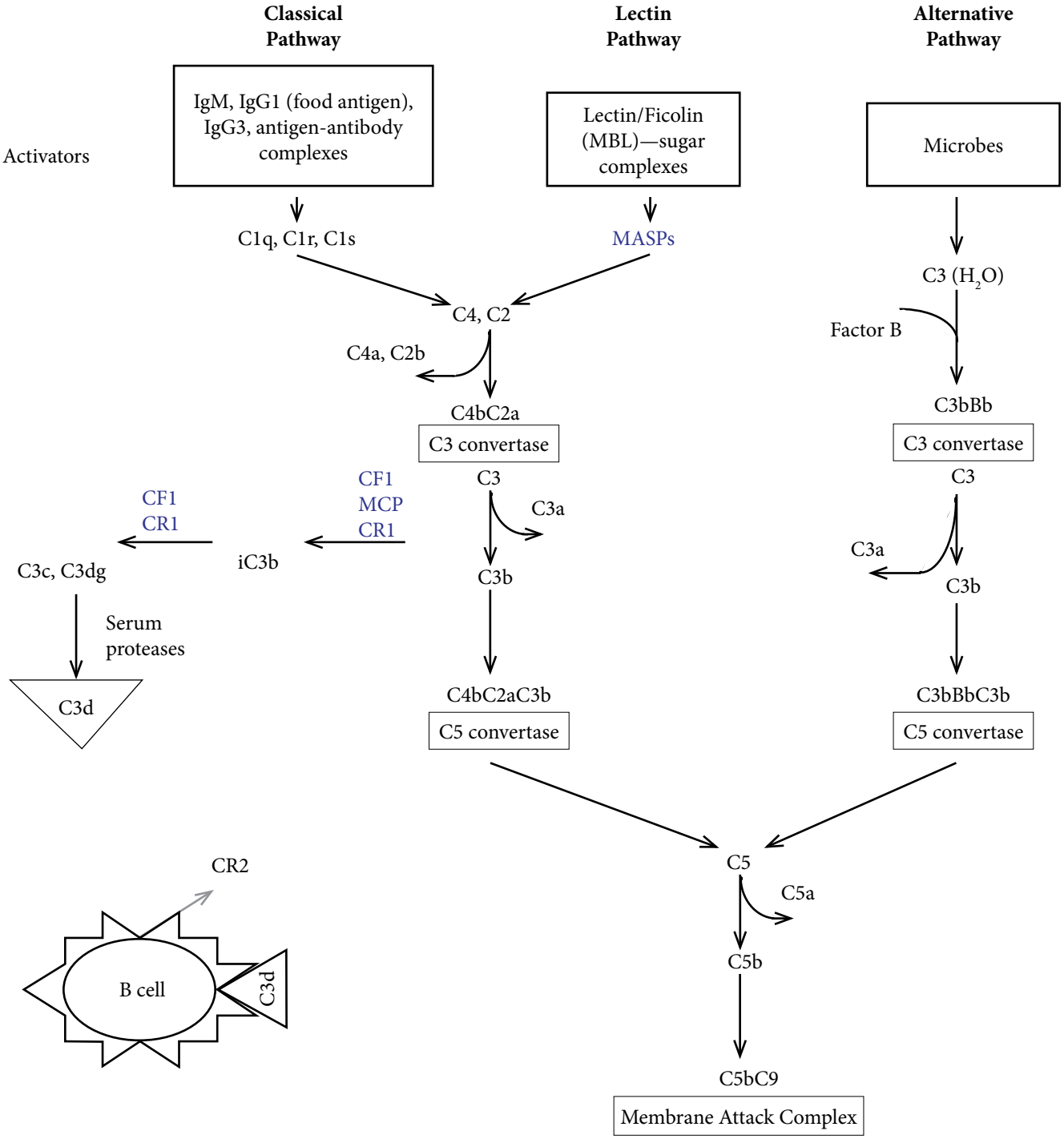
Figure 1. Classification of food sensitivity, with permission from the authors of *Food Allergies and Other Food Sensitivities*.[2] This figure was adapted initially from the Institute of Food Technologists (1985), a publication of the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition



Abbreviations: GI, gastrointestinal; IgE, immunoglobulin E; IgG, immunoglobulin G.

of antibody-dependent, cell-mediated cytotoxicity. Four IgG subclasses exist, all of which are measured within the IgG/C3d assay that the current study has used. IgG4 predominantly correlates with delayed food allergy. IgG1 responds to new food antigens; IgG2 and IgG3 react to cell-surface oligosaccharides of viruses, protozoa, and foods, which are potent allergic reactors.[3] Upon prolonged exposure to antigens, a class switch occurs from IgG1 to IgG4. IgG1 is able to activate the complement pathway through the flexible C_H2 domain, whereas IgG4 does not activate complement.[4] The complement pathway is a major effector mechanism of the innate immune system, with its primary functions being to destroy infectious agents, stimulate inflammatory response, and remove cellular debris (Figure 2).[5] It works collaboratively with the adaptive immune system.

Figure 2. Depicts the 3 pathways of the complement system. The dense, black arrows accentuate the classical pathway, which the current C3d/IgG study uses. Although all 3 pathways were able to generate the C3d fragment, the final cleavage product of C3, the figure shows only its production by the classical and lectin pathway for simplicity. C3d interacts with CR2, which is expressed on neutrophils, follicular dendritic cells, macrophages, and B cells.[6] When C3d binds CR2, it plays an important function in the cyclical control of B cells by lowering the threshold for B-cell activation, thereby contributing to proliferation of antibodies.[7]



Abbreviations: IgM, immunoglobulin M; IgG, immunoglobulin G; MBL, mannose-binding lectin. Written in blue are the correct abbreviations of important components of the complement cascade pathway: MASPs, membrane-associated serine proteases; CF1, complement factor 1; CR1, complement receptor 1; MCP, membrane cofactor protein.

Table 1. Differences Between Immediate and Delayed Food Allergies[1,4,5]

Characteristics	Immediate Food Allergy	Delayed Food Allergy
Ig	IgE-mediated	IgG-mediated
Ig half-life	1-2 d	21 d
Sensitivity	Tends to be permanent	Diminishes by avoidance
Onset of symptoms	Immediate phase: ≤8 h	Delayed: ≥24 h
Duration of symptoms	Acute	Chronic
Amount of exposure required for immunoreaction	Infrequent exposure	Frequent exposure
Mortality and morbidity	High probability of fatality (ie, anaphylactic shock); low morbidity	Low fatality, high morbidity
Dose	Not quantitative; all-or-none reaction	Dose dependent
Mechanism	Basophil/mast cell	Classical complement pathway
Chemical mediators	Histamine, leukotriene	Immune complex, helper T cells, cytokines
Effector function	Vascular permeability, smooth-muscle contraction	Inflammation, tissue damage

Abbreviations: Ig, immunoglobulin; IgE, immunoglobulin E; IgG, immunoglobulin G.

The inflammatory response is a recurring theme in all allergic reactions, resulting in release of various effector molecules, namely histamine, serotonin, tumor necrosis factor (TNF), and arachidonic acid metabolites.[8] Subsequently, vasodilation, increased vascular permeability, edema, smooth-muscle contraction, chemotaxis, and tissue damage are reactions to the effects of these chemical mediators. Immediate and delayed hypersensitivity reactions differ in that IgE causes an acute reaction, whereas IgG is insidious; hence, their names include "immediate" and "delayed," respectively. Table 1 lists the differences between the 2 types of food allergies that are classically observed.

The acute, IgE hypersensitivity reaction has been well studied and is highlighted by the most serious complication: anaphylactic shock. A delayed IgG reaction equally demands attention considering the wide range of symptoms it causes. The remainder of this article focuses on IgG; on C3d, a biomarker of the complement pathway; and on their significance in clinical interventions when a practitioner is managing patients with debilitating symptoms.

The pathogenesis of delayed food allergies is facilitated by intestinal permeability that results in a compromised gastrointestinal (GI) lining, contributing to the loss of tolerance and provocation of the immune system. For example, gluten, found in wheat, can be connected to the following issues: It (1) increases intestinal permeability and IgG; (2) elevates complement; and (3) has a temporal relationship with autoimmune disease, particularly, celiac disease (CD).[9] Among other foods, casein and whole milk are reported to contribute to the elevation of IgG antibodies in children with autistic spectrum disorders (ASD).[10] When children with ASD were monitored in a 2-stage, randomized, controlled study and placed on a gluten-free, casein-free diet, researchers reported dramatic improvements in behavioral patterns after 8 months.[11]

Within the last decade, much effort has been directed to attempt to elucidate the correlation between intestinal permeability and autoimmune diseases such as CD.[12] Recent studies suggest that an interplay between environmental factors and genetic susceptibility is an integral component in the establishment of certain autoimmune diseases. CD is characterized by chronic inflammation of the small intestine caused by ingestion of gluten, resulting in the destruction of villi, and subsequently, malabsorption. Individuals with the disease have either 1 or both classes of haplotypes of the major histocompatibility complex (MHC) 2 (ie, HLA DQ2 and HLA DQ8).[13-16]

After the discovery of zonulin, a marker of intestinal permeability, in April 2000 by Fasano et al,[17] the unilateral concept that molecular mimicry is the cause of autoimmune pathologies has given way to the emergence of a new paradigm focusing on that permeability. The C3d/IgG test has been validated in the management of patients with various symptoms who have intestinal permeability, which is an integral component in many disease processes. The test may prove to be useful in zonulin-related diseases, such as (1) autoimmune diseases: ankylosing spondytilitis, CD, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, and type 1 diabetes mellitus; (2) cancers: glioma tumors and breast, lung (adenocarcinoma), ovarian,

and pancreatic cancers; (3) neurological diseases: autism, multiple sclerosis, and schizophrenia; (4) infections: sepsis; and (5) metabolic disorders: obesity.[18-23] Zonulin is associated with obesity-induced, insulin-resistant metabolic disturbances and with elevations of interleukin 6, a cytokine of diverse functions.[24] In B lymphocytes, it promotes terminal differentiation; in plasma cells, it promotes antibody secretion; and in hepatocytes, it induces the synthesis of acute phase proteins, such as the complement component C3.[25-28] Also, a study documented by Klaus et al[29] shows a correlation between zonulin and sepsis.

In combination, the list of diseases above share a strong inflammatory component, which is measurable using the C3d/IgG tests because C3d is a robust indication of inflammation. C3d is a proteolytic fragment of C3dg that has the ability to function as a molecular adjuvant to augment humoral immune responses. It is a cleavage fragment of the complement cascade pathway. The related diseases are incurred through intestinal permeability and its multiple-organ involvement. Zonulin is the human analog of zonula occluden toxin, an enterotoxin from *Vibrio cholerae* that reversibly alters intercellular tight-junction permeability. Its study has shed light on the trafficking of macromolecules along a paracellular pathway, influencing the balance that exists between immune activation and tolerance.[19] When zonulin is upregulated through food antigens, particularly gluten, an increase in gut permeability occurs that sets the stage for inflammation that is driven by the increase in IgG and complement. Zonulin causes the disassembly of the tight junction, resulting in the loss of intestinal barrier integrity.[30]

Gluten, a proteinaceous antigen consisting of glutenins and gliadins, induces intestinal permeability, which occurs when gliadin binds the chemokine receptor CXCR3. This binding results in activation of the myeloid differentiation primary response (88) (MyD88) and promotes the epithelial release of zonulin when gliadin is exposed to the apical surface of the intestinal wall.[19,31,32] As a consequence of intestinal permeability, antigens that would otherwise be isolated to the lumen of the GI tract are afforded access to the submucosa, and, ultimately to the blood stream, resulting in immune activation.

Chronic inflammation is associated with asthma, chronic fatigue syndrome, depression, inflammatory bowel disease, and irritable bowel syndrome as well as inexplicable symptoms that are mentioned by patients to their medical practitioners.[1,33-35] The existence of zonulin provides evidence that tight junctions are regulated in a dynamic process, influencing physiological, developmental, and pathological activities.[12,36] Proteins from foods pass through tight junctions via a paracellular route, upregulating the immune response and, thereby, evoking an inflammatory reaction and creating the varied symptoms of general food sensitivity. When regulation has been lost and symptoms ensue, the most effective approach is elimination of the dietary antigens, resulting in upregulation of complements and IgGs. The removal of foods decreases the antigenic load and neutralizes the inflammatory response. Tight junctions close; the GI integrity of the mucosa is reestablished; and the individual's immune competence is restored.[13]

In addition to the GI system's digestive and absorptive functions, its unique anatomical and functional arrangements contribute to its ability to perform motility, neuroendocrine, and immunological functions.[37] The GI boasts a large intestinal mucosal surface, fortified by an impressive immune system; notably, the gut-associated lymphoid tissue has approximately 1 trillion lymphoid cells per meter of the small intestine.[38] Intraepithelial lymphocytes are positioned between epithelial cells, with a predominance of B lymphocytes in Peyer's patches located in the lamina propria.[13] The immunological function is of significant interest due to its involvement with inflammatory reaction, possibly causing intestinal and extraintestinal symptoms that are triggered by food antigens. Bloating, abdominal pain, diarrhea, constipation, fatigue, migraines, headaches, cognitive dysfunction, depression, myalgia, joint pain, sinusitis, and urticaria are a few of the symptoms described by patients.

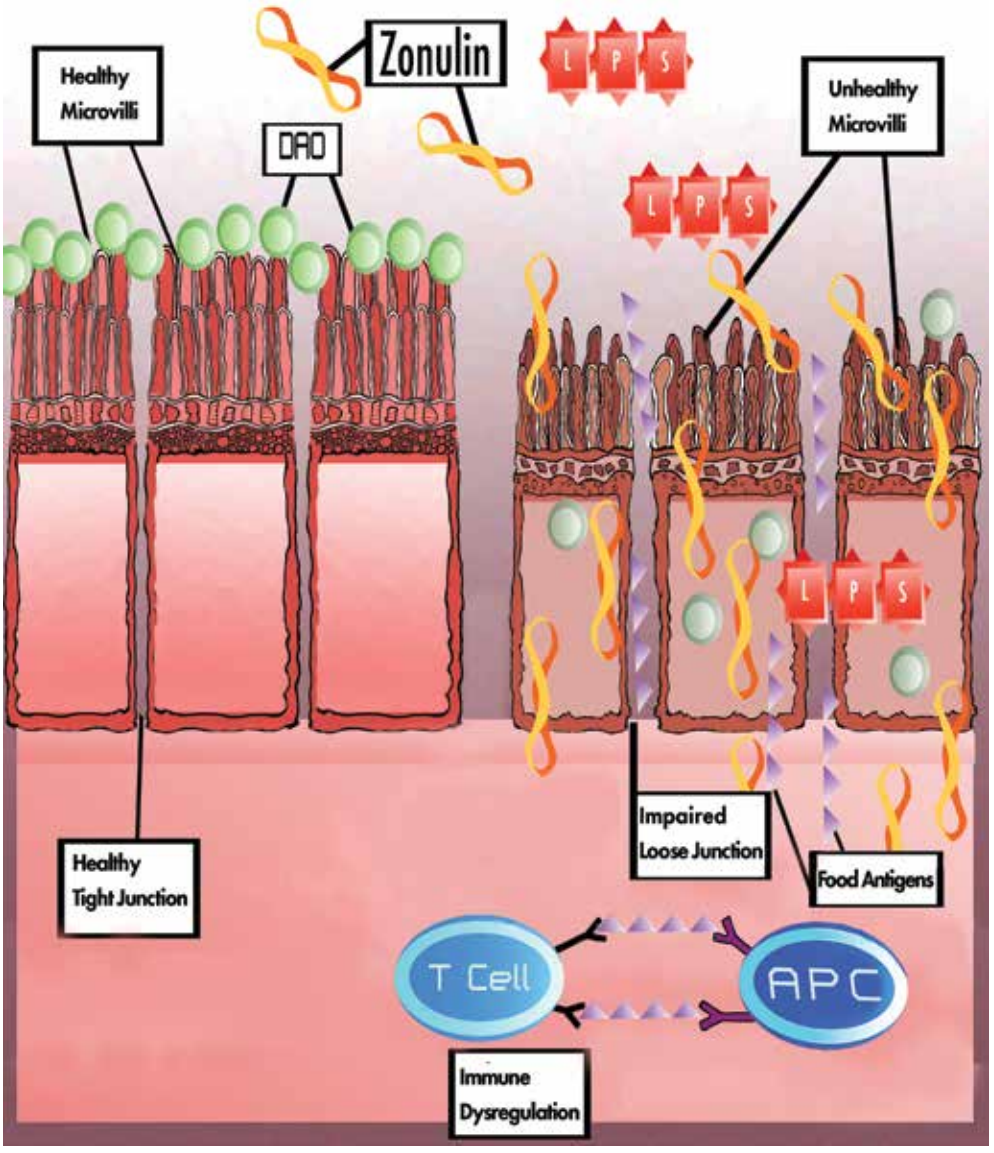
By measuring IgG and C3d, a delayed food-allergy reaction, also referred to as *non-IgE-cell–mediated reaction*, can be identified and quantified regarding the degree of permeability, and that information can direct the clinical intervention. Both facets of the immune system are measured, innate and adaptive, corresponding to C3d and IgG, respectively. C3d is measured in conjunction with IgG1, IgG2, IgG3, and IgG4 to quantify the severity of individuals' inflammatory responses to various offending foods.

In chronic disease states, such as hepatitis and rheumatoid arthritis, IgG levels are markedly high. Serum IgG levels also become elevated when food antigens permeate the intestinal wall at an abnormally high rate.[13] C3d is also elevated in patients with systemic lupus erythematosus, membranoproliferative glomerulonephritis, and hepatic cirrhosis from alcohol misuse.[39-43] As a consequence, increased serum IgG levels to multiple foods implicate intestinal permeability. Moreover, this change perpetuates a vicious cycle, because increased permeability puts a greater antigenic burden on the immune system, which in turn stimulates a hypersensitivity reaction to food antigens and components of the gut's flora. Inflammation continues, further adding insult to injury on the intestinal barrier and sustaining the destruction.[13,44,45]

The use of C3d as a quantifiable parameter in the C3d/IgG test enhances testing accuracy, because it is a reliable biomarker of tissue inflammation, linking the innate and adaptive immune response.[46-48] Its inclusion amplifies the sensitivity and improves the reproducibility of the test.

In 2000, Ross et al[49] demonstrated the ability of C3d to enhance antibody production to establish immunity against an attenuated virus. Hass et al[50] has demonstrated that C3d exerts its adjuvant-like activities by targeting antigens to the C3d receptor (CD21/35), which interact with CD19 to regulate B-cell activation, increasing antibody production.

Figure 3. The illustration shows the components that play a role in the mechanism of immunological tissue damage, which is set in motion by dietary antigens. The antigens gain access to the submucosa by the paracellular route due to intestinal permeability. This permeability is a result of relaxed tight junctions, predominantly caused by zonulin, or to a lesser extent, by lipopolysaccharide (LPS) in the event of bacterial exposure. The food antigen is first recognized and phagocytosed by antigen-presenting cells—dendritic cells, B lymphocytes, or macrophages, whereby the antigen is displayed on the surface of the cell via MHC 2 and presented to the $CD4^+$ helper T cells, the orchestrators of the inflammatory response. Also shown is diamine oxidase (DAO), a protein produced by enterocytes of the intestinal mucosa, whose function is to degrade histamine. In the absence of or through decreased production of DAO, the intestinal mucosa is prone to insults from histaminergic foods, resulting in the impairment of the microvilli.



Abbreviations: DAO, diamine oxidase; LPS, lipopolysaccharide; MHC 2, major histocompatibility complex II; APC, antigen-presenting cell.

Figure 3 depicts how food antigens enter the submucosa of the GI tract via a paracellular passage. This action is influenced by zonulin to incite an inflammatory process producing various symptoms from deposition of immune complexes in the tissue of organs. The conductor of this inflammatory process is the $CD4^+$ helper T cells, particularly the type 1 response that is characterized by the production of interferon gamma (IFN-γ) and TNF alpha (TNF-α) and by antibody production.[12]

Chronic exposure of immune cells to offending foods can result in overstimulation of immune cells that leads to antibody production, inflammatory reactions, and, subsequently failure of self-tolerance, whereby autoimmune disease ensues. In addition, intestinal and extraintestinal symptoms that are nonspecific to a definitive disease state can also occur. An overwhelming number of individuals battling these symptoms either remain undiagnosed, or they are incorrectly treated. Symptoms may be debilitating to the

patient, both physically and financially, and result in a loss of productivity. Table 2 enumerates the financial impact of several symptoms on the health care system in the United States.

MATERIALS AND METHODS

To determine its efficacy in determining each participant's food sensitivities, helping the medical practitioner to manage his or her various symptoms, the study used the C3d/IgG test.

Participants

Cases included in this study were selected from the database of patients seen at the Progressive Medical Center who presented with symptoms that included bloating; abdominal pain; diarrhea; constipation; fatigue; migraines; headaches; insomnia; cognitive dysfunction, such as poor memory or concentration; depression; and anxiety, myalgia, joint pain, sinusitis, and urticarial, which are known to be associated with food allergies. Additional criteria included as part of the chart review of the selected patients entailed a minimum of 2 C3d/IgG tests performed within 9 to 12 months of each other. Through the Progressive Medical Center authorization form, patients released their protected health information for research purposes.

The population of 30 participants ranged in age from 7 to 71 years and consisted of 9 males and 21 females.

Procedures

Each patient's medical record was analyzed, as available, from 2009 to 2013 to determine his or her chief complaints. At baseline and after an average of 10.7 months on a dietary regimen instituted during treatment, 2 C3d/IgG tests were performed on each patient's serum by the method of indirect enzyme-linked immunosorbent assay (ELISA).

A total of 132 foods were tested, including 8 of the most highly allergenic foods: milk; eggs; soy; fish; crustaceans (crab, lobster, shrimp); wheat; peanuts; and tree nuts. The method used for analysis of patients' blood samples was the ELISA. Samples were collected from the antecubital vein of a patient's preferred arm. Collection was performed by a phlebotomist competent in using serum separation tubes. The blood sample was allowed to sit for 10 minutes at room temperature and then was subjected to a relative centrifugal force of 3200 g for 20 minutes. Finally, the serum fraction was collected.

Serum samples were stored at -20°C for optimal stability. On the first day of sample preparation, all reagents and the microwell plates were brought to room temperature before use. The ELISA plates were coated with a microarray of the 132 food antigens. The foods used were organic and processed and were standardized to enable the coating of the ELISA plates. The plates and all the reagents were manufactured by Brendan Bioscience LLC (Hopedale, MA, USA) under patent No. 8 309 318. The serum sample was diluted 1:10 with 1× buffer—phosphate-buffered saline (PBS), pH 7.4, containing 0.5% bovine serum albumin (BSA) and 0.05% Tween 20—and gently mixed on a rotating rocker platform to ensure even distribution.

Table 2. Various Symptoms and Their Impacts on US Health Care Costs[47-52]

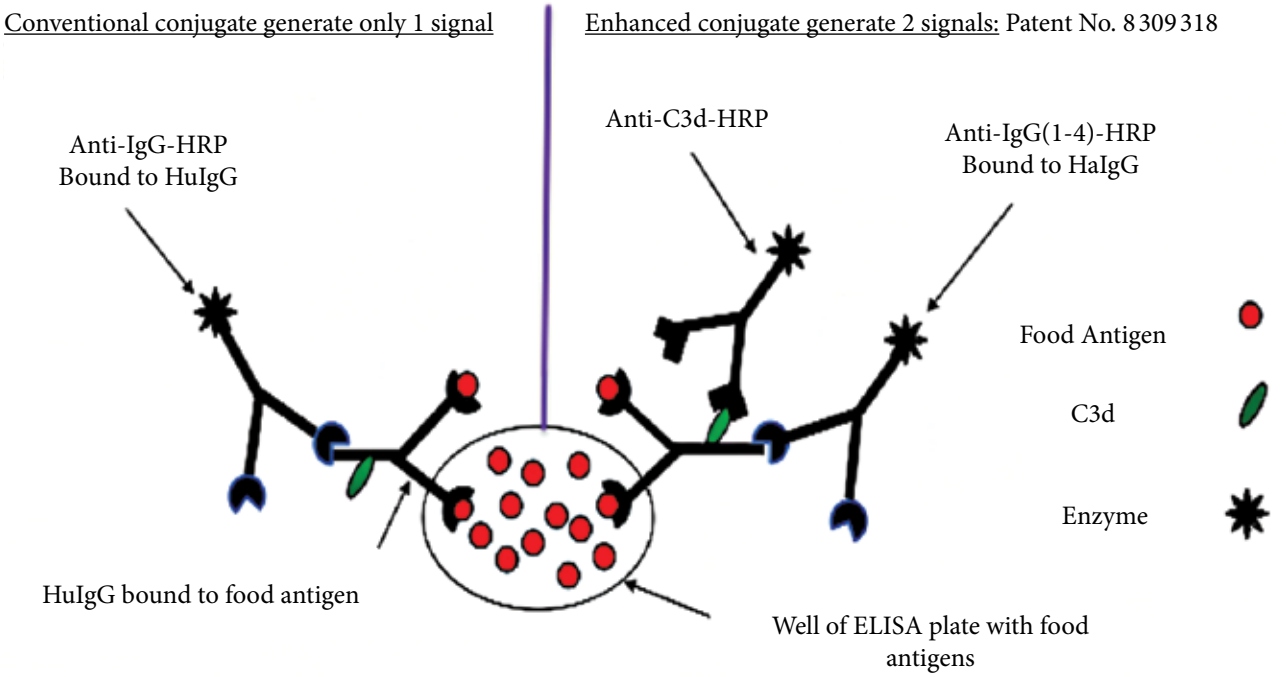
Symptoms	Annual Total, Direct, and Indirect Costs
Migraine	$12 billion
Atopic eczema	$364 million
Depression	$44 billion
Irritable bowel syndrome	$30 billion
Insomnia	$30 billion-$35 billion
Pain	$560 billion-$635 billion

Afterward, a portion of each patient's serum sample was added to each well (50 µL/well) of the 96-microplate, with the exception of the last 4 wells; 50 µL of 1× buffer was added in columns 11 and 12, reserved for the standard. The microplate was wrapped in parafilm and allowed to incubate for 18 to 20 hours. The standard curve used was obtained from the semiquantitative measure of human IgG (HuIgG): 0 ng, 25 ng, 7.5 ng, and 1.25 ng to standardize inter- and intra-assay readings.

During incubation, antibodies from the sample bind to food antigens on the ELISA plates. On the second day after application of the serum, the plates were washed 3 times with 1× buffer solution—phosphate-buffered saline (PBS), pH 7.4 containing 0.5% BSA and 0.05% Tween 20—to remove unbound antibody. Then the conjugate was added to each well (50 µL/well), including the last 4 wells in columns 11 and 12 that were designated for the standard. Next, the samples were allowed to incubate for 65 minutes. The conjugate—a secondary antibody, also known as the *labeled antibody*—contained monoclonal antihuman-IgG horseradish peroxidase (HRP) and monoclonal antihuman-C3d HRP.

Figure 4 highlights the distinctive features of the Ig/C3d test compared with a typical IgG test. The conjugate binds to the fragment's crystallizable region, which is conserved among all subclasses of HuIgG (1-4) and among HuIgG-C3d–containing immune complexes, therefore contributing to the specificity (lower cross-reactivity) of the test. Upon completing the 65-minute incubation with the secondary antibody, the plates were washed 2 times with 1× buffer solution to remove unbound conjugate. Then a chromogenic substrate—1 mg/mL o-phenylenediamine in 100 mM citrate, pH 5.0, 5 µL of hydrogen peroxide per 30 mL—and an enzyme catalyst mixture were added to each well (200 µL/well), including those designated as standards. This step of the assay was both light- and time-sensitive, requiring only 15 to 20 minutes of incubation. Last, 3N sulfuric acid was added to each well (100 µL/well) to stop the reaction. Adding the sulfuric acid brought each well, including those designated as standards, to a total volume of 300 µL. Plates were read at an absorbance of 492 nm using the Epoch microplate spectrophotometer (Biotek, Winooski, VT, USA) to visualize the bound, HuIgG, and HuIgG-C3d immune complexes. After analysis of the data, confidence limits were set that ensure a probability of

Figure 4. Comparison of conventional conjugate. Anti-IgG with enhanced conjugate: anti-IgG (1-4) and anti-C3d. The figure illustrates the differences between the conventional IgG and the IgG/C3d test.



Abbreviations: IgG, immunoglobulin G; HRP, horseradish peroxidase; Hu, human; ELISA, enzyme-linked immunosorbent assay.

≥95% that a sample was either positive or negative for a particular food antigen. The assay was conducted according to standard operating procedures.

Intervention

Using the baseline C3d/IgG test, foods causing elevated anti-C3d/IgG, with the exception of ones causing mild reactions, were identified and eliminated from each patient's diet. The offending foods were identified from the elevated C3d/IgG levels against the various food antigens in patients' sera. Each patient was made aware of the high-sensitivity foods through an official report identifying the list of foods to which they were reactive, along with the food's level of severity, and was advised to make appropriate dietary changes to avoid them. Afterward, patients spoke with a certified dietician to obtain ideas on establishing a new dietary plan, which excluded the reactive foods. Progress was obtained from each patient's chart, from reports documented by their physician during office visits, and messages sent via e-mail that were transcribed to a hard copy. Patients also underwent detoxification protocols and used various oral supplements intended to strengthen the GI mucosal lining, such as glutamine.

Outcome Measures

Changes in patients' symptoms were obtained by physicians during office visits, asking them to quantify how much their chief complaint(s) had improved in the period from baseline C3d/IgG testing to their second test. The second test was always done at least 6 months after the baseline test, because IgG exhibits a half-life of approximately 21 days, with residual effector functions of approximately 2 to 3 months. Further, IgG, combined with an antigen, forms an immune complex and may remain in circulation for an extended period. The amount of time ultimately depends upon the magnitude of the antigen load and the efficiency of the complement system in clearing immune complexes.[8] As in the case of cow's milk allergy, IgG antimilk can last for up to 9 to 12 months.[51]

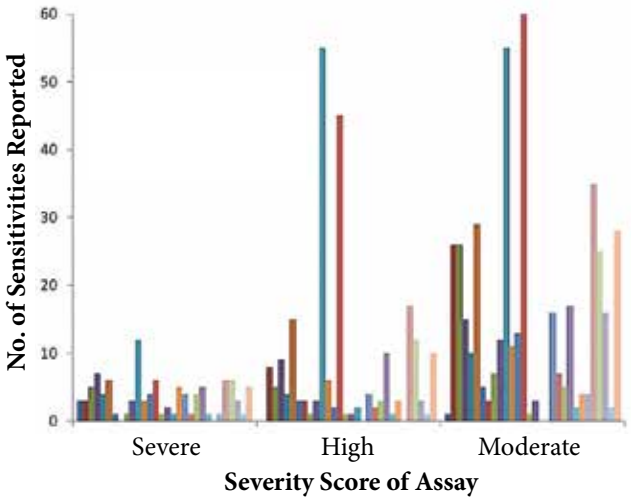
RESULTS

Patients' antibody production related to the offending foods decreased, and their symptoms either subsided or resolved. Among the 30 patients included in the study, 93% demonstrated sensitivity to 1 of the 8 foods that account for more than 90% of all allergic reactions that are documented worldwide. The 8 foods are cow's milk; eggs; fish (all species of finfish); crustaceans (lobsters, shrimp, crabs); peanuts; tree nuts (almonds, cashews, walnuts); soybeans; and wheat.

Initial and follow-up C3d/IgG tests were compared. The average time between the tests was 10.7 months. Figure 5 shows the scores of the initial C3d/IgG tests among all patients; the number of foods scored in the severe, high, and moderate ranges is reported. Figure 6 shows the corresponding scores for the second C3d/IgG test among all patients. The reduction of detected C3d/IgG sensitivity is clearly illustrated in the changes between Figure 5 and Figure 6.

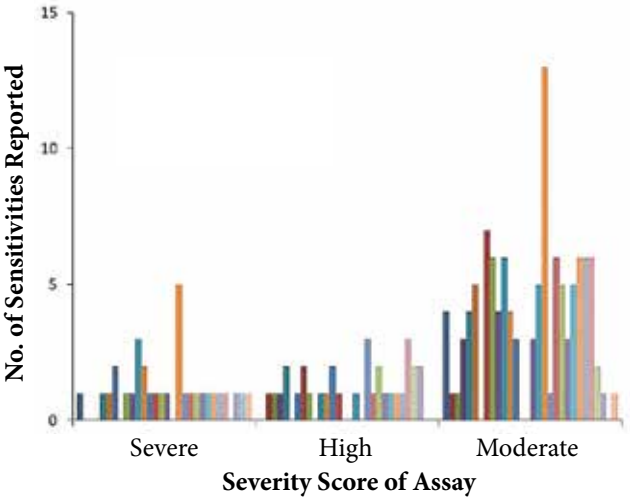
In some cases, a third test was performed on patients (n = 4). The results of the third test indicated that the

Figure 5. Scores of the baseline C3d/IgG tests across all patients. The number of foods scoring in the severe, high, and moderate ranges is reported.



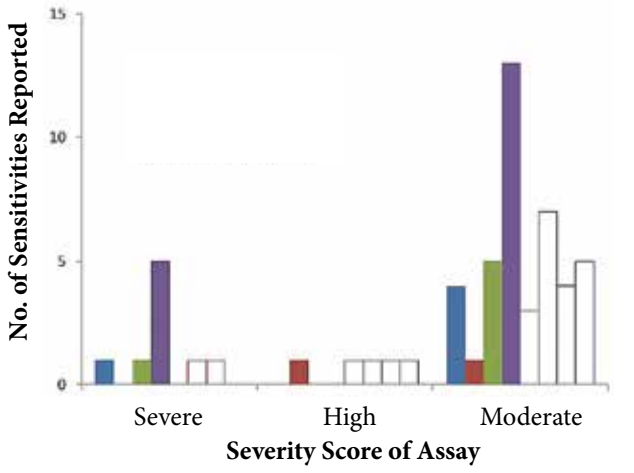
Note: Each colored bar represents a specific patient.

Figure 6. Scores of the second C3d/IgG test across all patients, an average of 10.7 mo later.



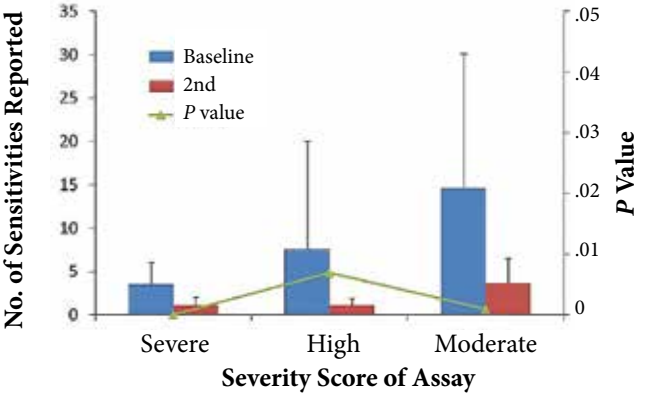
Note: Each colored bar represents a specific patient.

Figure 7. C3d/IgG test results for a subset of participants for whom a third test was also performed.



Note: Each colored bar represents a specific patient; Filled bars are the second test, and open bars are the third test.

Figure 8. Reduction of reported sensitivities. A summary of the average, standard deviations, and *P* values for C3d/IgG sensitivity scores across the baseline and second tests for paired patient results. Clinical manifestations were reduced from the baseline to the dates of the second test.



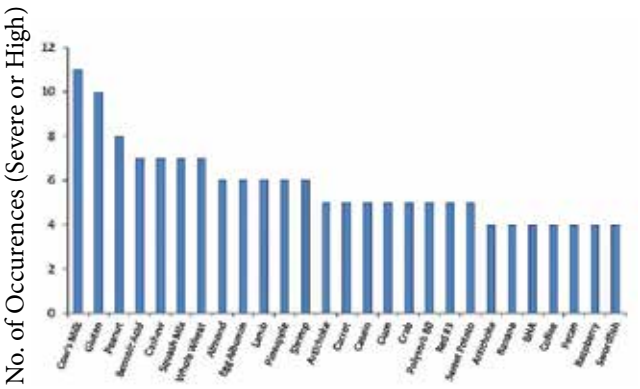
reduction in C3d/IgG sensitivity was maintained past the second test, as the dietary changes continued. Figure 7 shows the results of the second and third tests, respectively.

The reduction of detected C3d/IgG sensitivity between the initial and second test is again clearly illustrated in Figure 8, which summarizes the average and standard deviations across the 2 data sets. A *t* test statistical analysis using the 2-paired samples between the first and second tests was run to determine if the difference was significant. The *P* values were .000002, .007, and .001 for the severe, high, and moderate test results between the baseline and second pair. A *P* value below .05 indicates a strong presumption that the difference in the results was significant. Hence, the difference between the initial and second test results can be strongly presumed to be significant. Table 3 highlights the reduction in the number of symptoms that were reported by patients in the baseline test compared with those reported after the second test was performed as a result of the elimination of severely, highly, and moderately reactive foods.

Table 3. Scores of the Baseline and Second C3d/IgG Tests Across All Patients

	Baseline			Second		
No.	Severe	High	Moderate	Severe	High	Moderate
1	3	0	1	1	0	4
2	3	8	26	0	1	1
3	5	5	26	0	1	1
4	7	9	15	0	1	3
5	4	4	10	1	2	4
6	6	15	29	1	0	5
7	1	3	5	2	1	0
8	0	3	3	0	2	7
9	1	1	7	1	1	6
10	3	3	12	1	0	4
11	12	55	55	3	1	6
12	3	6	11	2	1	4
13	4	2	13	1	2	3
14	6	45	61	1	1	0
15	1	1	1	1	0	0
16	2	1	3	1	0	3
17	1	2	0	0	1	5
18	5	0	0	5	0	13
19	4	4	16	1	3	1
20	1	2	7	1	1	6
21	4	3	5	1	2	5
22	5	10	17	1	1	3
23	1	1	2	1	1	5
24	0	3	4	1	1	6
25	1	0	4	1	1	6
26	6	17	35	1	3	6
27	6	12	25	0	2	2
28	3	3	16	1	2	1
29	1	1	2	1	0	0
30	5	10	28	1	0	1

Figure 9. Food antigen occurrence (severe or high). Foods that ranked as severe or high reactivity with the greatest frequency (ie, >2 occurrences). milk; eggs; soy; fish; crustaceans (crab, lobster, shrimp); wheat; peanuts; and tree nuts.



The foods that demonstrated the greatest reactivity in the C3d/IgG test are shown in Figure 9 (ie, the foods that ranked as severe or high in reactivity with the greatest frequency).

DISCUSSION

The aim of the study was to assess the efficacy of C3d/IgG testing to guide food elimination to decrease various symptoms of patients. Participants who complied with the avoidance of anti-C3d/IgG dietary antigens demonstrated a statistically significant reduction in C3d/IgG sensitivity and a marked reduction in the symptoms that they had reported before beginning the diet. In addition to the elimination of the offensive foods, the research team had recommended that patients perform a detox and take supplements to enhance the rebuilding of the GI wall's integrity. The current study's results corroborate those of other studies, showing that elimination of the offending foods identified to be inflammatory using the C3d/IgG tests was effective in mitigating and treating chronic symptoms that are characteristic of various disease states.[52-55]

The high level of significance found in the current intervention was likely obtained through the use of a combined method of testing C3d and all 4 types of IgG simultaneously to determine the foods that the participants should avoid. With the complement accounting for the preponderance of the inflammation and causing patients' symptoms, the measurement of C3d, a cleavage fragment of C3, shows promise as a useful inflammatory biomarker in clinical applications. For example, patients with GI symptoms, involving the pain, diarrhea, constipation, and bloating typical of irritable bowel syndrome or the frequent headaches typical of migraine, observed noticeable improvement when placed on an individualized diet with elimination of the offending foods. This study supports the validity of the

C3d/IgG tests in identifying the severity of food allergies and its effectiveness as a tool in clinical intervention.

Despite the success of the test in showing the correlation between the magnitude of the C3d/IgG response and the reduction of symptoms after avoidance of the foods testing positive, unanswered questions remain. The C3d/IgG tests measures type 2 (IgG) and type 3 (IgG and complement) delayed reactions, whereas intradermal testing measures type 1 (IgE) immediate reactions that were not evaluated in this study. What would the results of testing C3d/IgG and IgE together? Some interesting data from Berrens et al[56] have shown that both IgG and IgE coexist in type 1 allergy; therefore, it would be reasonable to assume that type 1 and type 2 hypersensitivity reactions would also have an IgE component. In an elegant study by Aristo Vojdani,[57] the coexistence of IgE, IgM, IgA, and IgG specific for a particular food antigen was demonstrated. Moreover, Vojdani showed that this coexistence occurred for several different varieties of food antigens. The net result may be that simultaneous parallel testing of more than 1 antibody class would be superior to testing only C3/IgG alone. This methodology is worth considering for future prospective studies.

Another question is also worth addressing. Can it be proven with certainty that a patients' symptoms are immune-modulated (ie, the result of food allergy) versus nonimmune-modulated (ie, the result of food intolerance)? The answer is no, and this answer exposes the drawbacks of the current study. The term *food allergy* identifies an adverse health effect to an immune-mediated mechanism involving antibodies (IgE or IgG) or helper T cells.[58,59] On the other hand, food intolerance is an adverse health effect that does not involve an immune-mediated response.[58]

However, it is plausible in the current study that patients' symptoms were immune-modulated based on the time of onset of the symptoms and the inability of the patients to identify the food responsible for their symptoms, a strong characteristic of delayed reaction. Unlike a delayed reaction, symptoms caused by food intolerance appear relatively quickly, enabling a patient to link his or her symptoms to the causative foods. Food intolerances are caused by multiple factors. The most common are enzyme deficiency (eg, lactase deficiency, the cause of lactose intolerance); malabsorption secondary to inflammatory bowel disease; and pharmacological food reactions (eg, from tyramine in aged cheese and sulfites in preservatives). Food intolerances are predominantly associated with GI symptoms. The results from the current study indicate that patients with diverse symptoms, such as migraines, cognitive dysfunction, depression, and GI disorders improved or resolved after omission of the allergic foods that were identified with the C3d/IgG tests. To further substantiate the validity of this test, the research team anticipates implementing an oral-challenge phase in a new prospective study. This test would allow the team to determine whether reintroduction of the foods triggering the food allergies, identified by the C3d/IgG test, would cause the symptoms to reappear or worsen. This testing would further extend the study's findings.

Confounding variables in the current study included (1) some patients took pharmaceutical agents to alleviate symptoms; (2) socioeconomic challenges prevented patients from obtaining different foods and sustaining themselves on the recommended dietary regimen; (3) the level of the patient's knowledge and interest about their symptoms and disease and therapy affected their compliance; (4) symptom data were collected through patients' responses, which are subjective. Further prospective studies are warranted, with larger patient populations and a revised methodical approach to control for confounding factors and biases.

With numerous studies documenting the pathogenesis of various diseases associated with intestinal permeability, more attention should be placed on the contributive factors of this mechanism. In addition to antigenic foods, microbial pathogens, alcohol, stress, and inflammatory mediators are contributive factors to intestinal permeability. According to the United States Department of Agriculture (USDA), a study conducted in the course of 2006 to 2008 showed that the average American aged 15 years and older spent approximately 2.5 hours eating or drinking each day.[60] In another article released by the USDA's Economic Research Service, the average American consumed approximately 2000 pounds of food in 2011.[61] These data further provide a reference for how food could contribute to many symptoms and conditions, due to the sheer magnitude consumed, which constitutively keeps the immune system activated and perpetuates chronic inflammation. Given all the stratagems and treatment plans put forth in various formulations to treat symptoms of bloating, abdominal pain, diarrhea, constipation, fatigue, migraines, headaches, cognitive dysfunction, depression, myalgia, joint pain, sinusitis, eczema, and urticaria, this study has demonstrated strong, supportive data to warrant the use of C3d/IgG tests in clinical interventions in managing patients with diverse symptoms and in lowering total inflammatory load.

CONCLUSION

Patients who complied by avoiding C3d/IgG antifood antigens experienced significant improvements in their chief complaints. The C3d/IgG test may be an important clinical tool in the management of symptoms related to food sensitivities, such as bloating, abdominal pain, diarrhea, constipation, fatigue, migraines, headaches, cognitive dysfunction, depression, myalgia, joint pain, sinusitis, eczema, and urticaria. Overall, patients' food sensitivities were proportional to their symptoms; for example, high sensitivity would relate to a high level of symptoms. Patients felt better when their C3d/IgG food sensitivities were low, demonstrating that food removal based on the C3d/IgG test was an effective approach in patient care.

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