moleculera labs

Cunningham Panel™ Testing Results

Patient Name: Qemali, Albana
Patient DOB: 11/13/2013
Patient ID Number: V0043AQ
Date of Test Report: 08/24/2021

PATIENT REPORT

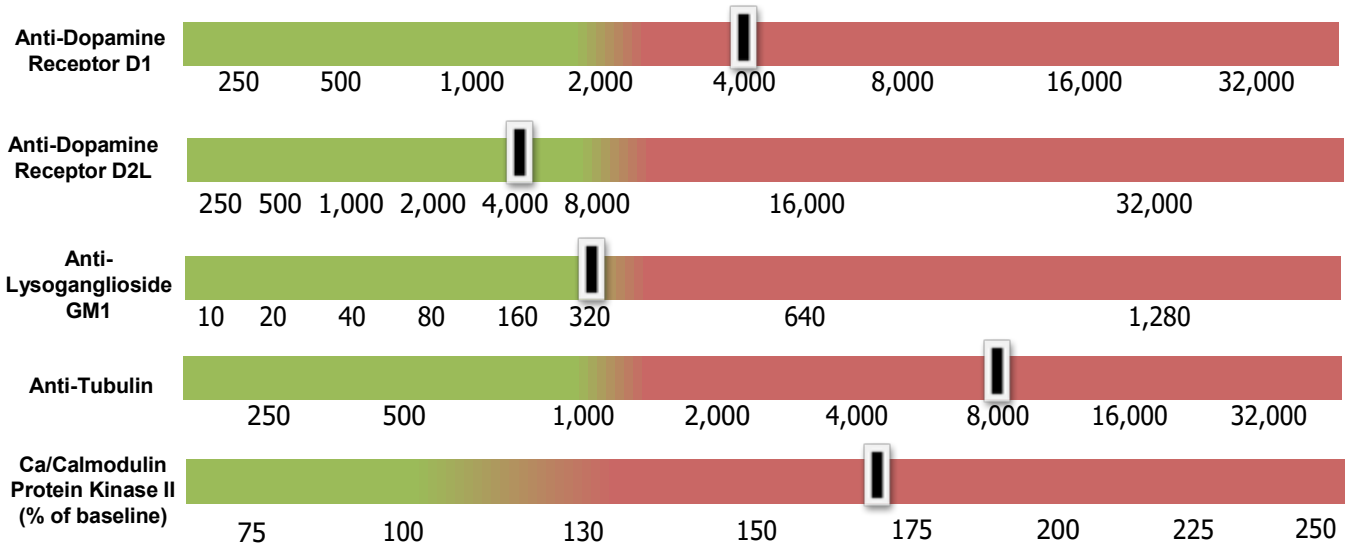
Submitting Prescriber: Volodymyr Stefanyshyn, Doctor
Date of Collection: 08/02/2021
Date of Receipt: 08/09/2021

LABORATORY TEST RESULTS COMPARED TO NORMAL RANGES

	Anti-Dopamine Receptor D1 (titer)	Anti-Dopamine Receptor D2L (titer)	Anti-Lysoganglioside GM1 (titer)	Anti-Tubulin (titer)	CaM Kinase II[1] (% of baseline)
Patient Result	1:4,000	1:4,000	1:320	1:8,000	170
Normal Ranges	500 to 2,000	2,000 to 8,000	80 to 320	250 to 1,000	53 to 130
Normal Mean	1,056	6,000	147	609	95
INTERPRETATION*	ELEVATED	NORMAL	BORDERLINE	ELEVATED	ELEVATED

***Report Guidance:** If any one (1) or more of these five (5) assay values is elevated, it may indicate a clinically significant autoimmune neurological condition. This is a condition in which the patient's autoantibodies cross-react and are directed against selected neuronal targets which are involved in normal neuropsychiatric and/or motor functions. It is important to note that the degree of elevation in assay values may not necessarily correlate with degree of symptom severity, as any value above normal ranges may correlate with symptomatology.

LABORATORY TEST RESULTS



The Cunningham Panel measures human serum Immunoglobulin G (IgG) levels by Enzyme-Linked ImmunoSorbent Assay (ELISA) directed against: Dopamine D1 Receptor (DRD1), Dopamine D2L Receptor (DRD2L), Lysoganglioside-GM1 (LYSO-GM1) and Tubulin (TUB). ELISA results are determined by measuring the colorimetric intensity at a specific wavelength which is directly proportional to the amount of antibody in the sample. The fifth assay of this panel measures the specific activity of calcium/calmodulin-dependent protein kinase II (CaM KII) induced by the patient serum in cultured human neuronal cell lines compared to controls. This panel measures the level of these antibodies, and the ability of the patient's sera to stimulate CaM KII at a single point in time. Results may vary depending on the patient's condition and status, whether they are on immunosuppressive agents, corticosteroids or other immune modulatory therapy, and the length of time post treatment.

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PANDAS and PANS are clinical diagnoses based upon defined clinical characteristics. The results from the Cunningham Panel are provided as an aid to the physician in their diagnosis of PANDAS or PANS. This test panel is used for clinical purposes and should not be regarded as investigational or for research. This test has been developed and its performance characteristics determined by Moleculera Labs, Inc. It has not been cleared or approved by the U. S. Food and Drug Administration. Moleculera Labs, Inc. and the Cunningham Panel are regulated under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) as qualified to perform diagnostic clinical testing. This test is physician ordered and is meant to be used in conjunction with a complete medical exam. ***There may be additional risk and diagnostic factors not measured by this test.*** The Cunningham Panel and Moleculera are trademarks of Moleculera Labs, Inc. This panel is patented in the US – US Patent 9,804,171 B2 . For more information please call (405) 239-5250 or e-mail us at customerservice@moleculera.com. CLIA Number: 37D2082408; COLA ID: 25744. Joseph Quashnock, PhD, HCLD/CC(ABB), FAACC, Clinical Laboratory Director (755 Research Parkway, Suite 448, Oklahoma City, OK 73104). Anti-Dopamine D1, Anti-Dopamine D2L, Anti-Lysoganglioside GM1, and Anti-Tubulin testing are performed at this location. Reference laboratory: Cunningham Clinical Laboratory, CLIA Number: 37D2052130; COLA ID: 24220. Kenneth Mueller, PhD, HCLD/CC(ABB), FAACC, Clinical Laboratory Director (975 NE 10th Street, Room 215, Oklahoma City, OK 73104). CaM Kinase II[1] indicates that the CaM testing was performed at the 755 Research Parkway location. CaM Kinase II[2] indicates that the CaM testing was performed at the 975 NE 10th Street location.

Reviewing Results

Anti-Neuronal Antibody Results

This report lists four anti-neuronal antibody test results which measure circulating levels of autoantibodies directed against specific neuronal antigens in the patient at the time the specimen was collected. These antigens include: Dopamine D_1 Receptor (DRD1), Dopamine D_{2L} Receptor (DRD2L), Lysoganglioside-GM1 (LYSO-GM1) and Tubulin (TUB). These laboratory results are expressed as the "titer" or final dilution at which an endpoint reaction was observed on an Enzyme-Linked ImmunoSorbent Assay format (ELISA). Eg. If the patient's titer is 1,000 that means that the patent's sample required a 1,000-fold dilution to reach a set endpoint for the particular anti-neuronal antibody measured. Each assay was performed in duplicate against each of the four (4) neuronal antigens. Directly below the patient's titer are the "normal ranges" in normal children without infection measured against each respective antigen. It is possible that "normal ranges" for adults may differ from normal ranges in children.

CaM Kinase II Activation

This laboratory value is a numeric score that reflects the percent of baseline CaM Kinase activity in a human neuronal cell line. Eg. If the patients score was 170, it means that the patient's sample stimulated CaM Kinase activity in a human neuronal cell line, at 170% over baseline activity. Directly below the laboratory value is the "normal range" value measured in normal children without infection. It is possible that "normal ranges" for adults may differ from normal ranges in children. CaM Kinase is an enzyme present in neuronal cells and is part of the activation pathway for the production of dopamine.

Other Factors

The laboratory results reflect the status of the patient at time their specimen was drawn. Certain treatments are believed to impact the results of these tests. These include: systemic steroids, IVIG and Plasma Exchange. For this reason we generally recommend that the patient wait to be tested approximately 6-8 weeks or longer after these treatments. However, it is possible that these treatments may not affect the results to the degree that an autoimmune condition would not be identifiable. Therefore, the timing of testing is best determined by the ordering physician and their assessment of the condition and status of the patient.

What is PANDAS?

PANDAS is an acronym for "Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infection." This condition was first described by Dr. Susan Swedo at the National Institutes of Mental Health in 1998[1]. PANDAS has been described as presenting with an acute onset of obsessive-

compulsive disorder (OCD) and/or motor tics in children with a recent streptococcal infection[1,2]. The clinical existence of PANDAS was published in a 2002 study[2] showing evidence of a temporal association of group A streptococcal tonsillopharyngitis and abrupt onset of OCD in 12 children. In all 12 children, treatment with antibiotics resulted in rapid reduction in OCD symptoms.

PANDAS is classified as one of the conditions on the spectrum of neuropsychiatric disorders caused by group A streptococcal infection and autoimmunity. The first condition described in this spectrum was Sydenham's Chorea[3] which demonstrates considerable clinical and immunological overlap with the presentation and mechanism of action of PANDAS. Studies from the NIH, as early as 1958, reported high rates of OCD behaviors in children with Sydenham's Chorea[4-6]. Neuropsychiatric symptoms predate the choreiform movements in this disorder. In addition, a subgroup of children with the onset of OCD and/or Tics was associated with a recent group A streptococcal infection and did not meet criteria for Sydenham's chorea was identified[7]. This subgroup was subsequently identified as PANDAS[8].

The immunological mechanism of Sydenham's Chorea has been identified as an antibody produced in response to a group A streptococcal infection, which cross-reacts with extra- and intracellular neuronal targets in the basal ganglia[9,10]. Specifically, anti-neuronal IgG antibodies, found in sera of patients with Sydenham's Chorea, target basal ganglia resulting in the disease state[10].
PANDAS appear to have a similar etiopathogenesis as Sydenham's Chorea[11]. The clinical symptoms of OCD and motor tics are reported to follow a child's contraction of a group A beta-hemolytic streptococcal infection, most commonly tonsillopharyngitis. The clinical characteristics of PANDAS are 1) prepubertal onset; 2) appearance of OCD symptoms and/or motor tics; 3) abrupt onset of OCD behaviors or motor tics and episodic course correlating with group A streptococcal infections[1,12,13]. PANDAS has also been reported in children with an existing tic disorder or Tourette's syndrome[14-17]. In this scenario, the baseline frequency of the child's tics may markedly increase when the child has a strep infection and return to baseline rates after antibiotic treatment[18,19].
The neuroanatomical correlates of PANDAS, as with tics, OCD and Sydenham's Chorea, are the basal ganglia and dopamine receptors[8]. Children with PANDAS have been found to have significantly larger basal ganglia structures on MRI than healthy age-matched controls[20]. There is evidence that both the basal ganglia and dopamine receptors are targeted by Group A streptococcal infection-associated autoantibodies in this condition[21-25]. Autoimmune reactions in Group A streptococcal infections are well known to occur in post-streptococcal glomerulonephritis and rheumatic fever[26].

Studies have shown children that meet the clinical criteria for PANDAS have higher levels or circulating antibodies targeting the caudate and putamen neuronal surface antigens in the midbrain compared to children with Tourette's syndrome or other tic disorders[22,23]. Specific antibodies against neuronal surface glycolytic enzymes, lysoganglioside and N-acetyl-β-D-glucosamine, are found to be in higher concentrations and more active in children with tics compared to those children with only Group A streptococcal pharyngitis and without tics[27,28]. Current findings support the hypothesis that there is an abnormal immunological response in those with the clinical symptoms of PANDAS[11,21]. These findings will allow a more specific diagnostic laboratory tool that will allow a more precise and rapid confirmation of PANDAS. Identification of autoantibodies directed against specific neurologic receptors may assist the clinician in making an accurate and timely diagnosis of PANDAS potentially allowing for effective treatment strategies to be implemented.

What is CANS and PANS?

Childhood Acute Neuropsychiatric Symptoms (CANS)[29] and Pediatric Acute-onset Neuropsychiatric Syndrome (PANS)[30] are proposed as a new, broader classification that would expand both the etiological infectious agents and the clinical manifestations, to the current description of PANDAS[30]. This is an important development since there are reported cases of patients fulfilling the clinical criteria of PANDAS but laboratory studies are negative for a recent group A streptococcal infection. Published reports have postulated that stress[31] as well as other types of infections can result in neuropsychiatric conditions,

which include Borrelia burgdorferi (Lyme disease), Mycoplasma pneumonia, herpes simplex, common cold and varicella viruses[32-36].

The classification of CANS is proposed to include individuals that present following an infectious illness, with an acute and dramatic onset of neuropsychiatric symptoms not limited to OCD and tics[29]. Identification of a specific infectious agent may be difficult in certain cases, but the immune response against the infection is proposed to be the cause of the symptoms[10,11]. It has been proposed that CANS may occur at any age and does not result in recurrent episodes of symptom exacerbations[29].

The definition of neuropsychiatric symptoms is broad, but current consensus is that many of the symptoms previously reported in PANDAS be included[29]. CANS would include the acute, post-infectious onset of OCD, anxiety, anorexia, conduct disorders, attentional and concentration deficits, depression, fine motor deficits, emotional lability, hyperactivity, irritability and sleep disorders. These symptoms may be isolated or in combination and not be the result of another medical condition.

Is There Treatment for PANDAS and PANS?

Reported treatments include eradicating the underlying infectious agent and reducing the immunological response that has been reported to cause the neuropsychiatric symptoms. The literature indicates that there are several proven effective PANDAS treatments that result in prompt resolution of both OCD and motor tics. Antibiotic treatment addresses the underlying group A streptococcal, or other bacterial infections and therefore reduce the immune system responses. Additional treatment methods specifically target the autoantibodies that cause PANDAS. Both IVIG and plasma exchange have been proven to be effective in significantly reducing symptoms in randomized control studies[37].

The effectiveness in treating group A streptococcal infections, with subsequent resolution of PANDAS symptoms, has been shown in several studies. One study reports confirmed resolution of OCD symptoms and tics following antibiotic treatment and subsequent negative throat cultures in patients the group-A streptococcal tonsillopharyngitis[2]. There are some children that are defined as group A streptococcal carriers and are asymptomatic, without signs of tonsillopharyngitis. These individuals may continue to generate the streptococcal related antibodies and harbor organisms in their nasopharynx[38-40].

With current opinion that post-infectious autoimmunity is the cause of neuropsychiatric symptoms, then it was hypothesized that plasma exchange and intravenous immunoglobulin (IVIG) treatment may be an effective treatment. There are published reports of successful resolution of symptoms associated with PANDAS treatment with plasma exchange and IVIG treatment[37,41]. There is one published case series of 4 adult patients presenting with an acute onset of OCD and tics following a documented group A streptococcal infection[42]. These patients would fulfill the criteria for CANS and were treated with plasmapheresis. In a separate placebo controlled study, adults with non- specific tics disorders did not show statistical improvement in symptoms with IVIG treatment[43].

It is reported that repeated group A streptococcal infections result in recurrent, episodic exacerbations of clinical symptoms[8] in PANDAS. During the affected period, the children may manifest concurrent new onset fine motor deterioration and personality/behavioral changes including abnormal movements of the head, neck and extremities. A range of personality and behavioral changes including hyperactivity, irritability, attentional problems and oppositional behaviors are also associated with the onset of PANDAS in children[13].

Although there are reports of successful resolution of PANDAS symptoms with antibiotics, plasma exchange and IVIG therapy, there are still concerns regarding the proper identification of patients that fulfill criteria for PANDAS and are candidates for therapy[29,44]. Early diagnosis and prompt treatment may prevent the significant social, emotional and educational toll that OCD and tics have on affected children and their families. Unnecessary stress and anxiety will be prevented with appropriate diagnosis and treatment of PANDAS.

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Evaluation of the Cunningham Panel™ in pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (PANDAS) and pediatric acute-onset neuropsychiatric syndrome (PANS): Changes in antineuronal antibody titers parallel changes in patient symptoms

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ABSTRACT

Objective: This retrospective study examined whether changes in patient pre- and post-treatment symptoms correlated with changes in anti-neuronal autoantibody titers and the neuronal cell stimulation assay in the Cunningham Panel in patients with Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infection (PANDAS), and Pediatric Acute-onset Neuropsychiatric Syndrome (PANS).

Methods: In an analysis of all tests consecutively performed in Moleculera Labs' clinical laboratory from April 22, 2013 to December 31, 2016, we identified 206 patients who were prescribed at least one panel prior to and following treatment, and who met the PANDAS/PANS diagnostic criteria. Patient follow-up was performed to collect symptoms and treatment or medical intervention. Of the 206 patients, 58 met the inclusion criteria of providing informed consent/assent and documented pre- and post-treatment symptoms. Clinician and parent-reported symptoms after treatment or medical intervention were categorized as "Improved/Resolved" (n = 34) or "Not-Improved/Worsened" (n = 24). These were analyzed for any association between changes in clinical status and changes in Cunningham panel test results. Clinical assay performance was also evaluated for reproducibility and reliability.

Results: Comparison of pre- and post-treatment status revealed that the Cunningham Panel results correlated with changes in patient's neuropsychiatric symptoms. Based upon the change in the number of positive tests, the overall accuracy was 86%, the sensitivity and specificity were 88% and 83% respectively, and the Area Under the Curve (AUC) was 93.4%. When evaluated by changes in autoantibody levels, we observed an overall accuracy of 90%, a sensitivity of 88%, a specificity of 92% and an AUC of 95.7%. Assay reproducibility for the calcium/calmodulin-dependent protein kinase II (CaMKII) revealed a correlation coefficient of 0.90

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($p < 1.67 \times 10^{-6}$) and the ELISA assays demonstrated test-retest reproducibility comparable with other ELISA assays.

Conclusion: This study revealed a strong positive association between changes in neuropsychiatric symptoms and changes in the level of anti-neuronal antibodies and antibody-mediated CaMKII human neuronal cell activation. These results suggest there may be clinical utility in monitoring autoantibody levels and stimulatory activity against these five neuronal antigen targets as an aid in the diagnosis and treatment of infection-triggered autoimmune neuropsychiatric disorders. Future prospective studies should examine the feasibility of predicting antimicrobial and immunotherapy responses with the Cunningham Panel.

1. Introduction

The biology behind many neuropsychiatric conditions remains elusive, but recent studies implicate immune dysregulation in some cases, particularly the presence of autoantibodies targeting neural tissue. Numerous studies have linked movement, behavior, and neuropsychiatric disorders to infections and the production of anti-neuronal autoantibodies (Kirvan et al., 2003; Cox et al., 2013; Kirvan et al., 2006a, 2006b; Kirvan et al., 2007; Singer et al., 2015; Brimberg et al., 2012; Cunningham, 2012, 2014; Garvey et al., 1998; Garvey et al., 1999; Gause et al., 2009; Greenberg, 2017; Murphy et al., 2007; Perlmutter et al., 1998; Taranta and Stollerman, 1956; Taranta, 1959; Swedo, 1994; Cox et al., 2015; Rhee and Cameron, 2012). Infectious triggers such as streptococcal and other infections, along with anti-neuronal autoantibodies similar to those associated with Sydenham chorea have been linked to childhood obsessive-compulsive disorder (OCD) and/or tics (Kirvan et al., 2003; Kirvan et al., 2006a, 2006b; Swedo, 1994; Swedo et al., 1998; Swedo et al., 1997; Swedo et al., 1993; Swedo et al., 1989). When symptom onset is abrupt, Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal Infection (PANDAS) and Pediatric Acute-onset Neuropsychiatric Syndrome (PANS) are two disorders used to describe these symptoms. The diagnostic criteria for PANS is defined as an abrupt onset of OCD *or* severely restricted food intake, and the presence of at least two of the following seven categories: (1) anxiety; (2) emotional lability and/or depression; (3) irritability, aggression, and/or severely oppositional behaviors; (4) behavioral (developmental) regression; (5) deterioration in school performance (related to attention deficit hyperactivity disorder-like symptoms, memory deficits, cognitive changes); (6) sensory or motor abnormalities; (7) somatic signs and symptoms, including sleep disturbances, enuresis, or increased urinary frequency (Swedo et al., 1997; Chang et al., 2015).

Since Sydenham chorea has a well-established biological mechanism connecting streptococcal infections with autoimmune-induced neuropsychiatric symptoms, it has been used as a biological model to better understand PANDAS and PANS (Cunningham, 2012, 2014). Central nervous system (CNS) autoimmune targets that were originally identified through multiple studies in patients with Sydenham chorea were applied to patients with PANDAS. Targets identified to overlap between Sydenham chorea and PANDAS were used to develop the Cunningham Panel, a set of blood tests utilized for measuring immune dysfunction, related to neuropsychiatric conditions associated with an infectious trigger.

1.1. Biological basis of assays in the Cunningham Panel

The Cunningham Panel includes five assays performed on a serum sample from blood collected in glass tubes free of any excipients. Four assays measure human serum Immunoglobulin G (IgG) levels by Enzyme-Linked ImmunoSorbent Assays (ELISA) directed against 1) Dopamine D1 Receptor (D1R), 2) Dopamine D2L Receptor (D2LR), 3) Lysoganglioside-GM1 and 4) Tubulin. A fifth assay is a cell stimulation assay which measures the ability of a patient's serum immunoglobulin G (IgG) to stimulate calcium/calmodulin-dependent protein kinase II (CaMKII) activity in human neuronal cells. We briefly review the biological basis for the selection of these five assays below. (See Fig. 1)

1.1.1. Anti-Lysoganglioside GM1 assay

Commercially available measurements of human group A streptococcal (GAS) antibodies (i.e., ASO and/or anti-DNase B antibodies) are sufficient for measuring GAS reactivity, but do not provide any relationship to GAS-related autoimmune reactivity and the CNS. Using human monoclonal antibodies derived from Sydenham chorea patients, Cunningham and Kirvan observed significant cross-reactivity against neurons in the human basal ganglia (Kirvan et al., 2003) and against *N*-acetyl-beta D-glucosamine (GlcNAc), the major constituent of the GAS cell wall, a carbohydrate epitope. They observed strong cross-reactivity against the neuronal surface antigen lysoganglioside GM1 but not against other gangliosides (Kirvan et al., 2003; Kirvan et al., 2007). Lysoganglioside GM1 antigen also blocked binding of these autoantibodies to human caudate and putamen, demonstrating specific neural targets in the brain center known to be involved in movement disorders such as chorea, tics and motor stereotypies, whereas these serum autoantibodies receded to normal levels during convalescence. In addition, IgG derived from the serum or cerebrospinal fluid (CSF) of Sydenham chorea and PANDAS patients was found to target human caudate and putamen brain tissue, and this reaction could be inhibited by lysoganglioside GM1 (Kirvan 2006; Kirvan et al., 2006a, 2006b).

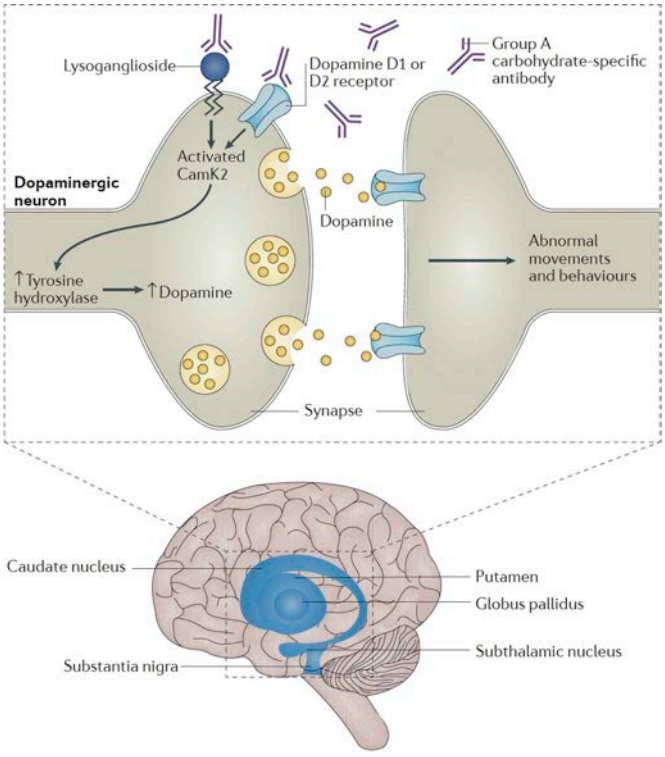


Fig. 1. Autoantibodies directed against Dopamine D1/D2 Receptors and Lysoganglioside GM1, and CaMKII activation. Reprinted by permission from Springer Nature: Nature Reviews Disease Primers, Carapetis et al. Volume 2," Acute Rheumatic Fever and Rheumatic Heart Disease." 2016.

1.1.2. CaMKII cell stimulation assay

Integrated into the Cunningham Panel is the measurement of CaMKII activation which is mechanistically important since activation increases the activity of tyrosine hydrolase, an enzyme that produces dopamine, resulting in increased dopamine output, a key neurotransmitter involved in movement disorders. Both IgG from sera and CSF of PANDAS patients were found to signal activation of CaMKII in human neuronal cells (Kirvan et al., 2006a, 2006b) . Supporting the specificity of GAS antibodies causing CaMKII activation, it was demonstrated that activation was blocked by streptococcal-associated GlcNAc or by depleting immunoglobulins from serum and CSF by affinity column adsorption (Kirvan et al., 2006a, 2006b). Additional data showed that CaMKII was activated 202% above basal level in Sydenham chorea and PANDAS patients during the acute phase, whereas convalescent serum collected in the absence of chorea showed no significant i ncrease i n C aMKII a ctivity (Kirvan 2 003) . F inally, additional studies have shown that patient symptom improvement was associated with a reduction in CaMKII activation.

CaMKII is also involved in the regulation of *N*-methyl-D-aspartate (NMDA) receptor excitability via glutamate transmission (Hell, 2014) which is being recognized in syndromes which include OCD, tics, and Tourette Syndrome (Marsili et al., 2017) , and being recognized as a treatment target in OCD (Laoutidis et al., 2016). Mutations in CAMK2A and CAMK2B, the genes that code for CaMKII, have been associated with intellectual disability (Kury et al., 2017) and ASD-related behaviors such as hyperactivity, social interaction deficits, and repetitive behaviors (Stephenson et al., 2017). Other studies have linked CaMKII to the pathogenesis and symptoms in a variety of mental and neurological illnesses, including learning disorders, cognitive impairment, schizophrenia (Robison, 2014; Kury et al., 2017), ischemia, Alzheimer's disease (Ly and Song, 2011; Ghosh and Giese, 2015) , epilepsy (Zhang et al., 2014; Robison, 2014) and Parkinson's disease (Zhang et al., 2014; Zaichick et al., 2017).

1.1.3. Anti-dopamine D1 and D2L receptor

The inclusion of dopamine D1 and D2L receptors as targets arose from studies that demonstrated autoantibodies directed against dopamine D1 and D2L receptors correlated with various neuropsychiatric symptoms (Ben-Pazi et al., 2013) . Two types of anti-neuronal human antibodies against the dopamine receptors induced an increase in dopamine neurotransmitter release (Kirvan et al., 2006a, 2006b). D1 and D2L receptor antibodies were elevated in patients with Sydenham chorea and PANDAS compared to controls (Kirvan et al., 2003; Kirvan et al., 2006a, 2006b; Kirvan et al., 2007; Cox et al., 2013; Cox et al., 2015; Cunningham and Cox, 2016) , and are likely important in pathogenesis of neuropsychiatric diseases associated with infection (Singer et al., 2015; Brimberg et al., 2012).

1.1.4. Anti-tubulin

Human monoclonal antibodies derived from patients with Sydenham chorea, reacted with human caudate and putamen brain sections and reactivity was blocked by anti-tubulin monoclonal antibodies. The reactive epitope of these brain proteins was found to be a N-terminal amino acid sequence with corresponding homology to β-tubulin (Kirvan et al., 2007) . Tubulin autoantibodies have also been identified in chronic inflammatory demyelinating polyneuropathy and Guillain-Barré syndrome (Connolly and Pestronk, 1997) , Graves' disease and Hashimoto's thyroiditis (Rousset et al., 1983).

1.2. Purpose of this study

The purpose of our study was to determine if, and to what extent, the assays of the Cunningham Panel parallel changes in PANDAS/PANS symptoms with treatment. Since immunomodulatory treatment for neuropsychiatric conditions can have positive (Perlmutter et al., 1999; Kovacevic et al., 2015) but also variable (Williams et al., 2016) outcomes, the ability to predict responsiveness to immunomodulatory treatments would be of high value. As a step towards this goal, this study examines whether the assays within the Cunningham Panel are potential biological markers that can be used to follow changes in symptoms with treatment, and could potentially be used to predict response to immunomodulatory treatment.

2. Methods

2.1. Subjects

All patients presented with various neuropsychiatric symptoms characteristic of the criteria for PANS/PANDAS and were either diagnosed with, or suspected of, PANS/PANDAS at the time of their first test requisition (Table 1). Fifty eight of 206 subjects who were identified to have had a Cunningham Panel from April 22, 2013 through Dec. 31, 2016, had two or more panels performed and met the inclusion criteria (see Fig. 2). Documentation of patient symptoms was received by direct

Table 1
Summary of symptoms of PANS/PANDAS patients included in this study by individual patients in Group 1: improved/resolved and Group 2: not improved/worsened.

Symptom	Group 1 Improved/resolved		Group 2 Not Improved/worsened		Combined All patients	
	Count (N = 34)	Percent	Count (N = 24)	Percent	Count (*N* = 58)	Percent
Decreased concentration	31	91%	22	92%	53	91%
OCD	34	100%	18	75%	52	90%
Emotional lability or depression	30	88%	19	79%	49	85%
Sensory symptoms	26	77%	22	92%	48	83%
Anxiety: general and/or Separation	26	77%	22	92%	48	83%
Sleep disorders	29	85%	15	63%	44	76%
Aggressiveness	27	79%	17	71%	44	76%
Tics	22	65%	21	88%	43	74%
Motor symptoms	19	56%	23	96%	42	72%
Developmental regression	23	68%	19	79%	42	72%
Dysgraphia	22	65%	18	24%	40	69%
Urinary urgency or frequency	15	44%	11	46%	26	45%
Chorea/choreiform movements	12	35%	13	54%	25	43%
Behavioral regression	8	24%	1	4%	9	16%
Anorexia or ARFID	3	9%	3	13%	6	10%
Psychosis	4	12%	1	4%	5	9%

OCD = obsessive compulsive symptoms; ARFID = avoidant/restrictive food intake disorder.

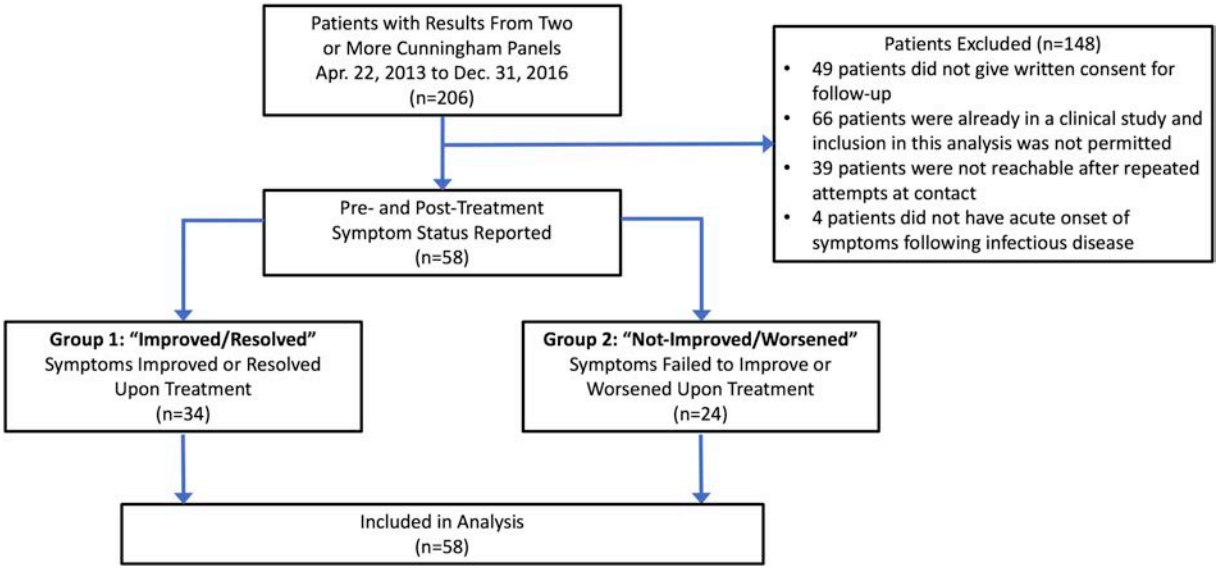


Fig. 2. Study Flow Chart

Table 2
Age and gender distribution and mean time between the first and second Cunningham panels.

Group	# Subjects	Age range (years)	Mean age (years)	Females	Males	Time between first and second test (weeks)	
						Mean	Median
1	34	5–21	12.2 (SD = 4.02)	13 (38%)	21 (62%)	68.1	48
2	24	2–23	12.1 (SD = 5.1)	9 (38%)	15 (62%)	66.2	62
All patients	58	2–23	12.2 (SD = 4.5)	22 (38%)	36 (62%)	67.3	50

phone and/or email communication with the prescribing clinician or parents, supported by documented call notes in our Laboratory Information Management System (LIMS). Clinician or parent assessments were specific to improvement or non-improvement compared to their initial presentation of symptoms when the first panel was performed. Based upon post-treatment assessments, patients were categorized into one of two groups: Group 1: "Improved/Resolved" ($n = 34$) or Group 2: "Not-Improved/Worsened" ($n = 24$) compared to pre-treatment symptoms. Informed consent/assent was reviewed and approved by the Western Institutional Review Board (WIRB). Since this was a retrospective analysis, parties making assessment were not necessarily blinded to Cunningham Panel results. All patients received medical attention, and 56 of the 58 patients received medications including therapy for infections and/or autoimmune disorders as potential causative factor(s) and/or psychotropic drugs for patient's neuropsychiatric symptoms. Of the two patients who did not receive medications between testing, one patient could not afford IVIg and therefore did not receive medication, and the other patient's medical treatment was watchful waiting or a "tincture of time."

2.2. Cunningham Panel assays

Whole blood or serum samples were received according to standard operating procedures, collected in red-top glass tubes without additives (Covidien, Monoject Red Stopper Blood Collection Tube, glass 7 mL draw, part #8881301512). Whole blood was spun to collect serum. Patient serum was tested according to standard operating procedures for Moleculera Labs' CLIA/COLA accredited laboratory (CLIA Number 37D2082408). Assay protocols have been described previously (Cox et al., 2013; Kirvan et al., 2003; Brimberg et al., 2012; Kirvan et al., 2006a, 2006b). The threshold for a positive response for the four ELISA assays was set at the mean value of normal controls plus two times the standard deviation, followed by rounding to the nearest titer. In each case, the selected threshold exceeded the 95% confidence interval for the t-distribution. A positive titer result for DR1 is 4000 or higher, for DR2 is 16,000 or higher, for lysoganglioside GM1 is 640 or higher, and for tubulin is 2000 or higher. A positive result for CaMKII activation in SK-N-SH cells was set at 130, or ≥30% above basal control sample. Initially, a population of 20 pediatric controls utilizing designated inclusion/exclusion criteria was obtained from the National Institute of Mental Health, Bethesda, MD (Courtesy of Dr. Susan Swedo) and the Yale Child Study Center, New Haven, CT (courtesy of Dr. James Leckman and Dr. Ivana Kawikova) (Singer et al., 2015). Since then, additional populations of pediatric controls have been tested with similar results.

2.3. Data and methods of analysis

Two distinct approaches were used to evaluate the results of these five assays. The first method (*Positive Test Count Method*) counted the change in the number of positive assays. The second method (*Magnitude of Change Method*) examined the quantitative change in each of the five assays using multivariate logistic regression analysis. The latter method examines the magnitude of change and is independent of the cutoff assigned by controls. Multivariate logistic regression analysis is effective when multiple test values may be better predictors compared to any single measure, and is used in other areas of medicine for predicting treatment outcome (Sparano et al., 2018; Hambardzumyan et al., 2015; Van Den Eeden et al., 2018).

2.3.1. Positive test count score

The Positive Test Count Score is the *difference* in the number of

Table 3A
Patients with Improvement in Symptoms (Group 1, N = 34).

Case #	Pretreatment					Post treatment					Number Elevated Markers	
	D1R	D2R	Tubulin	lyso-ganglioside G_{M1}	CaMKII	D1R	D2R	Tubulin	lyso-ganglioside G_{M1}	CaMKII	Pretreatment	Post Treatment
6	4000	8000	1000	160	125	2000	4000	1000	80	110	1	0
72	2000	2000	500	320	137	500	1000	250	80	117	1	0
55	1000	2000	500	80	138	1000	1000	250	10	93	1	0
1	1000	2000	500	160	142	2000	4000	1000	320	83	1	0
30	1000	4000	500	20	143	1000	2000	1000	80	98	1	0
3	500	1000	250	40	157	1000	2000	500	80	83	1	0
42	2000	4000	1000	40	167	1000	4000	1000	80	123	1	0
7	2000	4000	500	80	172	500	4000	1000	80	112	1	0
29	500	4000	1000	80	184	2000	4000	1000	80	113	1	0
26	1000	1000	250	40	271	1000	1000	250	40	134	1	1
5	2000	2000	2000	640	95	500	2000	250	40	112	2	0
12	2000	8000	4000	320	149	1000	8000	1000	20	113	2	0
37	4000	1000	1000	320	164	500	1000	250	40	92	2	0
39	8000	500	1000	640	123	1000	4000	1000	160	111	2	0
40	4000	4000	4000	320	122	250	2000	500	20	122	2	0
74	2000	8000	4000	40	133	1000	4000	500	160	116	2	0
41	8000	500	1000	320	177	2000	1000	250	20	83	2	0
16	2000	2000	2000	320	237	2000	8000	1000	320	132	2	1
19	1000	2000	4000	20	151	2000	4000	2000	80	128	2	1
43	16000	2000	8000	160	71	1000	4000	1000	80	148	2	1
71	1000	4000	2000	20	179	1000	4000	2000	320	131	2	2
66	8000	2000	2000	160	103	2000	8000	2000	320	138	2	2
4	8000	32000	4000	320	119	1000	8000	500	40	108	3	0
34	4000	16000	500	320	160	500	2000	500	40	118	3	0
54	8000	4000	4000	160	156	500	2000	500	10	89	3	0
35	4000	2000	16000	320	192	2000	4000	1000	80	119	3	0
2	2000	32000	4000	320	253	8000	16000	4000	320	120	3	3
45	8000	8000	4000	1280	143	2000	2000	1000	80	100	4	0
9	32000	4000	8000	640	153	2000	4000	1000	80	150	4	1
13	8000	8000	4000	640	176	1000	2000	2000	80	100	4	1
17	8000	2000	4000	640	139	500	2000	250	20	134	4	1
23	4000	16000	2000	320	139	4000	8000	1000	320	119	4	1
60	8000	16000	2000	80	179	1000	4000	500	80	165	4	1
79	4000	16000	2000	160	167	2000	16000	1000	160	110	4	1

Heat Map of the Results of the Cunningham Panel. Values for patients with improvement in, or resolution of symptoms after treatment (Group 1, $N = 34$). The results and the number of elevated tests before and after treatment are shown for each patient in this study. Elevated test values are highlighted in red; the intensity of color is indicative of magnitude of elevation above threshold. The count of elevated individual tests within each patient's panels before and after treatment are indicated by blue and orange horizontal bars, respectively.

positive tests pre-treatment, versus the number of positive tests post-treatment. An increase in the number of positive tests results in a *negative* Positive Test Count Score, an unchanged number results in a score of 0, and a decrease in the number of positive tests results in a *positive* score. We represent this by the following equation: Positive Test Count Score = $(X_{tub,pre} + X_{d1r,pre} + X_{d2r,pre} + X_{lyso,pre} + X_{CaMKII,\ pre}) - (X_{tub,post} + X_{d1r,post} + X_{d2r,post} + X_{lyso,post} + X_{CaMKII,\ post})$ where X = 1 for a positive test and 0 for a negative test, the subscripts "tub, "d1r", "d2r", "lyso" and "CaMKII" indicate the assay and the subscripts "pre" and "post" indicate whether the assay was conducted before or after treatment, respectively.

2.3.2. Magnitude of change score

The Magnitude of Change Score examines the magnitude of change in post-treatment test results compared to pre-treatment. This is defined by the equation: Magnitude of Change Score = $a_0 + a_1{*}DV_{tub} + a_2{*}DV_{d1r} + a_3{*}DV_{d2r} + a_4{*}DV_{lyso} + a_5{*}DV_{CaMKII}$ where DV = $\log_2(Titer_{post}/Titer_{pre})$ for each of the four ELISA assays and $DV_{CaMKII} = CaMKII_{value,post} - CaMKII_{value,pre}$ for the CaMKII assay. Logistic Regression Analysis, conducted in R with the glm2 (Fitting Generalized Linear Models) package (version 1.2.1), was used to determine the optimal values of the coefficients a_0, a_1, a_2, a_3, a_4 and a_5 and the probability of membership in either Group 1 versus Group 2 for each patient.

The value of a_0, the constant term, and coefficients a_{1-5} for tubulin, D1R, D2R, lysoganglioside and CaMKII DV values in the Magnitude of Change Score equation from our analysis are:

-0.862 ± 0.717 ($p = 0.229$), -1.284 ± 0.500 ($p = 0.010$), -0.361 ± 0.355 ($p = .310$), -0.738 ± 0.438 ($p = 0.093$), 0.063 ± 0.288 ($p = 0.826$) and -0.0461 ± 0.0172 ($p = 0.007$), respectively. Null deviance and residual deviance are 78.7 and 29.7 on 57 and 52 degrees of freedom, respectively. The values of the parameters used in the transformation are selected to provide maximal discrimination between Group 1 and Group 2. The probability (P) of membership in Group 1, the group with symptomatic improvement, is calculated from the individual's Magnitude of Change Score: $P = e^{Magnitude\ Score}/(e^{Magnitude\ Score} + 1)$. The resulting logistic regression model is then cross-validated using the "leave one out" method. Note that Magnitude of Change Score is independent of the thresholds used to determine individual assay results as positive or negative; instead, Magnitude of Change Score is a function combining the direction and magnitude of changes in all individual tests in the Cunningham Panel. We investigated other models in which selected assays in the Cunningham model were omitted; our findings were essentially unchanged from the full model.

2.4. Reproducibility of the Cunningham Panel

Extensive reproducibility testing was conducted using patient blood samples collected in validated glass tubes without excipients (Red Top glass tubes), and tested repetitively at random intervals over a period of 33 months. The reproducibility testing for D1R is representative of each of these tests. Note that a two-fold dilution scheme is used to determine titers. To assess robustness and ongoing assay reproducibility of the

Table 3B
Patients with no improvement in or worsening of symptoms (Group 2, N = 24).

Case #	Pretreatment					Post treatment					Number Elevated Markers	
	D1R	D2R	Tubulin	lyso-ganglioside G_{M1}	CaMKII	D1R	D2R	Tubulin	lyso-ganglioside G_{M1}	CaMKII	Pretreatment	Post Treatment
24	1000	8000	500	80	119	2000	8000	250	320	105	0	0
16	1000	4000	1000	20	124	2000	4000	1000	80	138	0	1
18	1000	2000	250	20	121	1000	1000	500	80	138	0	1
19	1000	250	250	160	115	2000	4000	2000	40	116	0	1
34	2000	2000	500	320	121	2000	8000	1000	20	145	0	1
77	500	2000	1000	20	126	1000	2000	2000	40	141	0	2
29	1000	2000	500	80	138	1000	2000	250	160	127	1	0
62	2000	4000	1000	80	216	2000	2000	500	80	123	1	0
64	500	2000	250	320	134	1000	4000	2000	80	125	1	1
104	2000	4000	1000	40	149	2000	4000	1000	40	175	1	1
45	2000	4000	500	80	217	4000	4000	2000	80	158	1	3
8	1000	8000	1000	40	219	4000	16000	1000	320	139	1	3
38	1000	1000	1000	160	179	8000	8000	4000	1280	140	1	4
48	1000	2000	2000	80	156	2000	8000	1000	160	145	2	1
36	2000	16000	1000	160	164	8000	8000	4000	160	123	2	2
122	500	8000	1000	640	136	2000	4000	4000	320	143	2	2
20	2000	2000	2000	40	164	4000	16000	8000	160	150	2	4
42	32000	4000	2000	80	112	4000	16000	2000	160	164	2	4
55	2000	8000	2000	320	160	4000	16000	8000	80	152	2	4
39	8000	250	2000	160	159	2000	16000	4000	80	162	3	3
26	4000	32000	8000	320	94	8000	16000	8000	1280	177	3	5
14	8000	4000	2000	640	148	8000	16000	16000	40	92	4	3
63	8000	32000	2000	320	142	32000	32000	8000	640	113	4	4
35	4000	32000	4000	160	130	8000	32000	4000	1280	149	4	5

Heat map of the results of the Cunningham Panel. Values for patients with no improvement in, or worsening of symptoms after treatment (Group 2, $N = 24$). The results and the number of elevated tests before and after treatment are shown for each patient in this study. Elevated test values are highlighted in red; the intensity of color is indicative of magnitude of elevation above threshold. The count of elevated individual tests within each patient's panels before and after treatment are indicated by blue and orange horizontal bars, respectively.

Table 4
Patient numbers by group: impact of treatment on Cunningham Panel tests, Comparison of the number of patients showing improvement in symptoms with those showing a decrease in the number of individual positive assays in the Cunningham Panel by populations described in Table 3A and 3B. The *p*-value was calculated using the Fisher exact test.

Group	N	Number Positive Tests Post-Treatment vs. Pretreatment				No Positive Tests (Pre- or Posttreatment)	Total Resolved or Decreased	Total Increased or Unchanged	Total Resolved or Decreased (%)	Total Increased or Unchanged (%)	p value
		Resolved (No positive test post-treatment)	Decreased Count (not resolved)	Unchanged (at least 1 positive test)	Increased						
1	34	21 (62%)	9 (26%)	4 (12%)	0 (0%)	0 (0%)	30 (88%)	4 (12%)			1.33E-07
2	24	2 (8%)	2 (8%)	6 (25%)	13 (54%)	1 (4%)	4 (17%)	19 (79%)			

CaMKII assay, routine repeat testing across multiple samples was performed.

3. Results

3.1. Patient demographics

Patients were either diagnosed with, or suspected of, PANS/PANDAS at the time of their test requisition. All 58 patients presented with various neuropsychiatric symptoms characteristic of the criteria for PANS/PANDAS (Table 1). Age and gender distribution for all 58 patients at the time of first testing ranged from 2 to 23 years, whereas the mean and median age for patients was 12.2 ± 4.5 and 12.0 years, respectively. The mean time between the first and second Cunningham Panels in Group 1 was 68.1 weeks, versus 66.2 weeks in Group 2 ($p = 0.87$). The median time between tests for Group 1 was 48 weeks, versus 62 weeks for Group 2. An interesting observation in examining symptom frequency is that patients who improved with therapy (Group 1) had a higher percentage of OCD, behavioral regression and sleep disorders, whereas those that did not improve with therapy (Group 2) had a higher percentage of tic and movement disorders.

3.1.1. Subjects in group 1: improved/resolved in symptoms (N = 34)

The group of patients reporting symptoms Improved/Resolved post-treatment consisted of 34 individuals ranging in age from 5 to 21 years with a mean age of 12.2 years. There were 13 females (38%) and 21 males (62%). See Table 2.

3.1.2. Subjects in group 2: not improved/worsened in symptoms (N = 24)

The group of patients reporting symptoms Not Improved/Worsened consisted of 24 patients ranging in age from 2 years to 23 years with a mean age of 12.1 years. There were 9 females (38%) and 15 males (62%), and there were no statistically significant differences in age or gender between Group 1 and Group 2. There were no statistically significant differences in the time between the first test and the second test in Group 1 versus Group 2 (Table 2).

3.2. Individual results and heat map

The heat-maps (Table 3A and 3B) display the Cunningham Panel

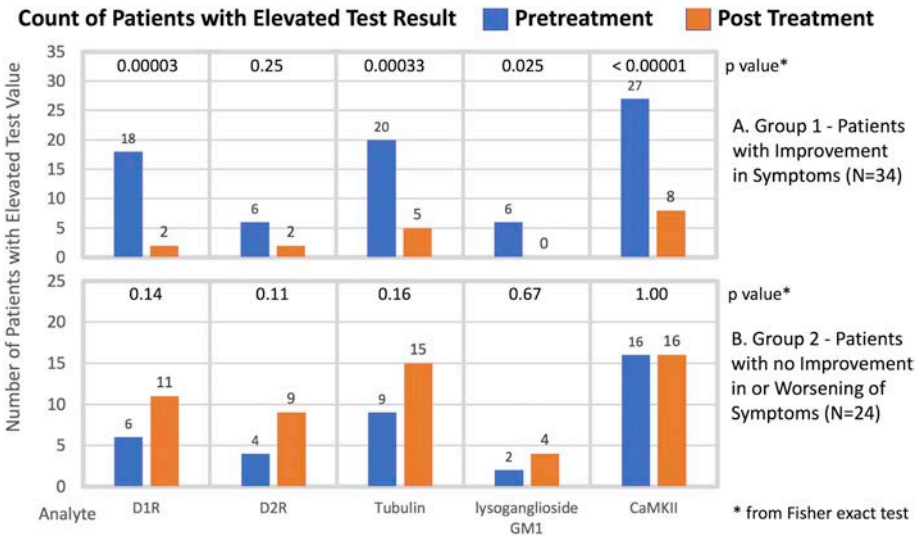


Fig. 3. Number of patients with elevated test result.

results pre- and post-treatment for all patients. Individual titers highlighted in red indicate a positive abnormal assay with the intensity of color indicating the extent of elevation. Titers without color highlighted indicate a normal assay value. The change in the Cunningham Panel results is highly associated with the reported change in symptoms following treatment as summarized in Table 4. The number of patients with elevated individual test results pre-treatment and post-treatment are shown in Fig. 3. The mean values for each of the individual tests in the Cunningham Panel before and after treatment are shown in Supplemental Fig. 1.

We evaluated the data for any association between changes in Cunningham Panel results and changes in patient symptoms after treatment or medical intervention using two distinct approaches: the Positive Test Count Method and the Magnitude of Change Method. Results from both analyses are summarized in the 2 × 2 contingency tables for sensitivity, specificity and accuracy (Fig. 4A and 5A), whereas the respective Receiver Operating Characteristic (ROC) curves are shown in Fig. 5A and 5B. The dot plots of individual scores by group and by analysis are shown in Fig. 4C and 5C.

4A. Sensitivity and Specificity Results
(Positive Test Count Score Method)

		Symptoms Improved			
		Yes	No		
Panel Positive	Yes	30	4	**Sensitivity**	88%
	No	4	20	**Specificity**	83%
				Accuracy	86%

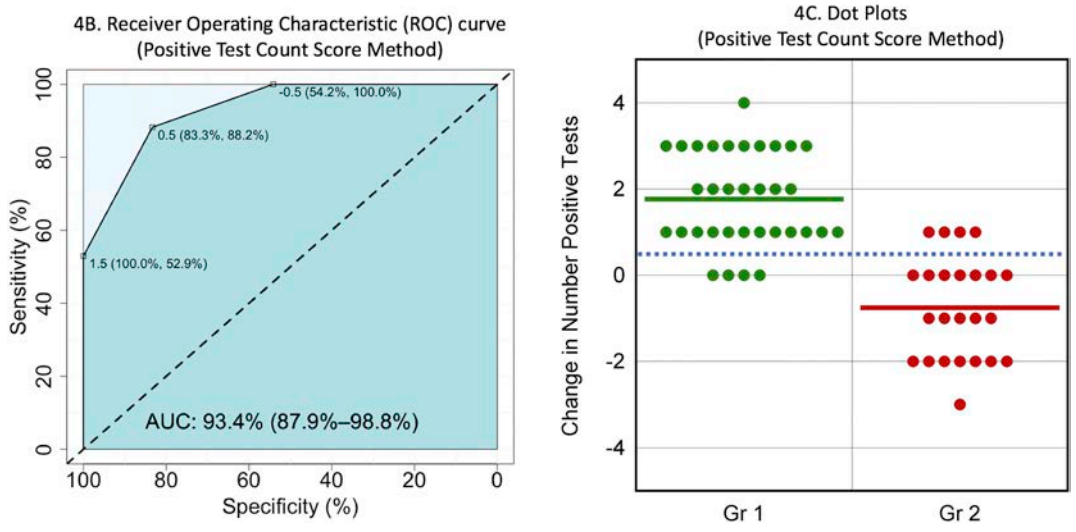
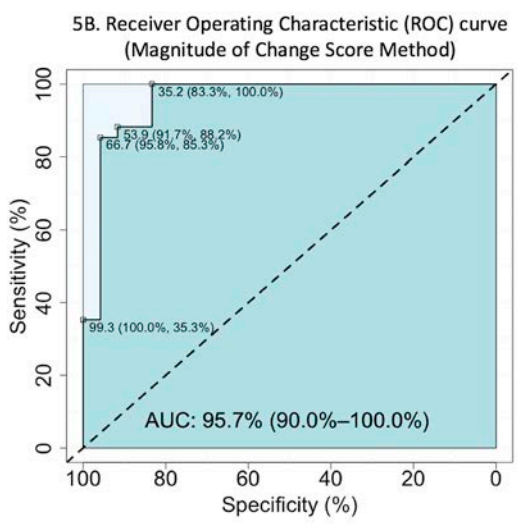


Fig. 4. Results for the Cunningham Panel Test (Positive Test Count Score Method).
4A. Sensitivity and specificity of Cunningham Panel assays: 2 × 2 matrix and calculation of sensitivity, specificity and accuracy using Change in Number of Positive Tests ≥0.5 as predicting membership in Group 1 (optimum from ROC curve).
4B. Receiver Operating Characteristic (ROC) curve of Cunningham Panel assay results.
4C. Dot Plots for Change in Number Positive Tests. Blue dotted line (y = 0.5) shows threshold used in assigning membership in 2 × 2 matrix. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5A. Sensitivity and Specificity Results
(Magnitude of Change Score Method)

		Symptoms Improved			
		Yes	No	**Sensitivity**	88%
				Specificity	92%
Panel Positive	Yes	30	2		
	No	4	22		
				Accuracy	90%



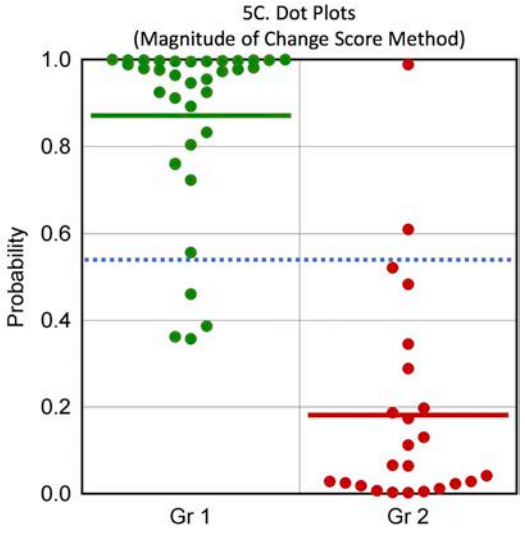


Fig. 5. Results for the Cunningham Panel Test (Magnitude of Change Score Method).
5A. Sensitivity and specificity of Cunningham Panel assays: 2 × 2 matrix and calculation of sensitivity, specificity and accuracy using Probability Threshold ≥0.539 as predicting membership in Group 1 (optimum from ROC curve).
5B. Receiver Operating Characteristic (ROC) curve of Cunningham Panel assay results.
5C. Dot Plots for Magnitude of Change scores. Blue dotted line (y = 0.539) shows threshold used in assigning membership in 2 × 2 matrix. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2.1. Positive test count method

This method consists of assessing change in Cunningham Panel results (positive or negative) upon treatment by tallying the number of positive tests for each of the five assays. A 2 × 2 contingency table analysis ($p = 5 \times 10^{-8}$, Fisher exact test) revealed an 88% sensitivity, 83% specificity, and an accuracy of 86% (Fig. 4A). The ROC curve had an AUC of 93.4% (Fig. 4B). Dot plots of individual scores revealed good separation by group (Fig. 4C).

In the patients reporting symptom improvement following treatment, 88% (30/34) demonstrated a decrease in the number of individual positive Cunningham Panel tests. Within these patients, 62% (21/34) had all tests become negative whereas 26% (9/34) showed reduction in the number of positive tests and 12% (4/34) demonstrated an unchanged number of positive tests. No patients in the Improved/Resolved group had an increase in the number of positive tests post-treatment (Table 4). For patients whose symptoms failed to improve or worsened post-treatment (Group 2), 79% (19/24) demonstrated either the same number of positive tests pre-treatment or an increase in the number of positive tests in their panel. Within these patients, 54% (13/24) revealed an increase in the number of individual positive tests, with 25% (6/24) having an unchanged number of individual positive tests, and 8% (2/24) with a decrease in the number of positive tests in their panel.

3.2.2. Magnitude of change method

Using linear logistic regression we created an optimal function derived from the magnitude of change in the Cunningham Panel test scores which separated the groups of patients who responded to treatment vs those that did not respond. This multivariate method analysis creates a linear transformation which reduces the five individual values comprising the Cunningham Panel into one composite or combined score. Null deviance and residual deviance are 78.7 and 29.7 on 57 and 52 degrees of freedom, respectively. Accordingly, $R^2 = 0.62$. In contrast to linear regression, R^2 is not related to any correlation coefficient, nor is it a percentage of variance explained by the logistic model, rather it is a ratio indicating how close the fit is to being perfect ($R^2 = 1$) or

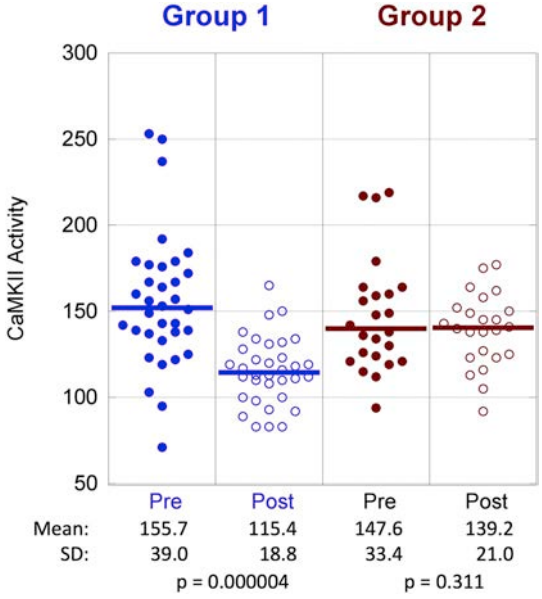


Fig. 6. Individual CaMKII assay results from the Cunningham panel before and after treatment and grouped by effect of treatment on patient symptoms. *P* values are from a paired sample *t*-test.

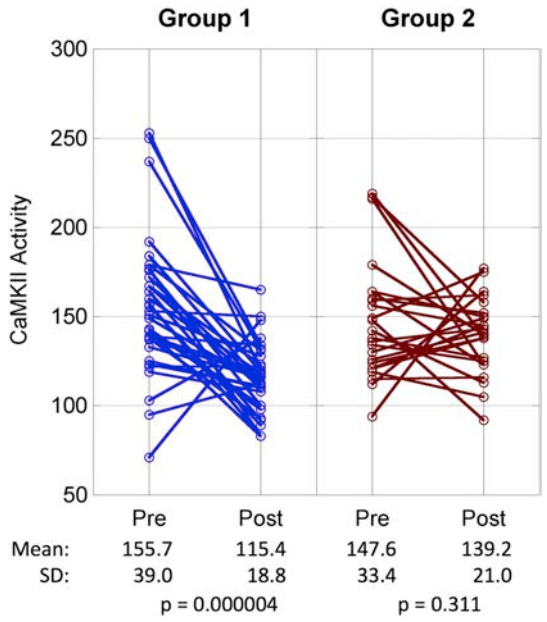


Fig. 7. CaMKII assay result trend lines of individual patent results in Group 1 and Group 2. Pre- and post-treatment values are connected by solid lines. P values are from a paired sample *t*-test.

the worst possible ($R^2 = 0$). As part of characterization of model quality, a summary of the values for all coefficients in "leave-one-out" analysis are shown in Supplemental Table 1. The probabilities for the misclassification in the original and leave-one-out validation are given in Supplemental Table 2.

Results in the 2 × 2 contingency table ($p = 6 \times 10^{-10}$, Fisher exact test) revealed an 88% sensitivity, 91% specificity with an overall accuracy of 90% (Fig. 5A). Values in 5A are taken from a threshold: $0.522 < \text{Probability} < 0.557$ which provides highest values per the ROC analysis in 5B. For example, if the threshold is set at 0.5, then the number of incorrect assignments of Group 2 to Group 1 increases from 2 to 3 of 24 whereas the number of incorrect assignments of Group 1 to Group 2 is unchanged at 4 of 34 (Supplemental Table 2) for an overall accuracy of 88%. The ROC curve used to define the threshold for predicting inclusion in Group 1 or Group 2 resulted in an AUC of 95.7% (Fig. 5B). Dot plots of individual scores revealed good separation by group (Fig. 5C). We find that there is good discrimination between Group 1 and Group 2 based on quantitative changes in the test results.

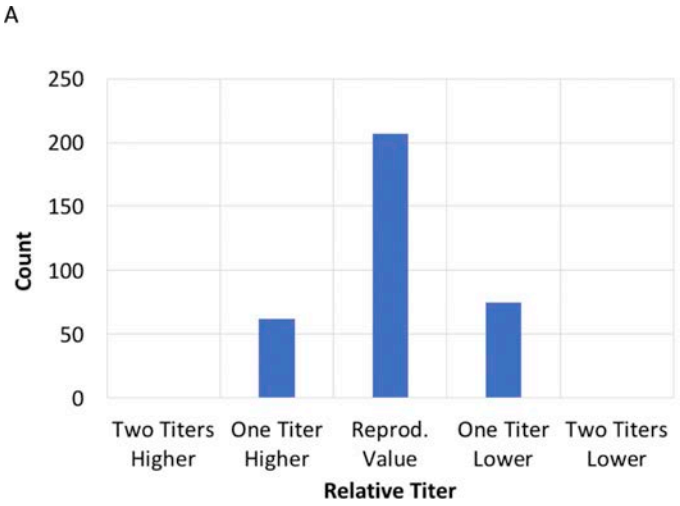
There is an interesting trend observed for patients in Group 1; CaMKII values were, more often than not, elevated prior to treatment and then significantly decreased in conjunction with a reduction in symptoms post-treatment ($p = 0.000004$), approaching levels associated with normal control populations (Fig. 6). In contrast, those patients in Group 2, defined as those whose symptoms did not improve post-treatment, tended to show elevated CaMKII levels both before and after treatment without any statistically significant change post-treatment ($p = 0.311$) (Fig. 7).

3.3. Summary of treatments and comparisons between groups

Retrospective evaluation of treatments revealed that of patients treated with immunotherapy (IVIg, plasmapheresis, Rituximab, or combination) 66.7% improved (Group 1), while just 33.3% failed to see improvement (Group 2). For patients who were treated with antimicrobial therapy alone, 70.6% of patients experienced symptom improvement vs. 29.4% failing to improve or worsening. Overall, treatment with multiple classes of therapeutic interventions in most cases showed modestly higher percentages of improvement, whereas in patients treated with psychotropic medications, dietary changes, or untreated, only 20% of patients reported symptom improvement. More specifically, four of four patients (100%) improved with combined IVIg and plasmapheresis, and one of two patients (50%) improved with combined IVIg and Rituximab. Only one of five (20%) patients improved who were treated with psychotropic medications, dietary changes, or no treatment (see Table 5). Note that in general, the utilization frequency of specific treatments is similar between Group 1 and Group 2 (Table 5). The use of "Immunotherapies Only" and "Antimicrobials Only" is somewhat higher in Group 1 than in Group 2, whereas the percentage of patients in Group 2 that received both classes of therapy is somewhat higher than in Group 1.

Table 5
Summary of treatments and outcomes. Binary classification of response to treatment (Improvement vs No Improvement / Worsened by therapeutic modality [i.e. antimicrobial therapy, immunotherapy, combination of these therapies, other]). * % Improved indicates the percent of patients whose symptoms improved with the corresponding therapy(ies). ** % of group receiving this therapy.

Treatment	Description	Group 1 Improved	Group 2 No improvement or worsened	% improved *
A	All therapies containing antimicrobials	19 (56%)**	13 (54%)	59.4%
B	All therapies containing immunotherapies	21 (62%)	15 (63%)	58.3%
C	Antimicrobials only	12 (35%)	5 (21%)	70.6%
D	Immunotherapies only	14 (41%)	7 (29%)	66.7%
	Individual Immunotherapies			
	IVIg	9	5	64.3%
	Plasmapheresis	1	1	50.0%
	Rituximab	1	0	100.0%
	Combination Immunotherapies			
	IVIg Plasmapheresis	4	0	100.0%
	IVIg Rituximab	1	1	50.0%
E	Antimicrobials + Immunotherapies	9 (26%)	8 (33%)	52.9%
	IVIg	6	7	46.2%
	Plasmapheresis	0	1	0.0%
	IVIg Plasmapheresis	3	0	100.0%
	IVIg Plasmapheresis Rituximab	0	1	0.0%
F	Antimicrobial, immuno- or combination thereof	33 (97%)	20 (83%)	62.3%
G	Other/none	1 (3%)	4 (17%)	20.0%
	Psychotropic medications only	0	1	0.0%
	Dietary changes only	1	1	50.0%
	No treatment	0	2	0.0%



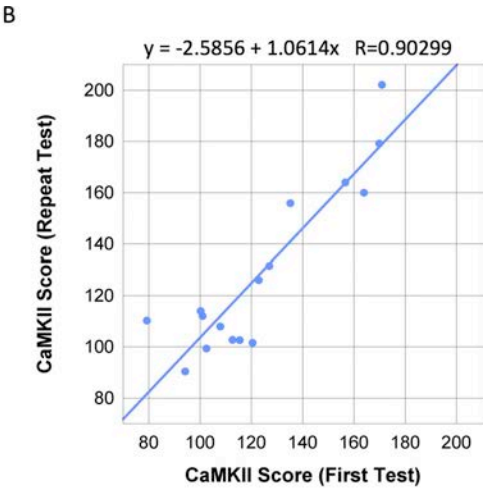


Fig. 8. Fig. 8A. D1R ELISA assay reproducibility. These are the summary of results for various control samples tested repeatedly between 3/29/2014 and 12/29/2016.
Fig. 8B. CaMKII assay reproducibility. Each data point represents a single specimen; the x value is the CaMKII score from the first measurement and y value is the CaMKII score from a second measurement. The solid line is the best-fit line to the experimental data.

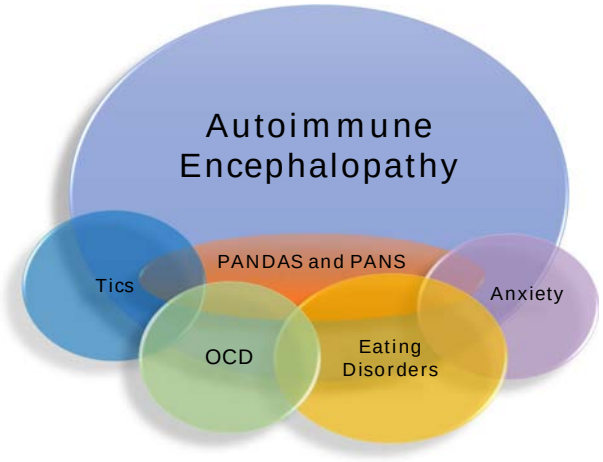


Fig. 9. Illustration of underlying etiology as the basis for symptoms or clinical diagnoses.

3.4. Reproducibility of ELISA testing in the Cunningham Panel

For repeated D1R assay we observed 207 readings at the same titer, 62 readings at one titer higher and 75 readings at one titer lower (Fig. 8), resulting in a distribution of 17.3%, 61.5% and 21.2%, respectively. No results were greater than one titer dilution away from the peak. When expanded to include mixed patient samples and additionally processed or concentrated patient samples, we saw rare instances (< 0.5%) where the assigned titer was two dilution steps from the peak. In no cases were any titers greater than two dilution steps from the peak.

3.5. Reproducibility of CAMKII assay results in the Cunningham panel

Seventeen samples underwent repeat CaMKII assay testing. The correlation between the original and the repeat test was 0.90 ($p < 1.67 \times 10^{-6}$) and the root-mean-square deviation (RMSD) is 13.8 (Fig. 8B). The deviation (Repeat Assay Value / Original Assay Value) was approximately 15% with no apparent dependence on the actual CaMKII activity over the range of values routinely measured. The baseline value by definition is 100, whereas the threshold for a positive in the CaMKII assay is set at 130 (30% above baseline values defined from measurements in pediatric controls).

In summary, repetitive testing of multiple samples, multiple times in the ELISA and CaMKII assays using patient samples demonstrates assay robustness and reproducibility.

4. Discussion

For PANDAS and PANS, there is a need to elucidate the pathophysiology and underlying disease mechanisms in order to improve the identification of patients, advance targeted therapies, and to clarify the characteristics of these disorders. The value of identifying a group of biomarkers that correspond to a disease etiology is that it can segment patients exhibiting similar symptoms into groups that can be administered different treatment modalities based upon their underlying disease etiology. In this 58 patient case series, we found that changes in assays of the Cunningham Panel parallel changes in patient symptoms following treatment. Using the change in the number of positive tests we were able to predict change in symptoms with an accuracy of 86% and sensitivity and specificity of 88% and 83%, respectively. Based upon the magnitude of change of individual test values of the Cunningham Panel, we were able to predict change in symptoms with an accuracy of 90%, a sensitivity of 88% and a specificity of 92%.

4.1. Comparison with other published studies

In a 2018 study of 80 patients having a diagnosis of ASD, in which 31 children with autoimmune encephalopathy received IVIg treatment, the Cunningham Panel predicted patient improvement and response to IVIg treatment with an accuracy of 81%, a sensitivity of 90% and a specificity of 67% based on the Aberrant Behavior Checklist (ABC) scores; with an accuracy of 88%, a sensitivity of 100% and a specificity of 75% based on the Social Responsiveness Scale (SRS) scores; and with an accuracy of 88% with a sensitivity of 100% and a specificity of 67% based on parental scores (Connery et al., 2018). The sensitivity and specificity of the Cunningham Panel in predicting IVIg responsiveness in children with autoimmune encephalopathy and diagnosed with ASD (81% to 88%) are similar to the performance accuracy we observed in this study (87% to 90%). Published literature reveals that children with ASD have strong family histories of immune dysregulation and inflammation, and that the literature supports a strong association of immune dysregulation and ASD (Rossignol and Frye, 2012). Because

current diagnostic criteria for ASD and PANDAS/PANS are based upon clinical symptom clusters (Chang et al., 2015) , it is possible that a common underlying etiology of these disorders, or a subset of these clinical syndromes, may be immune dysregulation with antineuronal antibodies directed against the basal ganglia and/or other CNS targets in the brain.

A 2017 study questioned the clinical utility of the Cunningham Panel (Hesselmark and Bejerot, 2017a) based upon a retesting study of 53 patients, with 46 patients having a repeat test. The estimated sensitivity for individual tests ranged from 15% to 60% and the estimated specificity ranged from 28% to 92%. Shortly thereafter, the authors submitted a Corrigendum (Hesselmark and Bejerot, 2017b) acknowledging they had unknowingly utilized invalid blood collection tubes containing clot activators and serum separator gels (BD Vacutainer® SST™ II Advance tubes, Gold Top) which are not acceptable for collecting Cunningham Panel samples. Specimens collected in blood tubes containing excipients or additives have the potential for irreproducibility of results, potential interactions with patient's specimens, and potential direct interference in the assays.

In the referenced study, the authors reported some “healthy controls” showing positive Cunningham Panel results. This could be a result of the invalid collection method or the exclusion and inclusion criteria utilized for their control population (Frye and Shimasaki, 2019) . Family history of psychiatric, autoimmune, or movement disorder was not investigated in the controls nor was the history of recent, chronic or recurrent infections. Since PANDAS/PANS is often triggered by an infection which can be subclinical or occult, transient molecular mimicry could have confounded test results. More importantly, although the patients were asked about a psychiatric or autoimmune diagnosis, symptoms of autoimmune or psychiatric disorders were not specifically investigated. This lack of detailed screening of the controls calls into question the extent to which the controls were verified as healthy.

4.2. Therapeutic interventions

Although this current study was not designed as a comparison of treatment interventions, within those whose symptoms improved post-treatment, a greater percentage received some form of immunomodulatory or antimicrobial therapy alone, compared to the patient group whose symptoms did not improve. It was observed that a higher percentage (70.6%) of patients improved who were treated with antimicrobial therapy alone, whereas 52.9% of patients treated with both modalities saw improvement. It is unknown whether those patients who required multiple therapeutic modalities may have had longer duration of illness or greater severity of symptoms. PANDAS/PANS patients typically have undergone multiple symptomatic-focused treatments prior to a recognition of their underlying infectious trigger and autoimmune etiology. It may be that prolonged duration of these conditions prior to proper diagnosis and treatment could impact the outcome of a course of therapy and the treatment duration required to observe symptom resolution in these patients.

A confounding issue that could potentially influence the outcome of certain immunomodulatory treatments with IVIg is the observation that in some IVIg lots we tested, we find varying, but significantly elevated levels of antineuronal antibodies against the biological targets and CaMKII activation in the Cunningham Panel (data not shown). Because IVIg is a product produced through the concentration of immunoglobulins from 1000 or more patients, it would not be unexpected to observe this. However, it is key that post-treatment testing with the Cunningham Panel be performed after significant clearance of potential exogenous antineuronal antibodies, as this may confound patient results. Estimates of half-life of some manufacturers' IVIg are as long as 40 days in patients (IVIg Manufacturer's Package Insert). Also, in a research setting where testing was performed at regular intervals during immunomodulatory treatment, completely normal testing results have been observed, due to the possibility of immunomodulatory treatment interfering with testing results, either by suppressing the production of these autoantibodies or direct interference. Because of potential for immunomodulatory treatment interference with test results, prescribers are advised to consider waiting at least six to eight weeks or longer following treatment to determine treatment effectiveness in reducing these antineuronal antibodies. Within this study the mean time from pre-treatment testing to post-treatment testing was 68.1 and 66.2 weeks respectively for Group 1 and Group 2.

4.3. Future directions

There is a clear need for more treatment practice guidance for post-infectious autoimmune neuropsychiatric disorders. The recently published treatment guidelines for patients with PANS/PANDAS (Swedo et al., 2017; Thienemann et al., 2017; Frankovich et al., 2017; Cooperstock et al., 2017) and clinical and treatment information on the PANDAS Physician Network (PPN) (‘PANDAS Physican Network", 2018) will help establish a standard for treatment of these patients. Additionally, in order to help guide treatment decisions, we are systematically analyzing our biobank of over 8000 annotated samples to study how baseline data may help stratify patient populations into diagnostic subgroups for predicting treatment response.

Other clinical reports have been published showing effective utilization of immunomodulatory treatment for autoimmune encephalitis based upon the presence of antineuronal antibodies directed against NMDA receptor (Dalmau et al., 2017) , voltage-gated potassium channel-complex (VGKC) (Vincent et al., 2011) , leucine-rich glioma inactivated-1 (LGI1) , astrocyte aquaporin-4 (AQP4) (Zekeridou and Lennon, 2015) , glutamic acid decarboxylase (GAD65) , gamma-aminobutyric acid-B receptor (GABAB) , and others (Mader et al., 2017; Platt et al., 2017; Mohammad and Dale, 2018; Lancaster, 2016; Dale et al., 2017) . It is plausible there could be additional antineuronal antibody targets that lead to symptoms of autoimmune encephalitis. Interestingly, in a study of 61 patients diagnosed with autoimmune encephalitis by confirmatory testing such as MRI, CSF inflammation, EEG and FDG-PET/CT, it was determined that the anti-neuronal antibodies in this referenced study, such as those listed above, did not account for 48% (29/61) of these patients presenting with autoimmune encephalitis (Probasco et al., 2017). A future study will include the examination of a broader range of anti-neuronal antibodies. Thus, while there are many known targets for autoantibodies in patients with autoimmune encephalopathies, antibodies to extracellular epitopes of dopamine D2 receptor have also been identified in patients with pediatric basal ganglia encephalitis (Dale et al., 2012) a related disorder with overlapping yet distinct clinical presentation to PANDAS/PANS.

5. Study limitations

Pre- and post-treatment symptom responses were carefully collected and documented by questionnaire and telephone follow-up with parents and physicians. However, because of the lack of a standardized instrument and a single individual assessing symptom severity in all these patients, there could be inter-patient variation in the self-reporting of symptom severity. Future studies would benefit from the utilization of a standardized rating scale in a single study utilizing a single independent assessor for measuring the severity of symptoms. Since PANS/PANDAS is a diagnosis of exclusion and patients currently may not receive this diagnosis during a primary medical visit, patients that are referred for testing may have visited multiple clinicians and received multiple therapies that have failed prior to their Cunningham Panel. Thus, there might be significant variability between symptom onset and initial testing with the Cunningham panel.

Other limitations to this study include the relatively small sample sizes of patients in each group, which was limited to the numbers of

patients that had pre- and post-treatment testing during this interval, and the relatively small number of carefully screened pediatric healthy controls from which the normal or basal levels were obtained.

Although published literature has demonstrated that anti-infectives and immune modulatory therapy can indeed be effective in PANDAS patients (Kovacevic et al., 2015; Perlmutter et al., 1999) , the exact treatment used, dose and duration of treatment and when the treatment was instituted was not controlled as part of this study and could have added to variability.

Interestingly, autoantibodies against certain biomarkers have also been identified in other inflammatory, movement, and/or neuropsychiatric conditions. Although autoantibodies against any individual target may not be diagnostic for PANDAS/PANS or Sydenham's chorea, the entire panel taken together may be a strong aid in a physician's clinical diagnosis of an autoimmune etiology. There also remains the possibility that autoantibodies against these targets may not necessarily be pathogenic but rather an epiphenomena associated with this disorder. Further research and clinical studies are in progress to assess this as a causal contribution or a downstream result of the disorder.

6. Conclusions

We report here for the first time a strong positive association between the change in anti-neuronal antibody titers and antibody-mediated CaMKII activation measured as part of the Cunningham Panel and change in neuropsychiatric symptoms in patients with PANDAS/PANS. These data provide supportive evidence that the change in levels of serum autoantibodies directed against neuronal pathophysiological processes may correlate with the change in neuropsychiatric symptoms in patients with certain neuropsychiatric disorders.

These observations and results support a potential common proposed mechanism where infection-triggered immune dysregulation may lead to antineuronal antibodies directed against specific targets in the brain resulting in neuropsychiatric symptoms (Labrie and Brundin, 2019) . A Danish study by Köhler-Forsberg et al. of over one million individuals provides compelling epidemiologic evidence that severe infections are linked to the onset of neuropsychiatric illnesses in children (Kohler-Forsberg et al., 2019). They observed an 8-fold increased risk for obsessive-compulsive disorder in teenagers, and the study identified a 1.5-fold to 5.6-fold elevated risk for neurodevelopmental delay, mental retardation, and behavioral and/or emotional disturbances in the young. If this mechanism proves to be true, there is the potential that subsets of other clinically-defined neuropsychiatric disorders and conditions may have a pathophysiology representing a broader category of disorders referred to as “Autoimmune Encephalopathies of Infectious Etiology,” (Fig. 9 Graphical Abstract).

In an era of precision medicine, there would be clinical advantages if targeted testing could segment patients having heterogeneous symptoms into discrete groups based upon responsiveness to a particular treatment modality. Although the historical identification of the anti-neuronal antibodies in the Cunningham Panel originated from patients diagnosed with Sydenham chorea and later PANS/PANDAS patients, these biomarker targets may help identify broader populations of patients experiencing neuropsychiatric symptoms triggered by a similar autoimmune etiology that may be responsive to immune modulation therapy. Testing results may also provide a clinician with the biological evidence for the diagnosis of an immune-mediated disorder which may alter their treatment modality.

Based upon this retrospective analysis, there is evidence to support that the Cunningham Panel may have value as an aid in a clinician's diagnosis and management of patients with PANDAS/PANS. Although it is not clear whether the presence of antineuronal antibodies to these targets may be causal or an associative response to a patient's neuropsychiatric symptoms, identifying such an objective measure is essential for performing future studies on treatment outcomes and to help elucidate a disease mechanism. The results of this study represent a step towards validating antineuronal antibody tools that may be utilized as an aid in a physician's diagnosis of PANDAS/PANS and support future prospective studies into the understanding of its etiology and pathogenesis. The validation of biological markers may also lead to the identification and development of more efficacious targeted therapeutics for treating patients with these neuropsychiatric disorders. Further, the data generated in this study suggests a larger analysis is warranted including more patients to explore whether baseline data can predict treatment response in patients with neuropsychiatric symptoms.

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Author Disclosures

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