Autism Insights

ORIGINAL RESEARCH

Initial Observations of Elevated Alpha-N-Acetylgalactosaminidase Activity Associated with Autism and Observed Reductions from GC Protein—Macrophage Activating Factor Injections

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Abstract

Background: Autism spectrum disorders (ASD) are developmental disorders affecting 1:88 children, and which appear to be associated with a variety of complex immune dysregulations including autoimmunity. The enzyme, alpha-N-acetylgalactosaminidase (Nagalase) deglycosylates serum Gc protein (vitamin D_3 – binding protein) rendering it incapable of activating macrophage defenses. Increased Nagalase activity has been associated with a variety of malignancies, immune disorders and viral infections. Macrophage activating factor (GcMAF) has been repeatedly published as an intervention to lower serum Nagalase activity for a variety of cancer and HIV patients. GcMAF is a naturally occurring protein with well-established safety and therapeutic benefit(s) supported by numerous human studies.

Methods: Initially, parents of 40 individuals with ASD sought testing for Nagalase serum activity as part of an evaluation of immune dysregulation. Nagalase enzyme activity measurement was performed by the European Laboratory of Nutrients (ELN), Bunnik, the Netherlands, using an end-point enzymatic assay of a chromogenic substrate. Some parents of patients with elevated Nagalase activity opted for weekly GcMAF injections provided by Immuno Biotech Ltd., Guernsey UK (www.gcmaf.eu). GcMAF is purified from human serum obtained from the American Red Cross using 25-hydroxyvitamin D3-Sepharose high affinity chromatography. The protein is then further diluted to obtain therapeutically appropriate levels for patients based on their clinical presentations.

Results: Individuals with ASD (32 males and 8 females, n = 40, ages: 1 year 4 months - 21 years 2 months) had initial and post treatment assessment of Nagalase activity. Dosing of GcMAF was recommended based on previously reported response curves adjusted by the treating clinician for age, weight, and Nagalase levels. The average pre-treatment Nagalase activity of the autism group was 1.93 nmol/min/mg of substrate. This was well above the laboratory reported normal range of <0.95 nmol/min/mg. For the ASD group the average level at the time of second testing was 1.03 nmol/min/mg, reflecting an average reduction of 0.90 nmol/min/mg ($P < 0.0001$). Apart from the likely immunological benefits of lowering the Nagalase activity of these individuals, uncontrolled observations of GcMAF therapy indicated substantial improvements in language, socialization and cognition. No significant side-effects were reported during the course of injections.

Conclusions: In this first report of Nagalase activity in patients with autism, it appears that most individuals have substantially higher levels than the expected healthy ranges. Although Nagalase is a nonspecific marker of immune dysregulation, its observed levels in autism may have both etiological and therapeutic significance. Importantly, this is also the first report of reduction of Nagalase activity in an autism population with GcMAF injections.

Keywords: autism, immune dysfunction, Nagalase, macrophage activation factor, biomarker

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Introduction

Autism is a complex neurodevelopmental disorder which appears in the first three years of life. Once a rare disorder, it is now approaching epidemic, if not pandemic, proportions. A recent report from the US National Center on Birth Defects and Developmental Disabilities revealed a variable range of state by state prevalence from 4.8 to 21.2 per 1,000 for children aged 8 years (data from 2008).[1] These data reflect a remarkable 23% increase over a mere two-year earlier evaluation. While no consensus exists, this trend in autism is at least suggestive of an infective pathogen. Within this context, various organisms have been postulated to be involved, including: gastrointestinal infections,[2] Polyomaviruses,[3] Chlamydophila,[4] Bornaviruses,[5] Paramyxoviruses,[6] and Borrelia burgdorferi.[7] While any of these may contribute to a small percentage of autism cases, it seems unlikely that any of them individually represents the origin of this epidemic.

Despite this uncertainty, growing evidence supports significant immune dysfunction, including autoimmunity, in autism.[8] Simultaneously, oxidative stress[9] and mitochondrial dysfunction[10] are common findings in this population. One possible explanation for the pattern of immune dysregulation and oxidative stress observed in autism spectrum disorders (ASD) could be persistence of active pathogens, perhaps from the perinatal or a subsequent period of child development.[11] Viruses are also known to subvert intracellular calcium regulation to their own functional requirements and in that process to disrupt mitochondrial activity.[12]

Measurement of biomarkers related to immune dysregulation and putative infectious agents is a routine part of the author's (JB) evaluation of ASD in a clinical setting. Recently, evaluation of the activity of alpha-N-acetylgalactosaminidase (Nagalase) has been made commercially available as a diagnostic laboratory measurement. Nagalase has been published as a biomarker associated with various types of cancer,[22–24,29,30] systemic lupus erythematosus (SLE),[13] influenza,[14] and human immunodeficiency virus infection (HIV).[31] It is enzymatically distinct from hepatic galactosaminidase and appears to be far more biologically active. It is therefore a nonspecific biomarker, which appears to be an important indicator of secondary immune dysregulation.

Nagalase is a component of viral hemagglutinin and is released by the action of trypsin on hemagglutinin.[14] Since hemagglutinin is a common glycan-binding lectin of many viruses (including influenza, paramyxoviruses and polyomaviruses), several viruses may individually or jointly contribute to hemagglutinin-derived Nagalase activity in the blood.[15]

In the absence of recent viral infection or malignancies, elevated Nagalase activity likely represents a marker of viral production of hemagglutinin protein being acted upon by inflammatory cell mediated trypsin activity; as such it may represent viral persistence, active transcription, and inflammation. Viral protein transcription is one potential mechanism of autoimmunity.[16,17] Beyond this, Nagalase is an enzyme that deglycosylates the Gc protein also known as vitamin D binding protein (VDBP), rendering it incapable of conversion to active GcMAF (Gc protein-derived Macrophage Activating Factor) and thereby preventing its regulation of macrophage activation.[18] It is noteworthy that vitamin D deficiency, either in pregnancy or during postnatal development, is an apparent risk factor for autism.[19] The impact of Nagalase on VDBP transportation of vitamin D is not known. However, vitamin D deficiency is a known risk factor for autoimmunity.[20]

In light of the possible involvement of immune abnormalities, autoimmunity, vitamin D deficiency, and potential viral persistence, Nagalase screening was added by JB and other physicians to the biomarker profile[21] of children presenting for biomedical evaluation of autism-related disorders and co-morbidities.

Methods

A retrospective chart review for analysis of routine Nagalase testing was accomplished on the initial cohort of patients tested by the clinician (JB). All records were reviewed by JB for confirmation of test results, confirmed diagnosis of autism, time intervals between testing, dosing of subsequent GcMAF used, and the observed clinical/parental/therapist/teacher responses. All patients met the criteria for autism (299.00 DSM-IV revised) and were diagnosed by either a child neurologist or developmental psychologist, in addition to receiving the evaluation of the clinician. No significant changes were made to the participants' treatments apart from the introduction of GcMAF during the timeframe reported in this study.

Additional pre-screening assessments

In addition to meeting DSM-IV revised criteria for autism and having independent determination of autistic severity performed by non-affiliated practitioners, the clinician in this study used an in-house severity scoring system. This consisted of 20 questions (Table 4) designed to evaluate relative severity in the standard domains required for the diagnosis of autism (ie, language, socialization, and stereotypies), as well as other meaningful determinants. Within each category the clinician and parents agreed on a score as follows: 1 = normal or near normal; 2 = mild; 3 = moderate; 4 = severe.

All of the participants assessed in this initial evaluation were in the 3–4 range for question 1 (General Impression of Autism Severity or Delayed Development). This was also true for the core domains of autism (questions 2, 4, and 10). Additionally, all participants scored a 4 (severe) for question 9, indicating a lack of imaginative or age-appropriate play. Significant variability was observed for most of the other domains in the questionnaire, especially in the areas related to motor (both fine and gross), where the greatest initial variability was observed. Specific assessments within each domain were beyond the limited scope of this initial retrospective analysis and would instead be appropriate for a future prospective investigation.

The parents provided written informed consent for phlebotomy and evaluation of potential medical comorbidities occurring in their children with autism. Specifically, Nagalase was discussed with the parents as a potential marker of immune dysregulation. Upon agreement by the parents, sufficient venous blood was withdrawn to fill a 9 ml EDTA tube, which was then immediately inverted at least 5 times. The tube was then centrifuged for 10 minutes at 3000 rpm to separate the plasma. After separation, approximately 3 ml of the clear plasma was transferred to the plasma collecting tube, which was then immediately frozen to −20 °C for at least 24 hours. The specimen was then shipped frozen overnight to an intermediary laboratory in New Jersey, United States. Collected specimens were kept frozen during further shipping to ELN.

The cohort in this initial study consisted of 40 patients whose records included both pre- and post-treatment Nagalase blood test results. Since this was a preliminary and retrospective evaluation of both Nagalase activity and the response to GcMAF, these initial data and observations reflect only the period between the first and second Nagalase testing for patients.

Of the 40 subjects, there were 32 males (ages at the point of first testing ranged from 1 year and 4 months to 21 years) and 8 females (ages ranged from 4 years 7 months to 18 years, median = 6.93 ± SD 5.08 years).

Nagalase assay

Following the procedure published by Yamamoto et al[22,23], Nagalase activity was determined by using an endpoint enzymatic assay using a chromogenic substrate. ELN established a reference range of 0.32–0.95 nM/min/mg of substrate based on serum collected from healthy volunteers, a range nearly superimposable to that previously reported which was between 0.35 and 0.65 nM/min/mg.[24]

GcMAF preparation

Patients obtained commercially available GcMAF from Immuno Biotech Ltd., Guernsey UK (http://www.gcmaf.eu). Immuno Biotech prepared the GcMAF according to the procedure described in their published materials.[24] Briefly, Gc protein was isolated from purified human serum obtained from the American Red Cross, using either 25-hydroxyvitamin D3-Sepharose high affinity chromatography or actin-agarose affinity chromatography. The bound material was eluted and then further processed by incubation with three immobilized enzymes. The resulting GcMAF was filter sterilized. The protein content and concentration was assayed using standard Bradford protein assay methods.[25] At the end of the production process, the GcMAF was checked for sterility in-house and externally by the UK Health Protection Agency and independent laboratories.[26]

GcMAF activity assay

Activity assay of GcMAF was based on a live macrophage cell proliferation method using water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, Calbiochem®). Cells were cultured in the presence of a range of GcMAF concentrations and incubated for 72 hours. The soluble MTT was added, and the cells incubated for a further period of time.

The resulting color change, due to the conversion of the MTT, was measured using a plate reader at 460 nm. The activity of the GcMAF could then be determined by comparison of the negative and positive controls using the RAW 264.7 murine macrophage cell line [Sigma-Aldrich®]. Positive macrophage activation was demonstrated by apoptosis of MCF7 breast cancer cells (Lawrence Berkeley National Laboratory). An internally known positive standard was also included in the assay as a control. Further testing of this GcMAF product's activity was determined measuring its biological effects in both human peripheral blood mononuclear cells[27] and human breast cancer cells.[28]

After appropriate written informed consent by the parents, GcMAF was injected subcutaneously on a weekly basis using a 31 gauge insulin syringe. In adults with HIV or several different types of cancer, the typical dose described for response given either intramuscular or intravenous administration has been a minimum of 100 ng/wk. Despite levels of Nagalase activity well into the range of many cancer or HIV patients, the clinician elected not to exceed 100 ng/wk so as to prevent putative over-stimulation of macrophages. All patients started on low doses that were increased gradually over the course of treatment. The doses ranged from 4 to 100 ng per wk and were adjusted based on clinical response, age, body weight, and the initial level of Nagalase activity.

To further assess the clinical responses, all parents were interviewed at intervals of no longer than a month using the improved Clinical Global Impression Scale (iCGI) defined by Kadouri et al and described below.[29]

Improved response format for the clinical global impression of improvement scale

The improved response format for the Clinical Global Impression of Improvement scale is as follows: 5 = Very considerable improvement; 4 = Considerable improvement; 3 = Moderate improvement; 2 = Slight improvement; 1 = Very slight improvement; 0 = State unchanged; −1 = Very slight deterioration; −2 = Slight deterioration; −3 = Moderate deterioration; −4 = Considerable deterioration; −5 = Very considerable deterioration; −6 = Maximum deterioration.

Statistical methods

Due to the relatively small population overall and only 8 females in this sample, the group was evaluated as a whole and not segregated based on gender or age. Statistical comparison between pre and post treatment levels of Nagalase was performed by two-tailed, paired difference *t*-test and by using standard formulas in Microsoft Excel® 2010. Since one subject had a Nagalase result significantly higher than the mean, consideration for skewing artifact was made. Adjusting for the skew effect changed the median of the group from 1.71 to 1.68, which was not statistically significant.

Results

The average pre-GcMAF treatment Nagalase activity was 1.93 nM/min per mg, with a median of 1.68 nM/min per mg (SD ± 1.21 nM/min per mg), and with a range of 0.90 nM/min per mg to 7.80 nM/min per mg (Table 1). At the point of time of subsequent testing (average interval 100 days, ±SD 32 days), the average Nagalase activity during GcMAF treatment was 1.03 nM/min per mg, with a median of 0.90 nM/min per mg (±0.67 nM/min per mg), and with a range of 0.44 to 4.40 nM/min per mg. This reflects an average reduction of 0.90 nM/min per mg ($P < 0.0001$). Of this original cohort only 2 of 40 (5%) were observed to be initially within the laboratory reference range (0.90 and 0.92 nM/min per mg).

Because of the standard laboratory turnaround time and the necessary time to discuss treatment options with the parents, the actual number of weekly injections was substantially less than the number of weeks in the interval of retesting. The average number of weekly injections was 14 (±4 weeks SD).

At the time of retesting, the Nagalase levels of 24 of the 40 patients (60%) had decreased to within the laboratory reference range of <0.95 nM/min per mg. In view of these results, a minimum of 16 patients (40%) would be considered not to have received adequate therapeutic effect and would therefore be candidates for continued intervention. Only 1 of 40 (2.5%) failed to respond with significant reduction of Nagalase activity (pre/post Nagalase difference of only 0.10 nM/min per mg). The families of the two patients whose initial Nagalase levels were within the upper part of the laboratory reference range both elected to initiate GcMAF therapy. Both patients experienced

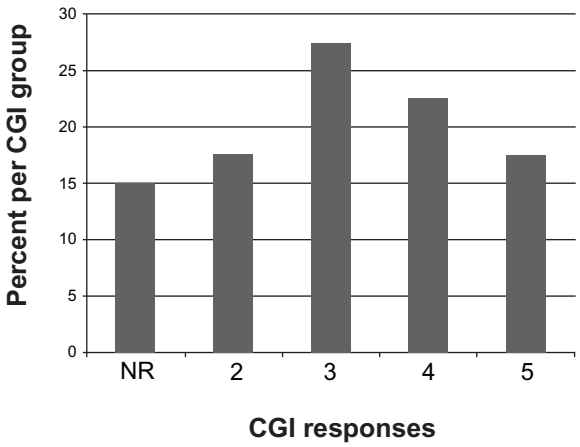
significant reductions in Nagalase activity, one with a considerable response (iCGI = 4), while the other was rated as a non-responder.

The initial levels of Nagalase activity in the group of patients that we studied ranged from the upper range of normal to beyond levels typically observed in metastatic cancer patients[30,31] and HIV-infected patients.[32] Despite concerns about autoimmunity in autism, none of the patients observed in this study experienced significant side-effects, and none were required to suspend

Table 1. Nagalase dataset for pre-post GcMAF therapy with iCGI response per subject.

Gender	Nagalase						Pre/post Nagalase difference	iCGI
Male/ female	Pre draw date	Age in years	Pre result	Post 1 draw date	Post 1 result	Days between pre—post 1		
m	6/21/2011	6.3	0.90	9/7/2011	0.47	76	–0.43	4
m	5/3/2011	21.2	1.00	7/22/2011	0.44	79	–0.56	5
m	4/28/2011	6.95	1.30	9/21/2011	0.72	143	–0.58	3
m	6/3/2011	9.75	2.20	12/23/2011	1.30	200	–0.90	2
m	5/3/2011	7.15	1.90	8/25/2011	0.76	112	–1.14	4
f	5/3/2011	11.1	1.90	8/25/2011	1.00	112	–0.90	5
f	5/3/2011	9.2	1.90	8/24/2011	1.20	111	–0.70	5
m	5/3/2011	4.3	1.70	8/25/2011	1.10	112	–0.60	4
m	4/29/2011	4.4	1.00	8/25/2011	0.76	116	–0.24	3
m	6/7/2011	6.05	1.20	8/16/2011	0.79	69	–0.41	4
f	6/15/2011	5.1	1.66	8/31/2011	0.40	76	–1.26	–1
m	8/25/2011	12.3	1.69	11/23/2011	0.47	88	–1.22	1
m	10/4/2011	11.5	7.80	11/30/2011	4.40	56	–3.40	3
m	6/24/2011	7	1.50	8/24/2011	0.90	60	–0.60	4
f	4/21/2011	12.05	1.98	8/5/2011	0.81	104	–1.17	3
m	6/16/2011	1.3	1.50	9/21/2011	1.00	95	–0.50	4
m	6/16/2011	5.6	2.60	9/21/2011	2.50	95	–0.10	2
m	5/11/2011	1	2.80	9/2/2011	1.80	111	–1.00	0
m	9/21/2011	18	1.30	11/30/2011	0.92	69	–0.38	3
m	5/6/2011	3.6	3.00	8/3/2011	1.00	87	–2.00	5
m	5/13/2011	16.5	1.20	9/21/2011	0.80	128	–0.40	4
m	5/13/2011	3.9	1.60	7/6/2011	1.10	53	–0.50	3
f	6/8/2011	4.6	0.92	9/21/2011	0.62	103	–0.30	0
m	6/29/2011	10.7	1.00	9/27/2011	0.90	88	–0.10	3
m	4/6/2011	4.85	1.40	7/21/2011	0.81	105	–0.59	3
f	4/13/2011	4.7	3.90	8/19/2011	1.60	126	–2.30	5
f	5/6/2011	3.65	1.10	8/5/2011	0.61	89	–0.49	2
m	4/19/2011	18.3	4.00	6/29/2011	1.40	70	–2.60	4
m	5/27/2011	3.05	2.60	8/18/2011	1.40	81	–1.20	3
m	5/19/2011	5.2	1.20	11/23/2011	0.96	184	–0.24	3
m	5/5/2011	9.1	1.79	8/10/2011	0.57	95	–1.22	2
f	6/22/2011	18.2	1.90	12/1/2011	1.20	159	–0.70	0
m	6/8/2011	16.4	1.82	8/25/2011	0.62	77	–1.20	2
m	5/19/2011	10.5	2.90	8/11/2011	0.93	82	–0.97	3
m	7/15/2011	6.8	1.73	8/23/2011	0.51	38	–1.22	4
m	5/6/2011	6.9	2.90	7/21/2011	1.20	75	–1.70	5
m	6/8/2011	10.15	1.20	9/21/2011	0.82	103	–0.38	1
m	6/8/2011	8.1	1.10	9/21/2011	0.68	103	–0.42	2
m	4/29/2011	4.1	1.00	8/26/2011	0.89	117	–0.11	2
m	4/6/2011	3.55	1.20	8/24/2011	0.91	138	–0.29	5
Average		8.33	1.93		1.03	99.63	–0.90	2.98
Median		6.93	1.68		0.90	95.00		3.00
S.D.		5.08	1.21		0.67	32.81	0.70	

Table 2. iCGI response to GcMAF after an average of 14 weekly subcutaneous injections.



Notes: Y-axis represents the percentage of total in each group. NR = non-responder (–1 to 1), 2 = slight improvement (generally described as better eye contact, increased interaction with the environment and/or family), 3 = moderate improvement (Based on parental and teacher/therapists input, there was improved communication, increased acquisition of new skills, vocabulary and noticeable increase in sociability), 4 = considerable improvement (To obtain this level of response, patients were noted by parents, teachers and the clinician to have substantially better communication, generally going from single word utterances to several word sentences, as well as showing evidence of improved academic processing), 5 = Very considerable improvement (This response was demonstrated at school, during therapies, home and outside the home as substantial improvement to the point that many or most of the criteria of autism were no longer present).

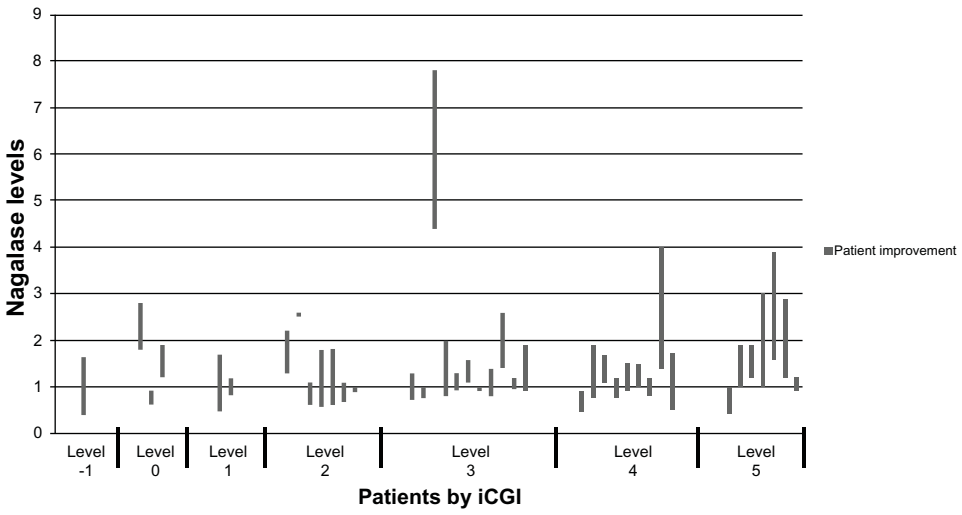
or drop out of treatment. During the first few weeks of treatment, 3 of 40 patients (7.5%) experienced low to moderate rise in body temperature, typically occurring 24 to 48 hours after the GcMAF injection and lasting less than 24 hours. Parents were instructed to use ibuprofen only if the temperature exceeded 102° F (approximately 39°C), and two were treated during the first few weeks. By the second month, no patients experienced significant febrile events. Interestingly, during the first 3 weeks, 6 of 40 patients (15%) were observed to have rashes compatible with viral exanthemas (generally on the trunk and in fine papules more commonly than maculae). Petechiae were not observed. These rashes could represent the manifestation of latent or persistent viral infections interacting with activated macrophages.

Discussion

Since this is an open-label, non-controlled, retrospective analysis, caution must be employed when ascribing cause and effect to any treatment outcome. However, the response to GcMAF was robust with regard to Nagalase reduction, as well as symptomatic improvements as shown by the iCGI. Despite the short observational time period, the result that 67.5% of the group responded in the 3 to 5 CGI-I range was unexpectedly substantial (Tables 2 and 3).

In this small population, it does not appear that an obvious association exists between the iCGI response and the change in the Nagalase activity (Table 3). Further statistical analysis was therefore not deemed appropriate.

Table 3. Pre-post Nagalase by iCGI response level.



Notes: The vertical line represents the change in Nagalase activity (all changed by going down over the time interval between pre-post testing). The smallest observed change in Nagalase activity was –0.1 nM/min per mg. The data are divided into groups based on the noted iCGI responses observed.

Table 4. Questionnaire designed to evaluate relative severity in the standard domains required for the diagnosis of autism.

1. General Impression of autism severity or delayed development?
2. Expressive language?
3. Difficulty following verbal commands?
4. Flapping or self-stimulation?
5. Sensory Issue (touch texture etc.)
6. Difficult transitions?
7. Tantrums?
8. Obsessive and/or compulsive behaviors?
9. Lack of imaginative or age appropriate play?
10. Lack of desire for social interaction?
11. Hyperactivity?
12. Inattention?
13. Lack of eye contact?
14. Problems Sleeping?
15. Sound sensitivity—ear covering?
16. Feeding problems?
17. Gross motor problems (abnormal walking or running gait)?
18. Fine motor problems (tripod grasp, buttons, zippers, snaps)?
19. Anxiety or panic around doctors or about bloodwork?
20. Problems with bowel movements?

Conclusion

The changes in Nagalase activity in response to GcMAF treatment in this ASD population reflected similar robust responses observed using GcMAF in the treatment of HIV infection and cancer. However, autism represents a developmental disorder with substantial delays in core domains of cognitive activity (language, socialization, and behavior) and is generally felt to be a life-long condition. Therefore, these initial observations give support to the notion that autism *per se* may be the consequence of treatable underlying pathophysiology. Given that ASD are now affecting more than 1% of US children, the observed response to GcMAF warrants urgent and further prospective evaluation.

Although Nagalase is a non-specific marker believed to be derived from viral hemagglutinin, it may be useful as a biomarker of therapeutic significance in ASD, and as such also warrants further investigation. Regardless of any immediate clinical improvement, the reduction of Nagalase to more desirable levels is of potential benefit to these patients, since Nagalase is known to impair immune defenses.

Author Contributions

Primary author, maintenance of the database, statistical analysis, and clinician: JB.Nagalase laboratory methodologies and assistance with statistical methods: EV. GcMAF preparation methods: LT.

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Competing Interests

JB prescribes testing and recommends therapies for children with autism. His son and stepson have autism. EV previously had a financial interest in ELN, the laboratory which tests measures Nagalase. LT is employed by Immuno Biotech, Ltd. (the laboratory isolating and purifying the GcMAF protein). However, in the case of ELN, EV had no knowledge of the therapies being used nor of the names of any patients whose data were being analyzed. Further, in the case of LT, neither she nor any employee of Immuno Biotech had any knowledge of the Nagalase results or the patient/parent names used in this study.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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