Study Protocol and Statistical Analysis Plan

Title: Sulforaphane Treatment of Children with Autism Spectrum Disorder (ASD).

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1) Title

Sulforaphane Treatment of Children with Autism Spectrum Disorder (ASD).

2) IRB Review History*

NA

3) Objectives*

We propose to conduct a phase I/II, randomized, double blind, placebo-controlled, one-arm crossover clinical trial to test the safety and efficacy of orally administered sulforaphane in young boys and girls with autism.

Our *long term research goal* is to discover and develop pharmaceuticals of low toxicity that treat core cellular mechanisms underlying behavioral features of ASD. Our *objective* in this application is to define the clinical effects of sulforaphane in children with ASD, along with the biomarkers and cellular mechanisms that may explain these effects.

Our *specific aims are:*
1. To determine if there are measurable effects on social responsiveness and problem behaviors during treatment with orally administered Sulforaphane-rich Broccoli Seed Powder (referred to as sulforaphane hereafter) in 3-12 year old boys and girls with ASD. Our *working hypothesis,* based on strong preliminary data from our recent trial of sulforaphane in young adults with ASD, is that sulforaphane will have similar and measurably greater clinical effects in children with ASD;

2. To determine if treatment with sulforaphane is safe and well tolerated. Our *working hypothesis,* based on our previous work and other studies, is that there will be few or no signs of toxicity;

3. To elucidate cellular biomarkers that support the hypothesized mechanism of action of sulforaphane in ASD. Our *working hypothesis* is that sulforaphane will increase levels of Nuclear factor-erythroid factor 2 (Nrf2), improve antioxidant capacity, upregulate expression of heat shock proteins, and modify mTOR signaling pathways and cytokine expression.

Our *overarching hypothesis* is that ASD results from underexpressed or abnormal genes that converge on common metabolic pathways which affect synaptic development and function. Our *long term goal* is to *discover* agents, such as sulforaphane, that are safe and effective in treating ASD, based on their known effects on these pathways, and to *define* these effects insofar as it is reasonably possible, in clinical trials. Sulforaphane exemplifies this goal, in that it is a nontoxic natural substance that has the potential to improve both underlying cellular abnormalities, as reflected in redox imbalance[1], Nrf2[2], heat shock proteins[3] and mTOR expression,[4] and their behavioral and cognitive effects in ASD. Furthermore, the beneficial effects of sulforaphane are likely to be more marked in children compared to adults with ASD, since they have greater potential for clinical

improvement due to the compounding effects of treatment during dynamic brain development in childhood.

4) **Background***

Despite progress in genetic research in ASD, direct treatment of underlying physiologic mechanisms is limited. Several findings in ASD suggest that different types of cellular dysfunction, including neuroinflammation[5], oxidative stress[1], mitochondrial abnormalities[6], and abnormal synaptic plasticity/connectivity[7] may involve a number of related, interacting metabolic pathways.

Fever effect: The widespread anecdotal reports that febrile illness dramatically but temporarily ameliorates the disordered behavior of a substantial fraction of autistic patients have been corroborated and may provide important clinical clues to cellular mechanisms in ASD[8]. The degree of improvement in these patients (mostly in reduced stereotypic behavior and speech) was unrelated to the severity of fever or of ASD. The mechanisms for this "fever effect" in ASD are unknown, but may include direct thermal effects, as well as indirect effects, such as increased cytokines, changes in the hypothalamic-pituitary-adrenal axis, locus coeruleus and central noradrenergic function[9], and increased expression of heat shock proteins (HSP) and cellular stress responses[3,10]. Fever stimulates the HSP and cellular stress responses that may ultimately lead to changes in synaptic function and increased long-range connectivity,[11] and thereby leads to behavioral improvements. The expression of gene transcription by NFE2L2 (Nrf2), which is reduced in ASD[2], may also be increased during fever, thereby increasing gene transcription. Sulforphane, by activating Nrf2, enhances antioxidant expression in astrocytes and protects against neurotoxicity in experimental herpes encephalitis[12]**.**

Sulforaphane, an isothiocyanate (1-isothiocyanato-4R-(methylsulfinyl)butane), is obtained from lyophilized extracts of 3-day-old broccoli sprouts or as its glucosinolate precursor glucoraphanin, from broccoli seeds. Both broccoli sprouts and seeds contain the glucosinolate precursor glucoraphanin; neither contains any sulforaphane, which is produced from glucoraphanin by the action of myrosinase in the gut. Sulforaphane induces HSP and Nrf2 with positive effects on redox regulation, DNA damage sensing and repair, molecular chaperones, fatty acid and lipid metabolism and energy metabolism[13,14]. Sulforaphane has been shown to suppress lipopolysaccharide induced inflammation in rat microglia[15] and may regulate neuroinflammation in degenerative disorders of the CNS through its induction of Nrf2[16]. Sulforaphane and related compounds are hormetic drugs that induce a general "cell-protective" response, as demonstrated in vitro in sickle cell disease as well as X-linked adrenoleukodystrophy[17,18], fragile X syndrome and spinal muscular atrophy[19]. Sulforaphane crosses the blood brain barrier and is bioavailable orally[20,21]. Recent evidence shows that dysregulation in the mTOR/AKT signaling and cell survival pathway is present in animal models of ASD and tuberous sclerosis with ASD[4]**.** The lack of suppression of mTOR leads to excessive synapse formation due to failure of autophagy and pruning of synapses during brain development, which may be improved by administration of rapamycin[22]. Sulforaphane

has been shown to induce cell cycle arrest and apoptosis in primary lymphoblasts from children with acute lymphoblastic anemia (ALL), and does so, in part, through its inhibition of the AKT and mTOR survival pathways[23]**.** Sulforaphane, therefore, has at least several possible modes of action that may benefit ASD through common cellular mechanisms that underlie its heterogeneous phenotypes.

Preliminary data: We completed a randomized, double blind, placebo-controlled pilot clinical trial at the Lurie Center/MassGeneral Hospital *for* Children from 01/2011 to 12/2013 (clinical trials identifier NCT01474993*)*[24] to test the efficacy of sulforaphane in 44 male adolescents and adults (13-30 years) with ASD. Fifteen participants were randomized to placebo and 29 to sulforaphane. Forty participants (14 on placebo and 26 on sulforaphane) completed the trial; of those 32 (80%) had a history of positive behavioral responses to fever. All participants were treated for 18 weeks with study visits at baseline, 4, 10 and 18 weeks, followed by a final visit at 22 weeks, 4 weeks after treatment ended. Parent/caregivers completed the Aberrant Behavior Checklist (ABC)[25] and Social Responsiveness Scale (SRS)[26] and study physicians completed the Ohio Autism Clinical Impressions Severity and Improvement Scales (OACIS-S and OACIS-I)[27,28] to document severity of autistic behavior at each visit. OACIS-S and -I evaluate severity and improvement of ASD symptoms in the following subdomains: general level of autism, social interaction, aberrant/abnormal behavior, stereotypical behavior, verbal communication, non-verbal communication, hyperactivity and inattention, anxiety/fears, sensory sensitivities and restricted interests. Participants were monitored with regular physical examinations and clinical laboratory studies.

Results from clinical trial in young adult males with ASD: Sulforaphane was well tolerated and there were no significant toxic effects. SRS scores decreased by an average of -15 units in the sulforaphane group compared to -3 units in participants on placebo ($p=0.02$); considering the 4 drop-outs as non-responders, 31% (9/29) sulforaphane participants vs 0% placebo participants had a 25% decrease in total SRS score from baseline ($p=0.018$). The average change in total ABC score was -18 units in the sulforaphane group as compared to -1 units in the placebo group ($p=0.0001$), and after considering the 4 drop-outs as non-responders, 52% (15/29) sulforaphane participants vs. 13% (2/15) placebo participants had a 25% decrease in their total ABC score from the baseline ($p=0.02$). On the OACIS-I, participants on sulforaphane were much improved or very much improved on the following subdomains: social interaction, 41% (12/29) sulforaphane participants vs. 0% on placebo ($p=0.003$); aberrant/abnormal behavior, 48% (14/29) on sulforaphane vs. 6.7% (1/15) on placebo ($p=0.007$); repetitive/stereotypical behavior, 21% (6/29) on sulforaphane vs. 0% placebo ($p=0.08$); and verbal communication, 38% (11/29) on sulforaphane vs. 0% placebo ($p=0.008$). Seventeen of the 26 participants on sulforaphane who completed the trial were much or very much improved on at least one subdomain of the OACIS-I. Ninety-four percent (16) of those 17 on sulforaphane had a history of positive behavioral effects during febrile illness. In comparison, of the remaining 9 participants who were taking sulforaphane but were not much/very much improved on OACIS-I, only 55% (5/9) had a history of positive effects of febrile illness ($p=0.03$).

5) Inclusion and Exclusion Criteria*

- Inclusion criteria
    - Autism (ASD) diagnosis. Quantitative autism traits and severity for diagnosis of autism will be assessed by clinical judgment corroborated by the ADOS-G and DSM-V checklist of symptoms. ASD symptoms moderate to severe, based on ADOS criteria.
    - Age from 3 through 12 years.

- Exclusion criteria for subjects will include:
    - Absence of a parent or legal guardian and consent
    - Inability to speak/understand English language
    - Seizure within **1** year of screening: This exclusion is based on the theoretical concern that cellular activation by sulforaphane might exacerbate seizures in patients with known seizure disorders. As previously noted in our previous trial of sulforaphane in young adult males, a seizure occurred in each of 2 participants: one during treatment (in a participant with a previously undisclosed seizure), the other 3 weeks after discontinuing sulforaphane.
    - Impaired renal function (serum creatinine > 1.2 mg/dl), impaired hepatic function (AST/ALT > 2x upper limit of normal), impaired thyroid function (TSH outside normal limits): This exclusion is based on a theoretical possibility of activation of underlying cellular metabolic abnormalities by sulforaphane. Current infection or treatment with antibiotics: this exclusion is to avoid complications of inter-current illness that may occur due to the clinical trial or obscure possible effects of sulforaphane.
    - Medications that may modify the course or testing of ASD parameters (e.g., prednisone): This exclusion is necessary in order not to interfere with or complicate effects of sulforaphane.
    - Chronic medical disorder (e.g., cardiovascular disease, stroke or diabetes) or major surgery within 3 months prior to enrollment**:** Serious medical illness in the child may be complicated by the clinical trial and make it difficult to discern a change in ASD associated with treatment.
    - Less than 3 years or more than 13 years of age: this age range was selected to cover the ages from usual diagnosis of ASD up to adolescence.
    - A diagnosis of autism spectrum disorder other than autism, for example, Asperger disorder, PDD-NOS, not consistent with moderate to severe autistic disorder, according to ADOS criteria.
    - Prisoners
    - Pregnant women

6) Study-Wide Number of Subjects*

NA

7) Study-Wide Recruitment Methods*

NA

8) Study Timelines*

Duration of intervention: 15 weeks in first (double blind, Phase 1), and 15 weeks in open-label (Phase2).

Prior to the main clinical trial, we will carry out a pilot study in 10 children with ASD, 6-12 years of age, who will receive sulforaphane, 2.2 micromoles/kg daily for 14 days (details below in "10: Procedures Involved (Study design)").

The entire clinical trial is estimated to last 3 years. The following tables below depict our estimated timelines for study procedures and subject recruitment:

SPECIFIC AIMS/TASKS	TIMELINE (UMASS)	TIMELINE (JH)
SPECIFIC AIMS 1 AND 2: Clinical trial: To determine if there are measurable effects on social responsiveness and problem behaviors during treatment with orally administered Sulforaphane-rich Broccoli Seed Powder (referred to as sulforaphane hereafter) in 3-12 year old boys and girls with ASD; To determine if treatment with sulforaphane is safe and well tolerated.		
Task 1: Obtain regulatory approvals for clinical trial (submit 3+ mo. before start date)		
Subtask 1.1: USAMRMC Human Research Protection Office	(-) 3-4 mo.	
Subtask 1.2: UMass and JH IRB application	(-3) mo.	(-) 3 mo.
Subtask 1.3: Apply to FDA for IND	(-) 3 mo.	
Task 2: Organize study staff: recruit Primary Care MD and Clinical Research Assistant, Training sessions to assure ratings reliability on CGI and review consent/assent procedures and patient privacy protections. Review clinical data collection methods and safeguards	0-1 mo.	
Task 3: Recruit for Pilot Study (10 subjects with ASD), administer SF (broccoli seed powder) for 2 weeks; collect blood and urine samples before and after BSP; process and ship samples to JH .	1-3 mo.	
Task 4: Recruit for rolling enrollment in main SF Clinical Trial (50 subjects)		
Subtask 4.1: Screen potential participants by phone, email or in person. Obtain informed consent and assent when possible at screening/enrollment visit. Explain the study in detail and answer all questions. Availability 24/7 with pager/phone. Perform ADOS, physical exam, phlebotomy for screening clinical labs and baseline CGI-S, ABC and SRS on each participant. Obtain SF-BSP/placebo from	2-24 mo.	

Pharmacy at dose specific for child, dispense but hold before starting until review of clinical lab studies, then call to start medication. 7-week visit: Review progress; Perform CGI-I; ABC and SRS by parents; physical exam; phlebotomy/urine for clinical and research lab studies. Dispense BSP. 15-week visit: Review progress; Perform CGI-I; ABC and SRS by parents; physical exam; phlebotomy/urine for clinical and research lab studies. Dispense BSP. 22-week visit: Review progress; Perform CGI-I; ABC and SRS by parents; physical exam; phlebotomy/urine for clinical and research lab studies. Dispense BSP. 30-week visit: Review progress; Perform CGI-I; ABC and SRS by parents; physical exam; phlebotomy/urine for clinical and research lab studies. Discontinue medication 36-week visit: Review progress; Perform CGI-I; ABC and SRS by parents; physical exam; phlebotomy/urine for research lab studies. Record exit comments on study.		
Task 5: Data storage and analysis	0-36 mo.	
SPECIFIC AIM 3: To elucidate cellular biomarkers that support the hypothesized mechanism of action of sulforaphane in ASD. Blood and urine samples will be collected at UMass in Pilot and Main clinical trial, processed, stored and shipped on dry ice to JH		
Task 1: Labwork at JH for Pilot Study of biomarkers: Nrf2, oxidative stress markers, heat shock proteins, mitochondrial functions, mTOR, cytokine	3-6 mo.	3-6 mo.
Task 2: Labwork at JH for Main Clinical Trial: biomarkers and mechanisms	6-36 mo.	6-36 mo.

ANTICIPATED TARGET ENROLLMENT (QUARTERLY):

	Year 1				Year 2				Year 3				
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	TOTAL
Target Enrollment Pilot Study	10	-	-	-	-	-	-	-	-	-	-	-	10
Target Enrollment Main Study (per quarter) (does not include the pilot study participants, who will not take part in the main study because they will no longer be sulforaphane naïve)	-	7	10	8	8	5	3	3	2	2	2	0	50

9) Study Endpoints*

a) Primary study endpoint:

i) Ohio Autism Clinical Impressions Scale – Improvement (OACIS-I) Average Score.

b) Secondary study endpoints:

i) OACIS-I response rate (much or very much improved) on aberrant behaviors

ii) OACIS-I response rate (much or very much improved) on social communication

iii) Change in total ABC score (as well as subscales of Irritability, lethargy and social withdrawal, stereotypic behavior, hyperactivity and inappropriate language) from baseline

iv) Change in total SRS score (as well as subscales of social awareness, social cognition, social communication, social motivation and autistic mannerisms) from baseline

v) Determination of Nrf2 levels

vi) Determination of oxidative stress biomarkers

vii) Determination of Heat Shock Response biomarkers

viii) Determination of mitochondrial function biomarkers

ix) Determination of mTOR signaling pathway markers

x) Determination of immune function and inflammatory biomarkers

c) Safety endpoints

i) Liver function tests (ALT/AST)

ii) Renal function tests (serum creatinine)

iii) Thyroid function (TSH levels)

iv) Complete blood counts (WBC count, RBC count, platelet counts)

v) Urinalysis

Description of surveys used as the primary and secondary study endpoints:

1. **Ohio Autism Clinical Impressions Scale - Severity (OACIS-S)[27,28]** is a 7 point, 10 domain scale that requires the clinician to rate the severity of the patient's ASD at the time of assessment, relative to the clinician's past experience with patients who have the same diagnosis. The questions are rated on a scale from 1 to 7 in the increasing order of severity, where "1" is normal (symptoms indistinguishable from typically developing children) and "7" is the most severe symptoms causing significant problems with individual functioning on a daily basis. The 10 items on the OACIS-S scale cover different domains of patients' behavior, including global autism severity, social interaction, aberrant behavior, repetitive or ritualistic behaviors, verbal communication, non-verbal communication, hyperactivity/inattention, anxiety, sensory sensitivities and restricted/narrow interests. These are rated in a similar way to the NIMH CGI Severity scale, but it

is focused on autism spectrum symptoms. This scale will be used to acquire clinician-assessed severity of autistic symptomatology in the study.

2. **Ohio Autism Clinical Impressions Scale – Improvement (OACIS-I)[27,28]** is a 7 point, 10 domain scale that requires the clinician to assess how much the patient's ASD has improved or worsened relative to a baseline state at the beginning of the intervention and rated as: "1", very much improved; "2", much improved; "3", minimally improved; "4", no change; "5", minimally worse; "6", much worse; or "7", very much worse. The 10 domains in which improvement is assessed are the same as those used in the OACIS-S scale. For this study, the proportion of patients who were rated as improved or very much improved on each of 10 domains of OACIS-I were compared between sulforaphane and placebo groups. This scale will be used to gauge the clinician assessment of improvement in autistic symptomatology at all follow up visits as compared to the previous study visits.
3. **Social Responsiveness Scale (SRS)[26]**: SRS is a parent- and/or teacher-reported 65 point scale covering 5 treatment subscales focusing on the social communication domain of ASD as observed in natural (non-clinical) settings. Each item on the scale inquires about an observed aspect of reciprocal social behavior that is rated on the questionnaire on a scale from "1" (never true), "2" (sometimes true), "3" (often true), and "4" (almost always true). Its quasi-interval, ordinal design facilitates assessing degrees of change and/or severity of symptoms in response to an intervention. The SRS scale scores is subdivided into 5 subscales (social awareness, social cognition, social communication, social motivation and autistic mannerisms). This scale will be used at all study visits and the scores (as well as change thereof from the baseline visit) will be used as one of the clinical outcome measures assessing social interaction skills in the study.
4. **Aberrant Behavior Checklist (ABC)[25]:** ABC is a 58-item rating scale was developed for persons with developmental disabilities living in the community, and to assess medication effects. Its 58 questions are rated by parents or teachers on a scale of 0 to 3, where a score of "0" for particular behavior is not a problem at all, "1" indicates that the behavior is a problem but slight in degree, "2" indicates that the problem is moderately serious, and "3" indicates that the problem is severe in degree. The ABC score is sub-divided into 5 subscales (Irritability, lethargy and social withdrawal, stereotypic behavior, hyperactivity and inappropriate language). This scale will be used at all study visits and the scores (as well as change thereof from the baseline visit) will be used as one of the clinical outcome measures assessing severity of aberrant/abnormal behaviors in the study.
5. **Autism Diagnostic Observation Schedule (ADOS)[29]:** ADOS is an instrument for diagnosing and assessing autism. The protocol consists of a series of structured and semi-structured tasks that involve social interaction between the examiner and the subject. The examiner observes and identifies segments of the subject's behavior and assigns these to predetermined observational categories. Categorized observations are subsequently combined to produce quantitative scores for analysis. Research-determined cut-offs identify the potential diagnosis of autism or related autism spectrum disorders, allowing a standardized

assessment of autistic symptoms. ADOS as performed by a research reliable psychologist will be used as one of the primary measures to ensure eligibility criteria for diagnosis of ASD in the study.

6. **Vineland Adaptive Behavior Scale (VABS)[30]:** VABS is a valid and reliable nonverbal test to measure a person's adaptive level of functioning. VABS aids in diagnosing and classifying cognitive impairment in ASD and developmental delays. The focus of VABS is the measurement of the adaptive behaviors, including the ability to cope with environmental changes, to learn new everyday skills and to demonstrate independence. VABS will be administered at all study visits to help assess changes in adaptive skills during the study.
7. **Leiter-3 scale[31]:** Leiter scale is used as a measure of nonverbal IQ. It will be used at the screening visit in order to classify study participants' nonverbal IQ.

10) Procedures Involved* (Study design):

Pilot Study to Determine Biomarkers: Prior to the main clinical trial, we will carry out a pilot study in 10 children with ASD, 6-12 years of age, who will receive sulforaphane, 2.2 micromoles/kg daily for 14 days. We will collect blood and urine samples before and at the end of treatment, in order to measure several parameters that are likely to demonstrate expected effects of sulforaphane, to standardize the assays and procedures, and to determine the most effective measures. Further details of the pilot study are discussed below.*

Study Design (Specific Aim 1): Following the pilot study for biomarkers, we will conduct a randomized, double-blind, placebo-controlled phase-2 clinical trial of sulforaphane in a single-arm crossover design in 3 phases, in 50 boys and girls (ages 3-12 years) with moderate to severe ASD (see Figure 1 below). In Phase 1 we shall treat participants for 15 weeks (50% with sulforaphane ((2.2 micromoles/kg body weight)), 50% with placebo of identical appearance). In Phase 2, all children will receive sulforaphane (2.2 micromoles/kg b.w.) on open label for an additional 15 weeks. In Phase 3 (6 weeks), participants will receive no sulforaphane, then return for followup at the end of the study. We project that treatment with sulforaphane will be associated with statistically significant improvements in physicians' global impressions, aberrant behaviors, social responsiveness and adaptive behaviors (see descriptions of outcome measures and statistical plan below).

Study variables will include clinician and parent assessments, and both clinical and scientific laboratory test measurements to evaluate for signs of toxicity and potential biomarkers of sulforaphane's effects. Our primary outcome measure will be the Ohio Autism Clinical Global Impression Scale (OACIS)[27,28]. Secondary outcome measures will include the Aberrant Behavior Checklist (ABC)[25], Social Responsiveness Scale (SRS)[26], and Vineland Adaptive Behavior Scale (VABS)[30]. The OACIS will be completed at each of 5 visits by clinicians; the ABC, SRS and VABS will be completed by the parents. All participants will have moderate to severe ASD, according to ADOS criteria as part of the screening process. Control subjects will be those children who

receive placebo for 15 weeks during Phase 1. All children will receive active sulforaphane during Phase 2.

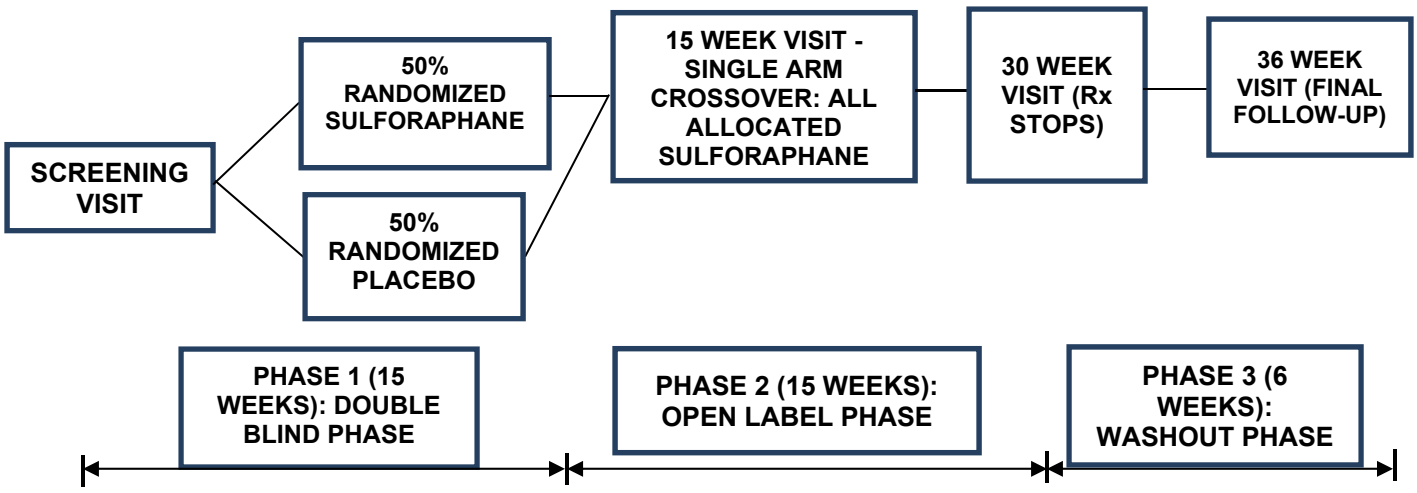

Figure 1: Flowchart of progress of study participants through the 3 phases of the clinical trial (flowchart not to scale)

At an initial screening visit, following informed consent by the parents and assent by the child if he or she is able, a complete pre- and postnatal, pediatric and neurodevelopmental history will be obtained. We will also review the child's history of behavioral responses to fever and illness, and rate the parents' recall of subjective impressions of changes in ASD according to the OACIS. Children will be tested using the Autism Diagnostic Observation Schedule (ADOS)[29] by a trained clinician. If the diagnosis of ASD is confirmed, nonverbal intelligence will be measured using the Leiter-3[31].

Monitoring for safety (Specific Aim 2): A complete physical exam will be performed at each of the 6 study visits. Phlebotomies will be performed 6 times, along with urine collection: 1) After screening and prior to randomization; 2) at 7 weeks after starting treatment in Phase 1; 3) at 15 weeks, at the end of Phase 1; 4) at 22 weeks; 5) at 30 weeks, at the end of Phase 2; and 6) at 36 weeks, at the end of the study. Blood samples will be sent for routine laboratory studies at screening, 7, 15, 22 and 30 weeks: complete blood count with differential white blood count; comprehensive metabolic profile, including AST and ALT; thyroid stimulating hormone, in order to monitor for possible adverse effects of sulforaphane in children. Portions of the blood and urine samples will also be processed and stored for research studies as outlined below at screening, 7, 15, 22, 30 and 36 weeks.
Following screening, participating children will be assigned to receive either sulforaphane (2.2 micromoles/kg b.w.) or placebo in Phase 1 by block randomization

using a table of random numbers, by the Research Pharmacy at the UMass Memorial Medical Center. Medication will be supplied to the Research Pharmacy at UMass by the Nutramax Laboratories.

The Research Monitor, Christy Stine, MD, is responsible to oversee the safety of the research and report observations/findings to the study DSMB and IRB of Record or a designated official. The Research Monitor will review all unanticipated problems involving risks to subjects or others associated with the protocol and provide an independent report of the event to the IRB of Record. The Research Monitor may discuss the research protocol with the investigators and the DSMB, shall have authority to stop the research protocol in progress, remove individual human subjects from the study, and take whatever steps are necessary to protect the safety and well-being of human subjects until the IRB can assess the monitor's report; and shall have the responsibility to promptly report their observations and findings to the IRB or other designated official and the HRPO.

We shall obtain an IND from the FDA, as well as approvals from the USAMRMC Human Research Protection Office, UMass and Johns Hopkins IRB for this study, and it will be listed on Clinicaltrials.gov. Study clinicians will remain blinded at all times to subject assignment until the last subject has completed the study. Blinding may be broken for any subject who develops adverse effects or illness, and every such event will be reviewed by the P.I. and DSMB. Stoppage will be determined by the P.I. in consultation with the DSMB, and appropriate medical care will be provided in consultation with the participant's primary care physician. Subjects will be entered into the study on a rolling basis, according to the time of their screening and entry into the study. All families will be followed on a regular basis and encouraged to call with questions or concerns regarding possible side effects of sulforaphane. A visit will take place at 7 weeks into Phase 1, as well as at 15 weeks, for clinical testing and phlebotomy. At 22 weeks, progress will be reviewed and clinical testing and phlebotomy performed. At the end of Phase 2 (30 weeks), all treatment with sulforaphane will end and a fifth phlebotomy will take place, for clinical and research lab studies. Testing of OACIS, ABC, SRS and VABS will be obtained at each visit. At the end of Phase 3 (36 weeks), an exit interview will be carried out, along with repeat OACIS, ABC, SRS and VABS, and the sixth phlebotomy for research studies. Subjects will receive a gift card for $15/visit for the 6 visits.

Monitoring of biochemical and molecular biomarkers of ASD (Specific Aim 3):
Over the last two decades many studies have shown that the ASD phenotypes (defined on the basis of behavioral manifestations) are associated with robust physiological abnormalities in a wide variety of systemic and central nervous system processes. These abnormalities include oxidative stress and reduced antioxidant capacity, depressed synthesis of reduced glutathione, dysfunction in mitochondrial energy capture, increase lipid peroxidation, immune dysfunction, and (neuro)inflammation[5,32-34]. Although it is unclear whether these ASD-associated anomalies are of etiological significance or secondary manifestations of ASD, it is clear that their correction often improves behavioral abnormalities substantially[35]. Quantifying these biomarkers before, during, and after sulforaphane intervention can therefore provide biomarkers for monitoring the

effects of therapeutic interventions and for correlating the effects with behavioral changes. They are also likely to provide important information as to the mechanisms of action of sulforaphane in ASD.

We propose to monitor selected examples of various types of biomarkers in peripheral blood mononuclear cells (PBMC), in plasma, and in urine of the patients undergoing sulforaphane treatment and compare them with matched placebo controls. Determinations will be made at the time of enrollment, at 7, 15, 30 weeks (end of treatment), and at 36 weeks, after discontinuation of the intervention.

***Pilot study:** Before running assays on the samples we collect from participants in the main study, we will first carry out a pilot study using administration of sulforaphane (1 micromole/lb or 2.2 micromoles/kg) to 10 children (6-12 years old) with ASD who donate samples of blood (PBMCs) and urine, before and after 14 days of daily treatment. By doing so, we will be able to identify the precise biomarker endpoints and the reproducibility of individual biomarker values and magnitudes of changes resulting from (short term) sulforaphane intervention. We will standardize the assays below and in the process will also determine the most effective and parsimonious group of assays to perform on samples from the children in our main study. Peripheral blood mononuclear cells will be obtained by differential centrifugation in Ficoll gradients using Vacutainer Cell Preparation Tubes (Becton Dickinson). The following will be studied in the Cullman Chemoprotection Laboratory at Johns Hopkins University School of Medicine:

1. ***Determination of Nrf2 Levels***. The transcription factor Nrf2 (Nuclear factor-erythroid factor 2) regulates a significant fraction of the genome (perhaps 4-5%) that codes mostly for cytoprotective gene products[14,36,37]. It orchestrates protective responses to a diversity of endogenous and exogenous stresses. Nrf2 levels are substantially depressed in ASD to 45% of typically developed children[2]. Determination of PBMC Nrf2 levels and the effects of sulforaphane treatment will be one of the primary biomarkers to be examined. Effects of sulforaphane treatment on the expression and activities of Nrf2-dependent enzymes such as nicotinamide nucleotide quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) will be also examined in PBMC of autistic children.

2. ***Oxidative Stress Biomarkers***. Most of the methods for these analyses are described in Rose (2014)[38]. These biomarker endpoints will be examined in PBMC, plasma, and urine, as appropriate. Biomarkers we will measure include: (a) levels of oxidized and reduced glutathione, and their ratios, (b) reactive oxygen species and their generation as determined by fluorescent probes, (c) plasma and urine F2 isoprostanes which are considered the most sensitive indicators of redox dysfunction[39], (d) plasma levels of 3-chlorotyrosine (measure of reactive nitrogen species and myeloperoxidase activity) and of 3-nitrotyrosine (measures of chronic immune activation and oxidative protein damage)[40], and (e) urinary levels of 8-hydroxydeoxyguanosine.

3. ***Heat Shock Response Biomarkers.*** The heat shock response is complex and evolutionarily conserved. Much evidence points to the neuroprotective role of heat shock proteins (HSPs), and the enhanced susceptibility of cells to damage when HSPs are depressed. HSPs are involved in sensing and repairing DNA damage, and function as chaperones for a number of misfolded proteins[41]. Since fever can dramatically but temporarily ameliorate abnormal symptoms in a substantial fraction of autistic patients[8], and fever is associated with activation of heat shock proteins[42], we will examine expression of heat shock proteins, focusing on heat shock factor 1 (HSF-1), HSP70 and HSP90, which are also upregulated by sulforaphane[3,43].

4. ***Mitochondrial Function Biomarkers.*** In the light of persuasive evidence for extensive mitochondrial dysfunction in autism[2,44], we will survey mitochondrial oxygen consumption and evidence for increased glycolysis (pyruvate and lactate levels and ratios in plasma). Determinations will also include measurements of mitochondrial NADH oxidase and pyruvate dehydrogenase activities, production of hydrogen peroxide, and mitochondrial DNA over-replication (compared to nuclear DNA).

5. ***mTOR signaling pathway.*** Synaptic dysfunction caused by aberrant protein synthesis is believed to be a key pathogenic mechanism for ASD[45]. The PI3K/AKT/mTOR signaling pathway plays central roles in synaptic protein synthesis[46], and its dysregulation results in many behavioral abnormalities, and may contribute to the pathogenesis of ASD[4]. Specifically, the phosphatase and tensin homolog on chromosome 10 (PTEN), the negative repressor of the PI3K/AKT/mTOR pathway, may be a significant regulator of this pathway in mediating the ASD phenotype. Deletion of PTEN results in autism-like behavioral deficits, hyperactivity of PI3K/AKT/mTOR pathway and alterations in synaptic scaffolding proteins[47]. We will therefore also examine biomarkers of this pathway in PBMCs, including gene expression of PTEN, AKT, and mTOR by real time PCR, and phosphorylated AKT and mTOR by Western blot.

6. ***Immune Function and Inflammatory Biomarkers.*** A number of studies have reported increases in cytokine expression, immune-related genes, and other biomarkers of inflammation and neuroinflammation in individuals with ASD[5,34,48]. Inflammation at birth may have long term detrimental effects on Nrf2 and with chronic neuroinflammation, Nrf2 is downregulated in some neurodegenerative diseases[49]. Sulforaphane can attenuate inflammation in a model of spinal cord injury by inhibiting the nuclear factor-κB (NF-κB) pathway, and the enzymatic activity of the proinflammatory cytokine macrophage inhibitory factor (MIF)[50]. Therefore, in addition to Nrf2 levels, plasma cytokine levels and cytokine gene expression, iNOS and COX-2 will be monitored in PBMC as well during the pilot study in order to determine their suitability for followup during the main study.

7. ***Bioavailability of sulforaphane:*** Our study drug is administered in the form of glucoraphanin rich broccoli seed powder with endogenous myrosinase.

Glucoraphanin (GR) is rapidly hydrolyzed by the enzyme myrosinase to sulforaphane[51]. Myrosinase is present in plant cells and is normally segregated from the glucoraphanin, until the cellular vacuoles are ruptured when the tissue containing glucoraphanin is ingested by mouth[52,53]. Sulforaphane, in turn, is metabolized rapidly by initial conjugation with glutathione, and successive steps of hydrolysis of the conjugates lead to the ultimate formation of the *N*-acetylcysteine derivatives (mercapturic acids). All of these conjugates are chemically designated dithiocarbamates (DTC) and can be quantified by the cyclocondensation reaction developed in our laboratory at Johns Hopkins as a complete and accurate measure of the bioavailability of sulforaphane[54]. We will collect a urine sample to perform measurement of Dithiocarbamate levels to assess the conversion of glucoraphanin to sulforaphane by the activity of endogenous myrosinase.

In consideration of Specific Aim 3, we will carry out studies of all 6 areas during the pilot study, in order to determine those that will be most important and repeatable. We expect, from previously published studies and our own experience with the effects of sulforaphane[55-57] that we will find changes in all 6 types of biomarkers above, however several may be more robust than others.

The pilot study will comprise of 2 study visits.

*** Pilot study visit 1 - Screening visit:** This visit will take around 2.5 hours to complete. All participants will undergo a pre-screen assessment to determine if basic eligibility criteria are met. Qualified study staff will ask questions related to the above described inclusion/exclusion criteria. The pre-screen may be completed by telephone or direct interview. Some subjects may require a more extensive examination to ensure that their autism meets the study's inclusion criteria. The subject will be enrolled after he and/or his legal guardian agree to a comprehensive informed consent. An informed consent will then be obtained. Autism diagnosis will be confirmed clinical judgment corroborated by administration of the Autism Diagnostic Observational Scale (ADOS)[29]. Those who have had an ADOS administered by an accredited professional within 3 years of study enrollment will not need to repeat this assessment if documentation of results can be provided. In that case, we will reconfirm diagnosis with a clinician check-list at enrollment (DSM-V checklist of symptoms).

At the visit, vital signs will be obtained. Then clinical laboratory tests (CBC and platelets, complete metabolic profile, including liver and renal function tests and thyroid stimulating hormone), blood for research laboratory tests (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and a urine sample for urinalysis will be obtained. The maximum amount of blood to be drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

If the blood tests done at the time of screening visit (liver function tests, renal function tests and thyroid function tests) are within study's acceptable limits (AST/ALT < 2x upper limit of normal, serum creatinine < 1.2 mg/dl, TSH within normal limits), we will

either mail the study drug to subjects or ask their parents to pick up the drug. We will provide the study participants a 14 day supply of study medication (sulforaphane – dosage details below). We will provide detailed instructions to subjects/parents/guardians on how to take the medication and include these instructions along with the medication package, and they will be made aware (and given written instructions) on how to recognize any side effects associated with the medication. They will be asked to call the study staff in case they have any questions or concerns.

*** Pilot study visit 2 – 14 day follow-up visit:** The second visit will take ~1 hour to complete. At this visit, vital signs will be obtained. Subjects will be asked about any adverse effects that they might have experienced. They will be asked to return the completed study medication diary and any unused medication. Also, a blood sample for research laboratory tests (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and safety labs (liver, renal and thyroid function tests) and a urine sample for urinalysis, isoprostanes and sulforaphane bioavailability will be obtained. The maximum amount of blood to be drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

Intervention details: Sulforaphane

Sulforaphane, an isothiocyanate (1-isothiocyanato-4R-(methylsulfinyl)butane), is obtained from lyophilized extracts of 3-day-old broccoli sprouts or as its glucosinolate precursor glucoraphanin, from broccoli seeds. Both broccoli sprouts and seeds contain the glucosinolate precursor glucoraphanin; neither contains any sulforaphane, which is produced from glucoraphanin by the action of myrosinase in the gut. Sulforaphane induces HSP and Nrf2 with positive effects on redox regulation, DNA damage sensing and repair, molecular chaperones, fatty acid and lipid metabolism and energy metabolism[13,14]. Sulforaphane has been shown to suppress lipopolysaccharide induced inflammation in rat microglia[15] and may regulate neuroinflammation in degenerative disorders of the CNS through its induction of Nrf2[16]. Sulforaphane and related compounds are hormetic drugs that induce a general "cell-protective" response, as demonstrated in vitro in sickle cell disease as well as X-linked adrenoleukodystrophy[17,18], fragile X syndrome and spinal muscular atrophy[19]. Sulforaphane crosses the blood brain barrier and is bioavailable orally[20,21]. Recent evidence shows that dysregulation in the mTOR/AKT signaling and cell survival pathway is present in animal models of ASD and tuberous sclerosis with ASD[4]**.** The lack of suppression of mTOR leads to excessive synapse formation due to failure of autophagy and pruning of synapses during brain development, which may be improved by administration of rapamycin[22]. Sulforaphane has been shown to induce cell cycle arrest and apoptosis in primary lymphoblasts from children with acute lymphoblastic anemia (ALL), and does so, in part, through its inhibition of the AKT and mTOR survival pathways[23]**.** Sulforaphane, therefore, has at least several possible modes of action that may benefit ASD through common cellular mechanisms that underlie its heterogeneous phenotypes.

Dosage and administration:

Sulforaphane (SF) will be administered in an approximate dosage of 1 μmol SF/lb (2.2 kg μmol/kg) body weight. This dosage roughly approximates the dosage that was used in our previous clinical trial of sulforaphane in male adolescents and adults with autism. The sulforaphane will be supplied as glucoraphanin (GR)-enriched broccoli seed extract tablets (manufacturing details follow). Each active tablet will contain 125 mg broccoli seed powder (source of glucoraphanin, equivalent to 34 μmol GR, which is equivalent to at-least ~ 15 μmol SF), 50 mg broccoli sprout extract (source of myrosinase), 15 mg ascorbic acid, 55.90 mg microcrystalline cellulose, and 4.10 mg silicon dioxide.
The total dose per day will depend of study participants' body weight:

- 30-50 lb 3 tablets (45 μmol/day)
- 50-70 lb 4 tablets (60 μmol/day)
- 70-90 lb 6 tablets (90 μmol/day)
- 90-110 lb 7 tablets (105 μmol/day)
- 110-130 lb 8 tablets (120 μmol/day)

If a child is unable to swallow tablets, parents will be asked to grind the tablets and mix the contents of capsules into small cups of applesauce. This method is commonly used to dispense medications in children. There has been extensive experience by the Cullman Chemoprotection Laboratory and others, in administering sulforaphane and its precursor glucoraphanin in a number of forms, both encapsulated and in various types of juice (mango, pineapple, lime)[58-62]**.** Placebo tablets identical in size and similar in appearance to the active tablets will be used.

Route and frequency of administration: The study drug (sulforaphane or placebo) will be administered orally, at approximately the same time, once a day, and preferably avoiding to take it with a heavy meal.

Duration of intervention: 15 weeks in first (double blind, Phase 1), and 15 weeks in open-label (Phase2).

Storage: prior to dispensing the study drug to the participants, the drug will be stored in temperature controlled conditions (-20C) at the UMass Memorial Investigational Pharmacy. During the study visits, we will directly dispense the medication to patients' parents (except the first visit where we will wait for the screening visit lab results before either mailing the study drug or asking the parents to pick it up). The parents will be instructed to keep the medication stored in their freezer for the duration of the study.

Concomitant medications allowed: Patients will continue taking previous psychopharmaceutical medications they are using to treat autism. However, we will request, insofar as possible, that no changes take place in the type or doses of their psychopharmaceutical medications for the duration of the study. They will also be allowed to continue taking any other medications, such as anti-epileptic medications for seizure control.

Study visits narrative:

The main study will consist of 2 phases of treatment, each lasting 15 weeks per patient. Phase 1 is the double blind phase, in which 50% of study participants will receive active drug (sulforaphane) and the other 50% will receive active placebo, according to the

dosing schedule as detailed above. In Phase 2, from 15 weeks until 30 weeks is an open-label phase during which all study participants will receive the active drug (sulforaphane). All safety laboratory tests will be performed in a CLIA certified laboratory at the University of Massachusetts Memorial Medical Center. Phase 3 will be from 30 to 36 weeks, without treatment, followed by a final follow up visit at 36 weeks.

The study comprises a total of 6 visits over a period of 36 weeks, with visits spaced around 6-8 weeks apart. In addition there will be two telephone surveys between visit 1 and 2, and between 3 and 4 to detect any possible adverse events. Following is a description of procedures that will be performed at each study visit:

1. **Screening/baseline visit (double blind Phase 1):** This visit will take around 2.5 hours to complete. All participants will undergo a pre-screen assessment to determine if basic eligibility criteria are met. Qualified study staff will ask questions related to the above described inclusion/exclusion criteria. The pre-screen may be completed by telephone or direct interview. Some subjects may require a more extensive examination to ensure that their autism meets the study's inclusion criteria. An informed consent will then be obtained. Autism diagnosis will be confirmed clinical judgment corroborated by administration of the Autism Diagnostic Observational Scale (ADOS)[29]. Those who have had an ADOS administered by an accredited professional within 3 years of study enrollment will not need to repeat this assessment if documentation of results can be provided. In that case, we will reconfirm diagnosis with a clinician check-list at enrollment (DSM-V checklist of symptoms). A Leiter-3 assessment will also be performed as a measure of nonverbal IQ[31].

   A complete, baseline history, physical and neurological exam will be performed. OACIS-S[27,28], SRS[26], ABC[25] and Vineland Adaptive Behavior Scale[30] will also be performed to quantify disease severity. Also, clinical laboratory tests (CBC and platelets, complete metabolic profile, including liver and renal function tests and thyroid stimulating hormone), blood for research laboratory tests (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and a urine sample for urinalysis will be obtained. The maximum amount of blood to be drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

   If the blood tests done at the time of screening visit (liver function tests, renal function tests and thyroid function tests) are within study's acceptable limits (AST/ALT < 2x upper limit of normal, serum creatinine < 1.2 mg/dl, TSH within normal limits), we will either mail the study drug to study participants or ask their parents/guardians to pick up the study drug. The subject will be enrolled after he and/or his legal guardian agree to a comprehensive informed consent.

At this visit, the study participant/s will already have been randomly assigned to either the sulforaphane or placebo group in the ratio of 1:1 by the study statistician. As mentioned above, after the safety labs come back normal, we will mail the study participants (or ask their parents to pick up) an 8 week supply of study medication (sulforaphane or placebo) that will last until the 7 week visit, plus a one week buffer. We will provide detailed instructions to subjects/parents/guardians on how to take the medication and include these instructions along with the medication package, and they will be made aware (and given written instructions) on how to recognize any side effects associated with the medication. They will be asked to call the study staff in case they have any questions or concerns.

2. **7 week visit (double blind Phase 1):** Enrolled subjects will then be asked to come for their 7 week (follow-up) study visit, where OACIS-I, SRS, ABC and Vineland Adaptive Behavior Scale will be administered. A complete physical examination will be performed and vital signs obtained. This visit will take ~ 1.5 hours to complete. They will be asked about any serious side effects they might have experienced. Also, clinical laboratory tests (CBC and platelets, complete metabolic profile (including liver function tests, renal function tests, thyroid function tests) and research laboratory tests blood - (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and urine (isoprostanes) will be obtained. Maximum amount of blood drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

   At this visit, we will give the study participants a further 7 week supply of study medication (sulforaphane or placebo) that will last until the next visit (15 week visit). Study participants will be asked to finish the medication from the previous lot before starting the latter batch. They will be asked to continue to take the study medication unless instructed otherwise. We will provide detailed instructions for subjects/parents/guardians on how to take the study medication along with the medication package, and they will be made aware (and given written instructions) on how to recognize any side effects associated with the study medication. They will be asked to call the study staff in case they have any questions or concerns.

   If the lab results are found to be abnormal (AST/ALT > 2x upper limit of normal, serum creatinine > 1.2 mg/dl, TSH outside normal limits), we will ask the study participants to discontinue study medication for 2 weeks. After 2 weeks, we will ask them to come for an interim study visit and draw their blood for liver function tests, renal function tests and thyroid function tests. If lab values have returned to within study's acceptable limits (AST/ALT < 2x upper limit of normal, serum creatinine < 1.2 mg/dl, TSH within normal limits), and as advised by the study Data Safety Monitoring Board, we will then ask them to resume the study medication.

After 2 weeks, if the lab tests are still abnormal (AST/ALT > 2x upper limit of normal; serum creatinine > 1.2 mg/dl; TSH outside normal limits), then the study participants will discontinue study medication permanently (although we will ask them continue to be followed up in the study).

3. **15 week visit (end of double blind Phase 1)**: Enrolled subjects will then be asked to come for their 15 week (follow-up) study visit, where OACIS-I, SRS, ABC and Vineland Adaptive Behavior Scale will be administered. A complete physical examination will be performed and vital signs obtained. This visit will take ~ 1.5 hours to complete. They will be asked about any serious side effects they might have experienced. Also, clinical laboratory tests (CBC and platelets, complete metabolic profile (including liver function tests, renal function tests, thyroid function tests), and research laboratory tests blood- (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and urine sample for isoprostanes will be obtained. Maximum amount of blood drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

   At this visit, we will give the study participants a further 8 week supply of study medication (sulforaphane) that will last until the next (22 week) visit plus a one-week buffer. We will provide detailed instructions for subjects/parents/guardians on how to take the study medication along with the medication package, and they will be made aware (and given written instructions) on how to recognize any side effects associated with the study medication. They will be asked to call the study staff in case they have any questions or concerns.

   If at this visit lab results are found to be abnormal (AST/ALT > 2x upper limit of normal, serum creatinine > 1.2 mg/dl, TSH outside normal limits), we will ask the study participants to discontinue study medication for 2 weeks. After 2 weeks, we will ask them to come for an interim study visit and draw their blood for liver function tests, renal function tests and thyroid function tests. If lab values have returned to within study's acceptable limits (AST/ALT < 2x upper limit of normal, serum creatinine < 1.2 mg/dl, TSH within normal limits), and as advised by the study Data Safety Monitoring Board, we will then ask them to resume the study medication.

   After 2 weeks, if the lab tests are still abnormal (AST/ALT > 2x upper limit of normal; serum creatinine > 1.2 mg/dl; TSH outside normal limits), then the study participants will discontinue study medication permanently (though we will ask them continue to be followed-up in the study).

4. **22 week visit (open label Phase 2)**: Enrolled subjects will then be asked to come for their 22 week (follow-up) study visit, where OACIS-I, SRS, ABC and Vineland Adaptive Behavior Scale will be administered. A complete physical

examination will be performed and vital signs obtained. This visit will take ~ 1.5 hours to complete. They will be asked about any side effects they might have experienced. Also, clinical laboratory tests (CBC and platelets, complete metabolic profile (including liver function tests, renal function tests, thyroid function tests), and research laboratory tests blood- (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and urine sample for isoprostanes will be obtained. Maximum amount of blood drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

At this visit, we will give the study participants a further 7 week supply of study medication (sulforaphane) that will last until the next (30 week) visit. We will provide detailed instructions for subjects/parents/guardians on how to take the study medication along with the medication package, and they will be made aware (and given written instructions) on how to recognize any side effects associated with the study medication. They will be asked to call the study staff in case they have any questions or concerns.

If at this visit lab results are found to be abnormal (AST/ALT > 2x upper limit of normal, serum creatinine > 1.2 mg/dl, TSH outside normal limits), we will ask the study participants to discontinue study medication for 2 weeks. After 2 weeks, we will ask them to come for an interim study visit and draw their blood for liver function tests, renal function tests and thyroid function tests. If lab values have returned to within study's acceptable limits (AST/ALT < 2x upper limit of normal, serum creatinine < 1.2 mg/dl, TSH within normal limits), and as advised by the study Data Safety Monitoring Board, we will then ask them to resume the study medication.

After 2 weeks, if the lab tests are still abnormal (AST/ALT > 2x upper limit of normal; serum creatinine > 1.2 mg/dl; TSH outside normal limits), then the study participants will discontinue study medication permanently (though we will ask them continue to be followed-up in the study).

5. **30 week visit (open label Phase 2):** Enrolled subjects will then be asked to come for their 30 week (follow-up) study visit, where OACIS-I, SRS, ABC and Vineland Adaptive Behavior Scale will be administered. A complete physical examination will be performed and vital signs obtained. This visit will take ~ 1.5 hours to complete. They will be asked about any side effects they might have experienced. Also, laboratory tests (CBC and platelets, complete metabolic profile, including liver function tests, renal function tests, thyroid function tests), and research laboratory tests blood - (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and urine sample for isoprostanes will be obtained. Maximum amount of blood drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg

in an 8 week period. At this visit, we will not give the study participants any further study medication.

6. **36 week final follow-up visit (Phase 3):** This will be the final study visit. OACIS-I, SRS, ABC and Vineland Adaptive Behavior Scale will be administered. A complete physical examination will be performed and vital signs obtained. This visit will take ~ 1.5 hours to complete. Participants and their caregivers will be asked about any side effects they might have experienced. Also, research laboratory tests for blood - (Nrf2, heat shock proteins, measures of oxidative stress and mTOR) and urine sample for isoprostanes will be obtained.

Following is a description of the telephone surveys:

1. **Phone survey 1**: This phone call will take place between visits 1 and 2 at approximately 3 weeks from the study drug start date. This will help us ensure that the study participants does not experience any unwanted side effects from taking the study drug. This survey will take around 15 minutes to complete. During this phone call we will ask them questions about their child's health since starting the study. We will ask them questions about any side effects that your child may have experienced since starting the study drug.

2. **Phone survey 2**: This phone call will take place between visits 3 and 4 at approximately 18 weeks from the initial study drug start date. This will help us ensure that the study participants does not experience any unwanted side effects from taking the study drug. This survey will take around 15 minutes to complete. During this phone call we will ask them questions about their child's health since starting the study. We will ask them questions about any side effects that your child may have experienced since starting the study drug.

Laboratory evaluations:
We will collect blood samples for safety evaluations as well as for biomarker evaluations.

1. Specimens to be collected, amount, schedule:
    a. Specimen to be collected: blood, urine
    b. Schedule: Blood and urine samples will be collected at screening visit, 7 week visit, 15 week visit, 22 week visit, 30 week visit and 36 week visit
    c. Amount: Blood ~ 13 ml (so long as it is not exceeding 2ml/kg body weight limit) each time (~4 ml for liver, thyroid and renal function tests, ~1.5 ml for complete blood counts, ~7.5 ml for biomarker measurement)
    Urine ~ 5 ml
2. Evaluations to be made:
    a. Safety evaluations: They will include liver function tests (SGOT/SGPT, Total bilirubin), renal function tests (serum electrolytes, serum creatinine), thyroid function tests (TSH).
    b. Biomarker measurement: Following biomarkers will be evaluated:

1. ***Determination of Nrf2 Levels***. The transcription factor Nrf2 (Nuclear factor-erythroid factor 2) regulates a significant fraction of the genome (perhaps 4-5%) that codes mostly for cytoprotective gene products[14,36,37]. It orchestrates protective responses to a diversity of endogenous and exogenous stresses. Nrf2 levels are substantially depressed in ASD to 45% of typically developed children[2]. Determination of PBMC Nrf2 levels and the effects of sulforaphane treatment will be one of the primary biomarkers to be examined. Effects of sulforaphane treatment on the expression and activities of Nrf2-dependent enzymes such as nicotinamide nucleotide quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) will be also examined in PBMC of autistic children.
2. ***Oxidative Stress Biomarkers***. Most of the methods for these analyses are described in Rose (2014)[38]. These biomarker endpoints will be examined in PBMC, plasma, and urine, as appropriate. Biomarkers we will measure include: (a) levels of oxidized and reduced glutathione, and their ratios, (b) reactive oxygen species and their generation as determined by fluorescent probes, (c) plasma and urine F2 isoprostanes which are considered the most sensitive indicators of redox dysfunction[39], (d) plasma levels of 3-chlorotyrosine (measure of reactive nitrogen species and myeloperoxidase activity) and of 3-nitrotyrosine (measures of chronic immune activation and oxidative protein damage)[40], and (e) urinary levels of 8-hydroxydeoxyguanosine.
3. ***Heat Shock Response Biomarkers.*** The heat shock response is complex and evolutionarily conserved. Much evidence points to the neuroprotective role of heat shock proteins (HSPs), and the enhanced susceptibility of cells to damage when HSPs are depressed. HSPs are involved in sensing and repairing DNA damage, and function as chaperones for a number of misfolded proteins[41]. Since fever can dramatically but temporarily ameliorate abnormal symptoms in a substantial fraction of autistic patients[8], and fever is associated with activation of heat shock proteins[42], we will examine expression of heat shock proteins, focusing on heat shock factor 1 (HSF-1), HSP70 and HSP90, which are also upregulated by sulforaphane[3,43].
4. ***Mitochondrial Function Biomarkers.*** In the light of persuasive evidence for extensive mitochondrial dysfunction in autism[2,44], we will survey mitochondrial oxygen consumption and evidence for increased glycolysis (pyruvate and lactate levels and ratios in plasma). Determinations will also include measurements of mitochondrial NADH oxidase and pyruvate dehydrogenase activities, production of hydrogen peroxide, and mitochondrial DNA over-replication (compared to nuclear DNA).
5. ***mTOR signaling pathway.*** Synaptic dysfunction caused by aberrant protein synthesis is believed to be a key pathogenic mechanism for ASD[45]. The PI3K/AKT/mTOR signaling pathway plays central roles

in synaptic protein synthesis[46], and its dysregulation results in many behavioral abnormalities, and may contribute to the pathogenesis of ASD[4]. Specifically, the phosphatase and tensin homolog on chromosome 10 (PTEN), the negative repressor of the PI3K/AKT/mTOR pathway, may be a significant regulator of this pathway in mediating the ASD phenotype. Deletion of PTEN results in autism-like behavioral deficits, hyperactivity of PI3K/AKT/mTOR pathway and alterations in synaptic scaffolding proteins[47]. We will therefore also examine biomarkers of this pathway in PBMCs, including gene expression of PTEN, AKT, and mTOR by real time PCR, and phosphorylated AKT and mTOR by Western blot.

6. ***Immune Function and Inflammatory Biomarkers.*** A number of studies have reported increases in cytokine expression, immune-related genes, and other biomarkers of inflammation and neuroinflammation in individuals with ASD[5,34,48]. Inflammation at birth may have long term detrimental effects on Nrf2 and with chronic neuroinflammation, Nrf2 is downregulated in some neurodegenerative diseases[49]. Sulforaphane can attenuate inflammation in a model of spinal cord injury by inhibiting the nuclear factor-κB (NF-κB) pathway, and the enzymatic activity of the proinflammatory cytokine macrophage inhibitory factor (MIF)[50]. Therefore, in addition to Nrf2 levels, plasma cytokine levels and cytokine gene expression, iNOS and COX-2 will be monitored in PBMC as well during the pilot study in order to determine their suitability for follow-up during the main study.

Following table depicts the study procedures that will be performed at each visit and also approximately how long each visit will take:

Visit	Informed consent, eligibility criteria (ADOS, Leiter-3)	Adverse event reporting	Medical history, vitals, physical exam	Autism symptom severity (OACIS-S, OACIS-I, ABC, SRS, Vineland Adaptive Behavior Scale)	Blood draw (LFTs, RFTs, TSH, CBC, HSP, mTor, Nerf), urine sample for isoprostanes	Study drug dispending	Visit duration
Double blind phase (50% participants receive sulforaphane and 50% receive placebo)							
Screening/ baseline visit	X		X	X	X	8 weeks	~ 2.5 hours
3 week phone survey		X					~ 15 minutes

7 week visit		X	X	X	X	7 weeks	~ 1.5 hours
15 week visit		X	X	X	X	8 weeks	~ 1.5 hours
Open label phase (all participants receive sulforaphane)							
18 week phone survey		X					~ 15 minutes
22 week visit		X	X	X	X	7 weeks	~ 1.5 hours
30 week visit		X	X	X	X		~ 1.5 hours
36 week visit		X	X	X	X		~ 45 minutes

11) **Data and Specimen Banking***

We plan to store leftover blood and urine samples in -80 C freezer in the Dept. of Pediatrics at UMass and the Cullman Chemoprotection Laboratory at Johns Hopkins for a period of 3 years after completion of the study. They will be accessed by the P.I. or study staff. The samples will continue to be de-identified and labeled with unique study I.D. numbers. The link between the study ID and identifiable information will only be accessible by the UMass personnel. At the end of 3 years the samples will be forever anonymized, and the link between study ID and identifiable information will be destroyed. We will make application to the IRB for any further studies to be conducted, by ourselves or others, using these specimens.

12) **Data Management***

Statistical Analysis: The OACIS-I, SRS and ABC data will be analyzed in a mixed model ANOVA with fixed effects for visit, treatment group, and their interaction and random participant-specific intercepts. The effect of sulforaphane on mean change in OACIS-I, SRS and ABC total scores from baseline will be estimated using linear contrasts. Estimates will be unbiased if any loss to follow-up is missing at random, conditional on the observed data and our model assumptions. Significantly greater improvement from baseline in mean scores among participants randomized to sulforaphane based on a two-tailed test at alpha = 0.05 will be taken as support of our primary hypothesis.

A secondary analysis assessing proportion of participants in both groups who have responded to the study drug will be performed by Fisher's Exact test. This analysis will be done by categorizing the OACIS-I, SRS and ABC data. Participants will be considered as responders to study drug if they experience either of the following: they are much/very much improved (a score of 2 or less) on any subdomain of OACIS-I scale, their total ABC scores at 15 weeks decrease by at least 25% from baseline score, and their total SRS scores at 15 weeks decrease by at least 25% from their baseline score.

Biomarkers (testing mechanism of action of SFN) will be tested both as outcome variables in mixed model analyses and as predictors of differences in behavioral symptoms using the models described above for behavioral symptoms. Markers that both respond to sulforaphane treatment and predict differences in behavioral symptoms will be interpreted as possible mediators of any benefit from sulforaphane treatment. Safety of sulforaphane treatment will be assessed based on absolute rates of liver, thyroid and renal toxicity among participants treated with sulforaphane and based on comparison of the proportion of participants with such toxicities between the two treatment groups.

Power Calculations: We plan to enroll a total of 50 participants in the study, randomized 1:1 to sulforaphane and placebo. With OACIS-I, ABC, and SRS assessments at 0, 7 and 15 weeks our proposed design will have at least 80% power to detect significance in the following outcome measures if the following sample sizes are considered: **OACIS-I average Score:** With a total of 40 patients (20 on placebo and 20 on sulforaphane) entering this two-treatment parallel-design study, the probability is 80 percent that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference of average OACIS-I score at 15 weeks between two treatments is 4 units. This is based on the assumption that the standard deviation of the average OACIS-I score is 4.9. **OACIS-I response rate on aberrant behaviors:** With a total of 44 patients (22 on placebo and 22 on sulforaphane) entering this two-treatment parallel-design study, there will be an 88% chance of detecting a significant difference at a two sided 0.05 significance level. This assumes that the response rate at 15 weeks of placebo is 9% and the response rate of sulforaphane is 54% on aberrant behavior subscale of OACIS-I. **OACIS-I response rate on social communication:** With a total of 44 patients (22 on placebo and 22 on sulforaphane) entering this two-treatment parallel-design study, there will be 82% chance of detecting a significant difference at a two sided 0.05 significance level. This assumes that the response rate of placebo is 7% and the response rate at 15 weeks of sulforaphane is 46% on the social communication subscale of OACIS-I. **Change in total ABC score from baseline:** With a total of 44 patients (22 on placebo and 22 on sulforaphane) entering this two-treatment parallel-design study, the probability is 81 percent that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference of change in baseline of total ABC score at 15 weeks between the two treatments is 19.4 units. This is based on the assumption that the standard deviation of the change in ABC score is 22.1. **Change in total SRS score from baseline:** With a total of 48 patients (22 on placebo and 22 on sulforaphane) entering this two-treatment parallel-design study, the probability is 81 percent that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference of change from baseline of total SRS score at 15 weeks between two treatments is 18.4 units. This is based on the assumption that the standard deviation of the change in SRS score is 22.0. Thus our proposed sample size of 50 participants, randomized 1:1 to sulforaphane and placebo will have more than enough power to detect significantly changes in majority of the outcomes listed above. The study will have 80% power to detect one or more toxicities expected to occur in at least 5.3% of sulforaphane-treated participants. The study is not powered to compare rates of safety outcome between sulforaphane and placebo treatment groups for a relevant non-inferiority threshold.

Study identifiers: We will make all efforts to preserve the privacy of the study participants. All data collected in the study will be identified by a random code and participant initials. We will use the following format to label the study participants: SF-15-001, SF-15-002 along with participant initials (eg. JD). No personally identifying information, such as complete name, hospital medical record number, date of birth etc. will be used on study documents.

Good Clinical Practices compliance will be maintained at all times during the study. All study staff will be trained in maintaining the study documentation according to the GCP guidelines. In addition, any electronic database solution we will use will be GCP guidelines compliant. University of Massachusetts Medical School has contracted the RedCap clinical trials software database solution. REDCap (Research Electronic Data Capture) is a secure web-based application built around HIPAA guidelines to support data capture for research studies. It allows users to build and manage online surveys and databases or a combination of both quickly. REDCap features automated export procedures for seamless data downloads to Excel, PDF, and common statistical packages (SPSS, SAS, Stata, R). Also included are a built-in project calendar, a scheduling module, ad hoc reporting tools, and advanced features, such as branching logic, file uploading, and calculated fields. The REDCap Software sits on a dedicated server behind a firewall to eliminate all nonessential access credentials. The data itself is isolated on a separate server that sits behind an additional firewall layer for added security. Interaction between REDCap and the client is encrypted via SSL.

In addition we will have an independent clinical trials monitor conduct regular visits to monitor the study for GCP compliance. This study monitor will have authority to stop a research study in progress, remove individuals from study, and take any steps to protect the safety and well-being of subjects until the IRB can assess. Please refer to the document titled “H00007832_ Research Monitor v2 111815” for further details about the roles and responsibilities of the Research Monitor.

In addition to the GCP visits, an independent data safety monitoring board (DSMB) of 3 persons will be constituted to evaluate the continued safety of sulforaphane. This DSMB will consist of one pediatrician (from the department of Pediatrics at UMass Medical School), one child psychiatrist (from the department of Psychiatry at UMass Medical School) and one biostatistician (from the department of Quantitative Sciences at UMass Medical School). This DSMB will meet twice a year and review all safety information collected in the past 6 months (see “13: Provisions to Monitor the Data to Ensure the Safety of Subjects” below).

Confidentiality: Strict confidentiality will be maintained at all times. As mentioned above, all subjects and clinical information associated with each subject will be assigned an identification number and will only be referred to by that number and subject initials. This number will be the only identifying information on test score sheets, data entry forms, and in the data files. Some data will require minimum protected health information (i.e. date of birth) recorded on the assessment sheets in order to be properly

scored. In this case, we will follow HIPAA limited data set guidelines. Source documents will be maintained by the study coordinator in a centralized, double-locked office accessible only by the study coordinator and involved members of the research team. Only initials and study number identify any specimens sent to laboratories outside of UMass.

We will collect some identifiable information, such as name, address, date of birth, contact information (mailing address, phone numbers, email address etc.) demographics (race, ethnicity, parental educational background) but this information will be kept separate from the other study files and documents. Documents containing this identifying information will be kept in a locked cabinet in a secure study location. Only study staff directly involved in interaction with the study participants and the study Principal Investigator will have access to this identifiable information. The Principal Investigator, or designee, will maintain a personal participant identification list (participant numbers with the corresponding names) to enable records to be identified. Other study staff (such as those working with biomarkers) whose role will not involve interacting with study participants will not have access to this identifiable information. Representatives of USAMRMC who may be entrusted with the responsibility of conducting site visits may be able to get access to the study records for review.

In terms of subject privacy protection, the researchers are aware of the implementation of the Health Insurance Portability and Accountability Act (HIPAA) and its impact on clinical research and electronic data collection activities. The database developed under this protocol will conform to national standards and procedures for the electronic storage and transmission of information to insure privacy and security of health information. This includes conformance to rules for transactions and code sets, the standards for privacy of individually identifiable health information and security standards.

Any blood samples sent to the laboratories at Johns Hopkins for analysis of heat shock proteins and mitochondrial study and measures of oxidative stress will not contain any direct identifiers and samples will be labeled with the subject's unique ID. Samples may be stored for future use with explicit consent of the subject and parents or guardians. Without such consent, samples will be destroyed following the study.

Data capture, verification, and disposition: Data will be primarily captured on paper records. Majority of the study instruments (such as Aberrant Behavior Checklist, Social Responsiveness Scale, Autism Diagnostic Observation Schedule, Vineland, Leiter scale) are provided as paper booklets and they will be used as the primary data capture devices. Additional information, such as physical examination findings, medical history records etc. will also be captured in paper records. After the study is complete, we will keep the study records for future regulatory purposes for a period of 7 years, at which time all study records will be destroyed. However the documents containing identifiable information will not be stored after the study ends; they will be destroyed after study is completed.

In addition, we will use a secure computer database to transfer the paper data records to an electronic format. University of Massachusetts Medical School has contracted the RedCap clinical trials software database solution. REDCap (Research Electronic Data Capture) is a secure web-based application built around HIPAA guidelines to support data capture for research studies. It allows users to build and manage online surveys and databases or a combination of both quickly. REDCap features automated export procedures for seamless data downloads to Excel, PDF, and common statistical packages (SPSS, SAS, Stata, R). Also included are a built-in project calendar, a scheduling module, ad hoc reporting tools, and advanced features, such as branching logic, file uploading, and calculated fields. The REDCap Software sits on a dedicated server behind a firewall to eliminate all nonessential access credentials. The data itself is isolated on a separate server that sits behind an additional firewall layer for added security. Interaction between REDCap and the client is encrypted via SSL. To ensure accuracy of transfer of data from paper to electronic format and to minimize data entry errors, all data that will entered to RedCap will be double entered by two independent study staff members. After the study is complete and all data has been collected and entered into the database, the database will be locked out from any further editing.

Storage of laboratory samples: The following storage specifications will be met:

a. For safety evaluations: The blood tubes/vacutainers will be labeled with study participant's unique ID number and shipped right away to the UMass Clinical Research Center's lab for analysis.
b. For biomarker evaluations: The vacutainer will be labeled with the ID number, and the blood processed within 2 hours of collection for isolation of monocytes. The monocytes so isolated will be labeled with the unique study ID number and frozen in an appropriate culture media at -80C for a period not exceeding 3 months in the -80 freezer at UMass Memorial's Clinical Research Center. These samples will then be shipped to Johns Hopkins University (Lewis B. and Dorothy Cullman Cancer Chemoprotection Center, and Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205) for analysis of biomarkers (details below). The tube containing urine will be labeled with the ID number and stored at -80C until shipment.

Shipping of laboratory samples and laboratories performing evaluations:

a. For safety evaluations: The samples will be tested at University of Massachusetts Memorial Medical Center Clinical Trials Unit Clinical Research Laboratory (located at Clinical Trials Unit, UMass Memorial Ambulatory Care Center, 1st Floor, 55 Lake Ave N, Worcester, MA 01655).
b. For biomarker evaluation: These evaluations will be conducted at Lewis B. and Dorothy Cullman Cancer Chemoprotection Center, and Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.
c. Transportation of samples: The blood and urine samples for biomarker evaluation will be transported frozen. To ensure that proper temperature is maintained during transport they will be shipped in special Styrofoam containers in dry ice. The

person arranging specimen packing and shipment will be trained in shipping and transportation of hazardous materials.

13) **Provisions to Monitor the Data to Ensure the Safety of Subjects***

Risk management and emergency response: Risks to subjects will be minimized by close follow-up and consideration of all reported symptoms and concerns of parents, guardians and caregivers. Regular examinations and laboratory testing of blood counts and serum chemistries, including liver function tests, renal function tests, and thyroid function tests will be performed at the screening visit, 7 week,15 week, 22 week, 30 week and 36 week visits, and reviewed by the study team and Data Safety Monitoring Board. The PI or physician colleague will be on call 24/7 for any concerns of adverse events during the study. Risks of phlebotomy will be reduced by behavioral preparation, use of topical anesthetic and carried out by phlebotomists experienced in working with persons with autism. Protection of subjects will include confidential treatment of all health related information, and all research data will be coded, the reference codes to be maintained under lock and key by the PI and study coordinator at all times.

If lab signs of toxicity are found (AST/ALT > 2x upper limit of normal; serum creatinine > 1.2 mg/dl; TSH outside normal limits), the patient will discontinue study medication for 2 weeks before resuming the study medication, once lab values return to within study's acceptable limits. At the end of 2 weeks, we will do lab tests again. If at that time, lab tests are still not within study's acceptable limits (AST/ALT > 2x upper limit of normal; serum creatinine > 1.2 mg/dl; TSH outside normal limits), subjects will discontinue permanently the study medication (though they will still continue to be followed-up in the study). Lab tests are required at screening, 7-week, 15-week, 22 week, 30 week and closeout visits: Hematology and complete metabolic profile (including liver, kidney, thyroid function tests); urinalysis for protein and hemoglobin. Possible clinical side effects of sulforaphane will be monitored, including: GI disturbances (flatulence, soft stool, and large bowel movement), and frequent urination[63]. Staff members will be available 24/7 for emergencies. Maximum amount of blood drawn at any stage during this study will not exceed 2 ml/kg body weight; also we will not exceed the minimal risk requirements of 3ml/kg in an 8 week period.

If any child in the study has a seizure while taking the medication, the study drug will be discontinued and study physicians and Data Safety Monitoring Board (DSMB) will be notified. The child will be evaluated by Dr. Zimmerman or his designee, pediatrician or family doctor, and treated and followed up appropriately for the duration of the study. We will ask the parents to return with the child for repeat safety lab studies and to return unused study drug.

In the event of significant shifts in behavior, based on the family's experience and judgment, the child will be examined by the study physician(s) or the child's pediatrician, the study drug will be temporarily discontinued for 2 weeks and DSMB notified. If the behavior(s) subside, the medication will be resumed on the previous schedule. Since

children with ASD often have cyclic behavior changes without respect to the medications they are taking, we will rely primarily on the caregivers' experience in assessing whether the behavior(s) in question are out of the norm for their child. However, we will be cautious in evaluating the child for possible medical reasons for the change in behavior (e.g., gastrointestinal discomfort, disordered sleep). If adverse behaviors do not subside after 2 weeks and no medical cause for the behaviors is found, the study medication may be restarted if the DSMB, study physicians and the parents agree. Otherwise, medication will not be restarted and we will ask that the family return the remaining study drug supply.

We will constitute study drug pause and study drug stop rules based on objectively measurable criteria. These criteria appear below:

Subject Drug Pause Criteria: Subjects will be asked to stop taking study medication for 2 weeks if:

1. At 7 week visit, subjects reported any serious side effects and subsequent laboratory tests are outside of study's acceptable limits - liver function tests (AST/ALT > 2x upper limit of normal); renal function tests (serum creatinine > 1.2 mg/dl); thyroid function tests (TSH outside normal limits).
2. At 15 week visit, subjects reported any serious side effects and subsequent laboratory tests are outside of study's acceptable limits - liver function tests (AST/ALT > 2x upper limit of normal); renal function tests (serum creatinine > 1.2 mg/dl); thyroid function tests (TSH outside normal limits).
3. At any time during the study, subjects reported any serious side effects and subsequent laboratory tests are outside of study's acceptable limits - liver function tests (AST/ALT > 2x upper limit of normal); renal function tests (serum creatinine > 1.2 mg/dl); thyroid function tests (TSH outside normal limits).
4. There is a potential interaction of study medication with another medication a subject is taking. We will ask the subject to temporarily stop taking study medication if the other drug is a short term medication (e.g., a course of antibiotics, or prednisone).
5. Intercurrent illness (e.g., infection, asthma, accident or serious injury), requiring medical attention (e.g., emergency room visit or hospital admission). All such incidents will be reviewed with study physicians and the DSMB as they arise, and we will consult with the child's physician regarding the advisability of pausing the study drug and continuing follow up of the child's illness.
6. In the event of significant shifts in behavior, based on the family's experience and judgment, the child will be examined by the study physician(s) or the child's pediatrician, the study drug will be temporarily discontinued for 2 weeks and DSMB notified. If the behavior(s) subside, the medication will be resumed on the previous schedule. Since children with ASD often have cyclic behavior changes without respect to the medications they are taking, we will rely primarily on the caregivers' experience in assessing whether the behavior(s) in question are out of the norm for their child. However, we will be cautious in evaluating the child for possible medical reasons for the change in behavior (e.g., gastrointestinal discomfort, disordered sleep). If adverse behaviors do not subside after 2 weeks and no medical cause for the behaviors is found, the study medication may be restarted if the DSMB, study

physicians and the parents agree. Otherwise, medication will not be restarted and we will ask that the family return the remaining study drug supply.

Subject Drug Stop Criteria: At the end of 2 weeks, we will do lab tests again. At that time, subjects will discontinue the study medication permanently if:

1. Subsequent laboratory tests repeated after 2 weeks are outside of study's acceptable limits - liver function tests (AST/ALT > 2x upper limit of normal); renal function (serum creatinine > 1.2 mg/dl); thyroid function (TSH outside normal limits).
2. At any point of the study, extreme exacerbation of behavioral symptoms is noted, as above and these behaviors do not subside after 2 weeks of study drug stoppage and no medical cause for the change in behavior is apparent (e.g., gastrointestinal discomfort, disordered sleep).
3. If any child in the study has a seizure while taking the medication, the study drug will be discontinued and study physicians and Data Safety Monitoring Board (DSMB) will be notified. The child will be evaluated by Dr. Zimmerman or his designee, pediatrician or family doctor, and treated and followed up appropriately for the duration of the study. We will ask the parents to return with the child for repeat safety lab studies and to return unused study drug.
4. At any time, if there is an interaction of study medication with another long term medication a subject is taking: In a case in which the other drug is the one which a subject is likely to require for a long period of time, subjects will discontinue study medication permanently if there is a possibility of adverse effects as a result of an interaction with that drug (for example, appearance of extrapyramidal signs in a subject taking risperidone).

Data Safety Monitoring Board: An independent data safety management board of 3 persons will be constituted to evaluate the continued safety of sulforaphane. This DSMB will consist of one pediatrician (from the department of Pediatrics at UMass Medical School), one child psychiatrist (from the department of Psychiatry at UMass Medical School) and one bio-statistician (from the department of Quantitative Sciences at UMass Medical School). This DSMB will meet twice a year and review all safety information collected in the past 6 months.

14) **Withdrawal of Subjects***

See study drug pause and stop criteria above.

15) **Risks to Subjects***

Sulforaphane Safety:
Cruciferous vegetables, including broccoli sprouts, are generally regarded as safe and are regular dietary components in many regions of the world. Previous estimates of the daily dietary intake of cruciferous vegetables vary regionally, averaging 40 g/day in Singapore[64], 11 g/day in the United States[65], 16 g/day in Canada[66], 30 g/day in the UK[67], and 112 g/day in Japan[68]. There have been numerous adult human and animal studies

assessing the safety of sulforaphane. These studies are summarized in the safety and efficacy section of the Investigator Brochure.

Singh et al recently conducted a double blind, placebo controlled, randomized, Phase II study of sulforaphane in 40 male adolescents and adults with moderate to severe autism[24]. The participants were dosed according to body weight: 50 µmol (1 capsule) of sulforaphane for <100 lb, 100 µmol (2 capsules) for 101-199 lb, and 150 µmol (3 capsules) for >200 lb. Sulforaphane treatment effectively improved core aberrant behaviors of ASD, and was safe and well-tolerated. Notably, none of the laboratory results were outside normal ranges at any time point. Unexpectedly, the sulforaphane group gained significantly more weight over the18-week period, compared to placebo. Thirty-six adverse events were noted during the trial. Vomiting, increased aggressions, abdominal pain, increased flatulence, irritability, constipation, diarrhea, fever, headache and exacerbation of seasonal allergies were reported in 12-19 percent of participants on sulforaphane; their incidence was the same in the placebo groups ($P > 0.10$). Two participants had single unprovoked seizures: one after 3 weeks on sulforaphane, with an undisclosed history of recent seizures; the other 3 weeks after discontinuing treatment and a past (more than 1 year) history of well-controlled seizures on treatment with anti-epileptic drugs. Although patients with ASD are predisposed to seizures[69,70], the possibility of seizures as a possible adverse effect of sulforaphane in ASD cannot be ruled out.

Although there have not been any studies designed explicitly to test the safety of sulforaphane in children, the overall weight of evidence as per our previous clinical trial of sulforaphane in adolescents with autism and as demonstrated by the numerous clinical as well as preclinical studies summarized in the Investigator Brochure strongly suggests that sulforaphane treatment is likely to be safe and without any serious adverse effects. Although we do not anticipate that children will show signs of toxicity to sulforaphane, it is for this reason that we have designed this clinical trial to carefully evaluate children for possible toxicity over 30 weeks of treatment.

Foreseeable risks: No severe adverse events have been observed in any trial with sulforaphane. In the case of ASD, however, we will be vigilant for any possible behavioral regression that could signify an unwanted interaction with concurrent medications or cause undue distress for the participant or guardian. Given the delicate balance between real events of this sort and those that could be due to a “reverse placebo effect” in the case of parental anxiety about new treatment, home reports of exacerbation will be confirmed by clinical review. If parental support for continuation is lacking and/or clinical review indicates a suspected undesired effect, censorship is the ethical policy even if definite proof of mechanism is lacking or it is possible that a worsened behavioral outcome would be transient.

A previous randomized, double-blinded phase I study of repeated administration (3 doses per day for 8 days) of sulforaphane-containing Broccoli Sprout Extract in healthy volunteers found no problems with safety and tolerance[71]. Another cross-over clinical

trial designed to look into bioavailability of sulforaphane reported no serious adverse effects[58]. Another animal study has shown that sulforaphane raised liver enzymes but only on very high dose of >500 μmol/day, which is much higher than our proposed dose[72]. We will be vigilant in treating subjects with autism, for behavioral regression that could signify an interaction with concurrent medications or other adverse effect.

In our recently completed, randomized, double blind, placebo controlled clinical trial of sulforaphane on 40 male adolescents and adults with autism, the study drug was remarkably well tolerated and very few adverse effects were noted[24]. Vomiting, increased aggressions, abdominal pain, increased flatulence, irritability, constipation, diarrhea, fever, headache and exacerbation of seasonal allergies were reported in 12-19 percent of participants on sulforaphane; their incidence was the same in the placebo group ($P > 0.10$). Two participants in our study had single unprovoked seizures: one after 3 weeks on sulforaphane, with an undisclosed history of recent seizures; the other 3 weeks after discontinuing treatment and a past (more than 1 year) history of well-controlled seizures with anti-epileptic drugs. Although patients with autism are predisposed to seizures, we cannot rule out the possibility of seizures as a possible adverse effect of sulforaphane in ASD. In consideration of this, we will not enroll any participant who has an active history of seizure within 1 year of participation.

Based on previously published data and data from our clinical trial, possible clinical side effects of sulforaphane include: seizures, insomnia, vomiting, abdominal pain, flatulence, constipation, diarrhea, large bowel movement, soft stools, weight gain, exacerbation of seasonal allergies and increased urination[63]. Caregivers will be advised to notify study staff should these side effects be experienced. Staff members will be available 24/7 for emergencies. We will diligently scrutinize each medication a subject is taking and will take precautions to monitor and/or discontinue the study medication if there is any sign of interaction, such as the appearance of exatrapyramidal signs in a subject taking risperidone. Additionally, we are not aware of any risks associated with stopping the study drug without any down titration.

Physical risks to subjects from phlebotomies include pain and discomfort, and bruising. Subjects and particularly children and mentally impaired subjects may experience pain and anxiety and become upset with phlebotomy. Physical risks will be minimized by having phlebotomy performed by appropriately trained and experienced personnel. Subjects will be asked to present for phlebotomy in an adequately hydrated state, so that it will be easier to identify and successfully puncture veins. All subjects will be offered the use of a local anesthetic (EMLA cream) prior to the blood draw, in order to minimize discomfort. Emotional risks will be minimized by utilizing personnel who are experienced in phlebotomy in pediatric and cognitively impaired populations, and ensuring that they have training in appropriate behavioral techniques. Maximum amount of blood drawn at any stage will not exceed 2 ml/kg body weight; also we will not exceed the minimal risk requirements of 3ml/kg in an 8 week period.

There is a small risk to privacy in answering questions. All the information will be kept strictly confidential and every known effort will be utilized to maintain confidentiality.

All records will be kept under lock and key or on encrypted and password protected digital media.

There is a small risk of emotional discomfort, distress and/or fatigue from completing the surveys and completing the assessment tasks. We will make all efforts to ensure that participants do not have to wait an inordinate amount of time while at the study site for completing the requisite surveys and assessments.

There is also a small risk of transient local reactions to EMLA cream such as paleness, erythema and edema.

Parents and/or guardians will be responsible for ensuring the timely and complete adherence to daily medication dosage. They will be asked to keep a log of the time of day and the number of capsules taken. They also will monitor for and report any adverse events, undesirable psycho-behavioral reactions throughout the duration of the study or until censorship, should that occur. Parents/guardians will be trained to ensure that the study medication is kept in the original, properly labeled, child-proof containers in a place that is secure from access by siblings, school mates, other children, mentally impaired adults, or pets. Any spilled or lost medication will be documented and reported so that replacement will ensure adherence to prescribed dosages. Parents/guardians will be reminded by telephone at-least 24 hours beforehand of their appointments. Those who miss an appointment will be contacted by telephone in order to elicit the cause and reschedule within one week.

16) **Potential Benefits to Subjects***

Potential benefits of sulforaphane may include demonstrable functional improvements in social responsiveness in subjects with autism. Treatment with sulforaphane may also increase mitochondrial function and decrease oxidative stress, both of which have been subjects of research in autism.

Current treatment standards for children with ASD include various therapy programs, including ongoing speech and language, occupational, physical and behavioral therapies. Many are also in special school programs with Individual Educational Plans. We anticipate that some of the children will be taking prescriptions of antipsychotic, antidepressant, antianxiety or stimulant medications. Approximately 10% will have a history of epilepsy and may be taking anticonvulsants. We will carefully evaluate each medication a subject is taking for potential interactions with sulforaphane. Subjects will continue to participate in their usual therapy programs.

Novelty: This trial of sulforaphane in children with autism provides a novel approach with a strong theoretical basis in clinical observations of the effects of fever in autism. Preliminary evidence from *in vitro* models supports the hypothesized cellular effects of sulforaphane on heat shock proteins Nrf2, mitochondrial functions, and the mTOR pathway.

There are increasing reports of successful "off target" therapies for genetic disorders that modulate cellular metabolic changes rather than directly ameliorating the underlying genetic cause [73,74]. The capacity of the general cellular stress response pathways to reestablish metabolic homeostasis allows treatment that is independent of -- and without knowledge of -- the primary genetic lesion. This has particular relevance for the treatment of ASD since it is characterized by genetic heterogeneity and interactive metabolic pathways.

Relevance to autism: Medical treatments that affect core features of ASD at the cellular level are needed. The approach to treatment presented here, based on clinical observations of improvements in patients with ASD during fever, and the known stimulation of the cellular stress proteome by sulforaphane *in vitro*, represents a relevant and rational approach to therapy. The findings of this study may provide the basis for further clinical trials and laboratory investigation at the cellular level in autism.

17) Vulnerable Populations*

Since our study involves children, we have reviewed the "CHECKLIST: Criteria for Research Involving Children".

18) Multi-Site Research*

This is not a multi-site study in the sense that all patient enrollment will be conducted at UMass. We are however collaborating with the Cullman Chemoprotection Laboratory at Johns Hopkins University and will perform the biomarker assessments for our study. The role of Johns Hopkins is limited to biomarker assessment only and storage of any leftover samples. Johns Hopkins will obtain its own IRB approval in order to conduct the biomarker analysis. We will hold regular phone conferences with Johns Hopkins investigators (once in 3 months) and again at any time if there are any changes to the biomarkers portion of the study plan and to discuss interim study results (if any available).

19) Community-Based Participatory Research*

NA

20) Sharing of Results with Subjects*

Since several of the safety lab test results (such as complete blood counts/liver/thyroid/ renal function tests) could potentially be clinically relevant to study participants or their primary care providers, upon request (and especially if the test results are abnormal/outside of reference range) we will provide a copy of these test results to study participants' health care providers.

21) **Setting**

The study will be conducted at UMass Medical School and UMass Memorial Medical Center. The Clinical Research Center and Child and Adolescent NeuroDevelopment Initiative (CANDI) at Shriver Center will be the main locations for conducting of study visits and procedures. Study visits may occasionally also be conducted in Benedict building Pediatric clinic offices if so preferred and convenient to the subjects. Clinical offices and laboratories are located in close proximity where ADOS, screening, examinations and phlebotomies will take place in this clinical trial. We will provide maps and arrange to meet families on the first visit to show them around the facility. There are quiet rooms available for resting and calming participants. The clinical laboratory that will run the routine lab studies is located in the hospital and will report results the same day. Two laboratories are available to us for centrifuging specimens and processing PBMCs from blood, and storing blood and urine specimens at -20°C and -80°C until shipment on dry ice to Johns Hopkins (Cullman Chemoprotection Lab).

UMass Research Pharmacy with expertise in clinical that will store and dispense sulforaphane and placebos, randomize participants and dispense the materials with proper labeling. Adequate office, clinic and lab spaces are available for this study through the department of pediatrics, along with various services and spaces in the UMMC. Adequate computer facilities, messaging and copying services are readily available.

The Cullman Chemoprotection Laboratory at Johns Hopkins School of Medicine, Baltimore, MD is a well-established and fully equipped investigational pharmacology laboratory. It is well staffed and equipped for all of the proposed research laboratory studies we have proposed, both in the pilot study and main clinical trial in this study. This laboratory will produce the broccoli seed powder, the source of sulforaphane, under approved protocols and certification of purity prior to transfer to UMass for this study. In other parts of the lab, clinical samples will be received, in close contact with the staff at UMass regarding processing, storage and shipment of samples.

22) **Resources Available**

The following is the study organization chart describing various staff involved with study procedures and their roles.

a. <u>Study Organization Chart</u>

<u>Site 1:</u> <u>University of Massachusetts Medical School, Worcester, MA</u>

Department: Pediatrics (Division of Pediatric Neurology)

1. Principal Investigator
2. PostDoc Associate
3. Primary Care Physician
4. Clinical Research Assistant (CRA)

Department: Child and Adolescent NeuroDevelopment Initiative (CANDI)

1. Psychologist

Department: Quantitative Health Sciences

1. Biostatistician

Site 2: Johns Hopkins University School of Medicine

Department: Pharmacology (Lewis B. and Dorothy Cullman Cancer Chemoprotection Center)

1. Johns Hopkins Principal Investigator
2. Johns Hopkins Co-investigator
3. Johns Hopkins Co-investigator

Quality Associates Inc.

1. External Good Clinical Practices (GCP) monitor

b. Study Personnel Description:

1. Andrew Zimmerman, MD.: Dr. Zimmerman is the Principal Investigator and a Professor of Pediatrics and Neurology at UMass Medical School. He is pediatric neurologist who has focused his research for over 20 years on novel immunological and therapeutic approaches to ASD. In this project, he plans to carry out a clinical trial of sulforaphane, a potent but safe natural substance in broccoli, in order to modify social and behavioral functioning in children. He will also evaluate underlying cellular effects of sulforaphane during the study, in collaboration with the collaborators at Johns Hopkins. He solely conceived this project and will be responsible for its overall administration and direction. He will supervise recruitment, enrollment, study implementation, monitoring of side effects, data management and ensure that the research is conducted in line with the ethical provisions of the University of Massachusetts Medical School (Aims 1-3). He will oversee and assure accurate data collection and analysis. He will present data at a national meeting, and prepare manuscripts for publication in peer-reviewed journals.

2. PostDoc Associate/Research Coordinator:. He will assist the PI in the planning and implementation of all aspects of the study. He is experienced in regulatory affairs and ethical considerations and will have a major role in obtaining IRB approval for this study and regular reporting to the IRB. He will oversee the day to day operation of the study, including recruitment, screening procedures, scheduling, coordinating physical examinations, outcome measures and administration of medication. He will coordinate efforts of the Primary Care Physician (TBN) for the recruiting, screening, physical examination and phlebotomies of subjects at each study visit. He will work with our collaborators at Johns Hopkins to supply the Research

Pharmacy with drug and placebos, and ensure timely delivery of medication to the participants. He will assist with phlebotomies, blood sample preparation and shipments to the Cullman Chemoprotection Laboratory at Johns Hopkins. He will be responsible for data collection and storage in accordance with FDA guidelines, with which he has extensive experience. He will oversee the collection of safety data and adverse event reporting for the Data Safety Monitoring Board (DSMB). He will also be a liaison to Dr. Maranda, Biostatistician, for data management and analysis.

3. Primary Care MD: The primary care MD will assist the PI in supervising and assisting with all medical aspects of the study, including review of medical histories during recruitment and screening, examinations of participants at each visit, and reporting of all potential side effects of treatment. He or she will review participants' pre- and postnatal histories and development, record and rate effects of fever on behavior, current and past medications and allergies; record clinical data; perform phlebotomies and respond to parents' calls or emails with respect to medical questions or concerns related to the study. This individual will communicate with participants' pediatricians regarding questions about and concerns during the study, e.g., intercurrent illnesses, and will report any concerns to the PI and DSMB.

4. Psychologist: A psychologist working at the Center for Autism and Neurodevelopmental Disorders (CANDO) clinic will assist with performing ADOS, Vineland and Leiter-3 assessments.

5. Biostatistician: A biostatistician from Quantitative Health Sciences, will be the primary statistician on the team. This person will have access to specialized statistical software, readily available to her for the conduct of this project's statistical analyses. These include SAS, SPSS, StatsDirect and STATA. For this project, he/she will assist in the planning for the clinical trial design, data handling and storage, and evaluation of outcomes.

6. Clinical Research Assistant: This individual will assist in recruitment and scheduling of appointments for screening, reporting and recording data from baseline assessments and outcome measures. He or she will work with other members of the team to communicate effectively with families in a timely manner, in order to assure both safety and compliance with the drug regimen and collection of data. The CRA will report any concerns of families to the PI, Research Coordinator and Primary Care MD. He or she will communicate with our collaborators at Johns Hopkins regarding drug shipments and coordinate with the Research Pharmacy, and arrange shipments of specimens to the laboratory in Baltimore.

7. Johns Hopkins Principal Investigator and Co-investigator: These persons will assist prepare and supply capsules of broccoli seed powder containing standardized quantities of glucoraphanin and similar placebo capsules containing

microcrystalline cellulose. They will also determine the glucoraphanin content. They will be responsible for assurance of purity of the medication.

8. Johns Hopkins Co-investigator: This person will make determinations of biomarker levels of oxidative stress, antioxidant capacity, and accumulation of reactive oxygen species, inflammatory biomarkers and heat shock proteins.

9. Good Clinical Practices Monitor: This person will conduct yearly site visits to ensure that the study is being conducted in compliance with Good Clinical Practices and will conduct yearly site visits and audits to ensure GCP compliance.

Our plan for ensuring the standardization of procedures among staff: We will have training sessions for all study staff to ensure internal consistency and replicability for OACIS scale. We will hold weekly meetings to review study progress, possible concerns of families and staff, review of study requirements, and compliance with the study plan. We will exchange de-identified data on a regular basis using encrypted email with the group at Johns Hopkins. Regular phone contacts and teleconferencing will take place, at least biweekly.

All Johns Hopkins investigators are under the jurisdiction of JHU.

23) **Prior Approvals**

In addition to obtaining the UMass IRB approval, we will also obtain approval from USAMRMC Human Research Protection Office. An Investigational New Drug (IND) approval from the Food and Drug Administration has been obtained. UMass Biosafety Committee has provided approval to perform the study. An IRB approval will also be obtained from Johns Hopkins University for the purposes of performing biomarker analysis at Johns Hopkins.

24) **Recruitment Methods**

Participants will be enrolled from Massachusetts and nearby New England states. The University of Massachusetts (UMass) Memorial Medical Center, Worcester is centrally located and has an excellent infrastructure for clinical trials as well as expertise in ASD with the Center for Autism and Neurodevelopmental Disorders (CANDO) Clinic. In our previous trial (at the Lurie Center, Massachusetts General Hospital for Children in Lexington, MA), we were readily able to enroll 44 participants in the span of 2 years despite restricting the population to male adolescents and young adults. Expanding the population to 50 younger children, both boys and girls, is a reasonable goal and will allow us access to an even greater target population than we had in the previous study. We will not restrict our study population to any race or ethnicity, and make all efforts to have this project meets the intent of the program as a clinical trial in ASD that is novel

and holds promise for a nontoxic approach to therapy of known biochemical and metabolic abnormalities in ASD.

Patients will also be recruited from the CANDO clinic at UMass Memorial Medical Center, developmental pediatrics, pediatric neurology, child psychiatry, genetics and general pediatrics clinics at UMass Memorial Medical Center, as well as related clinics in the area. Patients suitable for the study will be identified by their treating physicians (not study staff members). We will obtain permission from the treating physician to contact these patients. The treating physician will be invited to notify their patients of this research study by providing their patients with recruiting letters and study flyers. Any recruitment letters will include a signed letter from their treating physician. The majority of patients at the CANDO Clinic have idiopathic ASD, whereas others have known genetic, pre- and postnatal causes of ASD. There are also sizable patient populations with ASD in the Reliant Health System and private practices nearby, as well as in community schools and treatment centers.

Community based recruitment will also be done, and may include advertisements, electronic newsletters, and flyers to primary and subspecialty pediatric clinics within the participating institutions, local autism societies and support groups. Appropriate listservs and email outlets may be utilized. Advantages of UMass include its central location in New England and ready access with minimal traffic congestion, as well as a full-service academic medical center and pediatric hospital.

As noted above, participants will be recruited from central Massachusetts and New England. UMass Memorial Medical Center is ideally located and suited for this clinical trial of sulforaphane in children with ASD. It is centrally located in New England and is a full service academic medical center and children's hospital, as well as a Clinical Research Center. A strong presence in the field of ASD has developed at UMass in recent years with the founding of the CANDO Clinic, expansion of pediatric neurology and developmental pediatrics.

Participants will be screened by review of verbal histories and records on request as they are made available from their referral sources, following parental consent.

Participants will be compensated for their participation in the study. We will provide them with a gift card worth $15 for each completed study visit. We will also provide them with parking vouchers when families park their vehicle in the UMass Medical Center parking lot. Children will be given a small soft toy to keep.

Flyers describing the nature of sulforaphane and how it might help ASD will be used for study advertisement. These advertisements will make it clear that it is a research study and not a "free treatment program" for ASD, in order to remove any coercive or undue inducements for study participation. These flyers will be distributed to various autism advocacy centers for display purposes, and may also be distributed by email based listservs.

The recruitment process is similar for both the pilot study as well as the main clinical trial.

Screening Procedures:
Prior to coming for the formal screening visit, all subjects will undergo a pre-screen assessment to determine if basic eligibility criteria are met and the study procedures will be explained in detail. Qualified study staff will ask questions related to the above described inclusion/exclusion criteria. The pre-screen may be completed by telephone or direct interview. If a participant is found to be ineligible for the study, prescreening form will be destroyed.

At the screening visit, *after* the consent has been signed by parents/guardians and assent signed (if applicable), a complete, baseline medical history, physical and neurological exam will be performed. During that visit, ADOS-G, SRS, OACIS-Severity scale and ABC-W scales will be performed to confirm eligibility and to quantify disease severity. Also, following laboratory blood tests (complete blood count, comprehensive metabolic profile: liver function rests, renal function tests, thyroid function test (TSH)) will be performed. Maximum amount of blood drawn at any stage during this study will not exceed 2 ml/kg body weight; we will also not exceed the minimal risk limitation of 3 ml/kg in an 8 week period.

25) **Local Number of Subjects**

We will enroll 50 participants in the study. We anticipate that 400 to 500 families will be screened in order to attract 50 participants.

26) **Confidentiality**

Strict confidentiality will be maintained at all times. As mentioned above, all subjects and clinical information associated with each subject will be assigned an identification number and will only be referred to by that number and subject initials. This number will be the only identifying information on test score sheets, data entry forms, and in the data files. Some data will require minimum protected health information (i.e. date of birth) recorded on the assessment sheets in order to be properly scored. In this case, we will follow HIPAA limited data set guidelines. Source documents will be maintained by the study coordinator in a centralized, double-locked office accessible only by the study coordinator and involved members of the research team. Only initials and study number identify any specimens sent to laboratories outside of UMass.

We will collect some identifiable information, such as name, address, date of birth, contact information (mailing address, phone numbers, email address etc.) demographics (race, ethnicity, parental educational background) but this information will be kept separate from the other study files and documents. Documents containing this identifying information will be kept in a locked cabinet in a secure study location. Only study staff

directly involved in interaction with the study participants and the study Principal Investigator will have access to this identifiable information. The Principal Investigator, or designee, will maintain a personal participant identification list (participant numbers with the corresponding names) to enable records to be identified. Other study staff (such as those working with biomarkers) whose role will not involve interacting with study participants will not have access to this identifiable information. Representatives of USAMRMC who may be entrusted with the responsibility of conducting site visits may be able to get access to the study records for review.

In terms of subject privacy protection, the researchers are aware of the implementation of the Health Insurance Portability and Accountability Act (HIPAA) and its impact on clinical research and electronic data collection activities. The database developed under this protocol will conform to national standards and procedures for the electronic storage and transmission of information to insure privacy and security of health information. This includes conformance to rules for transactions and code sets, the standards for privacy of individually identifiable health information and security standards.

Any blood samples sent to the laboratories at Johns Hopkins for analysis of heat shock proteins and mitochondrial study and measures of oxidative stress will not contain any direct identifiers and samples will be labeled with the subject's unique ID. Samples may be stored for future use with explicit consent of the subject and parents or guardians. Without such consent, samples will be destroyed following the study.

Data will be primarily captured on paper records. Majority of the study instruments (such as Aberrant Behavior Checklist, Social Responsiveness Scale, Autism Diagnostic Observation Schedule, Vineland, Leiter scale) are provided as paper booklets and they will be used as the primary data capture devices. Additional information, such as physical examination findings, medical history records etc. will also be captured in paper records. After the study is complete, we will keep the study records for future regulatory purposes for a period of 7 years, at which time all study records will be destroyed. However the documents containing identifiable information will not be stored after the study ends; they will be destroyed after study is completed.

In addition, we will use a secure computer database to transfer the paper data records to an electronic format. University of Massachusetts Medical School has contracted the RedCap clinical trials software database solution. REDCap (Research Electronic Data Capture) is a secure web-based application built around HIPAA guidelines to support data capture for research studies. It allows users to build and manage online surveys and databases or a combination of both quickly. REDCap features automated export procedures for seamless data downloads to Excel, PDF, and common statistical packages (SPSS, SAS, Stata, R). Also included are a built-in project calendar, a scheduling module, ad hoc reporting tools, and advanced features, such as branching logic, file uploading, and calculated fields. The REDCap Software sits on a dedicated server behind a firewall to eliminate all nonessential access credentials. The data itself is isolated on a separate server that sits behind an additional firewall layer for added security. Interaction between REDCap and the client is encrypted via SSL. To ensure accuracy of transfer of

data from paper to electronic format and to minimize data entry errors, all data that will entered to RedCap will be double entered by two independent study staff members. After the study is complete and all data has been collected and entered into the database, the database will be locked out from any further editing.

27) **Provisions to Protect the Privacy Interests of Subjects (HIPAA)**

In terms of subject privacy protection, we are aware of the implementation of the Health Insurance Portability and Accountability Act (HIPAA) and its impact on clinical research and electronic data collection activities. The database developed under this protocol will conform to national standards and procedures for the electronic storage and transmission of information to insure privacy and security of health information. This includes conformance to rules for transactions and code sets, the standards for privacy of individually identifiable health information and security standards.

We will make all efforts to preserve the privacy of the study participants. All data collected in the study will be identified by a random code and participant initials. We will use the following format to label the study participants: SF-15-001, SF-15-002 along with participant initials (eg. JD). No personally identifying information, such as complete name, hospital medical record number, date of birth etc. will be used on study documents.

Any blood samples sent to the laboratories at Johns Hopkins for analysis of heat shock proteins and mitochondrial study and measures of oxidative stress will not contain any direct identifiers and samples will be labeled with the subject's unique ID. *Samples may be stored for future use with explicit consent of the subject and parents or guardians. Without such consent, samples will be destroyed following the study.*

28) **Compensation for Research-Related Injury**

No funds have been set aside for any compensation for research related injury.

29) **Economic Burden to Subjects**

Study participants will not have any costs associated with participating in the study – costs for all study related procedures and lab tests will be borne by the study funds. Participants will be compensated for their participation in the study. We will provide them with a gift card worth $15 for each completed study visit. We will also provide them with parking vouchers when families park their vehicle in the UMass Medical Center parking lot. Children will be given a small soft toy to keep.

30) **Consent Process**

A written consent will be obtained. We will follow the "SOP: Informed Consent Process for Research (HRP-802)" for consenting purposes.The consent will be obtained at the Clinical Research Center at the UMass Memorial Medical Center. We will allow participants/their parents or legal guardians a sufficient time interval (more than at-least 24 hours) to make up their mind before signing the consent form. After the telephone pre-screening with parents/guardians, we will mail a copy of the consent and assent form as well as study information flyers to their address and encourage them to thoroughly read the consent forms and call if they have questions. All potential participants for this study will be provided a consent form describing this study and sufficient information for subjects and/or their parent or guardian to make an informed decision about their participation in this study.

At the screening visit, the study investigator(s) will again discuss with each subject and his or her parents or guardian the nature of the study, its requirements, and its restrictions. At that time if they are interested in participating in the study, a written and witnessed informed consent will be obtained prior to the performance of any protocol-specific procedures. If however they need more time to make up their mind, they will be allowed to do so and we will reschedule the study visit to a date when they are ready to participate in the study and sign the consent form. Whenever possible, consent from both parents will be obtained.

Since all subjects in this study are children, permission will be obtained from their parent or legal guardian and will be documented in writing using the approved Consent Form. We will try to obtain the assent of subjects for participation in the research. This assent will be done on the approved assent form. If some participants are medically incapable of giving assent (those who are severely autistic and incapable of understanding the nature of the study) we may not obtain assent from these children. The Principal Investigator (who is a pediatric neurologist experienced in evaluating and treating children with ASD) will determine whether a patient is medically incapable of giving assent. In these and all cases, consent will be obtained from parents or legal guardians.

The formal consent, or assent, as applicable, using the IRB-approved forms will be obtained before that subject is submitted to any study procedure. The form(s) must be signed by the subject and/or parent/guardian for the child and the investigator-designated research professional witnessing the signatures. Every effort will be made to assure the safety of the subjects. A qualified person, such as a licensed physician on the study staff will obtain the consent/assent. A research coordinator will not obtain consent/assent from the study participants.

Since majority of instruments (such as the ADOS, Leiter-3) used in the study are in English language and validated in English language, we will only enroll English speaking participants in the study.

31) **Process to Document Consent in Writing**

We will be following "SOP: Written Documentation of Consent (HRP-803)."

32) **Drugs or Devices**

Prior to dispensing the study drug to the participants, the drug will be stored in temperature controlled conditions (-20C) at the UMass Memorial Investigational Pharmacy. During the study visits, we will store the study drug in a locked -20 freezer at the Clinical Trials Unit (to which only the study staff will have access to) and directly dispense the medication to patients' parents (except at the first visit where we will wait for the safety labs results and then either mail the drug to participants or ask their parents to pick it up). The parents will be instructed to keep the medication stored in their freezer for the duration of the study.

The firm/entity that will be hired to ensure GCP compliance will also monitor compliance with the FDA requirements.

We filed an IND application to the FDA to obtain permission for conducting this clinical trial and the FDA has approved the IND (IND number 127062). The IND is held by the study PI (Dr. Andrew Zimmerman). We will comply with the Sponsor-investigator requirements for IND studies (21 CFR 54, 210, 211, 312).

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