Document type: Protocol and Statistical Analysis Plan

Short Title: Sulforaphane in a New Jersey (NJ) Population of Individuals With Autism

Official Tittle: Sulforaphane in Autism: A Treatment Trial to Confirm Phenotypic Improvement With Sulforaphane Treatment in a New Jersey (NJ) Population of Individuals With Autism

ClinicalTrials.gov Identifier: NCT02677051

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***Title of Project*:** **Sulforaphane in autism. A treatment trial to confirm phenotypic improvement with sulforaphane treatment in a NJ population of individuals with autism.**

***Principal Investigator*:** **Steve BuyskePhD.**

***Funding Source(s)*:** The Governor's Council for Medical Research and Treatment of Autism of New Jersey

1. *Purpose/Specific Aims*

To complete a trial with sulforaphane in a NJ cohort of individuals with autism with the goal of independently replicating the findings that sulforaphane treatment is associated with improvement of core symptoms in autism.

Specific Aims:
Aim 1: To perform a double blind randomized control pilot study with sulforaphane in a NJ cohort of autism of at least 40 individuals with autistic disorder completing the study to replicate the previous findings of the Singh study.

Aim 2: To genotype specific functional variants in genes of sulforaphane metabolism to use as covariates to account for potential variability in treatment response.

Aim 3: To measure the pre- and post-treatment glutathione levels and glutathione status (e.g. how much is oxidized or reduced) to use as a covariate to account for potential variability in treatment response.

1.1 Objectives

The objectives of this study are:

- To replicate the study of Singh et al (Singh, Connors et al. 2014), that sulforaphane treatment results in improvements in core symptoms in autistic disorder (ClinicalTrials.gov Identifier NCT01474993).
- To account for potential variability in treatment response due to genetic variations in sulforaphane metabolizing enzymes.
- To correlate pre- and post- treatment glutathione levels with potential variability in treatment response.

1.2 Hypotheses

That t*reatment with sulforaphane will improve symptoms of autistic disorder as measured by the Aberrant Behavioral Checklist (ABC), the Social Responsiveness Scale (SRS) and the Clinical Global Impressions test (CGI).*

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Approval Date: 7/25/2023
Expiration Date: 7/18/2024

2. Background and Significance

One of the most promising findings in autism recently is that of Singh et al. (Singh, Connors et al. 2014) which reported results on a double blind study with the isothiocyanate, sulforaphane. They reported improvement by multiple metrics. Significant improvement was seen in behavior as measured by the Aberrant Behavioral Checklist (ABC) and by the Social Responsiveness Scale (SRS). In addition a significantly greater number of participants receiving sulforaphane had improvement in social interaction, abnormal behavior, and verbal communication as per the Clinical Global Impression (CGI). The Singh study needs to be replicated in an unrelated dataset to help determine if their results represent a real phenomenon and if a large scale clinical trial is warranted. In addition, there was some variability in response to treatment. We will attempt to replicate their study and try to account for some variability in response to sulforaphane treatment. The Singh study also demonstrated that treatment in autistic disorder using orally administered sulforaphane was well tolerated and safe.

Sulforaphane (1-isothiocyanato-4R- (methylsulfinyl)butane) is an isothiocyanate derived from the action of the plant enzyme myrosinase on glucosinolates including glucoraphanin. Cruciferous vegetables are an important source for glucosinolate and it is present at high levels in broccoli sprouts (Fahey, Zhang et al. 1997). We propose to administer Avmacol®. As far as we know Avmacol® is the only available product on the consumer market which co-delivers active myrosinase (a plant enzyme) along with its substrate (glucoraphanin), and is shelf-stable at room temperature. Avmacol® is a commercially-available over the counter supplement that is presently produced and marketed by Nutramax Laboratories, Inc., Edgewood, MD, USA, and has been on the market since 2013.

Sulforaphane is the most potent naturally occurring inducer of mammalian cytoprotective enzymes. Its therapeutic potential is based at least in part on their ability to upregulate genes responsible for removal of endogenous and exogenous electrophiles and to regulate immune and inflammatory genes through the Keap1–Nrf2 pathway. The transcription factor Nrf2 (Nuclear factor-erythroid factor 2) is an important regulator of a large fraction of cytoprotective gene products, and it orchestrates protective responses to a diversity of endogenous and exogenous stresses including those involved in neurodegenerative diseases (Gan and Johnson 2014). Importantly, Nrf2 levels are substantially depressed in ASD for 45% of typically developed children.

An important subset of these upregulated genes is that of the glutathione (GSH) metabolism genes some of which are relevant in sulforaphane metabolism. Sulforaphane is metabolized by glutathione-S transferases (GST's) through conjugation to GSH. Plasma levels and excretion of sulforaphane have been reported to be significantly different based on the presence or absence of the null genotype of *GSTM1* (Gasper, Al-Janobi et al. 2005, Lampe 2007, Steck, Gammon et al. 2007). In turn, GST's as well as other genes of glutathione metabolism and possibly other relevant

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IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

phase II genes, are upregulated by sulforaphane. The NAT2 slow-metabolizing genotype along with GSTM1 null modifies benefit of cruciferous vegetables. Sex specific differences in *GSTA1* up-regulation during cruciferous vegetable diet with *GSTM1*- and *GSTT1*- null genotypes has been described. It was postulated that these Null genotypes result in slower metabolism and therefore result in a stronger up-regulation of NRF2. GST's have overlap of substrate and it is hypothesized that the GSTT1 enzyme has little effect in GSTM1- not null individuals but is more important in individuals who are GSTM1-null. GSTP1 and GSTA1 may also be important in sulforaphane metabolism. It is therefore possible that genotypes of GST's that metabolize sulforaphane may contribute to the variability seen in the phenotypic response to sulforaphane treatment.

Interestingly GST's and other glutathione metabolism genes have been reported as risk factors for autism. We previously reported evidence that *GSTM1* and glutathione peroxidase-1 (*GPX1*) are risk factors for autism (Buyske, Williams et al. 2006, Ming, Johnson et al. 2009). Others reported data that support our *GSTM1* association(James, Melnyk et al. 2006). Other genes of glutathione metabolism implicated in autism by us and others include *GSTP1* (Williams, Mars et al. 2007), cystathionine gamma-lyase (*CTH*), the catalytic subunit of glutamate-cysteine ligase, catalytic subunit (*GCLC*), glutaredoxin-3 (*GLRX3*), alcohol dehydrogenase-5 (ADH5) and glutaredoxin (*GLRX*). Other important oxidative stress related genes that have been associated with autism are paraoxonase 1 (*PON1*) and glyoxalase 1 (*GLO1*). Interestingly, both enzymes are GSH dependent.

Oxidative stress and autism:
Inflammation and systems to combat oxidative stress has been repeatedly implicated in autism, including altered enzyme levels, altered metabolite and biomarker levels and genetic risk factors. Enzyme activity of several key anti-oxidant genes have been reported altered in autism including reduced enzymatic activities of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase. Another study reported significantly reduced levels of SOD and GPX and increased levels of malondialdehyde in subjects below the age of 6 but not above. Reduced taurine levels as well as carnosine levels have been reported (Ming, Stein et al. 2012). In addition, elevated nitrite concentrations have been detected in individuals with autism along with t-bars and xanthine oxidase activity in red cells. Increased t-bars, Na+/K+-ATPase, isoprostanes and hexanoyl-lysine adduct (HEL) have also been reported. Increased non-protein-bound iron and 4-hydroxynonenal protein adducts (4-HNE PAs) were also reported increased in autism.

Glutathione (GSH) levels have been shown to be altered in autism. Total GSH was reportedly decreased while plasma levels of GSSG were elevated; the tGSH:GSSG ratio was low (James, Cutler et al. 2004). A meta-analysis of oxidative stress-related biomarkers and genes showed associations with autism for levels of methionine, cysteine, and GSH, glutathione peroxidase but not homocysteine, SOD and cystathionine. Significantly reduced activity of key GSH metabolism enzymes was

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IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

reported in cerebellum of individuals with autism including glutamate-cysteine ligase, modifier subunit (*GCLM*), *GPX* and glutathione transferases (GSTs), as well as paroxanase 1 (*PON1*), an enzyme that is inactivated by oxidized GSH.

In addition to the previously mentioned relevant gene associations rare variants were reported in the 5' region of *SOD1* in a few cases of autism. Last, increases of mitochondrial DNA damage have been reported in autism and this is likely due to an increase in oxidative stress.

Other important functions of GSH consist of S-glutathionylation of proteins (most likely serving as a protective measure against oxidative stress), reduction of glutaredoxin, reduction of ascorbate and tocopherol, direct removal of free radicals and protection of protein sulfhydryls. GSH may also be important in cell signaling through glutathionylation and, based on its action on NMDA receptors, may modulate glutamate signaling. It has also been reported that GSH may be an NMDA receptor agonist. It is therefore possible that GSH levels may contribute to the variability seen in the phenotypic response to sulforaphane treatment. It is interesting to note that the GSH system and specifically the GST's are important metabolizers of both the pesticides and air pollutants that have been suggested to be risk factors for autism.

Significance:
Currently, there are only two FDA-approved medications for ASD that we know of and they are approved for irritability associated with autism. None so far addresses the core features (e.g., social responsiveness).

Sulforaphane is well tolerated and has shown negligible to no toxicity. Replication of the findings that sulforaphane may influence social interaction, abnormal behavior, and verbal communication in autistic disorder will lead to a large scale, multi-center, clinical trial in adolescents and adults with autism as well as trials in younger individuals with autism. Promising results seen in several early intervention studies suggest that there is a timeframe in which some may be rescued from lifelong symptoms. It is therefore possible that along with early intervention protocols or by itself sulforaphane treatment may give a better response and maybe a long term improvement when applied to those with very early diagnosis of autism.

Sulforaphane as a treatment for autistic disorder is intriguing since it intersects with several metabolites, systems and genes that we and others have previously implicated in autism.

3. Research Design and Methods

This is a study comparing Avmacol® and placebo as adjuncts in the treatment of autistic disorder. Forty adolescent or adult outpatients total, with age ranges from 13 to 30 years will be randomized into an 18-week (close out at 22 weeks) double-blind, placebo-controlled parallel treatment study in a 2:1 treatment to placebo ratio. The study Dr. , Eveline Traeger, MD, is an expert in autism and will perform study

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IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

assessments including lab work, scales such as the Aberrant Behavioral Checklist (ABC) while remaining blind to medication regimens. Study assessments will be administered at designated time points.

Research Design:
Subjects that express interest in the project will be called to see if they meet the inclusion and exclusion criteria. The studies Phone Script will be used for this call. Those that pass inclusion/exclusion and wish to be part of the project will undergo informed consent/assent. This will be done in a private location, preferably in the Staged Research Building room (SRB)115. Alternatives more convenient for the subject/family can be used as long as they are private. Consenting/assenting will be done by the Study Coordinator. When the study coordinator is not able to, the diagnostician or study doctor will perform informed consent/assent the subjects.

NDAR GUID (National Database for Autism Research Global Unique Identifier Web Service, from the National Institutes of Health) will be used to generate ID numbers. Only GUID numbers will be used on documentation, tests and sample tubes. Only the screening questionnaire page 2 the Study Visit form and the NDAR GUID for, will link GUIDs to the subjects. These questionnaires will be stored in a locked file cabinet separate from other study information and only GUIDs will be used for electronic data. The NDAR GUID helps to ensure that this information can be sent and stored securely, protecting the privacy of the individuals in the study and the confidentiality of their data. Using the NDAR GUID allows data collected on an individual from one study or site to be associated the data from another study or site without the risk of exposing PHI. The identifying information used to generate the GUID remains on our secure computer and is used to generate “hashtags” which are used by NDAR to generate the GUID. This unique ID number will be used to identify all samples, data and results. The Study Visit form will be kept by the study doctor in a locked cabinet/desk, in a secure office until the subjects has finished the study. The Study Visit form will then be destroyed.

Following informed consent/assent subjects will be tested with the studies diagnostic tools, ADOS-2 and DSM-IV. by one of the two study diagnosticians. Drs. Mars or Patel. These tests will be done in the SRB room 115. Those that are not eligible for the study will be informed of this and will no longer be part of the study. Study tests and documents will be stored securely in SRB room 107.

Subjects that are diagnosed with moderate to severe autistic disorder will be invited to participate. Subjects will receive evaluations and medical tests by the study team, including the study doctor, Eveline Traeger, MD, in either the Clinical Academic Building or the Clinical Research Center (CRC). This may include a clinical blood draw and urine collection to measure liver, thyroid and kidney function which will be done in the Clinical Research Center (CRC). It may include one 5ml draw for serum and one 5ml EDTA draw and 5ml of urine.

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

A checklist will be used for each subject visit. Additionally, two exam forms will be used for each visit. The first is to be used by the study Dr. called “Exam for study doctor” and the second to be used at the CRC called the “Exam form CRC” will be used for each visit.

For those subjects that allow a blood draw, 2ml of the blood samples will be used for research and will be used to test biomarkers and as a source of DNA. Glutathione levels will be measured at baseline and weeks 18 and 22. Glutathione levels will be used to try to account for variability in response to sulforaphane. DNA will be used to genotype a specific group of genes of sulforaphane metabolism to try to account for variability in response to sulforaphane. A cheek swab will be used for collecting DNA for the subset of subjects that will not undergo a blood draw.

Baseline study metrics Clinical Global Impression (CGI), Aberrant Behavioral Checklist (ABC) and by the Social Responsiveness Scale (SRS) will be performed in the Clinical Academic Building Suite 6100 6th FL or the CRC

The CRC pharmacist will randomize subjects in a 2:1 sulforaphane:placebo ratio. The medication or placebo is subsequently started. Enough study medication/placebo will be given until the next scheduled visit plus 1 week.

If any subjects are found to have clinical blood/urine test results outside normal limits, we will ask the subject to discontinue study medication. If after 2 weeks the lab values return to within the study’s acceptable limits and the subject wish to remain in the study and the study doctor agrees we will then ask the subjects to resume the study medication. Any adverse effects will be logged on the adverse effects form.

If the lab tests remain abnormal the subject will discontinue study medication permanently (though will continue to be followed-up in the study).

Follow up visits with the study doctor will be at weeks 4, 10, 18 and 22 (please see outline below). In each week the study doctor will perform a screen and ask questions to see if there are any adverse effects. In each of these weeks the study doctor will perform the CGI, the ABC and the SRS.

In weeks 4 and 10 additional study medication/placebo will be dispensed.

At weeks 4 and 18 Examination by study doctor and query for adverse effects will be done and phlebotomy may be done for test of liver, thyroid and kidney function,

Study medication/placebo will be completed in week 18 and some of the blood draw may be used to see if there are changes in biomarker levels.

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

The final visit will be in week 22. Examination for adverse effects and phlebotomy will be done for test of liver, thyroid and kidney function, Blood may be drawn for testing of glutathione levels.

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APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Schedule of Study Evaluations:

Time Line	Informed consent /assent	Diagnosis, ADOS DSM	Study medication	Tests of autistic disorder symptoms	Physical exam and possibly clinical blood/urine tests	Screening / questioning for any adverse effects	Sample for biomarke analysis
Screening /Enrollment visit:	Y	Y	N/A	N/A	N/A	N/A	N/A
Baseline visit:	N/A	N/A	y	CGI, ABC and SRS.	y	N/A	y
4 week (follow-up) visit:	N/A	N/A	y	CGI, ABC and SRS	y	y	n
10 week (follow-up) visit:	N/A	N/A	y	CGI, ABC and SRS	n	y	n
18 week (treatment stop) visit:	N/A	N/A	n	CGI, ABC and SRS	y	y	y
22 week (final/closeout) visit:	N/A	N/A	n	CGI, ABC and SRS	y	y	y

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Methods:
Glutathione analysis:
Pre- and post-treatment plasma samples will be analyzed for total glutathione (tGSH) and reduced glutathione (GSH). While there are multiple commercially available kits available for these assays a general problem is lower sensitivity and susceptibility to interference from contaminants. More recently HPCL and LC-MSMS techniques have been developed. The latter are preferable because they are not subject to contamination, are very specific and are sensitive. Therefore we will do the analyses for GSH and GSSG by LC-MSMS for this study. Dr. Stein will use the method of Squellerio et al (Squellerio, Caruso et al. 2012). We have the necessary equipment and do isotope dilution assays of biomolecules routinely in our laboratory. These assays will be completed by Dr. Peter Stein at Rowan – SOM in when the last samle is collected. All samples will be stored in SRB until all subjects have completed the study. Samples will then be sent to Rowan for analysis as a single batch to avoid possible confounding factors. Due to a Rowan requirement that we must have an IRB approval before he can apply for an approval at Rowan neither he nor Rowan are listed as part of this project in the initial application. Once we receive IRB approval we will give a copy of the approval letter to Rowan for their IRB review. Once approved or determined to be exempt / non-human research we will amend our protocol to add Dr. Stein and Rowan to our protocol.

Genotyping:
DNA will be extracted at pre-treatment using established protocols. Genotyping of SNPs of alleles of genes of sulforaphane metabolism will be done by TaqMan assays as per the manufacturer's protocol. Genotyping of the *GSTM1*- and *GSTT1*-null alleles (whole gene deletions) will be done by a quantitative method to account for all three genotypes as opposed to standard methods that only account for null and not null. We will use qPCR TaqMan assays that we have previously used.

3.1. Duration of Study
This is a project. Each subject will be randomized into an 18-week double-treatment study with a closeout visit at 22 weeks.

3.2 Study Sites

Steven G. Busyke PhD. . ***PI / Principal Investigator and study sponsor.***
Rutgers University
Associate Research Professor of Statistics
110 Frelinghuysen Road Room 559 Hill Center Bldg
Department of Statistics
Piscataway New Jersey, 08854

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Study Coordinator – Data Steward: Edward S. Stenroos
Department of Neurology
Rutgers-RWJMS
675 Hoes Lane The Staged Research Building Room 107.
Piscataway, NJ 08854
Subject diagnosis will be done at;
Rutgers-RWJMS
675 Hoes Lane The Staged Research Building Room 115.
Piscataway, NJ 08854

The study doctor will see the subjects at
Rutgers RWJMS Department of Neurology
Clinical Academic Building
125 Paterson Street, Suite 6100 6th FL
New Brunswick, NJ 08901

We will use the CRC pharmacy and labs for phlebotomy ;
RWJ East Tower, 8th floor
125 Paterson St., New Brunswick, NJ 08901
New Brunswick, NJ 08901

Biomarker testing will be done at Rowan-SOM by T. Peter Stein PhD Professor of Surgery.
Please note, biomarker testing will not be done until the last sample is collected. Since Rowan University will not review Dr. Stein's IRB application until Rutgers has approved the IRB protocol Dr. Stein will not initially be part of the project with respect to this IRB application. Once we have received an approval letter we will send a copy to Dr. Stein for his IRB application at Rowan. Once approved we will amend our protocol to add Dr. Stein and Rowan to the project. Text referring to Dr. Stein has been left in for clarity of study design and methods.

2 Medical Center Drive Science Center 1-106
Stratford Camden New Jersey, 08084
Department of Surgery
Rowan - School of Osteopathic Medicine

3.3 Sample Size Justification
Forty child, adolescent or adult outpatients with autistic disorder will be randomized. This project is designed to confirm a previous study (Singh, Connors et al. 2014) with the goal of justifying a large scale clinical trial. This number is therefore based on both the design of the Singh study and cost constraints of this award.

Power Analysis:

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Based on the observed standard deviations for change observed in Singh et al (2014), the designed sample size with 10% attrition, and two-tailed tests with α = 0.05 as described above, we estimate that with a simple t-test the proposed study would have 80% power to detect differences in change scores as small as 20.4 and 20.5 in ABC or SRS, respectively. The published standard errors of the mean in Singh et al (2014), however, from which the standard deviations were derived, were not derived in the mixed effects model; the paper indicates that model-derived estimates would be smaller, suggesting power to detect small differences in change scores. The observed, unadjusted change scores in Singh et al (2014) were 19.4 for ABC and 18.4 for SRS.

3.4 Subject Selection

The inclusion and exclusion criteria of this study are dictated by the previously reported study of Sing et al (Singh, Connors et al. 2014). Additional variables are avoided with the hopes that replication will result in a large scale trial with a broad inclusion including females and individuals with autistic disorder of younger ages and possibly those with a diagnosis of autism spectrum disorder other than autism.

3.4.1 Inclusion Criteria

- Autistic disorder diagnosis.
- Age between 13-30 years.
- Male gender.

3.4.2 Exclusion Criteria

- Absence of a parent or legal guardian and consent/assent,
- Those that can not or will not complete all visits and adherence to study regimen.
- Seizure within 2 years of screening,
- History of chronic kidney, liver or thyroid disease.
- Impaired renal function (serum creatinine> 1.2 mg/dl).
- Impaired hepatic function (> 2x upper limit of normal).
- Impaired thyroid function (TSH outside normal limits).
- Current infection or treatment with antibiotics.
- Chronic medical disorder (e.g., cardiovascular disease, stroke or diabetes) or major surgery within 3 months prior to enrollment.
- Less than 13 years or more than 30 years of age.
- Female gender.
- A diagnosis of autism spectrum disorder other than autistic disorder, for example, Asperger, PDD-NOS etc.

4. Study Variables

4.1 Independent Variables or Interventions

This is a double blind placebo-controlled treatment trial in autistic disorder.

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

4.1.1 Drug or Device Interventions

Dose Rational:

Broccoli sprout extracts have previously been used as a delivery agent for the isothiocyanate sulforaphane and its precursor glucoraphanin, in clinical studies conducted in the US and China (Shapiro, Fahey et al. 2006, Fahey, Talalay et al. 2012, Fahey, Wehage et al. 2012, Egner, Chen et al. 2014, Singh, Connors et al. 2014). Mean bioavailability of glucoraphanin preparations lacking active myrosinase is roughly 10% of dose, whereas when active myrosinase is included in the dose bioavailability increases to about 50%, with a concomitant reduction in within- and between-subject variability(Shapiro, Fahey et al. 2006, Fahey and Kensler 2007, Fahey, Talalay et al. 2012, Fahey, Wehage et al. 2012) }. Using glucoraphanin rich broccoli seed powder containing active myrosinase will allow us to deliver sulforaphane in predictable doses in a delivery vehicle that is highly stable and thus unlikely to lose potency over time. Nutramax Laboratories, Inc. has had a broccoli extract supplement on the market that has been tested by independent labs and has been found to be of high quality. They have now manufactured a version of their supplement, modified to the studies specifications, (Avmacol®) and it will be send to us accompanied by appropriate placebo capsules, to be used in clinical trials. This supplement will be provided to us in the form of tablets that will contain a mixture of broccoli seed powder with 30 mg of glucoraphanin (equivalent to 68 µmoles of glucoraphanin) and enough active myrosinase enzyme to fully hydrolyse glucoraphanin to sulforaphane. About 50% of ingested glucoraphanin is recovered as sulforaphane metabolites in 24 hour urine collections(Ye, Dinkova-Kostova et al. 2002). Nutramax will send Avmacol® and the placebo to the CRC pharmacy in bulk (500 pill jars) once IRB approval has been obtained. All pills will be from a single batch. Avmacol® is stable at room temperature for up to two years and does not need special consideration. Once opened, pills will be kept in a refrigerator to avoid potential loss of sulforaphane bioavailability. If expiration of the study medication occurs, Nutramax will send a second batch.

FDA:

We have prepared and submitted an IND application to the FDA for use of Avmacol® in autism. We received a “Study May Proceed “ letter from the FDA. IND 127543.

Dose:

We will use the same dose considerations as the Singh study which is based on 100umol/day for average weight subject (set at 68kg / 150lbs). For this study we will administer 1.47umol/kg/day.

Each pill has ~ 37umol GR/ tablet which produces ~14.8umol bioavailable sulforaphane/ tablet (based on an observed 40% conversion efficiency).

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Therefore;

Body weight	Body weight	Dose of sulforaphane	
45 kg	100 lb.	~ 50 µmol	3
68 kg	150 lb.	~ 100 µmol	6
102 kg	225 lb.	~ 150 µmol	9

Pill grinders will be supplied for those that have problems taking pills. The ground up pills can be added to food.

4.2 Dependent Variables or Outcome Measures

We will test for phenotypic change by the Clinical Global Impression (CGI), Aberrant Behavioral Checklist (ABC) and by the Social Responsiveness Scale (SRS) at baseline, week 4, week 10, week 18 and at a 22 week close out visit to assess phenotypic change in male individuals with autistic disorder receiving daily supplementation with Avmacol® .

Kaufman Brief Intelligence Test (KBIT):

All potential subjects will undergo the Kaufman Brief Intelligence Test (KBIT) to determine ability to undergo informed consent/assent. An IQ score greater than 2 SD from mean (IQ score < 70) will be used as a cutoff point. This test will take 15 – 30 minutes.

Diagnosis:

We will include male subjects with moderate to severe autistic disorder using the same two diagnostic criteria used in the Singh study, The Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV), and The Autism Diagnostic Observation Schedule 2(ADOS-2). We will also score the more up to date tool DSM-5 for possible additional analysis after the study is complete.

Drs. Audrey Mars or Brenda Patel will assess phenotype of subjects with ADOS and DSM-IV and score DSM-5. Due to the inclusion criteria it is not likely that many potential subjects will have been recently diagnosed for other studies and will therefore need to be re-evaluated. If subjects have been recently diagnosed we may review these test results/records with the goal of relieving the burden on subjects and families. Drs. Mars and Patel will do so if they feel it is appropriate. Administration time is approximately 45 minutes.

Outcome Measures:

Outcome measures will be performed by Drs. Traeger and Nallapati.
As in the Singh study the primary tests metrics will be the Aberrant Behavior Checklist (ABC) and Social Responsiveness Scale (SRS). A secondary metric will be the Clinical Global Impression Improvement Scale (CGI-I).

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Administration time is approximately 45 minutes.

These tests are standard, proprietary tools or measures and are included in Appendix A., Some of these tests contain internal scoring (e.g. carbon paper) and were copied for review and are watermarked as “Sample”.

We will test for differences in the average change in ABC and SRS scores from baseline to 18 week, and their reversion to baseline at 22 week between those receiving sulforaphane and placebo. As a secondary test we will look at the change in the CGI-I scores from baseline to each follow-up time point for each participant.
Administration time is approximately 45 minutes.

Testing for possible treatment variability:
Biomarker: As previously mentioned we will be testing for differences in pre- and post-treatment glutathione levels in plasma samples and will measured for total glutathione (tGSH) and reduced glutathione (GSH).

Genetic data: DNA will be extracted from the pre-treatment sample. Genotyping of SNPs and InDel alleles of genes of sulforaphane metabolism will be done genotyped.

4.3 *Chart Review Selection*
Not applicable.

4.3.1 Electronic Medical Records
Not applicable.

4.4 Risks of Harm
One risk to subjects is the potential side effects of sulforaphane. Sulforaphane is derived from eating cruciferous vegetables. The form used in this study is well tolerated and has shown no toxicity at the doses to be used in this study. We will carefully monitor subjects for adverse effects or clinical laboratory results outside of range.

Possible clinical side effects of sulforaphane include: flatulence, weight gain, GI disturbances (large bowel movement, soft stool), and increased urination. There was no higher prevalence of these side effects in the treatment arm vs. placebo arm in the Singh study with the exception of weight gain (4.31 vs. 0.31, 9 = 0.056 (Singh, Connors et al. 2014).

These possible clinical side effects are relatively minor when compared to the possible improvement of core symptoms of autism.

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

As with all studies there is a small risk of the loss of confidentiality. All efforts will be made to keep personal information and research records confidential, but total confidentiality cannot be guaranteed. All data including screening data, physical results, blood test results, phenotypic data and test results will be securely sent to or brought to the Data manager/study coordinator. Data will be stored in both electronic files and hard copy source documents. The source documents will be coded and will be stored in locked file cabinets in a locked room. Any electronic data will be stored on password protected computers on a secure network within the University and not accessible by outside individuals.

Sample collection tubes will not contain PHI. Each collection tube will be labeled with the subject's unique ID number. Data analysis and reporting will be done with the Unique ID number only.

These risks are relatively minor when compared to the possible improvement of core symptoms of autism.

4.5 Potential for Benefit

Confirmation that sulforaphane treatment may influence social interaction, abnormal behavior, and verbal communication in male individuals with autistic disorder without serious side effects would be a major step in helping families and individuals with autistic disorder and may benefit the subject directly. Follow up studies will be done that may benefit females with autism as well younger individuals with autism. However, it is possible that subjects might receive no direct personal benefit from taking part in this study.

5. Subject Recruitment and Enrollment Considerations

5.1 Subject Recruitment

We will attempt to give as many potential subjects the opportunity to enter this study as possible. Only IRB-approved recruitment tools will be used. As previously mentioned, we expect that few subjects will be verbal. We therefore expect that recruitment will be through parents or legal guardians. Those subjects/parents/guardians that learn of our study and express interest will be contacted at their best convenience. Those that wish to enter the study will be scheduled for informed consent/assent. No information will be kept for those that decide they are not interested. Those that may be interested in future studies will be informed of the NJ ACE website which may help inform them of such studies.

Study patients will be recruited in several ways including local pediatric and pediatric neurology clinics, reference from other physicians, postings of study, invitation to those from previous studies that expressed a wish to be notified about future studies. Recruitment from local clinics, including pediatric, neurological and psychiatric. We will reach out to schools for individuals with

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

autism and other community groups serving the autism spectrum community as well. We will also use local advertising if necessary. In addition, we will have the benefits of the NJ ACE coordinating center at Montclair State University. We expect the coordinating center's expertise and experience will be a significant asset in recruitment and completing this project. Based on previous experience we expect to need to recruit about 70-100 subjects to have at least 40 subjects that pass inclusion/exclusion criteria, screening and complete the study. Additional recruitment tools will be sought out throughout the study and added if appropriate.

Recruitment will be done by the study coordinator.

5.2 Consent Procedures

Subjects will be between 13 and 30 years old. . Determination of capacity to undergo informed consent/assent is crucial. All potential subjects, not clearly verbal will undergo the Kaufman Brief Intelligence Test (KBIT) to determine ability to undergo informed consent/assent.

Any subjects that are clearly verbal will undergo informed consent if 18 or older and informed assent if between 13 and 17 years old prior to KBIT and diagnostic testing. Those that assent and turn 18 during the study will undergo informed consent at the next study visit.

All subjects determined to be verbal and can understand and have the capacity will undergo informed consent/assent.

Those that are not verbal and under 18 will be consented for by the parent / legal guardian.

Those that are not verbal and over 18 and their parent / guardian has/have been granted legal guardianship will be consented for by the parent / legal guardian.

Those that are not verbal and over 18 and their parent / guardian has/have not yet been granted legal guardianship will be consented for using the surrogate consent process. An attending physician that is not part of/related to this project will assess ability to consent. A person, with no connection to this study will witness the surrogate consent process and sign the Surrogate Consent form. The person will be the chaplain at RWJ or someone identified prior to the need for consent that can attest that the requirements for informed consent to enter this study have been satisfied. If a subject expresses resistance or dissent they will be removed from the study. The subject will undergo informed consent if they become verbal and gain the ability to consent for themselves during the study.

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Subjects will be assessed for improvement at each study visit. Any non-verbal subjects over 18 that become verbal during treatment will undergo the Kaufman Brief Intelligence Test (KBIT) to assess ability to undergo informed consent. Those determined to have sufficient improvement and gain the capacity to undergo informed consent and that are literate will undergo informed consent.

Those that have been consented for and turn 18 during the study and have gained the capacity to understand and undergo informed consent as described in the previous paragraph will undergo informed consent at the current or next scheduled visit.

Any subjects that gain the capacity to undergo informed consent that dissent to participation in the study will be removed from the study as soon as safe termination can be affected.

The consent/assent will be made available to the subject/family by mail prior to the first appointment if desired, or at the time of the first appointment.

Each subject and a parent / legal guardian will read through the consent/assent with the study member and have a dialogue about the study procedures, risks and potential benefits. Time will be provided, and if necessary, another appointment will be scheduled if a potential subject/parent/legal guardian requires further time for their decision or would like consultation with another individual prior to signing the consent/assent. All subject/parent/legal guardians will be reminded prior to signing a consent/assent that the study is voluntary, and they have the right to withdraw from the study at any time for any reason.

All individuals providing consent/assent must be literate. Consenting/assenting will be done in a private location, preferably in the Staged Research Building room (SRB)115. Alternatives more convenient for the subject/family can be used as long as they are private. Consenting/assenting will be done by the Study Coordinator. When the study coordinator is not available/able to, the diagnostician or study doctor will perform informed consent/assent of the subjects.

Withdrawal: Subjects/parents/legal guardians that wish to withdraw from the study will be removed as soon as a safe termination can be affected. Any that wish to have data already collected destroyed/deleted must request this in writing. NDAR will be informed by the PI to delete the subject's data. Any data already submitted to other PIs by NDAR will not be retracted as per NDAR policy. Loss of confidentiality is not likely since no link will be present.

5.3 Subject Costs and Compensation

There is no cost to participate in the study other than travel expenses. There is a small reimbursement for travel to and from the research site. Each subject will

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

receive $5 for each scheduled visit. All study costs (e.g. study medication, tests, etc.) are covered by the grant from The Governor's Council for Medical Research and Treatment of Autism of New Jersey.

6. ***Data Handling***

NDAR GUID (National Database for Autism Research Global Unique Identifier Web Service, from the National Institutes of Health) will be used to generate ID numbers. Only GUID numbers will be used on research documentation and tests from this point forward. Only the screening questionnaire page 2, the Study Visit form and the NDAR GUID from will link subject's names to IDs. All other documents will contain only GUID numbers. These will be stored in locked file cabinet separate from other study information. Only GUIDs will be used for research related electronic data. The NDAR GUID helps to ensure that this information can be sent and stored securely, protecting the privacy of the individuals in the study and the confidentiality of their data. Using the NDAR GUID allows data collected on an individual from one study or site to be associated the data from another study or site without the risk of exposing PHI. The identifying information used to generate the ID is encrypted. This unique ID number will be used to identify all samples, data and results. The Study Visit form will be kept by the study doctor in a locked cabinet/desk, in a secure office until the subjects has finished the study. The Study Visit form will then be destroyed.

Information needed to generate the NDAR GUID, including name as it appears on birth certificate, birthday, sex and town or municipality of birth as on the birth certificate will be kept on the "NDAR GUID form" and stored separately and securely from all other study documents in a locked file cabinet in room 107 of SRB. This form/data/link will be kept until data has successfully been uploaded to NDAR and then it will be destroyed. For those that do not wish their data to be uploaded to NDAR destruction of this form//data/link will occur immediately after the GUID has been generated successfully.

Only data from those that indicate on the consent/assent form that their data may be sent to NDAR will be uploaded to NDAR.

All other forms, including screening questioners, consent/assent forms and diagnostic material will be kept in a locked file cabinet in room 107 of SRB. Only the study coordinator and PI will have access to these documents. Clinical documents and phenotypic tests will be brought to SRB room 107 the day of the visit when possible or stored in the CRC until the study coordinator can bring to SRB room 107 and placed in the locked cabinets. Dr. Stein will receive sample with NDAR GUID's only. No PHI will be placed on sample tubes. Dr. Buyske the study statistician will not receive PHI. Study data including glutathione levels and genotypes will be stored in the project database which will be specific to this project and will not contain identifying information. The database will be password-

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

protected on a password-protected computer in room 107. We have storage space on a University secured shared drive (Core) for storage. Subject and regulatory data will be kept for a minimum of 6 years. The link to identifying information used to generate the NDAR GUID will be destroyed as described above. Following this only the screening questionnaire and the Study Visit form will contain the link between name and GUID. Name and contact information will be part of the subject records and will be kept for a minimum of 6 years. Screening questionnaires for those that do not enter the study will be destroyed.

7. Statistical Analysis

Our statistical methods mirror those of the original Singh study. There is an unfortunate tendency, known as "researcher degrees of freedom" or (less generously) "p-hacking" by which investigators, perhaps subconsciously, make analysis decisions that favor significant results. This process has been proposed as a partial mechanism for the phenomenon of studies that do not replicate.

Since the proposed study is a replication of an existing study, we plan on a statistical analysis that fully matches the analysis in the original study. In particular, for Aim 1, we will use as our primary outcome the change in Aberrant Behavior Checklist (ABC) and Social Responsiveness Scale (SRS) scores from baseline to 18 weeks and the change from 18 to 22 weeks. Changes in Clinical Global Impression Improvement Scale (CGI-I) scores, as well as changes in all scores from baseline to other time points, will be treated as secondary analyses. We will use a mixed effects general linear model, with fixed effects for both week and the interaction of post randomization week and treatment group and random effects for intercept and slope of participants. Unstructured covariance will be used. As shown in (Liang KY 2000), the shared baseline implied by the lack of a main effect for treatment corresponds to the undifferentiated (with respect to treatment) population before randomization and reduces variance much as adjusting for baseline does in ANCOVA. The mixed effects model allows for all participants with at least one post randomization visit to be included in the analysis. An intent-to-treat analysis will be done to supplement the per-protocol analysis.

Test statistics will be based on contrasts corresponding to differences between treatments in the baseline-to-visit scores, except for the test of behavior regression, which will be based on a contrast from week 18 to week 22. We will use two-tailed tests with alpha = 0.05, both to match the original study and to follow the usual practice of a study replicating an established result.

Power Analysis:

We plan on recruiting sufficient amount of subjects to have 40 participants finish the trial. That said based on the same power calculation assumptions in Singh et al. (2014), the designed sample size with 40 participants, 10% attrition, and two-tailed tests with alpha = 0.05 as described above, we estimate that with a simple t-test the proposed study would have 80% power to detect differences in change scores as small as 16.3 for ABC or SRS. The more refined mixed effects model should have even greater power (and so the ability to detect significance for smaller change score differences). For

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

context, the observed change scores in Singh et al. (2014) were 19.4 for ABC and 18.4 for SRS.

8. Data and Safety Monitoring

While this study does not put the subjects at greater than minimal risk we will assemble a Data and Safety Monitoring Board.

The Data and Safety Monitoring Board will be composed of no less than three persons with one being a physician (MD) familiar with treatment of autism and a statistician, none with any affiliation to the study. The monitoring board shall determine safe and effective conduct and recommend conclusion of a trial if significant risks develop or the trial is unlikely to be concluded successfully. The DSMB will conduct an open session prior to the initiation of the trial. The DSMB will have been provided all material (protocol, consents, and procedures) associated with the trial, and have had sufficient opportunity to review the documents.

Specifically, the Data and Safety Monitoring Board shall be responsible for:

- Reviewing the research protocol and plans for data and safety monitoring.
- Monitoring that will take place on a regular basis: every quarter.
- Evaluating the progress of the trial, including periodic assessments of data quality and timeliness, participant recruitment, accrual and retention, participant risk versus benefit, and other factors that may affect study outcome. Monitoring may also consider factors external to the study when interpreting the data, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the study.
- Inquiring for further information as necessary to accomplish their mission.
- Maintaining confidentiality during all phases of the trial including the monitoring, preparation of interim results, review and response to monitoring recommendation.
- Generating a report that will be provided to the PI and the IRB.

The Principal Investigator of the trial, Steve Buyske, shall be responsible for:

- Evaluation of any members of the DSMB for a conflict of interest or financial stake in the outcome of the trial.
- Delegation of the ongoing monitoring of the trial to the DSMB.
- Ensuring that monitoring is timely and effective and the DSMB is composed of individuals with appropriate expertise to accomplish their tasks.
- Overseeing the monitoring activities.
- Responding to and with the study doctor, addressing recommendations that result from monitoring activities.
- Provision of adverse event reports and other safety data to the DSMB, as well as any changes in the trial or annual reports to the IRB and DOD.
- Contributing to the report that the DSMB generates, which will be distributed to the IRB.
- Submitting the DSMB report to the IRB.

RUTGERS | eIRB
APPROVED
IRB ID: Pro20150001884
Approval Date: 7/25/2023
Expiration Date: 7/18/2024

Protocol deviations:
Protocol deviations will be reported to the IRB, the DSMB as well as the funding institutions as required.

Withdrawal from the Protocol:
All the individuals and parents/guardians will be reminded at the time they sign consent/assent that the study is voluntary and they have the right to withdraw from the study at any time for any reason. If they wish for samples or data to be withdrawn we will request they inform us of their decision, in writing. NDAR will be informed by the PI to delete the subject's data. Any data already submitted to other PIs by NDAR will not be retracted as per NDAR policy. Loss of confidentiality is not likely since no link will be present.

9. Reporting Results

9.1 Individual Results
The study physician will report abnormal clinical test results received from the CRC with the subject/parents/guardian as soon as received and they will be asked to stop taking the study medicine/placebo immediately.

9.2 Aggregate Results
Results of the study will be made available for individuals in the study once results have been analyzed and published. Individuals will not be identified in any of the study publications. Details about study medication and response will be made available to those subjects/families to those that request after data has been analyzed.

9.3 Professional Reporting
Results of this study will be published in peer review journals. Presentations will be made at appropriate seminars and conferences including appropriate conferences held by the Coordinating Center of Montclair State University (CC-MSU) – NJ Autism Center of Excellence. No identifying information will be used in publications.

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RUTGERS | eIRB
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RUTGERS | eIRB
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RUTGERS | eIRB
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