Sulforaphane treatment of autism spectrum disorder (ASD)

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Autism spectrum disorder (ASD), characterized by both impaired communication and social interaction, and by stereotypic behavior, affects about 1 in 68, predominantly males. The medico-economic burdens of ASD are enormous, and no recognized treatment targets the core features of ASD. In a placebo-controlled, double-blind, randomized trial, young men (aged 13–27) with moderate to severe ASD received the phytochemical sulforaphane (n = 29)—derived from broccoli sprout extracts—or indistinguishable placebo (n = 15). The effects on behavior of daily oral doses of sulforaphane (50–150 μmol) for 18 wk, followed by 4 wk without treatment, were quantified by three widely accepted behavioral measures completed by parents/caregivers and physicians: the Aberrant Behavior Checklist (ABC), Social Responsiveness Scale (SRS), and Clinical Global Impression Improvement Scale (CGI-I). Initial scores for ABC and SRS were closely matched for participants assigned to placebo and sulforaphane. After 18 wk, participants receiving placebo experienced minimal change (<3.3%), whereas those receiving sulforaphane showed substantial declines (improvement of behavior): 34% for ABC ($P < 0.001$, comparing treatments) and 17% for SRS scores ($P = 0.017$). On CGI-I, a significantly greater number of participants receiving sulforaphane had improvement in social interaction, abnormal behavior, and verbal communication (P = 0.015–0.007). Upon discontinuation of sulforaphane, total scores on all scales rose toward pretreatment levels. Dietary sulforaphane, of recognized low toxicity, was selected for its capacity to reverse abnormalities that have been associated with ASD, including oxidative stress and lower antioxidant capacity, depressed glutathione synthesis, reduced mitochondrial function and oxidative phosphorylation, increased lipid peroxidation, and neuroinflammmation.

Autism spectrum disorder (ASD) includes neurodevelopmental abnormalities characterized by impaired ability to communicate and interact socially and by restricted and repetitive patterns of behavior, interests, and activities (1). The prevalence of ASD in the United States is about 1 in 68 among children aged 8 y, with marked male (4.5:1) preponderance (2). No validated pharmacological treatments for the core symptoms of ASD are available. We report here that in a placebo-controlled, double-blind, randomized clinical trial, daily treatment with sulforaphane for 4–18 wk resulted in significant improvements in aberrant behavior and social impairment in a majority of young males diagnosed with moderate to severe autism, and that this improvement regressed upon cessation of treatment. Physician and parent/caregiver impressions of clinical improvement were evaluated by behavioral outcome measures.

Sulforaphane is an isothiocyanate derived from broccoli. Its therapeutic potential is based on its potent activity in transcriptionally up-regulating genes that control mechanisms whereby aerobic cells protect themselves against oxidative stress, inflammation, DNA-damaging electrophiles, and radiation (3, 4). Under basal conditions, these protective systems do not operate at maximal capacity but can be induced to higher activity levels by sulforaphane, thus reducing the risks of developing malignancies and other chronic diseases (5–10). Sulforaphane is now in widespread clinical evaluation (10).

The decision to test sulforaphane to treat ASD was based on four premises. First, extensive evidence shows that sulforaphane counteracts many of the same biochemical and molecular abnormalities associated with ASD, including oxidative stress and reduced antioxidant capacity, defects in glutathione synthesis, mitochondrial dysfunction and low oxidative phosphorylation, increased lipid peroxidation, and neuroinflammation (11–16). Although it is unclear whether these anomalies are etiological or secondary manifestations, their correction often improves ASD behavior (17).

Second, a variety of small molecules including sulforaphane can ameliorate a number of unrelated genetic disorders by activating the "stress proteome," which regulates many of the aforementioned damaging processes. Sulforaphane, as well as hydroxyurea,

Significance

Autism spectrum disorder (ASD), encompassing impaired communication and social interaction, and repetitive stereotypic behavior and language, affects 1–2% of predominantly male individuals and is an enormous medical and economic problem for which there is no documented, mechanism-based treatment. In a placebo-controlled, randomized, double-blind clinical trial, daily oral administration for 18 wk of the phytochemical sulforaphane (derived from broccoli sprouts) to 29 young men with ASD substantially (and reversibly) improved behavior compared with 15 placebo recipients. Behavior was quantified by both parents/caregivers and physicians by three widely accepted measures. Sulforaphane, which showed negligible toxicity, was selected because it upregulates genes that protect aerobic cells against oxidative stress, inflammation, and DNA-damage, all of which are prominent and possibly mechanistic characteristics of ASD.

Author contributions: K.D.S., P.T., and A.W.Z. designed research; K.S., S.L.C., and A.W.Z. performed research; J.W.F. and P.T. contributed new reagents/analytic tools; K.S., E.A.M., J.W.F., P.T., and A.W.Z. analyzed data; K.S., K.D.S., J.W.F., P.T., and A.W.Z. wrote the paper; and J.W.F. and P.T. supplied sulforaphane-rich broccoli sprout extract.

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Conflict of interest statement: U.S. patent applications have been filed by The Johns Hopkins University (inventors K.D.S., P.T., and A.W.Z.). P.T. and A.W.Z. have divested themselves from all potential financial benefits. The sulforaphane-rich broccoli sprout extract is not a commercial product. Broccoli sprouts and seeds rich in glucosinolates have been licensed by Johns Hopkins to Brassica Protection Products LLC (A. Talalay, son of P.T., is chief executive officer).

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phenylbutyrate, and trichostatin A, have been shown in vitro to have therapeutic potential to reestablish cellular homeostasis in a number of unrelated genetic disorders (18).

Third, sulforaphane is a dietary phytochemical, derived from its precursor glucosinolate glucoraphanin, that is widely consumed in cruciferous plant-rich diets, and qualifies for consideration as a food, a dietary supplement, or a drug, depending on its intended use. Sulforaphane is therefore justifiably considered to be of low toxicity, and its administration to humans is well tolerated (10, 19, 20).

Fourth, widespread anecdotal reports have suggested that fever can dramatically but temporarily ameliorate the disturbed behavior of many autistic patients (21). Notably, the degree of improvement (mostly in stereotypic behavior and inappropriate speech) was unrelated to the severity of fever or of autism (21). This study explicitly suggested that elucidation of the fever response might provide insight into the mechanisms of ASD and point to new therapeutic approaches (21, 22). Fever up-regulates heat-shock proteins and related mechanisms central to multiple cellular processes in the CNS, including synaptic transmission (23, 24), and may improve long-range cerebral cortical connectivity that is depressed in ASD (25). Sulforaphane also up-regulates expression of the heat-shock response (26).

We hypothesized that daily treatment with sulforaphane at levels achieved by diet might reduce the severity of socially impaired behavior in ASD. Behavior was quantified directly by three widely validated behavioral outcome measures at the periods before, during, and after intervention (Fig. 1). Parents/caregivers completed the Aberrant Behavior Checklist (ABC) (27) and the Social Responsiveness Scale (SRS) (28). Study physicians completed the Clinical Global Impression Severity (CGI-S) and the Clinical Global Impression Improvement (CGI-I) scales (29, 30).

Results

Participant Characteristics. More than 90% of all scheduled tests were completed on the 40 participants who received placebo or sulforaphane treatment and returned for the first return visit (week 4). Twenty-two participants (6 placebo, 16 sulforaphane) were also tested at 22 wk, 4 wk, after treatment ended (Table 1 and Fig. S1). Four participants (one placebo, three sulforaphane) were lost to follow-up before their first on-treatment visit.

Participants, all male, were 13–27 y old at enrollment (median: 17 y). A history of behavioral improvements with fever was given by a large majority (32 of 40; 80%) of participants. Participants in sulforaphane and placebo groups were well matched, and did not differ at baseline with respect to various demographic, behavioral and clinical features, behavioral outcome score measures, abnormalities in physical examination, blood chemistries, hematology, and urinalysis (Table S1).

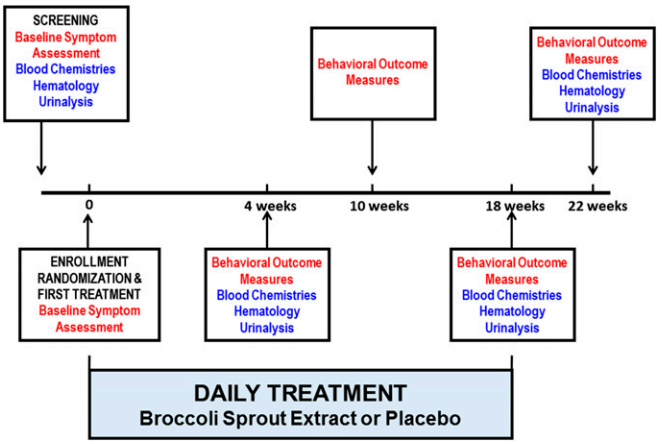

Fig. 1. Schedule for study of the effects of sulforaphane in ASD.

Analysis of Outcome Measures. The total and the changes in total ABC and SRS behavioral scores of the 26 sulforaphane-treated and 14 placebo recipients from enrollment to the 18-wk end of treatment and after a 4-wk recovery period are shown in Figs. 2–4 and Tables 1 and 2. Treatment group mean ABC scores differed significantly at 4, 10, and 18 wk (Fig. 2 *B* and *E* for ABC and SRS, respectively). At 18 wk there was a 34% reduction in ABC and a 17% reduction in SRS scores, and these trended toward nonsignificant differences upon cessation of treatment (Fig. 2 *B* and *E* and Tables 1 and 2).

Significantly greater improvement was observed among participants randomized to sulforaphane at 4, 10, and 18 wk for irritability, lethargy, stereotypy, and hyperactivity subscales of the ABC, and in awareness, communication, motivation, and mannerism subscales of SRS (Fig. 3 and Tables 1 and 2). After stopping sulforaphane treatment, both ABC and SRS subscores tended to revert toward baseline.

On subscale analysis of CGI-I scale scores at 18 wk (Tables 1 and 2), 46% (12 of 26), 54% (14 of 26), and 42% (11 of 26) of sulforaphane recipients were much or very-much improved on social interaction, aberrant behavior, and verbal communication, respectively, compared with 0% (0 of 11; $P = 0.007$), 9% (1 of 11; $P = 0.014$), and 0% (0 of 11; $P = 0.015$), respectively, for placebo recipients.

Individual changes in total ABC and SRS scores from basal levels to 18 wk are shown in Fig. 4. A positive response was defined post hoc as a 30% decrease from baseline in total ABC and SRS scores. Thirty-five percent (9 of 26) of participants on sulforaphane had a positive response on SRS compared with 0% (0 of 11) on placebo (Fisher's exact test $P = 0.036$), and 60% (15 of 25) of participants receiving sulforaphane had a positive response on ABC compared with 20% (2 of 10) on placebo ($P = 0.059$).

Our clinical impressions during the study, although blind to group assignment, were that 13 of the 40 participants improved noticeably with respect to sociability and behavior, usually observable by 4 wk; all were receiving sulforaphane. In queries to families and caregivers, before unblinding, 17 of 26 whose sons had taken sulforaphane reported gradual changes within the first month of treatment and correctly surmised their group assignment, whereas the remaining 9 on sulforaphane—and all but 1 of 14 who received placebo—were not improved, and believed that their sons had not received sulforaphane. Positive responses to sulforaphane were spontaneously reported by parents and caretakers, who commented (before disclosure of treatment category) on improved social responsiveness, behavioral compliance, and calmness in the subjects with ASD who were taking the active compound.

Safety and Adverse Events. Sulforaphane treatment effectively improved core aberrant behaviors of ASD, and was safe and well-tolerated (Table S2). Notably, none of the laboratory results were outside normal ranges at any time point (Dataset S1). Unexpectedly, the sulforaphane group gained significantly more weight over the 18-wk period, compared with placebo (4.31 vs. 0.31 lb, $P = 0.056$). Pulse rate was lower in the sulforaphane group both at baseline and during the study. 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% 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 wk on sulforaphane, with an undisclosed history of recent seizures; the other 3 wk after discontinuing treatment and a past (more than 1 y) history of seizures well-controlled with antiepileptic

Table 1. Effect of sulforaphane treatment on Aberrant Behavior Checklist (ABC) and Social Responsiveness Scale (SRS) scores

	Total and changes in mean total scores				
	Time of observations (wk)				
Scale and treatment	0	4	10	18	22
ABC					
Placebo					
Baseline	60.14	59.77	58.85	58.10	57.67
Intervention point	60.14	60.54	62.15	56.10	55.83
Change*	0	0.77 ± 1.84	3.31 ± 3.50	−2.00 ± 4.59	−1.83 ± 6.60
n	14	13	13	10	6
Sulforaphane					
Baseline	62.77	62.77	62.34	63.88	69.16
Intervention point	62.77	50.08	42.73	42.44	58.44
Change*	0	−12.69 ± 4.17	−19.61 ± 5.95	−21.44 ± 4.34	−10.72 ± 5.07
n	26	26	22	25	16
P value (between treatments)†	–	0.035	0.002	<0.001	0.33
SRS					
Placebo					
Baseline	120.21	120.21	118.85	119.55	122.00
Intervention point	120.21	112.43	117.46	117.55	115.33
Change*	0	−7.79 ± 3.09	−1.38 ± 3.72	−2.00 ± 3.46	−6.67 ± 3.82
n	14	14	13	11	6
Sulforaphane					
Baseline	120.15	120.88	118.26	120.96	116.91
Intervention point	120.15	106.12	103.78	100.56	109.88
Change*	0	−14.76 ± 3.79	−14.48 ± 5.72	−20.40 ± 4.54	−7.03 ± 4.20
n	26	25	23	25	16
P value (between treatments)†	–	0.29	0.080	0.017	0.87

ABC and SRS total scores of participants who completed at least one postintervention measurement ($n = 40$). The ABC and SRS scores and changes thereof from baseline are the raw, unadjusted values and the *P* values are from the linear mixed model adjusting for repeated measures.

*Individuals' scores at 4, 10, 18, or 22 wk were subtracted from the same individual's scores at time 0 ("Baseline"); differences were averaged, and are presented as means ± SEM. Because the number of individuals for whom scores were obtained (*n*) at each time period varied, so did the baseline score used to calculate each change.

†*P* values as determined from mixed-effects general linear model.

drugs. Although patients with autism are predisposed to seizures (31, 32), we cannot rule out the possibility of seizures as an adverse effect of sulforaphane in ASD.

Discussion

The behavioral outcome measures (ABC, SRS, CGI-I) and clinical observations by study physicians and many parents/caregivers, all before unmasking, indicated that many of the participants who were treated with sulforaphane in this study had statistically significant and clinically meaningful improvements during treatment with sulforaphane. The substantial improvements of individual ASD patients' trajectories were conspicuous and suggest that further investigation of sulforaphane in ASD is promising.

Although we observed consistent and large improvements in behavior in the majority of sulforaphane-treated ASD, this was a single-site, limited dose-range study of only 44 male, predominantly Caucasians, aged 13–27 y, 4 of whom dropped out of the study before their first follow-up visit. Although we did not test specifically for adaptive or cognitive skills at baseline, our cohort included subjects with moderate and severe ASD with substantial variability in total baseline SRS and ABC scores (Fig. 4 and Tables 1 and 2). Because of considerable heterogeneity in the etiology, pathogenesis, and symptomatology of ASD, generalization of our findings requires confirmation.

Nevertheless, this study may shed light on the basic pathophysiology of at least a subset of ASD. Most clinical studies and medications aim to restrain ASD's troublesome symptoms. In contrast, this study was, to our knowledge, one of the few designed to target core clinical features as well as the fundamental biochemical abnormalities of ASD (oxidative stress and antioxidant deficiency, increased susceptibility to electrophile toxicity, and inflammation) by the administration of sulforaphane.

Our suggestion that participants with ASD whose behavior improved during fever would also respond to sulforaphane could not be confirmed because of the unusually high prevalence of fever responders (80%) in our cohort compared with most ASD populations (35%) (33). Unlike the rapid onset of changes in behavior during fever in ASD, responses to sulforaphane in this study appeared over several weeks. This finding suggests that sulforaphane may cause increases in gene transcription in multiple underperforming cell-signaling pathways (34, 35). Sulforaphane may be only one of several small molecules that will ameliorate deficiencies that lead to abnormal functioning in the whole organism. Further studies of sulforaphane's effects at the cellular level, if confirmed, could guide discovery of new drugs with similar underlying mechanisms of action in ASD.

Activation of the cellular stress response, in addition to its therapeutic potential, is known to protect cells from environmental toxins. Recently, we demonstrated the efficacy of sulforaphane as an environmental detoxicant (36). Together with sulforaphane's capacity to activate the Keap1–Nrf2 cytoprotective signaling pathway (10), it may therefore protect against both environmental and endogenous risk factors that affect brain development in ASD (37). Given its favorable safety profile, future

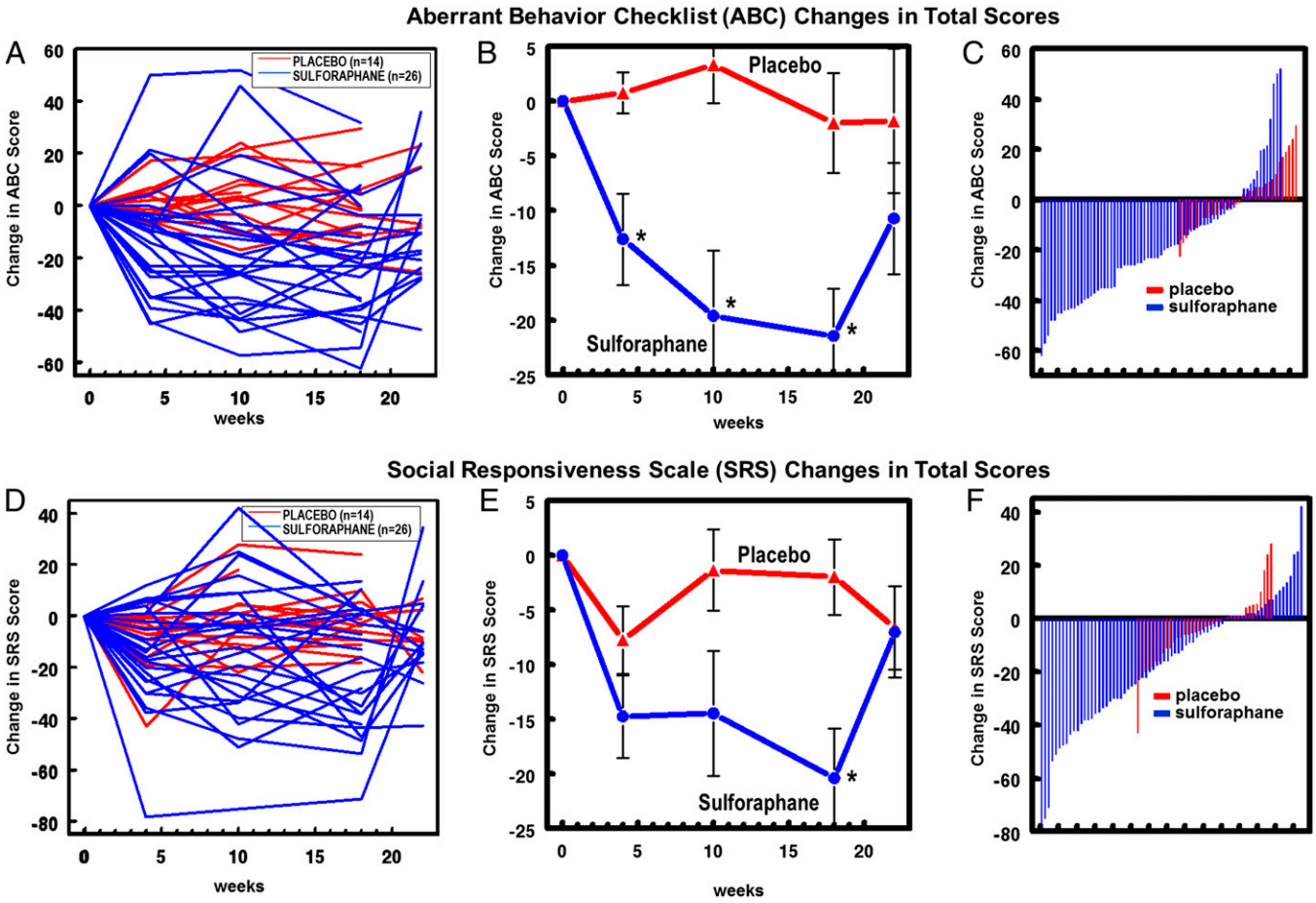

Fig. 2. Changes in total ABC and SRS scores. Forty male ASD participants who were treated daily with either placebo (initially $n = 14$) or sulforaphane (initially $n = 26$) for 4, 10, and 18 wk, followed by a terminal 4-wk untreated period (22 wk). Panels *A* (ABC) and *D* (SRS) show all observations. Means of changes in raw, unadjusted total scores (±SEM) at 4, 10, 18, and 22 wk are shown in *B* for ABC and *E* for SRS. Reductions in ABC score upon sulforaphane treatment were −20.2% ($P = 0.035$), −31.5% ($P = 0.002$), and −33.6% ($P < 0.001$), at 4, 10, and 18 wk, respectively. The corresponding changes in SRS were −12.2% ($P = 0.29$), −12.2% ($P = 0.080$), and −16.8% ($P = 0.017$). Panels *C* (ABC) and *F* (SRS) show the changes in total scores at all timepoints for placebo- and sulforaphane-treated participants. All changes were calculated from the initial values for each individual participant at time 0 (the means of the two values obtained at screening and at enrollment).

studies should address sulforaphane's potential benefits for the prenatal prevention of ASD as well as for the early treatment of young children with this disorder.

Materials and Methods

Study Protocol. This study was conducted at the Lurie Center for Autism of the Massachusetts General Hospital (MGH) for Children with approval of the

Table 2. Clinical Global Impression-Improvement (CGI-I) scores at 18 wk for the 37 subjects for whom scores were available

	Number of subjects' scored as either "much improved" or "very much improved" after 18 wk/total number of subjects (% of total number evaluated)		
Subscore	Placebo	Sulforaphane	*P* for-difference*
Overall level of autism	0/11 (0%)	0/26 (0%)	–
Social interaction	0/11 (0%)	12/26 (46.2%)	0.007
Aberrant/abnormal behavior	1/11 (9.1%)	14/26 (53.8%)	0.014
Repetitive and stereotypical behavior	0/11 (0%)	6/26 (23.1%)	0.15
Verbal communication	0/11 (0%)	11/26 (42.3%)	0.015
Nonverbal communication	1/11 (9.1%)	5/26 (19.2%)	0.65
Hyperactivity and inattention	0/11 (0%)	3/26 (11.5%)	0.54
Anxiety	0/11 (0%)	2/26 (7.7%)	>0.99
Sensory sensitivities	0/11 (0%)	6/26 (23.1%)	0.15
Restricted and narrow interests	0/11 (0%)	0/26 (0%)	–

*By Fisher exact test.

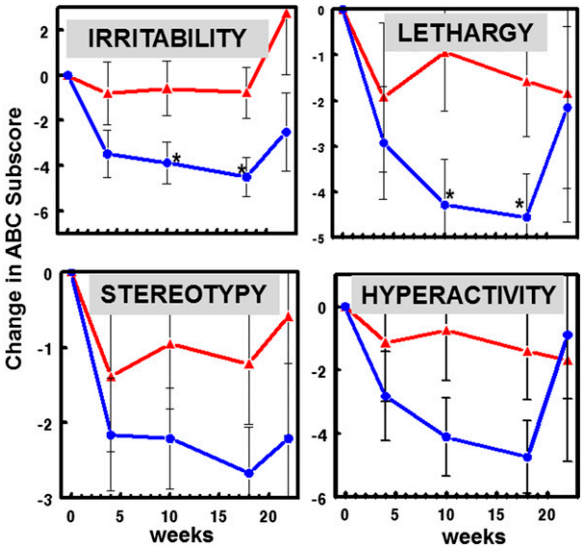

Fig. 3. Changes in ABC subscores for irritability, lethargy, stereotypy, and hyperactivity. After 4, 10, and 18 wk of treatment with sulforaphane or placebo, and a 4-wk untreated recovery period (22 wk). Raw, unadjusted mean values of changes (±SEM) for sulforaphane- and placebo-treated participants are shown. Changes were significant at the 95% confidence level (*) for both irritability and lethargy at 10 and 18 wk of treatment.

MGH and Johns Hopkins University Institutional Review Boards, and was registered at ClinicalTrials.gov (NCT 01474993 under Food and Drug Administration IND 113542). All participants who were able, and parents or caregivers, gave written informed consent. All participants met criteria for autistic disorder (1). Forty-four male ASD patients were enrolled from February 2011 to July 2013. The Autism Diagnostic Observation Schedule, performed by a trained psychologist/tester (in 43) or DSM-4 (1) checklist of symptoms performed by a trained physician (two participants), were used to confirm the diagnosis of autism at the screening visit. All participants were moderately to severely autistic on the CGI-S, with varied cognitive capacity (Table S1).

Eligibility criteria included male sex, age 13–30, no intercurrent chronic illness, no history of active seizures within 1 y, and normal liver, renal, and thyroid functions. Participants continued their regular medications, if any, during the study.

Participants were assigned by the MGH Research Pharmacy to receive either placebo or sulforaphane according to computer-generated randomly permuted blocks of three assignments, with sulforaphane and placebo treatments allocated in a 2:1 ratio in two strata defined by parent-reported history of improvement in behavior during febrile illness. Physicians and study staff were blind to group assignment. Forty-four subjects were selected to provide at least 80% power to test the primary hypothesis for the SRS using a two-tailed two-sample t test with $\alpha = 0.05$ and assuming that the true difference in average change in SRS was 15 units with a SD of 16 units. This is roughly twice the average magnitude of natural change observed over 1 y among male children and adolescents with ASD (38).

The study comprised seven visits: screening, randomization, and start of treatments, at 24 h, and at 4, 10, and 18 wk after the first dose. Treatment was discontinued after the 18-wk visit, and participants returned at 22 wk. Medical history, physical examination including vital signs, adverse event reporting, and SRS, ABC, and CGI-I were performed (Fig. 1). At the 4-, 18-, and 22-wk visits, hematology, chemistry, and urinalysis were also obtained.

All families were contacted after the final participant completed follow-up and asked for their impressions of the study and their child's progress while under treatment. Families were then informed whether he received sulforaphane or placebo.

Administration of Medication and Protocol Schedule. Capsules of sulforaphane-rich broccoli sprout extracts were maintained at −20 °C, and checked periodically microbiologically and for sulforaphane titer (*SI Materials and Methods*) (8). Indistinguishable placebo capsules contained microcrystalline cellulose. Sulforaphane or placebo was administered daily for 18 wk. The participants were dosed according to body weight: 50 μmol (one capsule) of sulforaphane for <100 lb, 100 μmol (two capsules) for 101–199 lb, and 150 μmol (three capsules) for >200 lb. Placebo recipients received equivalent numbers of capsules according to their weight. Capsules were dispensed to participants in sealed bottles by the MGH Research Pharmacy, with instructions to keep them in a household freezer.

Behavioral Outcome Measures. The ABC is a parent- or caregiver-reported 58-item questionnaire designed to assess medication effects; each item is scored on a scale of increasing severity from 0 to 3 (27). ABC also assesses several subdomains (irritability, lethargy, stereotypy, and hyperactivity).

The SRS is a parent- or caregiver-reported 65-point social communication questionnaire that covers five subscales (awareness, cognition, communication, motivation, and autistic mannerisms) (28). Each SRS item is rated on a scale of 1–4; the total score was our primary efficacy endpoint.

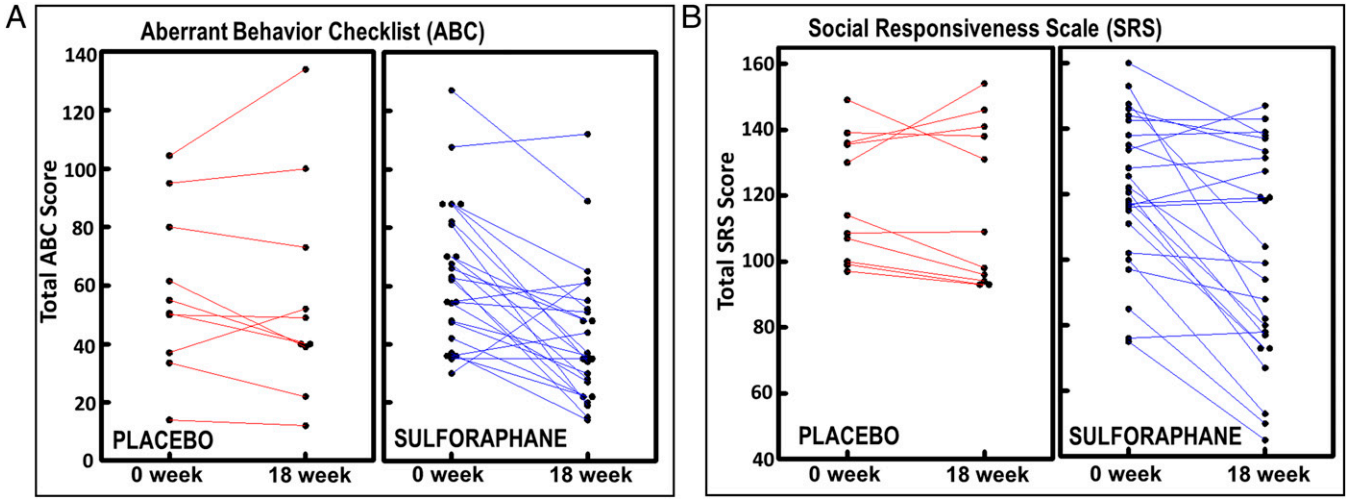

Fig. 4. Total scores for (*A*) ABC and (*B*) SRS of individual placebo- and sulforaphane-treated participants at baseline and after 18 wk. At 18 wk, total ABC scores were available for 35 (10 placebo and 25 sulforaphane) and total SRS scores for 37 (11 placebo and 26 sulforaphane). Only the differences for sulforaphane treatment were significant at 18 wk, thus a change in score of from 62.4 to 45.0 on the ABC scale (*A*) was significant ($P < 0.001$), and a change in score of from 121.5 to 105.2 on the SRS scale (*B*) was significant ($P < 0.001$). Means for the subjects shown, at 1 and 18 wk respectively, for placebo treatment, were 62.4 and 62.6 on the ABC scale, and 121.5 and 117.5 on the SRS scale.

The Ohio Autism Clinical Global Impression Severity Scale (CGI-S, also designated OACIS-S, and only measured at screening) (29, 30) is a clinician-rated assessment of the severity of autistic behavior (in increasing order of severity from 1 to 7) and includes the following subdomains: global autism severity, social interaction, aberrant behavior, repetitive or ritualistic behaviors, verbal and nonverbal communication, hyperactivity/inattention, anxiety, sensory sensitivities, and restricted/narrow interests. The Ohio Autism Clinical Global Impressions Improvement Scale (CGI-I or OACIS-I) (29, 30) is a clinician-rated assessment of how much the patient's behavior has changed during an intervention.

Statistical Evaluation. Forty-four subjects were originally enrolled and randomized to sulforaphane treatment (n = 29) or placebo (n = 15); four subjects discontinued participation in the study before the first (4-wk) return visit. Behavior scores for the remaining 40 participants, who completed at least part of the outcome measure evaluations (14 placebo and 26 sulforaphane), are described in our primary results and shown in Figs. 2–4 and Tables 1 and 2. To compensate for incidental changes in ABC/SRS scores resulting from normal fluctuation, we obtained these scores at both screening and randomization visits, and used their averages to compare with subsequent ABC/SRS scores. Our primary analysis used the differences between scores of individuals at 4, 10, 18, and 22 wk from their respective average pretreatment values. The test of our hypothesis was the difference between the sulforaphane and placebo treatment groups in the change in ABC and SRS scores from baseline to 18 wk, and their reversion to baseline at 22 wk.

Each outcome was modeled in a shared-baseline mixed-effects general linear model with fixed effects for visit and the interaction of postrandomization visit and treatment group and random participant-specific intercepts and slopes with unstructured covariance. The absence of a main effect for treatment (i.e., a "shared baseline") properly reflects the true state of the population sampled before randomization and has the advantage of adjusting for any chance differences at baseline in a manner similar to ANCOVA (39). Linear contrasts of least-square means were used to estimate changes from baseline between treatment and control groups at each follow-up visit. Given its assumptions, the mixed model yields estimates that are unbiased as long as loss to follow-up, and missing test scores are predictable from observed scores under assumptions of the model. An intention-to-treat analysis that included all 44 participants led to similar conclusions (*SI Materials and Methods*).

Statistical analyses were performed with SAS v. 9.3 software (SAS Institute), and Stata v.11.2 (Statacorp).

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