CLINICAL PROTOCOL

A Placebo-Controlled, Randomized, Double-Blind Study to Assess the Safety, Tolerability and Efficacy of Folinic Acid Administered to Pediatric Subjects with Autism Spectrum Disorder (ASD)

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SIGNATURE PAGE

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Revision History

Version	Author	Date	Description (if after study onset)
1.0	Aran A	February 8, 2026	First draft
2.0	Aran A	March 23, 2026	First revision before ethic approval
3.0	Aran A	April 28, 2026	Correction of minor inconsistencies prior to study onset
4.0	Aran A	May 7, 2026	Change of study phase
5.0	Aran A	June 8, 2026	Clarification of the randomization process and addition of maternal blood collection for FRAA testing

Short Synopsis

Title	A Placebo-Controlled, Randomized, Double-Blind Study to Assess the Safety, Tolerability and Efficacy of Folinic Acid, Administered to Pediatric Subjects with Autism Spectrum Disorder (ASD)
Short Title	A placebo-controlled trial of folinic acid in children with ASD
Running Head	Folinic acid in ASD
Protocol Number	0038-26-SZMC
Study Center	Shaare Zedek Medical Center, Jerusalem, Israel
ClinicalTrials.gov - Number	NCT07642661
- **Registration Date**	June 07, 2026
Ethic Approval Date	May 10, 2026
Trial Commencement	TBD
Planned Number of Participants	Total: 150; At this site:100; Age 3-6 years
Study duration per participant	Placebo-controlled phase: 9 weeks. After completion, participants will be randomized to receive folinic acid at one of two doses for an additional 8 weeks.
Study Interventions	Folinic acid (2 mg/kg/day; maximum dose 50 mg/day). Study interventions will be administered as an add on to any existing treatment.
Primary outcome	ASD-associated behavioral problems, using the Aberrant Behavior Checklist Irritability Subscale (ABC-I) score - change from baseline to week 9.

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A placebo-controlled trial of folinic acid in children with ASD

Background: Autism spectrum disorder (ASD) encompasses a wide range of neurodevelopmental disorders with diverse etiologies and clinical manifestations. Currently, there is no established pharmacological treatment for the core symptoms of ASD, and antipsychotic medications approved for associated irritability have limited tolerability. Folinic acid has been suggested as a potential treatment for core ASD symptoms and associated irritability in several placebo-controlled studies using different outcome measures.
Objective: To assess the impact of folinic acid in children with ASD in a multicenter, randomized, double-blind, placebo-controlled trial.
Specific aims:
The primary aim is to assess the effect of high-dose folinic acid compared to placebo on behavioral problems, using the Aberrant Behavior Checklist Irritability Subscale (ABC-I) score - change from baseline to week 9.
The main secondary aim is to assess the effect of folinic acid, compared with placebo, on change from baseline to week 9 in (a) communication and (b) socialization, as measured by the Vineland™ Adaptive Behavior Scales, Third Edition (VABS-3) Communication and Socialization Domain standard scores.
Other secondary aims include: (1) To assess the tolerability and safety of folinic acid compared to placebo, using detailed questionnaires, physical examination, blood count, and liver enzyme tests. (2) To assess global change in ASD-associated symptoms since baseline using the Clinical Global Impression- Improvement (CGI-I) and the Caregiver Global Impression of Change (CGIC) assessments – at week 9. (3) To assess changes in verbal communication, core ASD symptoms, social withdrawal, stereotypic behavior, hyperactivity, noncompliance, inappropriate speech, quality of life, sleep parameters, and gastrointestinal problems using the MacArthur-Bates Communicative Development Inventories (MB-CDIs), Social Responsiveness Scale 2nd edition (SRS-II), Aberrant Behavior Checklist (ABC-C), Quality of Life in Autism Questionnaire (QOLA) – Parent version, the Children's Sleep Habits Questionnaire (CSHQ) and a modified gastrointestinal sign and symptoms inventory (GISSI) - change from baseline to week 9. (4) To compare the effects of high- and low-dose folinic acid using the above assessments, based on change from baseline to weeks 9 and 17.
Study design:
In this phase 2, multicenter, randomized, double-blind, placebo-controlled, parallel-group trial, 150 children with ASD will be randomized in a 1:1 ratio to receive either placebo or high-dose folinic acid (2 mg/kg/day; maximum dose 50 mg/day). Treatment will be administered as an add on to any existing therapy for 9 weeks, with dose up-titration over 5 days. After completion of the initial 9-week period, participants will be randomized to receive one of two folinic acid doses (1 or 2 mg/kg/day; maximum dose 50 mg/day) for an additional 8 weeks**.**
Inclusion criteria: A diagnosis of ASD according to the DSM-5 criteria, confirmed by ADOS-2; Age 3-6 years; Bodyweight between 11.45kg and 27kg; CGI-Severity score ≥ 4; ABC-I score ≥ 12; and SRS-II total T score ≥ 66.
Exclusion criteria: Seizures or changes in anticonvulsive therapy within the last 8 weeks; Any change in the pharmacological or behavioral treatment in the 4 weeks prior to randomization; Participants or caregivers are unlikely to comply with all trial requirements.
Significance:
Studies suggest that folinic acid may reduce disruptive behavior and improve core symptoms in children with ASD. However, the level of evidence for efficacy, safety, and tolerability remains relatively low and does not justify its recent widespread use in children with ASD. The current study will assess the impact of high- and low-dose folinic acid in a multicenter trial.

Introduction

There is no established pharmacological treatment for the core symptoms of autism spectrum disorder (ASD), persistent deficits in social communication, and repetitive, restrictive patterns of behavior.[1] Risperidone and aripiprazole have been approved by the U.S. Food and Drug Administration (FDA) to treat comorbid irritability[2] but these medications often cause obesity and metabolic syndrome.[2,3]

Folate is a water-soluble B vitamin that is essential for development[4] and particularly neurodevelopment.[5] Folic acid is a synthetic, oxidized, and water-soluble form of folate. Folinic acid is a naturally occurring, reduced form, of folate commonly known in clinical practice as leucovorin. The folate cycle is essential for a wide variety of developmental and metabolic pathways such as DNA synthesis and methylation (epigenetic regulation), neurotransmitter synthesis, and redox metabolism.[6] Maternal folic acid supplementation is associated with a reduced risk of autism.[7]

The cerebral folate receptor alpha (FRα) transports 5-methyltetrahydrofolate (5-MTHF) into the brain. Autoantibodies to FRα (FRAAs), which may bind to or partially block the receptor, are associated with reduced cerebrospinal fluid levels of 5-MTHF, a condition known as cerebral folate deficiency.[8] In affected children, oral folinic acid at doses of 0.5 to 2 mg/kg/day, which can bypass FRα blockade via the reduced folate carrier, normalized cerebrospinal fluid 5-MTHF levels and led to clinical improvement.[8] Children with non-syndromic ASD frequently have FRα autoantibodies and may also exhibit low cerebrospinal fluid levels of 5-MTHF.[9,10]

Several placebo-controlled studies have demonstrated that high-dose folinic acid (2 mg/kg/day, up to 50 mg/day) administered for 10–12 weeks can improve verbal communication, social reciprocity, and some behavioral symptoms compared to placebo, with medium-to-large effect sizes.[11-14] There is initial evidence suggesting that the most robust improvements are observed in children who are FRAA positive, supporting a potential mechanistic link to cerebral folate deficiency.[11,13] However in the study of Frye et al[11], the apparent greater improvement in FRAA positive children was driven by a higher placebo response in the FRAA negative group, likely reflecting variability related to the small sample size (n = 48) rather than a true differential treatment effect of folinic acid. In addition, the study by Panda et al[13] was recently retracted by the journal editor.

Genetic polymorphisms affecting folate metabolism (e.g., MTHFR, MTRR) may also predict greater response to folinic acid.[14] Overall, folinic acid has been well tolerated, with no significant increase in adverse effects compared with placebo in published studies.[11-14]

In addition to clinical effects, folic acid has been shown to modulate inflammatory responses in lipopolysaccharide activated BV-2 microglial cells through multiple signaling pathways. These include upregulation of the anti inflammatory cytokine IL-10 mediated by p38 MAPK phosphorylation, upregulation of suppressors of cytokine signaling SOCS1 and SOCS3, downregulation of TNF-α, IL-1β, and inducible nitric oxide synthase expression with reduced nitric oxide production, and downregulation of phosphorylated IκBα and JNK, reflecting reduced NF-κB and JNK pathway activation.[15]

In recent years, folinic acid has gained substantial popularity among families of children with ASD, leading to widespread off-label use in clinical practice. This increased demand is largely driven by early positive findings and extensive dissemination through parent networks and social media. However, despite this growing use, the available evidence is derived primarily from relatively small studies employing heterogeneous outcome measures. Consequently, there is insufficient high-quality evidence to firmly establish

efficacy across the broader ASD population or to adequately assess long-term efficacy, safety, tolerability, and longitudinal dosing requirements, underscoring the need for large, well-powered confirmatory trials.

The primary outcome measure in this study is the Aberrant Behavior Checklist, Irritability Subscale (ABC-I). Of the four previous placebo-controlled studies that evaluated high-dose folinic acid, two used the ABC-I as a primary or secondary outcome measure and reported signals of efficacy ($p<0.1$).[11,12] The irritability subscale of the ABC-C has demonstrated high reliability[16] and is widely used as a primary outcome measure in interventional studies in autism spectrum disorder (ASD).[17,18]

Additional outcome measures in which folinic acid demonstrated potential efficacy in prior studies included verbal communication,[11] other subdomains of the ABC-C,[11,12] the Childhood Autism Rating Scale (CARS, retracted article),[13] and the Psycho-Educational Profile, specifically the social reciprocity domain.[14] Accordingly, in the current study, the main secondary outcome measures focus on communication and socialization, assessed using the Vineland Adaptive Behavior Scales, Third Edition (VABS-3) Communication and Socialization Domain standard scores.

Working hypothesis and expected significance:

The present study evaluates folinic acid for its potential to alleviate disruptive behavior and core symptoms in children with ASD. Folinic acid is a reduced form of folate with a favorable safety and tolerability profile and a well-established role in neurodevelopmental processes, including methylation, neurotransmitter synthesis, and redox metabolism. Preliminary placebo-controlled studies suggest potential benefit of folinic acid in ASD, although robust confirmatory evidence is still lacking.

Folinic acid is thought to exert its effects, at least in part, by improving cerebral folate availability and modulating neuroinflammatory and metabolic pathways implicated in ASD. Given the heterogeneity of ASD and variability in underlying folate related mechanisms, including cerebral folate deficiency and folate pathway polymorphisms, different dosing strategies may be required to optimize clinical response.

We hypothesize that treatment with folinic acid will lead to meaningful improvement in disruptive behavior and core ASD symptoms compared with placebo, with possible dose dependent effects. The use of a standardized pharmaceutical grade formulation and a randomized, double blind design ensures consistent treatment exposure and enables rigorous evaluation of efficacy, safety, and tolerability.

Even modest improvements in core and associated ASD symptoms may have a meaningful impact on the quality of life of affected children and their families and may contribute to a reduction in long term public health burden.

Methods

Study design

This is a phase 2, multicenter, randomized, double-blind, placebo-controlled, parallel-group study, involving 150 children with ASD and behavioral problems, age 3-6 years. Participants who pass screening will be randomized (in a 1:1 ratio) to receive either placebo or folinic acid (1mg/kg twice daily). The treatment will be given orally as an add on to any existing treatment, for 9 weeks. The treatment dose will be up titrated over 5 days.
After completing the first 9-week period, participants will be randomized (in a 1:1 ratio) to receive one of two folinic acid doses (1 or 2 mg/kg/day; maximum dose 50 mg/day) for an additional 8 weeks**.**

First phase (placebo-controlled phase)

Five times during the first 9-week period:
Parents or primary caregivers will rate adverse events and document: any use of medical services, any change in the doses of the study intervention and concomitant medications, any change in the regular diet and intake of food supplements, and any change in the home or school routine or in the behavioral or emotional treatments.

At the onset and end of the first 9-week period:

1. physicians will assess physical examination and BMI
2. Parents will rate behavioral symptoms on the Aberrant Behavior Checklist Community (ABC-C)
3. Parents will rate their child's: (a) *Communication, Socialization and maladaptive behavior* using the Vineland Adaptive Behavior Scales (VABS-III; a structured interview); (b) *Verbal Communication* using the MacArthur-Bates Communicative Development Inventories (MB-CDIs); (c) *ASD core symptoms* using the Social Responsiveness Scale – second edition (SRS-2); (d) *Behavior* using the emotion dysregulation inventory (EDI) and the multidimensional assessment of disruptive behavior (MAP-DB); (e) *Severity of core symptoms* using the autism impact measure (AIM); (f) *Sleep habits* using the Children's Sleep Habits Questionnaire (CSHQ); (g) *Health-related quality of life of themselves* using the Quality of Life in Autism Questionnaire (QOLA) – Parent version; and (h) *Gastrointestinal symptoms* the gastrointestinal symptoms a modified gastrointestinal sign and symptoms inventory (GISSI)
4. At baseline only: Serum levels of folate, vitamin B12 and both blocking and binding folate receptor-α autoantibodies (FRAA). Maternal serum levels of FRAA.
5. At baseline and end of the first phase: Complete blood count (CBC), liver transaminases (LTs), and total bilirubin; Plasma homocysteine, proteome, and ratio of free reduced glutathione (fGSH) to free oxidized glutathione disulfide (fGSSG); RNA-sequencing of whole blood samples (transcriptome); Gut microbiota.

At baseline, a buffy coat obtained from plasma collection tubes will be stored frozen for the potential performance of future NGS panels assessing polymorphisms in folate metabolism genes. Any genetic analyses will be conducted only after obtaining specific genetic ethics approval and written informed consent from the parents, in accordance with that approval. The gene panel will include the following genes: MTHFR, MTR, MTRR, DHFR, FOLR1 (folate receptor alpha), SLC19A1 (reduced folate carrier), TYMS, SHMT1, PCFT and CBS.

Additionally, at the end of the first phase physicians and caregivers will rate the global change in ASD-associated symptoms using the Overall Function Clinical Global Impression- Improvement (CGI-I) and the Caregiver Global Impression of Change (CGIC) assessments. Parents or primary caregivers will complete an Assessment of Blinding Questionnaire.

Second phase: Assessments at the end of the second phase will include: CGI-I and CGIC (global change in ASD-associated symptoms); adverse events; ABC-C, EDI, and MAP-DB (behavioral symptoms); SRS-2 and AIM (ASD core symptoms); CSHQ (sleep habits); QOLA (quality of life); modified GISSI (gastrointestinal symptoms) and Assessment of Blinding Questionnaire.

A telephone follow-up will also be conducted between the visits (Table 2) and six months after completion of the study. The same parent will be required to complete the questionnaires throughout the entire study period.

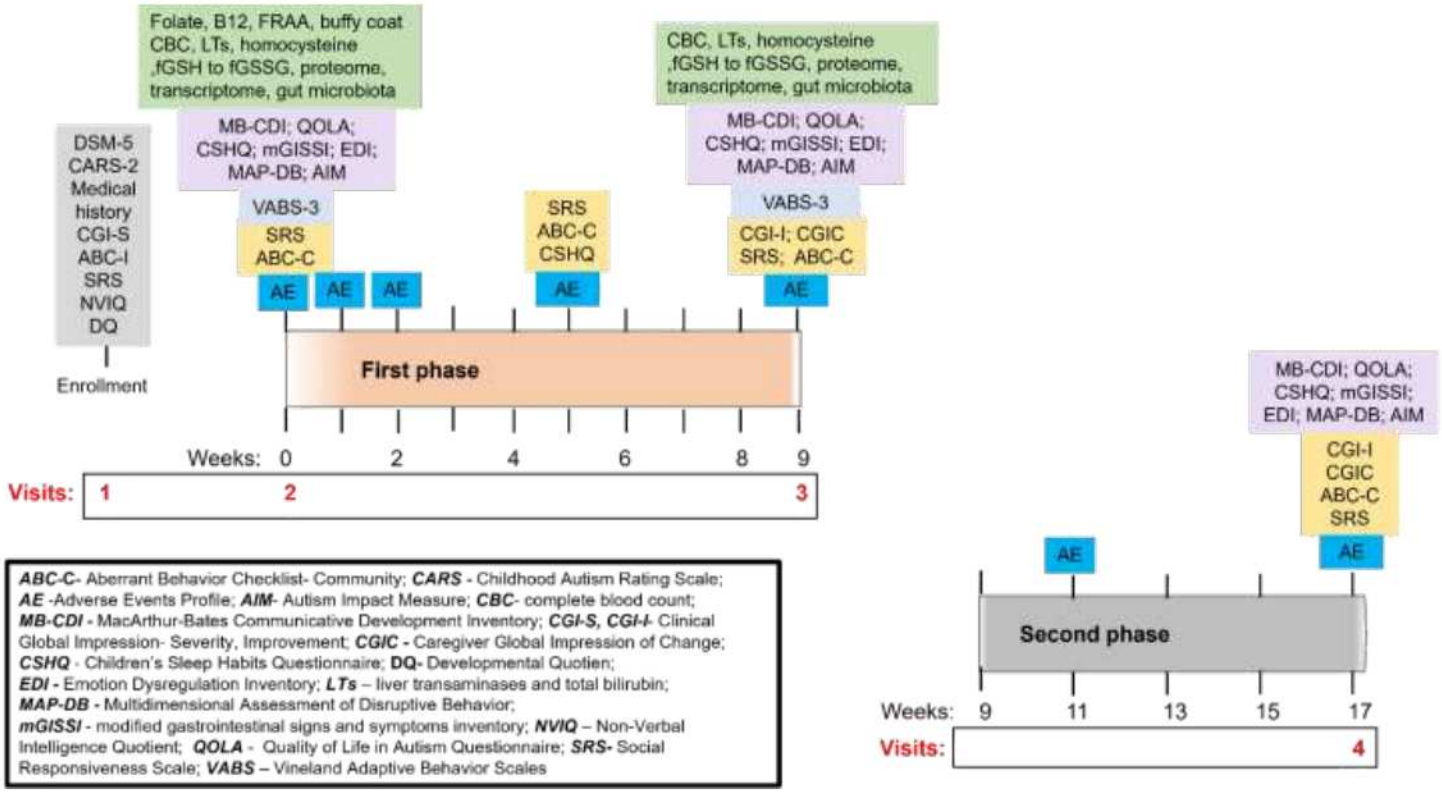


Figure 1: Study design

Participants

One hundred and fifty participants will be recruited across five sites in Israel. Written informed consent will be obtained from a parent or legal guardian prior to participation, following approval by the local institutional review board.

Inclusion criteria are:

- Children aged 3 to 6 years.
- A diagnosis of ASD based on the Diagnostic and Statistical Manual of Mental Disorders [Fifth Edition; DSM-V], confirmed by ADOS-2.
- A diagnosis of ASD was made at least 6 months before screening.
- Non-syndromic ASD, defined as ASD occurring in the absence of a known genetic syndrome, chromosomal abnormality, major congenital anomaly, or identifiable metabolic or neurological disorder.
- Moderate or greater ASD-associated symptoms as measured by a rating of moderate or higher (≥4) on the Overall Function Clinical Global Impression–Severity (CGI-S).
- Aberrant Behavior Checklist Irritability Subscale (ABC-I) score of 12 or higher.
- Social Responsiveness Scale 2nd edition (SRS-II) total score of 66T or higher.

Exclusion criteria are:

- Bodyweight is below 11.45kg or above or equal 27 kg.
- A seizure or change in the antiepileptic medications in the 8 weeks prior to randomization.
- A physical exam and laboratory results are out of the normal range for individuals with ASD, including a significant impairment in heart, liver, or renal function.
- Any treatment with folinic acid in the 3 months prior to randomization.
- Any change in the pharmacological or behavioral treatment or change in the home or school settings (other than school holidays) in the 4 weeks prior to randomization or planned changes during the study.
- Predicted low compliance to the study procedures.
- Use of antifolate medications or other agents known to interfere with folate metabolism (e.g., methotrexate, trimethoprim).

Criteria for termination of participation:

- The Principal Investigator believes that continued participation endangers the participant.
- Upon a decision of the regulatory bodies: IRB or MOH.
- Upon request of one of the legal guardians
- Initiation of antifolate medications.

Terms for termination of participation: After termination of participation – participants will continue to receive the same treatments they received before termination, excluding the study treatment. These treatments will not be affected in any way by their decision to terminate their participation in the study

Procedures

Screening: ASD diagnosis will be determined using a structured assessment of DSM-5 criteria, confirmed by *Autism Diagnostic Observation Schedule-2* (ADOS-2) and *Childhood Autism Rating Scale – Second edition (CARS-2).*

Caregivers will complete a medical history questionnaire tapping demographic parameters,

etiological factors (perinatal, genetic, and imaging), comorbidities (epilepsy, allergies, immune mediated disorders, and sleep disorders), autistic regression, diet including the amount of cow milk consumption, food supplements, behavioral treatments and impact of high fever on social deficits. Other assessments will include CGI-S, ABC-C, CDIs, SRS, DQ, and NVIQ (as detailed below).

Blood samples- (15 ml) will be collected at 0 and 9 weeks.

- Complete blood count (CBC), liver transaminases (LTs) and total bilirubin will be measured as part of the safety analyses.
- Serum levels of both blocking and binding folate receptor-α autoantibodies (FRAA)[19]
- RNA-sequencing of whole blood samples (transcriptome)[20]
- Plasma levels of homocysteine, proteome, and ratio of free reduced glutathione (fGSH) to free oxidized glutathione disulfide (fGSSG) will be assessed to explore possible markers of treatment response. GSH/GSSG ratio is a reliable marker of oxidative stress, a postulated factor in the pathophysiology of ASD.[21] Treatment with folinic acid [11,19] is associated with lower ratios of fGSH/fGSSG.

Stool samples will be collected at 0 and 9 weeks.

- Gut microbiota was demonstrated to be associated with ASD as well as symptom severity in multiple studies.[22-24] It is also related to the glutathione redox status.[24,25]

Maternal Blood samples (5 ml, serum) will be collected at baseline to assess FRAA.

Study Treatment

Participants will receive calcium folinate hydrate (leucovorin calcium) as a 5 mg/mL oral solution or a matched placebo for 9 weeks during the first phase of the study. The placebo will consist of a solution containing only inactive aqueous excipients, matched in appearance, taste, and smell to the folinic acid solution.

The target dose of calcium folinate hydrate is 2 mg/kg/day (maximum 50 mg/day), administered in two divided doses.

The dose initiation and titration schedule will be as follows:

• 0.5 mg/kg twice daily for 4 days

• 1 mg/kg twice daily thereafter (maximum 50 mg/day)

Second Phase

At the end of the initial 9-week phase, participants will discontinue the study treatment received during the first phase and will be randomized in a 1:1 ratio to receive calcium folinate hydrate at one of two doses: 1 or 2 mg/kg/day (maximum 50 mg/day), for an additional 8 weeks. Participants and study personnel will remain blinded to the assigned dose. No dose titration will be performed during the second phase.

Study visits

On-site visits: 4 visits: Screening (visit #1), Baseline (visit #2), 9 weeks (visit #3), and 17 weeks (visit #4). Phone visits to document compliance and adverse events: Two visits at study onset- (days 8, and 15) and another 2 visits on weeks 5, and 11.

Outcomes and measures:

Baseline evaluations (at screening):
Childhood Autism Rating Scale – Second Edition (CARS2):[26] CARS-2 is a validated tool for screening and classifying ASD, based on direct behavioral observation across 15 items (14 specific domains such as communication, body use, and response to stimuli, plus one global severity rating). Each item is scored from 1 (normal) to 4 (severely abnormal). Total scores of 30–36.5 indicate mild to moderate ASD, and scores >36.5 indicate severe ASD. The High-Functioning Form (CARS2-HF) is used for children ≥6 years with IQ >80, while the Standard Form (CARS2-ST) is used for all others.

Nonverbal Intelligence Quotient (NVIQ): NVIQ will be assessed using Raven's Colored Progressive Matrices (RPM), an untimed measure of problem-solving and abstract reasoning. The RPM consists of colored pattern sets (A, Ab, B) of increasing difficulty to assess observational and fluid intelligence. Test–retest reliability is typically >0.80. The RPM is generally considered to provide a more accurate estimate of NVIQ in children with ASD compared to the WISC or WPPSI.[27-29]

Outcome Measures
ABC-C: The ABC[30,31] is a caregiver-completed questionnaire with 58 items, organized into five subscales. The ABC-I (irritability) subscale consists of 15 items that measure the emotional and behavioral symptoms of ASD, including aggression toward others, deliberate self-injuriousness, temper tantrums, depressed mood and rapidly changing moods. The ABC-I has been accepted as the gold standard for measuring irritability in medication trials for ASD[32] and therefore, we will use it as the primary endpoint in this study. Individual items are rated from 0 (never a problem) to 3 (severe problem). Other subscales include Hyperactivity, Noncompliance (16 items), assessing overactivity, impulsivity, distractibility, and difficulty following instructions; Lethargy, Social Withdrawal (16 items), measuring reduced social engagement, apathy, and diminished initiation; Stereotypic Behavior (7 items), capturing repetitive and ritualistic behaviors; and Inappropriate Speech (4 items), evaluating excessive, repetitive, or contextually inappropriate verbalizations. Each subscale yields a summed score, with higher scores indicating greater symptom severity.

VABS-III [33]: The VABS is a semi-structured caregiver interview designed to assess functional skills in three domains: Communication, Socialization, and Daily Living Skills. It also contains a short Maladaptive Behavior Domain. The Adaptive Composite Score estimates overall adaptive behavior. Vineland items are scored based on an open-ended question as 0 (behavior not performed), 1 (performed sometimes), or 2 (performed on a regular basis). The instrument provides Standard scores (mean = 100, SD = 15) and Age Equivalent scores

MB-CDI (Hebrew version):[34,35] This instrument is based on parent report of the words used by their children from structured, closed vocabulary lists. The MB-CDI has been adapted into more than 100 languages and has demonstrated strong validity and reliability for assessing lexical development in toddlers.[36]
When feasible, children will also undergo evaluation by a speech and language therapist using an age- and ability-appropriate standardized instrument, including the *Clinical Evaluation of Language Fundamentals preschool-2* (*CELF-P2*; standard score range 45–155, mean 100, standard deviation 15)[37] or the *Preschool Language Scale-5* (*PLS-5*; standard score range 45–155, mean 100, standard deviation 15).[38] Both are validated instruments sensitive to subtle changes in verbal communication abilities.

Calculation of language level from ADOS-2: Language level at baseline will be also derived from the ADOS-2 using the language item, based on the method described by Visser et al.[39] Participants will be categorized according to expressive language ability as reflected in the ADOS-2 language item, allowing standardized comparison of language development trajectories across participants.

SRS (Hebrew version) [40,41]: The SRS is a 65-item caregiver or teacher questionnaire with five subscales: Social Awareness, Social Cognition, Social Communication, Social Motivation, and Autistic Mannerisms. Items are rated on a 4-point Likert scale (0–3, total 0–195), with higher scores indicating greater impairment. Subscales yield T-scores; a Total T-score of 60–75 indicates mild to moderate impairment, and >75 indicates severe impairment. Caregivers will complete the SRS based on behavior during the previous 6 weeks. The SRS is widely used in ASD research. [42-47]

CGI [48]: this is a 7-point scale designed to measure the severity of illness (CGI-S) and, an overall improvement from baseline (CGI-I). This measure has been used in many clinical trials in ASD.[49-51] Scores in the severity scale range from 1 (normal) through to 7 (amongst the most severely ill patients); in the improvement scale, scores range from 1 (very much improved) through 4 (unchanged) to 7 (very much worse).

CGIC: this is a 7-point scale designed to measure the overall improvement from baseline. This measure is completed by the primary caregivers and is similar to the CGI-I

Autism Impact Measure (AIM)[52,53]: This is a 41-item caregiver-reported questionnaire designed to characterize core symptoms of ASD in individuals aged 3–18 years

Multidimensional Assessment of Preschool Disruptive Behavior (MAP-DB)[54]: This 40-item caregiver-reported questionnaire assesses behavior across four domains: Temper Loss, Noncompliance, Aggression, and Low Concern for Others. It is available in three age-specific versions: 3-5 years, 6-8 years, and 9-10+ years.

Emotion dysregulation inventory (EDI)[55]: This 30-item caregiver-reported questionnaire assesses observable signs of poor emotion regulation in children with autism.

Children's Sleep Habits Questionnaire (CSHQ)[56]: this is a 33 items Hebrew validated questionnaire. A higher score reflects more significant sleep disturbances (range: 33–99, a score of ≥41 classifies a "poor sleeper). The CSHQ has eight subscales: bedtime resistance; sleep onset delay; sleep duration; sleep anxiety; night waking; parasomnias; sleep-disordered breathing; and daytime sleepiness. This questionnaire has been used extensively in previous research in ASD

Quality of Life in Autism Questionnaire (QOLA) – Parent version:[57]The QOLA is a 48-item tool with two subscales to measure quality of life in parents of children with ASD. Part A (QoL subscale) includes 28 items assessing parents' overall quality of life, with scores ranging from 28 to 140 (higher scores indicate greater QoL). Part B (impact of ASD symptoms subscale) includes 20 items evaluating how problematic a child's ASD-related difficulties are for the parent, with scores ranging from 20 to 100 (higher scores indicate fewer problems).

Modified gastrointestinal signs and symptoms inventory (GISSI): This 15-item questionnaire was adapted to this study based on the GISSI-17,[58] a brief parent-report screen for common gastrointestinal problems in children with ASD.

Adverse events (AE) will be monitored using a 31-item modification of the Dosage Record Treatment Emergent Symptom Scale. Recent health concerns, use of medical services, and change in study treatment dose and concomitant medications will be recorded as well. BMI, pulse rate, and blood pressure will be assessed at each clinic visit. Reports of new adverse events or worsening of previously reported events will be rated mild (present, but not persistent or requiring intervention), moderate (requiring intervention), or severe (posing a severe problem and requiring immediate intervention). Hospitalization will be documented as a serious adverse event. The PI will systematically evaluate all AEs for timing, biological plausibility, alternative causes, dose–response relationship, and prior knowledge to determine relatedness to the study drug, categorized as: not related, unlikely, possibly, probably, or definitely related.

Table 1: Primary, secondary, and exploratory outcomes

Primary outcome measure: Change in the Aberrant Behavior Checklist Irritability Subscale (ABC-I) score from Baseline to week 9.

Main secondary outcome measures:
1) Change from baseline to week 9 in the Vineland ™ Adaptive Behavior Scales (VABS3)-Socialization Domain Score
2) Change from baseline to week 9 in the Vineland ™ Adaptive Behavior Scales (VABS3)-Communication Domain Score

Other secondary measures:
1) Number of participants with severe, moderate, and mild treatment-emergent adverse events during the 9-week first phase.
2) Number of participants with clinically significant abnormal values in the CBC, liver transaminases, or total bilirubin or clinically significant abnormal values in the vital signs or physical examination at week 9
3) Change from baseline to week 9 in the BMI
4) Clinical Global Impressions-Improvement since baseline at week 9.
5) Change from baseline to week 9 in the Communicative Development Inventory.
6) Change from baseline to week 9 in the Social Responsiveness Scale (SRS-II) total raw score.
7) Change from baseline to week 9 in the ABC-C subdomains scores
8) Change from baseline to week 9 in the Vineland ™ Adaptive Behavior Scales (VABS3)-maladaptive behavior Domain Score
9) Change from baseline to week 9 in the emotion dysregulation inventory (EDI)
10) Change from baseline to week 9 in the multidimensional assessment of disruptive behavior (MAP-DB)
11) Change from baseline to week 9 in the autism impact measure (AIM).
12) Change from baseline to week 9 in the Quality of Life in Autism Questionnaire (QOLA) – Parent version – part A: parents' overall perception of their QOL- subscale score; and (c) part B: impact of ASD symptoms on parents' QOL- subscale score
13) Change from baseline to week 9 in the Children's Sleep Habits Questionnaire (CSHQ)
14) Change from baseline to week 9 in the gastrointestinal signs and symptoms inventory.
15) Caregiver Global Impression of Change (CGIC) since baseline at week 9.

To compare the effects of high- and low-dose folinic acid after initial high-dose treatment or after initial placebo- treatment, the above assessments, will be used- change from baseline to weeks 9 and 17:
16) Clinical Global Impressions-Improvement since baseline at week 17
17) Change from baseline to week 17 in the Communicative Development Inventory.
18) Change from baseline to week 17 in the Vineland ™ Adaptive Behavior Scales (VABS3)-Socialization, Communication and Maladaptive Behavior Domains Scores
19) Change from baseline to week 17 in the emotion dysregulation inventory (EDI)
20) Change from baseline to week 17 in the multidimensional assessment of disruptive behavior (MAP-DB)
21) Change from baseline to week 17 in the autism impact measure (AIM).
22) Change from baseline to week 17 in the Quality of Life in Autism Questionnaire (QOLA) – Parent version – part A: parents' overall perception of their QOL- subscale score; and (c) part B: impact of ASD symptoms on parents' QOL- subscale score
23) Change from baseline to week 17 in the CSHQ -total score

24) Change from baseline to week 17 in the gastrointestinal signs and symptoms inventory.
25) Caregiver Global Impression of Change (CGIC) since baseline at week 17.

Exploratory outcomes

1) Correlations between 'biomarkers' at baseline [Serum levels of folate, vitamin B12 and both blocking and binding folate receptor-α autoantibodies, plasma levels of homocysteine, proteome, and ratio of fGSH to fGSSG, transcriptome, and gut microbiota] and treatment outcomes, in the active treatment group.
2) Correlations between change in 'biomarkers' [plasma levels of homocysteine, proteome, and ratio of fGSH to fGSSG, transcriptome, and gut microbiota] from baseline to 9 weeks and treatment outcomes, in the active treatment group, in the placebo group, and in the entire cohort.
3) Correlations between 'child characteristics' at baseline [age, sex, level of function (DSM-5 levels of support required, CARS, VABS, CGI-S, ABC-C, SRS, DQ, NVIQ), etiological factors, comorbidities, history of autistic regression, diet, behavioral treatments and impact of high fever on social deficits status], and treatment outcomes, in the active treatment group.
4) Correlations between 'child characteristics' at baseline and 'biomarkers' at baseline.
5) Effects of folinic acid compared to placebo on 'biomarkers'[plasma levels of homocysteine, proteome, and ratio of fGSH to fGSSG, transcriptome, and gut microbiota] - change from baseline to week 9.
6) Correlations among 'biomarkers' [Serum levels of folate, vitamin B12 and both blocking and binding folate receptor-α autoantibodies, plasma levels of homocysteine, proteome, and ratio of fGSH to fGSSG, transcriptome, and gut microbiota]: at baseline (entire cohort), at 9 weeks (if data available; active treatment group, placebo group, and entire cohort), and when combining data from baseline and 9 weeks (entire cohort).
7) Correlations between each 'biomarker' and each behavioral assessment score (i.e., symptom severity) at baseline (entire cohort), at 9 weeks (active treatment group, placebo group, and entire cohort), and when combining data from baseline and 9 weeks (entire cohort).
8) Correlations between treatment outcomes in the placebo group ('placebo response') and the family characteristics: 'child characteristics' (age, IQ, severity of core symptoms, experience with previous oral treatments and perceived ability to understand the study goals) and 'parent characteristics' (age, education, and parental expectations and believes regarding pharmacological and natural treatments as assessed by the parental expectations and believes 29-item questionnaire).
9) Correlations between gut microbiota and gastrointestinal symptoms as assessed by the 30-item Health State Questionnaire (adverse events questionnaire) – when combining 0- and 9-weeks (entire cohort).
10) Correlations among all assessments including: 'treatment outcomes', 'child characteristics', and 'parent characteristics' (e.g., child QOL, parent QOL, severity of ASD symptoms, etc.).

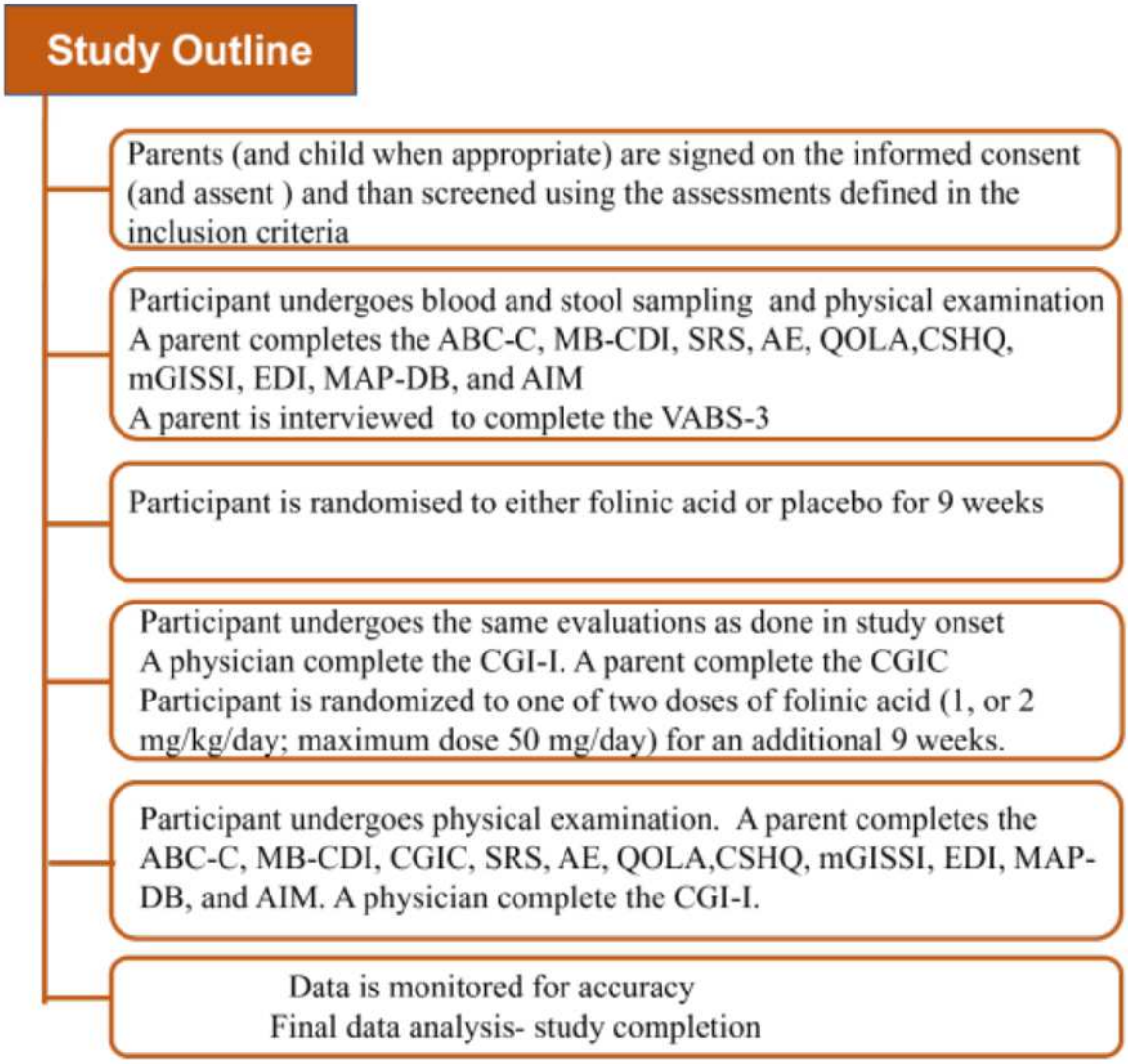
Table 2: Assessment Schedule

Visit Number	**1** Screening	**2** Baseline	**a**	**b**	**c** 5W	**3** 9W	**d** 11W	**4** 17W
Day Number (Visit Window)	**-90 to 1**	**1**	**8±2**	**15±2**	**35±3**	**63±3**	**77±3**	**119±3**
Informed consent/assent	X							
Eligibility criteria[$]	X	X						
Demographics & Medical history	X							
ASD criteria - DSM-5	X							
- CARS-2	X							
Overall severity – CGI-S	X							
Behavioral problems: ABC-I	X	X			X	X		X
Core symptoms: SRS-2	X	X			X	X		X
Nonverbal IQ and DQ	X							
Vital signs	X	X				X		X
Physical exam (including BMI)	X	X				X		X
Concomitant Medications	X	X	X	X	X	X	X	X
Parent expectations and believes		X						
Adverse Events		X	X	X	X	X	X	X
Compliance with Intervention			X	X	X	X	X	X
Blood sampling*		X				X		
Gut microbiota		X				X		
Core symptoms – VABS-3		X				X		
MB-CDI; QOLA; EDI; MAP-DB		X				X		X
Gastrointestinal problems; AIM		X				X		X
Sleep Habits – CSHQ		X			X	X		X
Physician impression of overall change - CGI-I						X		X
Caregiver impression of overall change – CGIC						X		X
Assessment of Blinding Questionnaire						X		

[$] A diagnosis of ASD based on DSM criteria and ADOS-2, core symptoms cannot be explained by the level of intellectual disability (NVIQ), age 3-6 years, weight 11.45-27 kg, CGI-S ≥4, ABCI-I score ≥12, SRS-2 total T score ≥ 66, predicted compliance with study requirements including blood tests and tolerability to taste and smell of the study interventions.

[*] CBC, liver transaminases, total bilirubin; Plasma levels of homocysteine, proteome, and ratio of free reduced glutathione (fGSH) to free oxidized glutathione disulfide (fGSSG); RNA-sequencing of whole blood samples (transcriptome)
At baseline only: Serum levels of folate, vitamin B12 and both blocking and binding folate receptor-α autoantibodies (FRAA).

Figure 2: Study Flow Chart



Statistical Analyses

Statistical Analysis Plan:
A detailed Statistical Analysis Plan (SAP) will be finalized prior to database lock and unblinding.

Sample Size Determination:
The primary endpoint is the change from baseline to week 9 in the Aberrant Behavior Checklist Irritability (ABC-I) score during the placebo-controlled phase. The study is powered to detect a standardized between-group effect size of 0.45 at week 9 with 80% power and a two-sided alpha level of 0.05, using a mixed model for repeated measures (MMRM) incorporating post-baseline assessments at 1 and 2 months.

Based on these assumptions, 65 participants per group are required. To account for an anticipated dropout rate of approximately 15%, 75 participants per group will be enrolled, for a total sample size of 150 participants.

Analysis Populations:
The primary efficacy analysis will be conducted in the modified intent-to-treat (mITT) population, defined as all randomized participants who receive at least one dose of study medication and have at least one post-baseline efficacy assessment. A per-protocol analysis will be conducted as a sensitivity analysis.

Primary Analysis:
The primary endpoint will be analyzed using an MMRM with fixed effects for treatment group, visit, treatment-by-visit interaction, and baseline ABC-I score. The primary treatment effect will be estimated as the between-group difference in change from baseline to week 9, with corresponding 95% confidence intervals and two-sided p values.

Missing Data:
Missing data will be handled under the missing-at-random assumption inherent to the MMRM framework. Sensitivity analyses may include per-protocol analyses and multiple imputation methods.

Secondary Analyses:
Secondary continuous outcomes will be analyzed using similar longitudinal models. Binary outcomes, including the proportion of participants achieving the minimal clinically important difference (MCID), will be analyzed using logistic regression or chi-square tests, with results summarized as odds ratios and 95% confidence intervals. Nominal p values will be presented for secondary and exploratory outcomes.

Additional Analyses:
Vineland Adaptive Behavior Scales, Third Edition (VABS-III), will be analyzed using Growth Scale Values (GSVs), with appropriate handling of age transitions. The second 8-week active-dose phase will be analyzed separately as a secondary outcome phase.

Data Management and Confidentiality

Identifiers- We will use a mathematic manipulation on the participants' ID number (Xor with a 4-digit secret code) to assign a unique 'participant number'. We will use the participant number to avoid exposing personally identifiable information (PII). Only the Principal Investigator and data coordinating manager will have the list connecting the identified data with the participant number. The list will be kept in a locked desktop computer protected by two passwords and in a locked safe in Shaare Zedek Medical Center. Representatives of the IRB and MOH may have access to this identified list upon specific request.

Confidentiality: To protect the privacy of human subjects and maintain the confidentiality of study data we will use a strict separation between identified and non-identified data.
Identified data: consent forms and contact information will not contain 'participant number'. It will be kept in a locked cabinet at Shaare Zedek Medical Center- SZMC. Electronic files of contact information and scans of consent forms will be kept in a locked desktop computer protected by two passwords.
ADOS-2 assessments (if performed) will be videotaped (with legal guardians' consent) to allow evaluation by two independent ratters. Files will be kept in external hard disks at a locked safe. All video files will be completely removed within 12 months after study completion.
All other data, records, and biological samples will be captured and stored un-identified, using the participant number as the sole identifier.
Hard copies of all outcome questionnaires and assessment tools will be stored un-identified (participant number only) in locked cabinets at the study site (SZMC). Biological samples (sera, plasma, and stool samples) will be stored un-identified (participant number only) in locked freezers at the study site. Identified and non-identified data will be kept in different rooms. Only the study PI and the data coordinating manager will have access to study records, data, and specimens. Representatives of the IRB, and MOH are eligible to review study records.

Data capture, verification, and disposition: Study data will be captured using paper and pencil forms that will not contain identified data. Forms will be uploaded to the web application REDCap. REDCap is a secure web application for building and managing online surveys and databases. It can be used to collect any type of data including 21 CFR Part 11, FISMA, and HIPAA-compliant environments. REDCap supports online and offline data capture for research studies and the REDCap Consortium is composed of thousands of active institutional partners in over one hundred countries.
All data (hard copy and electronic) will be stored for 15 years.

Sharing study results: The results of all baseline assessments (CARS-2, nonverbal IQ), blood work, and other data regarding study participation (including study assignments and individual results) will be shared with the primary care providers.

Procedures Description:

The principal investigator (PI) for the clinical investigation is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported in the eCRFs and in all other reports during the clinical investigation

The Site Clinical Coordinator for the clinical investigation is responsible for:

1 Maintaining all of the study-related files: This includes maintaining all logs such as:
   - Ethics Approvals and all communications with the ethic committees (such as protocol amendments and serious adverse events reports)
   - Protocol Deviation Log
   - Subject Informed Consent (SIC) Signature Log
   - Investigation Team Log
2 Correct filling of the eCRFs based on source documentation and live documentation
3 Maintaining contact with study subjects, and coordinating study visits

At the start of the clinical study, the Clinical Coordinator will make sure that all study files for the following:

- IRB/EC/Helsinki Committee Documents.All IRB Correspondence is on file.The study staff are IRB approved prior to performing any study procedures.Adverse events and deviation procedures for reporting to IRB per current guidelines are in place.All versions of the IRB protocols and informed consent forms are on file.Signed Good Clinical Practice compliance form, financial disclosure and CV for the PI and sub investigators.

Other documents:

- CVs for all study staff are on file and updated within the last 2 years
- Medical licenses for the Sub-Investigators are on file and updated prior to the expiration
- Delegation Log is updated and procedures for updating the log with new staff are in place
- All other essential documents have been prepared and completed as appropriate

Before, during, and at the close of the study period, study documentation will be monitored by the Clinical Coordinator for the following:

- Informed Consent Form (ICF)
    - Ensure that subject identification is on all pages of the ICF, if applicable
    - Documentation of subject receiving a copy of the consent form is present
    - Clinic note documents informed consent process
    - The parents or a legal guardian of the subject and study representative signed and dated the consent form for their self
    - Note to file completed for any informed consent deviations.
    - Ensure a valid (current version date) copy of the consent form was used

Protocol:

- Confirm that the study staff is conducting the study in compliance with the protocol approved by IRB/CE/Helsinki Committee and are trained in Good Clinical Practices (GCP).
- The protocol deviations (exceptions and violations) are documented in the subject chart and reported to IRB/CE/Helsinki Committee as required.
- Ensure any deviations are logged in the form *'Protocol Deviations'*

Source Documents:

- Review subject charts to ensure accuracy, completeness, and legibility of the data

- Any correction made to the source documents is dated, initialed, and explained. The original entry should not be obscured.
- The protocol-specific source documents are on file.
- Source documents are completed in ink or electronically.
- Note to files are generated for missing or incomplete data, and to explain any discrepancies or additional comments.

Case Report Forms:

- Ensure that the data reported on the CRF is consistent with the source documents.
- Discrepancies between the source documents and eCRF are explained in a note to file or captured in a comment in the eCRF.

Biological samples

Biological samples will be labeled with participant numbers and stored in locked freezers. The legal guardians will decide in the consent form if the samples will be kept after the termination of the study for possible use in further studies or be destroyed upon termination of the study.

Blood samples

Blood (6 ml in tubes with procoagulant, 6 ml in tubes with EDTA, and 3 ml in Tempus™ Blood RNA Tube) will be collected at the baseline (9-11 AM) and at 2-month (on 9:00- 11:00 AM, 3-4 hours post treatment administration).
Assessments will include:

- CBC, liver transaminases, total bilirubin
- Plasma levels of homocysteine and ratio of free reduced glutathione (fGSH) to free oxidized glutathione disulfide (fGSSG)
- Serum levels of folate, vitamin B12 and both blocking and binding folate receptor-α autoantibodies (FRAA).
- RNA-sequencing of whole blood samples (transcriptome), and plasma proteomics will be performed pre and post-treatment to discover treatment-responsive biomarkers which correlate with behavioral improvement.

Stool samples

Stool samples will be collected using OMNIgene GUT sample collection kit (DNA Genotek, Canada). This kit homogenizes the sample and stabilizes the DNA for up to 60 days at room temperature. Participants will receive the kits in advance: a first kit during the screening visit, a second kit during the 2nd or 3rd. Participants will be instructed to use the kits within 2 weeks prior to their next visit (baseline, 9 weeks) and bring them with them to the next visit. Stool samples will not be collected if the participant had received any antibiotic treatment during the 4 weeks prior to the sampling day. Any antibiotic treatment, special diets, and use of probiotics during the 4 to 8 weeks prior to the sampling day - will be documented.

Informed Consent Process

Subjects' legal guardians interested in having their children participate in the clinical investigation will be screened by the participating physician.
Subjects' legal guardians will then be given a copy of the Patient Informed Consent with ample time to read and review the document and ask questions before signing.
Subjects will meet with one of the investigators to discuss investigational participation and any questions they may have regarding the investigation, the investigational device, and the informed consent.
The informed consent will be signed by the legal guardian, during the meeting with one of the participating investigators.
A photocopy of the signed patient informed consent form will be provided to the legal guardian prior to the procedure.
Subjects requesting to withdraw consent will be disqualified from the investigation at no cost or consequence

All subjects in this study are pediatric subjects (3-6 years) with ASD and are therefore unable to sign an informed consent. A legal guardian will sign the informed consent. Assent form will be obtained from any participant who is able to give it.

Vulnerable Population

This clinical investigation will be conducted on pediatric (3-6 years old) ASD populations. Vulnerable patients' legal guardians will be responsible for patient informed consent.

Data and Safety Monitoring Board (DSMB)

An independent Data and Safety Monitoring Board (DSMB) will oversee the conduct of the trial. The DSMB is responsible for monitoring study progress, including data quality, recruitment, retention, and the overall risk–benefit balance. It will also consider external developments relevant to participant safety and research ethics. The DSMB will review interim analyses as needed, although no formal stopping rules are defined for this study, and will ensure that all trial data remain confidential. The DSMB will provide regular reports to the Bazelet Group regarding participant safety and trial progress, and will make recommendations on whether the study should continue as planned, be modified, or be terminated. In addition, the DSMB will advise the Bazelet Group on issues related to study conduct, enrollment, or data integrity. The DSMB will conclude its responsibilities upon completion of follow-up for the final participant.

Randomization and blinding

Eligible subjects will be assigned an enrollment number in sequential order beginning with 01. Randomization will be performed using a computer-generated stratified permuted block randomization scheme generated by an external unblinded biostatistician. Stratification will be based on sex and developmental quotient (DQ; ≥ 70 or <70), resulting in four strata. Within each stratum, permuted blocks of varying sizes will be used to maintain allocation balance while preserving allocation concealment.
Two separate computer-generated randomization lists will be prepared for each stratum: one for Treatment Period 1 and one for Treatment Period 2. These lists will be generated independently, allowing each participant to receive allocations for both treatment periods while maintaining balance within each stratum and preserving independence between allocations in the two treatment periods.
For Treatment Period 1, participants will be randomized in a 1:1 ratio to receive either folinic acid at a target dose of 2 mg/kg/day (maximum 50 mg/day) or matching placebo. For Treatment Period 2, participants will undergo a separate and independent 1:1 allocation to receive folinic acid at either 2 mg/kg/day (maximum 50 mg/day) or 1 mg/kg/day.

The study active treatments as well as the placebo treatments will be packed in similar bottles and packages and will have similar appearance, taste, and smell.

The code key for the active/ placebo labels will be kept by the external biostatistical until completion of the study and database lock. Neither the principal investigator nor any other team member will have access to the treatment codes until study-end.

To assess the success of blinding , caregivers and researchers will complete an Assessment of Blinding Questionnaire at the end of each treatment period.

Amendments to the Protocol

The study coordinator will ensure that
1 Amendments to the Clinical Investigation Plan are documented, using the form 'Amendment and Revision Sheet'
2 Approval is documented, using form 'Clinical Investigation Document Approval Log''
The amended protocol is sent to all investigators and the EC/IRB/Helsinki Committee after approval

Deviations from the Protocol

Neither the Principal Investigator nor any of the sub-investigators are authorized to deviate from the protocol, except as specified in 4.5.4 b of ISO 14155:2011 and 21 CFR 812.150 (a)(4), specifically requests for deviations, and reports of deviations, if the deviation affects subject's rights, safety, and wellbeing, or the scientific integrity of the clinical investigation.
Under emergency circumstances, deviations from the protocol to protect the rights, safety, and well-being of human subjects may proceed without prior approval of the sponsor and the EC/IRB/Helsinki Committee. Such deviations shall be documented and reported to the sponsor and the EC/IRB/Helsinki Committee as soon as possible.
The research coordinator will prepare the EC/IRB /Helsinki Committee Notifications letter using the EC Notification Form if any reportable serious deviations or adverse events were recorded and send them to the Ethics Committee/Institutional Review Board/Helsinki Committee and Principal Investigator, according to this protocol.
All notifications will be carried out within the time frames specified by local regulatory authorities.

Statements Of Compliance

1	This clinical investigation will be conducted in accordance with ethical principles stated in the Declaration of Helsinki and in compliance with all national and local regulatory requirements for the safety and protection of human subjects.
2	This clinical investigation will be implemented only upon receiving all final notification/authorizations from local and national regulatory and Helskinki/EC/IRB authorities, as appropriate.
3	This clinical investigation will comply with any additional requirements set forth by the Helsinki/EC/IRB and/or regulatory authorities.
4	This clinical investigation provides clinical study medical insurance for study participants as required by local and national regulatory authorities.
5	This protocol was written in accordance with the ISO 14155:2011 Standard for Clinical investigation of medical devices for human subjects – Good Clinical Practice

Early Termination or Suspension of the Clinical Investigation

Grounds for suspension or premature termination of the clinical investigation include but are not limited to:

- Non-compliance of the investigational site personnel to the protocol and or investigational procedures
- Serious Adverse Events that demonstrate danger to the patient population
- Unethical performance on the part of any of the investigators
- Failed audit of investigation with serious flaws to study design, performance or outcomes
- Business/Financial issues that cannot be resolved
- Other unforeseen events that give the PI reasonable doubts to the safety of the participants which cannot be resolved in a reasonable time

Publication Policy

The results of this clinical investigation may be published in a mainstream peer-reviewed professional journal as per Chapter I. of the agreement between the Shaare Zedek Medical Center and the Sponsor

Adverse Events

An **adverse event** is any undesirable or unintentional or unexpected event (sign, symptom, illness, abnormal laboratory value, or other medical events) that occurs during the course of the study, whether or not considered related to the study interventions. This definition includes events related to the investigational treatments and also includes events related to the procedures involved.

Regardless of the relationship to the investigational treatments, all moderate to serious adverse events occurring from the time that the informed consent is signed must be recorded in the patient's CRF.

A **mild adverse event** is one that the symptom does not influence performance, require drug treatment or prevent the patient from carrying on with normal life activities.
A **moderate adverse event** is one that the symptoms make the patient uncomfortable and causes some impairment to normal life activities and require treatment for symptom(s).
A **serious adverse event** is an adverse event that:

- Led to death
- Led to a serious deterioration in the health of the subject, that either resulted in
    - A life-threatening illness or injury, or
    - A permanent impairment of a body structure or body function, or
    - In-patient or prolonged hospitalization, or
    - Medical or surgical intervention to prevent life-threatening illness or injury or permanent impairment to a body structure or a body function

In the event of an emergency resulting from the clinical investigation, all emergency treatment will be provided at no cost to the patient. Clinical Investigation Insurance has been provided to ensure all costs be covered if necessary.

The principal investigator will notify the sponsor immediately (within 24 hours) of any serious adverse event. All reports to the appropriate authorities will be made by the principal investigator as set forth in the standard clinical practices outlined in the 21 CFR (§ 812.150(a)(1), § 812.46(b) and § 812.150(b)(1))/ MEDDEV 2.7/3 Dec 2010 and ICH-GCP Guidelines.

Adverse Events will be reported and treated as per the standard clinical practices outlined in the 21 CFR (§ 812.150(a)(1), § 812.46(b) and § 812.150(b)(1))/ MEDDEV 2.7/3 Dec 2010 and ICH-GCP Guidelines

Anticipated adverse events include rare insomnia, irritability, and intolerance of the taste and smell.

Appendix 1 – non-generic questionnaires

The Hebrew version of the following questionnaires appears in the investigator brochure.

Demographics and medical history questionnaire…….……31

Health State Questionnaire (adverse events) ……...……...34

Parental expectations and beliefs……...…...……35

Assessment of Blinding Questionnaire ……36

Gastrointestinal signs and symptoms inventory …….....37

Folinic acid treatment in children with ASD – Demographic questionnaire

Date: _____ GUID: _____ Informant: Mothe / Father / Guardian

1. **Has your child regressed in his/her development or communication?** No/Yes
   Please describe at which age and the nature of the regression: _____
   _____

2. **When your child has a high temperature, does she/ he have a substantial improvement in communication?** No/Yes (Please specify) _____
   _____

3. **Has your child had a genetic testing?** No/Yes
   CMA (chip) - No/Yes /unknown; **Exome sequencing** - No/Yes /unknown

4. **Does your child have a genetic diagnosis?** No/Yes _____

5. **Does your child suffer from a syndromic ASD?** (Such as Down syndrome or Angelman syndrome, or substantial deficits in motor function or cardiac problems, or unusual facial features) – No/Yes (Please specify _____

6. **Does your child have a sibling or an uncle with ASD (even mild)?** No/Yes (Please specify) _____

7. **Does your child have a sibling or an uncle with intellectual disability?** No/Yes (Please specify) _____

8. **Birth week**: _____: Normal / 35-36/ 32-34/ 29-31/ 23-28

9. **Birth weight:** _____ Appropriate for Gestational Age /
   Small for Gestational age/ Large for Gestational age

10. **Curse of delivery:** Normal / Cesarean / Vacuum/ Forceps, **APGAR**: _____

11. **Was your child transferred to NICU after the labor?** No / Yes-less than 24 hours / 2-7 days / 8-13 days / 2-4 weeks / longer than 4 weeks _____

12. **Was you child ever hospitalized in ICU:** No/Yes (Please specify)
    _____

13. **Was there a serious problem in pregnancy or childbirth or a serious illness / accident in the first two years of life? (**Severe complications of prematurity, meningitis or encephalitis with prolonged hospitalization in intensive care, drowning or suffocation, severe accident) No / Yes_____

14. **How old was your child when he was first diagnosed with ASD?** _____

15. **How old was your child when he/she started attending a special education program?**
    _____

16. **Did your child suffer (for more than 6 months) from any of the following problems?** (Please mark all that applies)
Significant behavioral difficulties / Anxiety/ Significant sleep disorder / ADHD / Epilepsy / High sensitivity to noise, smell or touch /Unusual seeking of stimulations: smell, touch, sight / Low motor skills / GI problems / Food allergy / Food sensitivity

17. **Does your child have any other chronic diseases?** No/Yes _____

18. **Has your child ever taken any medications on a regular basis (for more than a month)**? No/Yes (Please mark all that applies)

Antipsychotics: Neuleptil (Pericyazine) / Rispond, Risperdal (Risperidone) / Abilify, Ariply (Aripiprazole) / Entumine (Clothiapine) / Zyprexa (Olanzapine) / Promethazine (Prothiazin) / Seroquel (quetiapine) / Ziprasidone (Geodon) /
Nozinan (levomepromazine) / clozapine

Stimulants: Ritalin, Concerta, Focalin / Attent, Vyvanse

SNRI: Atomic, Strattera

alpha-2 agonists: Clonirit (clonidine), Intuniv (Guanfacine)

Mood stabilizers: Depalept (Valproic acid) / Tegratol, Timonil (Carbamazepine)

Benzodiazepines: Rivotril, Clonex (Clonazepam) / Xanax (alprazolam)

SSRIs: Cipralex, Lexapro (escitalopram), Prozac, Prizma, Flutine (fluoxetine), Favoxil (fluvoxamine), Seroxat (paroxetine), Lustral, Serenada, Zoloft (sertraline)

Other: _____

19. **Is your child currently taking any medications on a regular basis?** No/Yes

1.Name: _____ daily dose _____ since _____
2.Name: _____ daily dose _____ since _____
3.Name: _____ daily dose _____ since _____
4.Name: _____ daily dose _____ since _____
5.Name: _____ _____ daily dose _____ since _____

20. **Has your child ever taken any nutritional supplements (for more than a month)?** No/Yes (Please mark all that applies)

Vitamins: Folic Acid / Folinic Acid / B12 / B6 / Vitamin D
Minerals: Magnesium / Iron / Calcium
Other: Carnitine (L-carnitine, Acetyl carnitine) / Sulforaphane, Glucoraphanin
Other: Melatonin / Probiotics/ Omega – 3
Other: _____

21. **If any of the mentioned nutritional supplements were discontinued in the past 3 months, please specify which and when**: _____

22. **Has your child received antibiotics in the past two months**?
No/Yes (Please specify Name, daily dosage and for how long) _____
_____

23. **Has your child currently taking any nutritional supplements?** No/Yes

1.Name: _____ daily dose _____ since _____
2.Name: _____ daily dose _____ since _____
3.Name: _____ daily dose _____ since _____
4.Name: _____ daily dose _____ since _____
5.Name: _____ daily dose _____ since _____

24. **Please specify the amount of cow milk (not dairy products but actual milk) that your child consumes per day:**
Not at all / Up to half a glass / about one glass / about two glasses / 3 glasses or more

25. **Is your child currently on a special diet?** (Gluten-free / Low Gluten / Lactose free / Low Carbs (modified Atkins)
No/Yes (Please specify Which and for how long) _____
_____

26. **Has your child ever taken medical Cannabis / CBD / Cannabis products?**
No/Yes (Please specify for how long he/she took it and when was it discontinued)

1. Licensed Medical Cannabis which mostly contains CBD (20:1, 15:3, 24:0, 28:1) for _____ months. Discontinued _____ years and _____months ago.
2. Medical Cannabis which mostly contains THC (such as: night oil, Erez, Alaska) for _____ months. Discontinued _____ years and _____months ago.
3. Other: _____
for _____ months. Discontinued _____ years and _____months ago.

27. **Is your child currently receiving behavioral/emotional therapy?** No/Yes

1. _____. Hours per week: _____. Since _____
2. _____. Hours per week: _____. Since _____
3. _____. Hours per week: _____. Since _____
4. _____. Hours per week: _____. Since _____
5. _____. Hours per week: _____. Since _____

28. **Please specify all the schools/ education programs your child attended:**

Age _____. Program: _____
Age _____. Program: _____
Age _____. Program: _____
Age _____. Program: _____
Age _____. Program: _____
Age _____. Program: _____
Age _____. Program: _____

29. **Parents education (years)**: Mother: _____ Father: _____

30. **Parents occupation**: Mother: _____ Father: _____

Health State Questionnaire (modified Dosage Record Treatment Emergent Symptom Scale)

Date: _____ GUID: _____ Informant: Mothe / Father / Guardian

During the last 2 weeks (or 4 days, if at study onset), has your child had any of the problems listed?
If you didn't mark 'No', please indicate if the problem was mild, moderate, or severe. If you think that this problem is related to the study treatment, please mark it as well.

	Health Problem	**No**	**Rarely**	**Sometimes**	**Always**	**mild**	**moderate**	**Severe**	**Treatment related**
1	Dizziness								
2	Drowsiness								
3	Restlessness								
4	Aggression								
5	Increased tantrums								
6	Emotional liability								
7	Hair loss								
8	Rash								
9	Blurred vision								
10	Nausea								
11	Vomiting								
12	Abdominal pain								
13	Concentration problem								
14	Dry mouth								
15	Tremors								
16	Headache								
17	Insomnia								
18	Involuntary movements								
19	Depression								
20	Memory problems								
21	Constipation								
22	Loss of appetite								
23	Gain of appetite								
24	Diarrhea								
25	Nasal congestion								
26	Sweating								
27	Weakness								
28	Increased salivation								
29	Increased anxiety								
30	Confusion								
31	Suicidal thoughts								

During the last 2 weeks (or 4 days, if at study onset) Please indicate:
Any other important health problems: _____
Any use of medical services: _____
Any change in the study treatment dose: _____
Any change in the regular medications: _____
Any change in the regular diet or intake of food supplements (including probiotics): _____
Any change in the home or school routine or in the behavioral/ emotional treatments: _____

Do you think the current treatment is helpful: Yes / No
If you answered Yes, please specify in what way: _____

Parental expectations and beliefs

The following table presents various statements. Please indicate to what extent do you agree with each statement (your answers will have no effect on your participation in the study)

#	Statements	Strongly Agree	Quite agree	Not so agree	Not at all agree
1	When I give my child medicine (for example to relieve pain) he/she usually understands that the treatment may help him/her.				
2	My child can understand that the treatment given in the study may improve his / her behavior.				
3	My child can understand that the treatment given in the study may improve his / her communication difficulties.				
4	My child's condition in the last month is worse compared to his average condition				
5	It is hard for me to deal with my child's behavioral difficulties (I am losing patience)				
6	When I am calm and patient my child is usually calmer too				
7	I believe that the treatment given in the study may **greatly** improve my child's communication difficulties.				
8	I believe that the treatment given in the study may **greatly** improve my child's behavioral difficulties				
9	I personally know a family that treatment similar to the one given in the study - helped her a lot.				
10	In the last year, my child received natural or medicinal therapy that helped him/ her with his/ her behavioral difficulties.				
11	In the last year, my child received natural or medicinal therapy that helped him/ her with his/ her communication difficulties.				
12	In the last year my child received natural or medicinal treatment for the behavioral difficulties which was discontinued due to side effects				
13	My child is relatively sensitive to side effects of medications				

Questions for the child participating in the study

- What do you see in the picture?
- Why do you think the child is taking medicine? (Maybe his tummy hurts and they want to help him feel better, maybe he has trouble behaving nicely and they want to help him)
- How can the medicine help the child?
- Do you sometimes have trouble behaving nicely?
- We want to give you a treatment that will help you feel calmer and behave more nicely. Do you think that will help you?
- We also believe the treatment can help you with your communication, like talking, understanding, and playing with others. Do you think that will help you?

Rank (from 1-10):
A. The child's ability to understand that the medicine given to him will improve his behavioral difficulties _____
B. The child's expectations that the treatment will help with his behavior problems _____

Assessment of Blinding Questionnaire

1) **Please select the option that best reflects your beliefs regarding the treatment that your child received in the study**:
   A) I am convinced that the treatment was 'real treatment'
   B) It seems to me that the treatment was 'real treatment'
   C) It seems to me that the treatment was 'placebo'
   D) I am convinced that the treatment was 'placebo'
   E) I really do not know

2) **If you chose A, B, C, or D, please tell us why you chose this option (you can choose more than one option)**
   A) Due to the appearance, smell, or texture of the treatment. please specify: _____
   _____
   B) Due to the effectiveness of the treatment
   C) Due to the inefficiency of the treatment
   D) Due to the side effects of the treatment. Please indicate which side effects: _____
   _____
   E) I have such a feeling but I cannot point to the reason

Gastrointestinal signs and symptoms inventory (modified GISSI-17)

Participant research ID_____

Your relationship to this child: ☐Mother ☐Father ☐Other: _____

Most of the questions on this form are about THE LAST SIX WEEKS.
Please put a check (V) in the box that best describes your child.

In the last 6 weeks, has your child experienced any of the following gastrointestinal (tummy) symptoms:

	Yes	No	Unsure
1. Abdominal (belly) pain	☐	☐	☐
2. Nausea	☐	☐	☐

3. **In the last 6 weeks,** how often did your child usually have BMs?
   a. ☐ Less than once a week
   b. ☐ 1-2 times a week
   c. ☐ 3-6 times a week
   d. ☐ Once a day
   e. ☐ 2-3 Times a day
   f. ☐ More than 3 times a day
   g. ☐ Unsure

4. **In the last 6 weeks**, what were your child's BMs usually like?
   a. ☐ Very hard
   b. ☐ Hard
   c. ☐ Not too hard and not too soft
   d. ☐ Very soft or mushy
   e. ☐ Watery
   f. ☐ Unsure

In the last 6 weeks, did your child:	Yes	No	Unsure
5. Appear to feel pain when having a BM?	☐	☐	☐
6. Have to rush to the bathroom for a BM?	☐	☐	☐

In the last 6 weeks, has your child:	Yes	No	Unsure
7. Spit up ≥ 2x per day?	☐	☐	☐
8. Experienced retching?	☐	☐	☐
9. Tilted his/her head to the side and arched back?	☐	☐	☐

In the last 6 weeks, has your child missed activities due to:	**Yes**	**No**	**Unsure**
10. pain and/or discomfort?	☐	☐	☐
11. Vomiting	☐	☐	☐
12. problems with BMs	☐	☐	☐
13. **In the last 6 weeks**, did your child push his abdomen with his/her hands or your hands, push his/her abdomen against or lean forward over furniture?	☐	☐	☐
14. **In the last 6 weeks**, did your child choke, gag, cough, or sound wet during or after swallowing or with meals?	☐	☐	☐
15. **In the last 6 weeks**, has your child started to refuse many foods that he or she would eat in the past?	☐	☐	☐

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