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Research Paper

# A Double-Blind, Randomized, Placebo-Controlled Clinical Study of Trofinetide in the Treatment of Fragile X Syndrome

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ABSTRACT

*Background:* We analyze the safety and tolerability of trofinetide and provide a preliminary evaluation of its efficacy in adolescent and adult males with fragile X syndrome.
*Methods:* This study was an exploratory, phase 2, multicenter, double-blind, placebo-controlled, parallel group study of the safety and tolerability of orally administered trofinetide in 72 adolescent and adult

Conflicts of interest: E.B.-K. participated in the Neu-2566-FXS 001 trial, has been a consultant for Seaside Therapeutics, Novartis, Roche, Alcobra, Neuren, Cydan, Fulcrum, GW, Neurotrope, Marinus, Zynerba, BioMarin, and Ovid Pharmaceuticals regarding conduct of clinical trials in FXS and other rare neurological diseases, is on the Scientific Advisory Board for Vtesse/Mallinckrodt to provide guidance on clinical trials in Niemann-Pick type C (NP-C), has received research funding to conduct clinical trials in FXS from Novartis, Roche, Seaside Therapeutics, Alcobra, Neuren, Ovid, and Zynerba to conduct a clinical trial in NP-C from Vtesse/Mallinckrodt, and to develop FMR1 testing standards from Asuragen. She was also a participating principal investigator on a clinical trial in Rett syndrome sponsored by Neuren Pharmaceuticals. She has research finding through grants from NIH, CDC, FRAXA Research Foundation, International Rett Syndrome Foundation, and the Phelan McDermid Foundation. She was consultant to GCC on the project funded by Neuren Pharmaceuticals for the psychometric analysis of the FXRS. J.P.H. was an employee at Neuren Pharmaceuticals, Inc. during the conduct of the research. He is currently an employee at AMO Pharma Ltd., which was not involved in the clinical trial reported in this manuscript. N.T. participated in the Neu-2566-FXS 001 trial and has received research funding from NICHD and CDC, and for industry-sponsored clinical trials in autism and/or fragile X from Seaside Therapeutics, Roche, Neuren, Alcobra, Zynerba, and Ovid. She has consulted with Zynerba and Ovid on clinical trial design and outcome measures in FXS. R.H. participated in the Neu-2566-FXS 001 trial and receives research funding from NICHD, Azrieli Foundation, Curemark, and Zynerba currently regarding studies in FXS, autism, or premutation involvement. She has consulted with Zynerba, Fulcrum, Ovid, Novartis, and Roche in the past and has carried out studies funded by Marinus, Roche, Novartis, Seaside Therapeutics, Forest, and the National Fragile X Foundation in the past. A.K. participated in the Neu-2566-FXS 001 trial and receives research funding from the National Institute of Neurological Disorders and Stroke, New York Community Trust, AMO Pharma, and the Seaver Foundation. He has been a consultant for Ovid Therapeutics, Genentech, Supernus Pharmaceuticals, Fulcrum Therapeutics, Coronis, 5AM Ventures, Labcorp, and sema4. C.A.E. participated in the Neu-2566-FXS 001 trial. He has received current or past funding from Confluence Pharmaceuticals, Novartis, F. Hoffmann-La Roche Ltd., Seaside Therapeutics, Riovant Sciences, Inc., Fulcrum Therapeutics, Neuren Pharmaceuticals Ltd., Alcobra Pharmaceuticals, Neurotrope, Zynerba Pharmaceuticals, Inc., and Ovid Therapeutics Inc. to consult on trial design or development strategies and/or conduct clinical trials in FXS or other neurodevelopmental disorders. C.A.E. is additionally the inventor or coinventor on several patents held by Cincinnati Children's Hospital Medical Center or Indiana University School of Medicine describing methods of treatment in FXS or other neurodevelopmental disorders. S.H. participated in the Neu-2566-FXS 001 trial. M.S. was a consultant for Neuren Pharmaceuticals for the reported study. He is an employee of AMO Pharma Ltd., which was not involved in the presently reported study. A.Y. is a Biostatistician for Vital Systems, Inc., a contract research organization utilized by Neuren for the trial described in this article. G.S. is President of Vital Systems, Inc., a contract research organization utilized by Neuren Pharmaceuticals for the conduct and analysis of this clinical trial, the Rett-001 and Rett 002 trials of trofinetide in Rett syndrome, and trials in traumatic brain injury sponsored by Neuren. L.G. and N.E.J. are executives at Neuren Pharmaceuticals Ltd.

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males with fragile X syndrome. Subjects were randomly assigned in a 1:1:1 ratio to 35 or 70 mg/kg twice daily trofinetide or placebo for 28 days. Safety assessments included adverse events, clinical laboratory tests, vital signs, electrocardiograms, physical examinations, and concomitant medications. Efficacy measurements were categorized into four efficacy domains, which related to clinically relevant phenotypic dimensions of impairment associated with fragile X syndrome.

*Results:* Both 35 and 70 mg/kg dose levels of trofinetide were well tolerated and appeared to be generally safe. Trofinetide at the 70 mg/kg dose level demonstrated efficacy compared with placebo based on prespecified criteria. On the basis of a permutation test, the probability of a false-positive outcome for the achieved prespecified success was 0.045. In the group analysis, improvement from treatment baseline was demonstrated on three fragile X syndrome–specific outcome measures.

*Conclusions:* Trofinetide was well tolerated in adolescent and adult males with fragile X syndrome. Despite the relatively short duration of the study, a consistent signal of efficacy at the higher dose was observed in both caregiver and clinician assessments, based on a novel analytical model incorporating evaluation of multiple key symptom areas of fragile X syndrome. This finding suggests a potential for trofinetide treatment to provide clinically meaningful improvement in core fragile X syndrome symptoms.



## Introduction

Fragile X syndrome (FXS) is a genetically determined neurodevelopmental disorder with a prevalence of about 1 in 4000 males and 1 in 6000 females.[1] FXS is characterized by intellectual disability, autism spectrum disorder (ASD), emotional dysregulation, and a characteristic behavioral phenotype.[2] The behavioral phenotype in FXS can be distinguished from intellectual disability of differing etiology[3] with characteristic dysfunction including attention deficit hyperactivity, hyperarousal, and anxiety, as well as perseverative and aggressive behaviors.[4,5] Up to two thirds of males and a quarter of females also meet criteria for ASD.[6,7]

FXS is caused by expansion of a cystosine, guanine, guanine repeat (more than 200 repeats) in the promoter region of the *FMR1* gene on the X chromosome, triggering partial or complete transcriptional silencing and partial or complete lack of the fragile X mental retardation protein.[8] Patients with FXS show altered neuronal dendritic spine morphology.[9,10] Dendritic spine morphology is under the control of the phosphoinositide 3-kinases-Akt-mammalian target of rapamycin and Ras-mitogen-activated protein kinases signaling pathways,[11] which are aberrantly activated in patients with FXS.[12,13] Loss of *Fmr1* function in astrocytes induces the FXS neuronal phenotype in mouse models, and normal astrocytes can rescue this abnormality.[14]

Findings from FXS mouse models have demonstrated that trofinetide treatment modulates disease symptoms.[15] Trofinetide (glycyl-L-2-methylprolyl-L-glutamic acid) is an analogue of the amino-terminal tripeptide of insulin-like growth factor 1 (IGF-1[1-3]). It is postulated to diminish neuroinflammation, reduce microglial activation and astrogliosis, normalize protein synthesis and dendritic morphology, and restore homeostasis of excitatory and inhibitory neuronal signaling.[15-18] In a study of trofinetide in the *Fmr1* knockout phenotype, untreated mutant mice displayed hyperactivity, social behavior dysfunction, macroorchidism, reduced levels of brain IGF-1, and overactivation of extracelluar signal-regulated kinases and Akt, intracellular signaling molecules playing a crucial role in synaptic plasticity.[15] Trofinetide (100 mg/kg intraperitoneal for 28 days) normalized the *Fmr1* knockout phenotype in all behavioral, morphologic, and biochemical measures assessed. It rescued abnormal dendritic morphology, neuroinflammation and glial activation, normalized brain IGF-1 levels, and reduced abnormal activation of the Ras-mitogen-activated protein kinase-extracelluar signal-regulated kinases and phosphoinositide 3-kinases-Akt-mammalian target of rapamycin signaling pathways. No significant effects were observed in wild-type animals.

Here we report the first clinical study of trofinetide for the treatment of FXS. The study examined the safety, tolerability, bioavailability, and efficacy of treatment with trofinetide. It provided insight into efficacy measures for future studies and models for analyzing treatment effect across multiple domains.

## Methods

### *Study design*

The present study was an exploratory, phase 2, multicenter, double-blind, placebo-controlled, parallel group study of the safety and tolerability of oral treatment with trofinetide in adolescent and adult males with FXS (aged 12 to 41 years). After the screening period subjects were randomly assigned (1:1:1) to 35 mg/kg trofinetide, 70 mg/kg trofinetide, or placebo twice daily for 28 days. All subjects were first administered single-blind treatment with placebo for 14 days. At the completion of this run-in period, subjects began 28 days of double-blind treatment based on their randomization group (Table 1).

The study design and subject disposition are shown in Fig 1.

The study enrolled participants aged 12 to 45 years. All subjects had a clinical diagnosis of FXS and a molecular confirmation of the full *FMR1* mutation (more than 200 cystosine, guanine, guanine repeats). See the clinicaltrials.gov listing and Supplementary Table S1 for detailed eligibility criteria (NCT01894958). Caregivers recorded medications, behavioral treatments, and seizure frequency in a paper diary to ensure that these had been stable for at least four weeks before the first dose of study medication. Baseline assessments occurred after a subject's eligibility was confirmed and before the first dose of single-blinded study medication. Guardians of all patients signed informed consent before any study

**TABLE 1**
Dosing Schedule

| Day of Study | 35 mg/kg b.i.d. | 70 mg/kg b.i.d. | Placebo b.i.d. |
|---|---|---|---|
| 1-14 | Placebo b.i.d. | Placebo b.i.d. | Placebo b.i.d. |
| 15-42 | 35 mg/kg b.i.d. | 70 mg/kg b.i.d. | Placebo b.i.d. |
| 43-56 | No treatment | No treatment | No treatment |

Abbreviation:
b.i.d. = Twice daily

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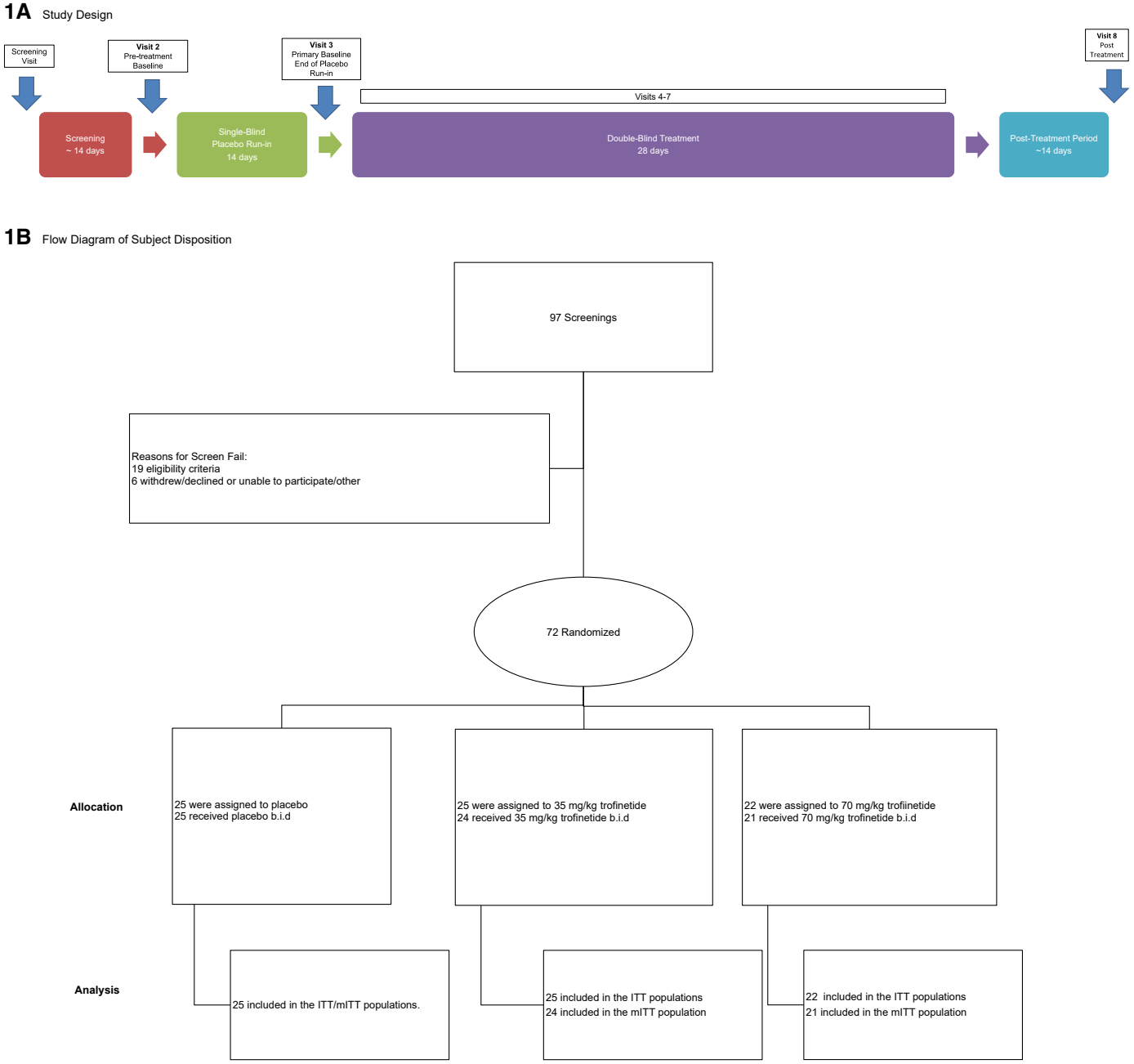


**FIGURE 1.** (A) Study design and (B) subject disposition. ITT, intent to treat; mITT, modified intent to treat. The color version of this figure is available in the online edition.

procedures, and patients signed assent as appropriate. The study was approved by a central or local institutional review board for all participating sites.

### *Safety assessments*

Safety assessments included adverse events (AEs), laboratory tests (urinalysis, hematology, chemistry, [hemoglobin A1c, electrolytes, minerals, protein, lipids, and thyroid, renal, and liver function]), vital signs, electrocardiograms (EKGs), physical examinations, fundoscopy and tonsil size, and concomitant medications. Dosing compliance, seizures, changes in tolerability, and concomitant medications were monitored in a caregiver diary.

### *Efficacy measurements and end points*

Outcome measures assessed clinically relevant dimensions of impairment associated with FXS.

The efficacy variables used in this study were categorized into five domains and for statistical analysis, further characterized into priority levels of core, secondary, and exploratory as shown in Table 2.

### *Core efficacy end points*

The primary efficacy analysis was conducted on core end points, which were prespecified in the Statistical Analysis Plan before unblinding of treatment codes. Core end points included five measures from three efficacy domains, as described subsequently.

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**TABLE 2**
Outcome Variables by Efficacy Domain

| Efficacy Domain | Description | Type of Variable | Variable |
|---|---|---|---|
| 1 | Clinician-completed syndrome-specific symptom measures | Core | • FXSRS total score<br>• FXSDSC total score |
| | | Secondary | • FXSRS subscale scores<br>• FXSDSC individual domain scores |
| 2 | Clinician-completed syndrome-specific global measures | Core | • CGI-I score |
| | | Secondary | • CGI-S score |
| 3 | Clinician-completed non−syndrome-specific measures | Secondary | • CYBOCS-ASD score<br>• VABS-II composite and domain scores |
| 4 | Caregiver-completed measures | Core | • Caregiver top three concerns total score<br>• ABC-$C_{FX}$ total score |
| | | Secondary | • ABC-$C_{FX}$ subscale scores<br>• CASI-20 score |
| 5 | Functional and physiological measures | Secondary<br>Exploratory | • KiTAP subtest scores<br>• Change in pupil diameter<br>• Time spent on eyes/face<br>• Number of fixations to eyes/face<br>• ELS narrative variables<br>• ELS conversation variables<br>• Blood biomarkers (peripheral Akt and extracelluar signal-regulated kinases) |

Abbreviations:
ABC-$C_{FX}$ = Aberrant Behavior Checklist-community version, fragile X scoring
CASI-20 = Child and Adolescent Symptom Inventory-20 item anxiety subscale
CGI-I = Clinical Global Impression-Improvement
CGI-S = Clinical Global Impression-Severity
CYBOCS-ASD = Children's Yale-Brown Obsessive Compulsive Scale-autism spectrum disorders
ELS = expressive language sampling
FXSDSC = fragile X syndrome domain-specific concerns
FXSRS = fragile X syndrome rating scale
KiTAP = Kinderversion der Testbatterie zur Aufmerksamkeitsprüfung (children's test of attention)
VABS = Vineland Adaptive Behaviors Scales, version II
VAS = visual analog scale

Although a number of validated scales have been used in FXS clinical trials, no gold-standard scale validated specifically to measure core FXS symptoms is available, particularly in relationship to treatment.[19] To complement existing measures used in prior studies in FXS, two novel clinical measures designed to assess the core symptoms of FXS were developed: the fragile X syndrome rating scale (FXSRS) and the fragile X syndrome domain-specific concerns (FXSDSC) visual analog scale (VAS).

The FXSRS[20,21] is a clinician-completed scale including 34 items comprising three subscales: core phenotype, ASD, and associated phenotypic features. The FXSRS with core phenotype includes 10 items that have sensitivity and specificity for FXS compared with intellectual disability more generally. The FXSRS-ASD includes six items assessing symptoms enriched in individuals with comorbid ASD. The FXSRS with associated phenotypic features includes 18 items not specific to FXS but which are of clinical significance based on natural history data. Ratings were made on a Likert scale of 0 to 3 based on either frequency or severity of symptoms. Total and subscale scores were calculated with decreases indicating improvement.

The FXSDSC[20,21] is a clinician-completed scale that assesses domains in core symptom areas of FXS: repetitive behaviors; speech and language; anxiety, phobias and social withdrawal; motor performance; sensory oversensitivity; and cognition. Verbatim terms are chosen by the investigator to denote specific phenotypic "concerns" for each subject, and the same concerns are evaluated at baseline and subsequent visits. The severity of each concern is scored using a 10-cm VAS by the number of centimeters from the left margin, with anchors of "not at all severe" (left side of the line, 0 cm) and "very severe" (right side of the line, 10 cm). The score is reported as a percentage of the actual length of the line. The total score is the sum of the percent scores for the six domains.

For the Clinical Global Impression-Improvement (CGI-I) Scale the clinician rates how much the subject's illness has improved or worsened relative to a baseline state[22] on a seven-point scale: 1 = very much improved; 2 = much improved; 3 = minimally improved; 4 = no change; 5 = minimally worse; 6 = much worse; and 7 = very much worse. The CGI-I was scored using a standardized rubric specific to the clinical features of FXS. Day 14 assessments were made relative to the pretreatment baseline visit. For all subsequent visits, assessments were made relative to the day 14 visit (primary baseline).

The ABC-community version (ABC-C)[23] is a caregiver-completed rating scale for assessing problem behaviors in children and adults, which has robust psychometric properties in intellectually impaired and developmentally delayed populations.[24,25] The ABC-C has 58 items rated on a Likert scale from 0 ("not at all a problem") to 3 ("the problem is severe in degree") with decreases indicating improvement. The ABC-C has been shown to be sensitive to treatment change in previous clinical trials of FXS.[19,26]

Although the ABC was not designed as an FXS-specific measure, a scoring rubric has been validated specifically for FXS (ABC-$C_{FX}$).[27] The ABC-$C_{FX}$ was scored using this rubric, in which 55 of the 58 items resolve into a total score and six subscale scores: irritability, socially unresponsive-lethargy, stereotypic behavior, hyperactivity, inappropriate speech, and social avoidance.

The caregiver top three concerns VAS is a syndrome-specific measure of three symptoms identified by caregivers at baseline, which they would like to see improve.[28] Concerns are identified on a per subject basis and could be from any symptom domain related to the subject's FXS. The severity of each concern is scored by caregivers using a 10 cm VAS as described previously for the FXS-DSC.

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*Statistical methodology*

*Safety analysis*

Safety analyses were conducted for the intent to treat (ITT) population (all subjects randomized). Most comparisons between trofinetide and placebo were descriptive. AEs were summarized by system organ class and preferred term (number and percentages). Separate summaries were completed for treatment emergent adverse events occurring during the single-blind period, double-blind dosing period, and after dosing was completed. Clinical laboratory results, vital signs, EKG result, abnormal fundoscopy, and tonsil findings were summarized by time point and dose group. Concomitant medications were summarized by the preferred term and dose group at baseline (numbers and percentages). This listing counted the number of unique concomitant medications used by the subjects, so subjects who had multiple administrations of the same medication were counted once for that medication.

*Efficacy analysis*

Efficacy analyses evaluated both group and individual responses using an analytical methodology previously reported in a phase 2 study in Rett syndrome.[29] This approach was based on the concept that concordant trends in multiple, biologically meaningful, and syndrome-specific end points from different efficacy domains may provide strong evidence of efficacy in small studies.[30] Determination of the efficacy of trofinetide was made using a prespecified definition of benefit between active treatment groups and placebo as shown in Fig 2.

As shown in Fig 2, the overall study outcome was considered indicative of efficacy *only* if efficacy was detected as defined previously at the group-level *or* subject-level, *and* if at least numerical superiority was evident in the other analysis. Note that $P < 0.2$ (versus usual $P < 0.05$) for any single end point was not specified, *a priori*, as a criterion for study success; the success definition was based on a combination of the previously described criteria to control the probability of a false-positive study outcome. If study outcomes met or exceeded the minimal requirements, a permutation test was performed to determine the probability of obtaining the overall study results by chance alone.[31] An attractive aspect of the permutation test is that it preserves the correlation structure in the study data and does not require additional assumptions.

*Group-level efficacy analysis*

The primary efficacy analysis was conducted for the modified intent to treat (mITT) population (all subjects receiving at least one dose of double-blind medication). For this analysis, missing data were imputed with the median value for the assigned dose group at that visit. The general linear model (GLM) was used for the analysis. If normality and homogeneity of variance assumptions were violated, an appropriate nonparametric test would be performed instead. Subjects who responded to single-blind placebo treatment were identified in the analysis as described subsequently based on data at the day 14 visit, which was defined as the primary baseline for the efficacy analysis. All subjects randomized were scheduled to continue in the study after placebo run-in and their safety and efficacy data were included in the analysis as defined subsequently.

Results were considered indicative of efficacy if a difference in favor of the active group over placebo ($P < 0.2$) was demonstrated for at least two core efficacy variables from two different efficacy domains, with no clinically significant worsening in any other core efficacy variable.[30] If an active treatment group did not show an actual worsening from baseline for a particular variable (i.e., improvement or no change was observed), the criterion for "no clinically significant worsening" was considered met. If worsening in a core variable was observed for an active group, mean changes were compared between the active and placebo groups to determine whether the difference met the criterion for clinically significant difference. Except for CGI, a minimal clinically significant difference was defined as 20% of the mean primary baseline value for all subjects. For CGI-I, the minimal clinically significant difference was defined as 0.5. These thresholds were defined based on input from clinical experts in neurodevelopmental disorders and are aligned with contemporary best practices in other therapeutic areas.[32,33]

The group level comparison involved mean changes from primary baseline to end of treatment (EOT) between active groups and placebo for the core efficacy variables using a GLM. For CGI-I, actual values at EOT were compared because the CGI-I score itself represents an assessment of change and there are no baseline values for CGI-I, by definition. Data were summarized by time point using least square means with dose group as a main effect and primary baseline value as a covariate. If primary baseline was not statistically significant at 0.1 (two-sided significance), it was dropped from the model. The analysis was performed with a run-in placebo response (PR) indicator variable and its interaction with treatment group included in the GLM. Because the CGI-I represents a clinician-assessed global improvement across FXS symptomatology, it was used as the primary definition of run-in PR, which was a CGI-I score = 1, 2, or 3 at day 14. On the basis of this definition each subject was identified for the analysis as a run-in placebo responder (PR = 1) or a placebo nonresponder (PR = 0). Effect sizes were also determined using Cohen's *D* for each of the core variables.

*Subject-level efficacy analysis*

In the subject-level analysis, each patient's primary baseline value was compared with their EOT value for each core end point, and a numeric score was calculated as shown in Table 3.

The total subject-specific efficacy score for each subject was a sum of these scores for a maximum score of 5 (improvement on all five core end points) and a minimum of −5 (worsening on all core five end points). The mean of the subject-specific efficacy scores at EOT was compared between each active group and placebo. Results were considered indicative of efficacy if a difference in favor of an active group over placebo ($P$ value <0.2) was demonstrated.

## Results

*Demographics*

A total of 72 male subjects from 15 study sites were randomized. All 72 subjects were included in the ITT population (safety analysis) and 70 were included in the mITT population (efficacy analysis). The mITT excluded one subject, who was randomized but discontinued before receiving any study medication, and a second subject who discontinued during the placebo run-in. Demographics were generally comparable among the trofinetide and placebo groups as shown in Table 4. The mean age of all subjects was 23.5 years (12 to 41 years), most subjects were white (89%), and mean body mass index was 26.6 kg/m$^2$.

*Safety*

Both dose levels of trofinetide (35 and 70 mg/kg twice daily) were generally safe and well tolerated. No deaths and serious AEs were reported during the study. Four subjects in total withdrew from the study before completing the final visit (day 56), including

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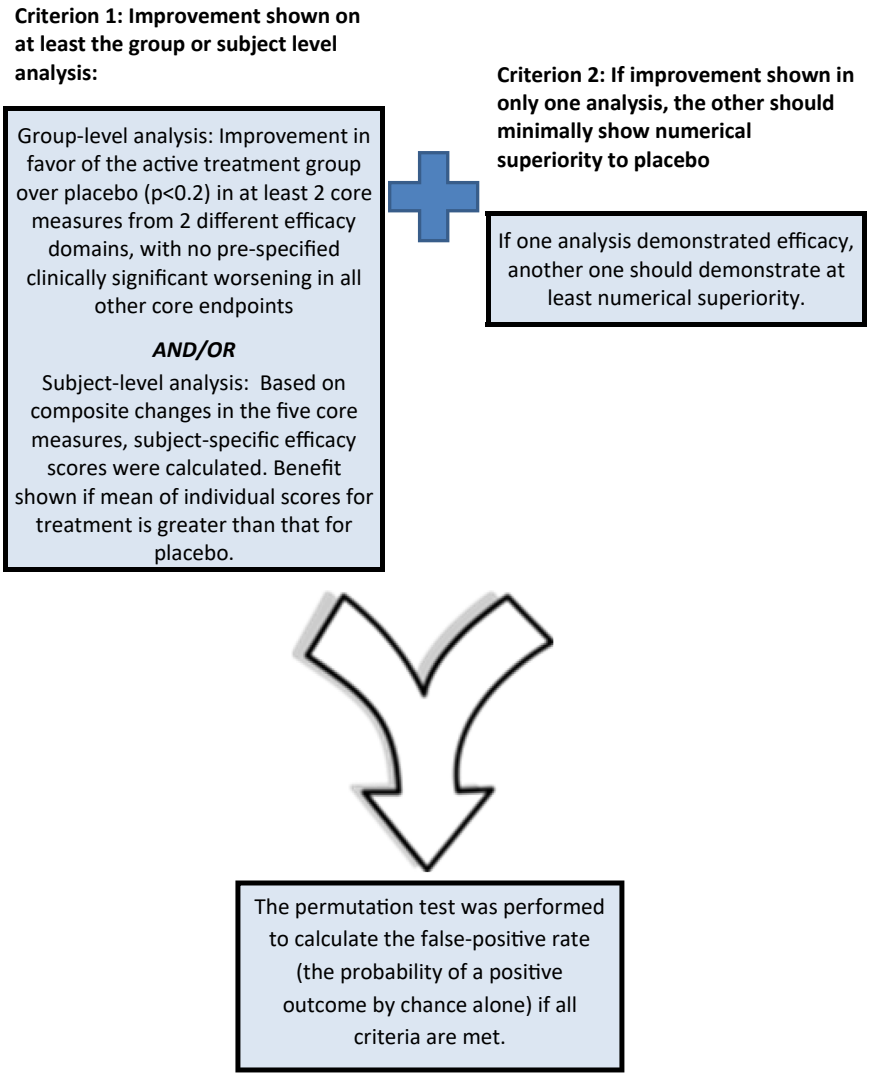


**FIGURE 2.** Prespecified criteria determining overall efficacy. The permutation test was conducted under the assumption of no difference between any of the trofinetide doses and placebo, thereby determining the false-positive rate based on the actual study results.[31] Randomly simulated allocations of patients to trofinetide and placebo were repeated 1000 times and positive outcomes (predefined study successes) were counted from these iterations. The permutation test, by design, takes into account the multiplicity of outcomes (three treatment groups, multiple end points, and so forth). The color version of this figure is available in the online edition.

two during the double-blind period. One subject was withdrawn during the double-blind period because of protocol violation (change in the dose of a concomitant medication). One completed the entire treatment period but was lost to follow-up before the post-treatment visit. One subject was withdrawn at the request of the parent or guardian because of an AE experienced during the

**TABLE 3**
Minimal Clinically Significant Differences for the Subject-Level Analysis

| Variable | Minimal Clinically Significant Difference From Baseline | Efficacy Score |
|---|---|---|
| CGI-I score at end of treatment | CGI-I score of 1, 2, or 3 (improvement) | +1 |
| | CGI-I score of >4 (worsening) | −1 |
| | CGI-I score of 4 (no change) | 0 |
| FXSRS total score | Decrease from primary baseline value for an individual subject ≥20% of that subject's primary baseline value (improvement) | +1 |
| Clinician domain-specific concerns total VAS score | Increase from primary baseline value for an individual subject ≥20% of that subject's primary baseline value (worsening) | −1 |
| ABC-$C_{FX}$ total score | | |
| Caregiver top three concerns total VAS score | Change from baseline value for an individual subject <20% of that subject's primary baseline value (no change) | 0 |

Abbreviations:
ABC-$C_{FX}$ = Aberrant Behavior Checklist-community version, fragile X scoring
CGI-I = Clinical Global Impression-Improvement
FXSRS = fragile X syndrome rating scale
VAS = visual analog scale

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**TABLE 4**
Demographics by Treatment Group (mITT Population)

| Characteristic | Number (%) of Subjects | | | |
|---|---|---|---|---|
| | Placebo b.i.d. (N = 25) | 35 mg/kg b.i.d. (N = 24) | 70 mg/kg b.i.d. (N = 21) | Total (n = 70) |
| Age, years | | | | |
| Mean (S.D.) | 20.9 (7.58) | 24.6 (8.71) | 25.5 (8.95) | 23.5 (8.52) |
| Median | 18.6 | 25.5 | 24.6 | 20.7 |
| Minimum, maximum | 12, 41 | 13, 41 | 13, 39 | 12, 41 |
| Sex, N (%) | | | | |
| Female | 0 | 0 | 0 | 0 |
| Male | 25 (100%) | 24 (100%) | 21 (100%) | 70 (100%) |
| Ethnicity, N (%) | | | | |
| Hispanic or Latino | 1 (4%) | 1 (4%) | 3 (14%) | 5 (7%) |
| Not Hispanic or Latino | 24 (96%) | 22 (92%) | 18 (86%) | 64 (91%) |
| Not reported | 0 (0%) | 1 (4%) | 0 (0%) | 1 (1%) |
| Race, N (%) | | | | |
| White | 22 (88%) | 21 (88%) | 19 (90%) | 62 (89%) |
| Black or African American | 2 (8%) | 1 (4%) | 0 (0%) | 3 (4%) |
| Asian | 0 (0%) | 0 (0%) | 1 (5%) | 1 (1%) |
| Other | 1 (4%) | 2 (8%) | 1 (5%) | 4 (6%) |
| BMI, kg/m$^2$ | | | | |
| Mean (S.D.) | 26.2 (5.40) | 24.4 (5.43) | 29.6 (5.39) | 26.6 (5.74) |
| Median | 26.2 | 24.6 | 28.6 | 26.6 |
| Minimum, maximum | 16, 39 | 16, 40 | 19, 43 | 16, 43 |

Abbreviations:
b.i.d = Twice daily
BMI = body mass index
mITT = modified intent to treat

placebo run-in, and one was randomized but was lost to follow-up before receiving any study medication.

A summary of AEs during the double-blind period is shown in Table 5. Incidences of AEs were generally comparable between the trofinetide and placebo groups, with most AEs being reported in one or two subjects in any treatment group. From the start of the double-blind period, the most common AEs (more than 5% of subjects overall) were upper respiratory tract infection (7%) and diarrhea (6%). For the 35 mg/kg group, the most common AEs occurring more than placebo were diarrhea (8%), vomiting (8%), and headache (8%). In the 70 mg/kg group, diarrhea (9%) and fatigue (9%) were the most common AEs. Most AEs were mild or moderate in intensity. There was no evidence of withdrawal effects when study drug was discontinued. Clinical laboratory tests, EKGs, vital signs, and physical examinations (including fundoscopy and tonsil hypertrophy) indicated no time- or dose-dependent patterns.

*Efficacy analysis*

*Group-level efficacy analysis*

Results from the group-level analysis are shown in Table 6. PR based on the CGI-I score at primary baseline (day 14) was found to be an influential modifier so the PR indicator was included as a covariate in the GLM. Trofinetide at the 70 mg/kg twice daily dose level exceeded the minimum requirement for efficacy based on the prespecified criteria. Trofinetide was associated with benefit over placebo ($P < 0.2$) in each of three core variables from two different efficacy domains:

(1) FXSRS total score, indicating improvement in major symptoms of FXS.
(2) FXSDSC total VAS score, indicating improvement in most concerning areas of FXS-related impairment for each subject.

**TABLE 5**
Incidence of Treatment-Emergent Adverse Events in at Least Two Subjects Overall During the Double-Blind Period and Post-treatment (Days 15 to 56) (ITT Population)

| System Organ Class Preferred Term | Number (%) of Subjects | | | |
|---|---|---|---|---|
| | Placebo b.i.d. (N = 25) | 35 mg/kg b.i.d. (N = 25) | 70 mg/kg b.i.d. (N = 22) | Total (N = 72) |
| Reported at least 1 event, N (%) | 10 (40%) | 14 (56%) | 11 (50%) | 35 (49%) |
| Gastrointestinal disorders | | | | |
| Diarrhea | 0 (0%) | 2 (8%) | 2 (9%) | 4 (6%) |
| Vomiting | 0 (0%) | 2 (8%) | 1 (5%) | 3 (4%) |
| General disorders and administration site conditions | | | | |
| Fatigue | 0 (0%) | 0 (0%) | 2 (9%) | 2 (3%) |
| Infections and infestations | | | | |
| Upper respiratory tract infection | 2 (8%) | 2 (8%) | 1 (5%) | 5 (7%) |
| Investigations | | | | |
| Blood bilirubin increased | 0 (0%) | 1 (4%) | 1 (5%) | 2 (3%) |
| Blood triglycerides increased | 0 (0%) | 1 (4%) | 1 (5%) | 2 (3%) |
| Glycosylated hemoglobin increased | 2 (8%) | 0 (0%) | 0 (0%) | 2 (3%) |
| Nervous system disorders | | | | |
| Headache | 0 (0%) | 2 (8%) | 1 (5%) | 3 (4%) |
| Skin and subcutaneous tissue disorders | | | | |
| Rash | 1 (4%) | 1 (4%) | 0 (0%) | 2 (3%) |

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**TABLE 6**
Group-Level Analysis (mITT Population)

| Efficacy Domain Core Variable | Change From Primary Baseline | | |
|---|---|---|---|
| | Placebo b.i.d. | 35 mg/kg b.i.d. | 70 mg/kg b.i.d. |
| Clinician-completed syndrome specific measures (efficacy domain 1) | | | |
| FXSRS total score | | | |
| N | 25 | 24 | 21 |
| Day 42 | −6.19 | −8.58 | −10.26 |
| *P* value versus placebo* | | 0.466 | 0.199† |
| Fragile X domain-specific concerns total VAS score | | | |
| N | 25 | 24 | 21 |
| Day 42 | −34.80 | −68.25 | −69.02 |
| *P* value versus placebo | | 0.220 | 0.193† |
| Clinician-completed syndrome-specific global measures (efficacy domain 2) | | | |
| CGI-I score | | | |
| N | 25 | 24 | 21 |
| Day 42 | 3.07 | 3.03 | 2.83 |
| *P* value versus placebo | | 0.861 | 0.373 |
| Caregiver-completed measures (efficacy domain 3) | | | |
| ABC-$C_{FX}$ total score | | | |
| N | 25 | 24 | 21 |
| Day 42 | −3.64 | −8.04 | −11.63 |
| *P* value versus placebo | | 0.366 | 0.095† |
| Caregiver top three concerns total VAS score | | | |
| N | 25 | 24 | 21 |
| Day 42 | −37.51 | −47.31 | −47.49 |
| *P* value versus placebo | | 0.568 | 0.546 |

Abbreviations:
ABC-$C_{FX}$ = Aberrant Behavior Checklist—community version, fragile X syndrome
CGI-I = Clinical Global Impression-Improvement
FXSRS = fragile X syndrome rating scale
mITT = modified intent to treat
N = number of subjects
VAS = visual analog scale

* *P* values are based on a general linear model with dose group as a main effect and primary baseline and placebo effect indicator as covariates. If primary baseline *P* value exceeded the 0.1 two-sided threshold, this covariate was dropped from the model. The placebo response term (based on CGI-I at primary baseline) was included in the model.

† Met the prespecified threshold ($P < 0.2$).

(3) ABC-$C_{FX}$ total score, indicating improvement in common behavioral problems associated with FXS.

The predefined criteria for no clinically significant worsening were met for the remaining two core variables, CGI-I and caregiver top three concerns. Although the prespecified criteria were not met at the 35 mg/kg twice daily dose level, a dose-related response was observed for all five core variables.

*Dose- and time-dependent patterns of efficacy*

As shown in Fig 3, at the end of 28 days of treatment (day 42), the three end points exhibited a time-dependent pattern of increasing improvement compared with placebo. After cessation of treatment (approximately 14-day post-treatment assessment), these trends were reversed for the FXSRS and the ABC-$C_{FX}$.

The 70 mg/kg (twice daily) group showed improvements in symptoms across the phenotype on the FXS-specific measures as indicated by the Cohen's *D* effect sizes (Fig 4).

On the FXSRS, items that showed numerical improvement in favor of the 70 mg/kg twice daily group were observed across all the subscales (see Supplementary Figure S1). Symptom areas where Cohen's *D* values for the items were ≥0.3 included social avoidance, communication, stereotypic and repetitive movements, hyperactivity, insomnia, and sensory regulation. As shown in Fig 5, on the FXSDS-VAS, improvements were observed in key symptom areas including anxiety and social withdrawal, motor impairments, and communication. Improvements in sensory oversensitivity were particularly notable.

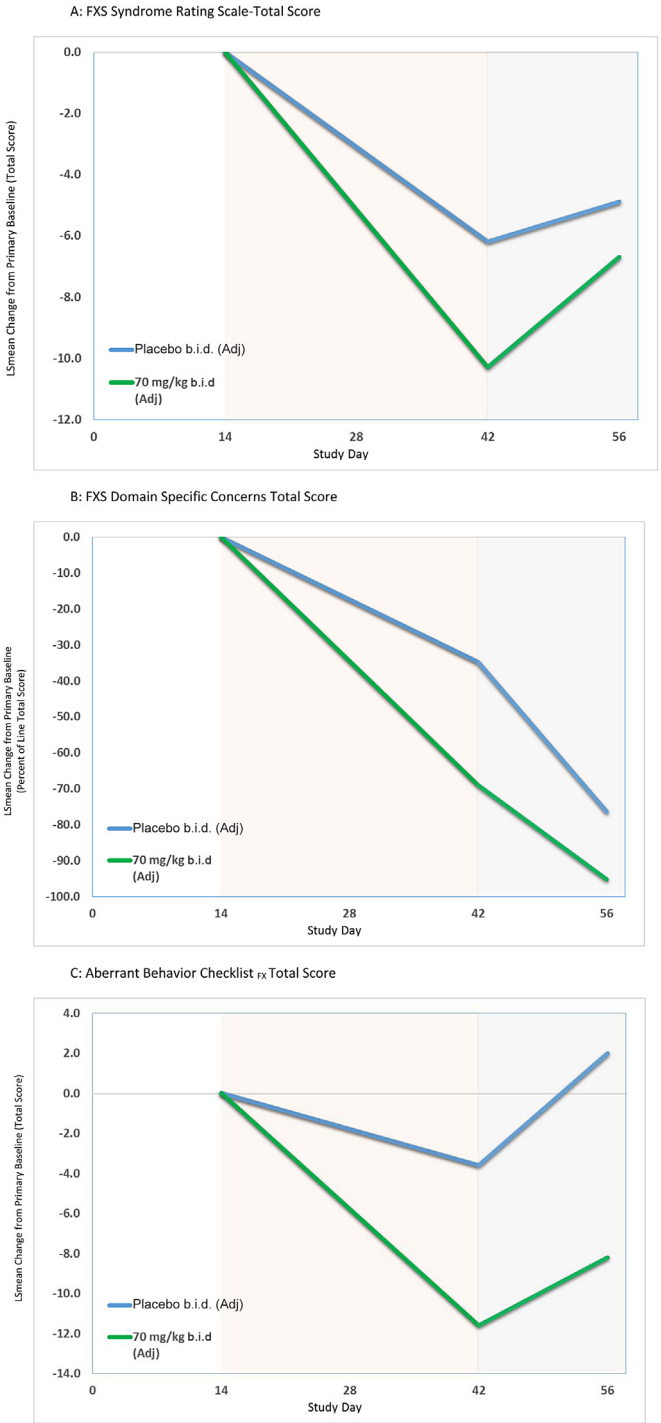


**FIGURE 3.** Core efficacy measures demonstrating improvement compared with placebo. Mean change from primary baseline of the 70 mg/kg group compared with placebo in the mITT population for the (Panel A) FXSRS, (Panel B) FXS domain-specific concerns visual analog scale, and (Panel C) ABC-$C_{FX}$. EOT was measured at day 42 and post-treatment follow-up at day 56. In Panels A and C, improvement is a decrease in score and in Panel B, improvement is a decrease in total score (sum of percent of line for all domains). Least square means adjusted for primary baseline when $P < 0.1$ and for placebo response. ABC-$C_{FX}$, Aberrant Behavior Checklist-community version, fragile X scoring; EOT, end of treatment; FXSRS, fragile X syndrome rating scale; mITT, modified intent to treat. The color version of this figure is available in the online edition.

As shown in Fig 6, improvements directionally in favor of the 70 mg/kg twice daily group were seen on all symptom areas assessed on the ABC-$C_{FX}$.

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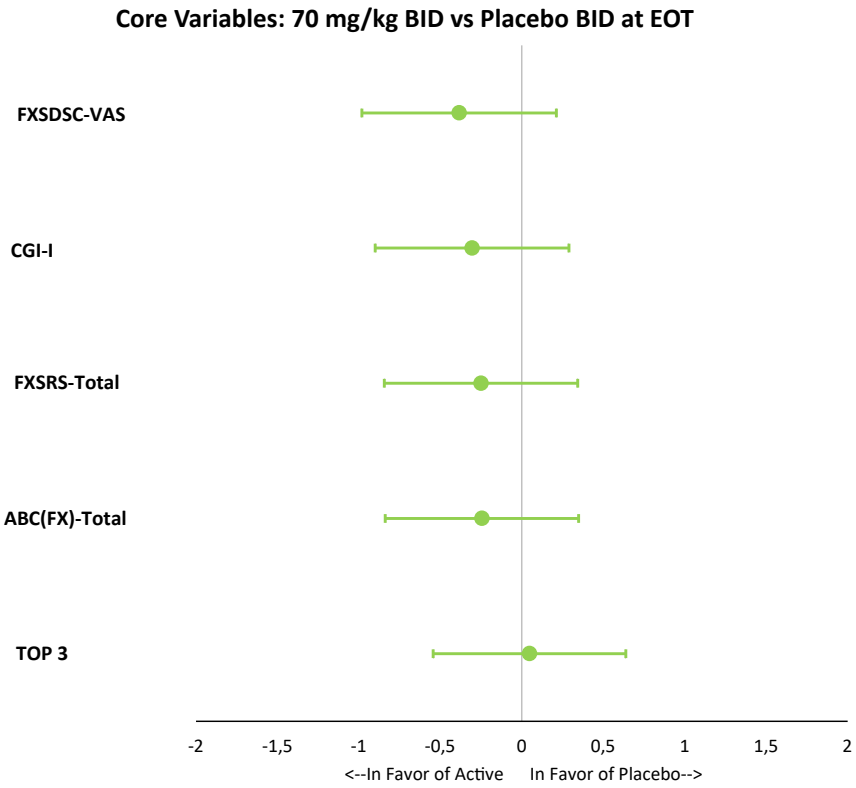


**FIGURE 4.** Cohen's *D* effect sizes and 95% confidence intervals at EOT in core variables (70 mg/kg versus placebo twice daily; mITT population). ABC-$C_{FX}$, Aberrant Behavior Checklist-community version, fragile X scoring; CGI-I, Clinical Global Impression-Improvement; EOT, end of treatment; FXSDSC-VAS, fragile X syndrome domain-specific concerns visual analog scale; FXSRS, fragile X syndrome rating scale; mITT, modified intent to treat; TOP 3, caregiver top three concerns. The color version of this figure is available in the online edition.

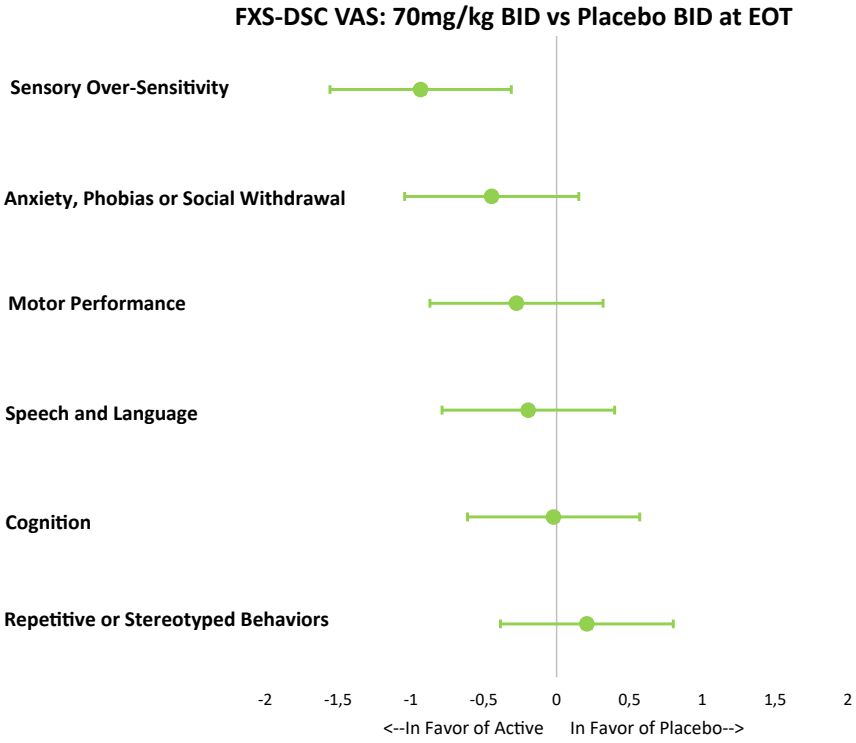


**FIGURE 5.** Cohen's *D* effect sizes and 95% confidence intervals for individual domains of the Fragile X Domain-specific Concerns at day 42 (EOT); 70 mg/kg versus placebo twice daily; mITT population. EOT, end of treatment; mITT, modified intent to treat. The color version of this figure is available in the online edition.

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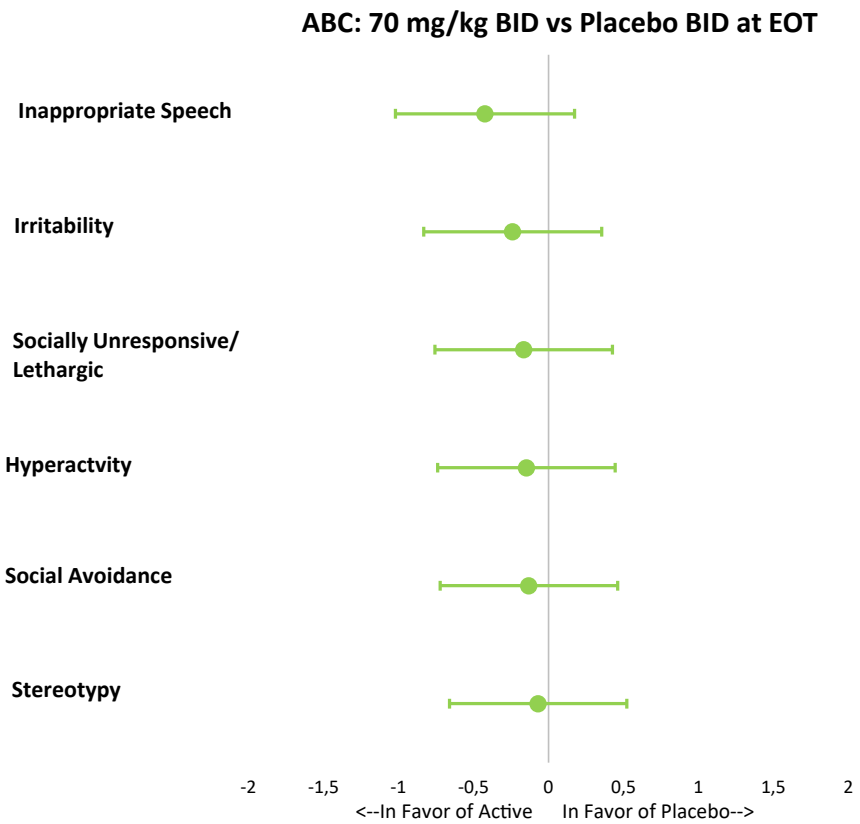


**FIGURE 6.** Cohen's *D* effect sizes and 95% confidence intervals for the subscales of the Aberrant Behavior Checklist$_{FX}$ at day 42 (EOT); 70 mg/kg versus placebo twice daily; mITT population. Subscale name shown on the axis. EOT, end of treatment; mITT, modified intent to treat. The color version of this figure is available in the online edition.

*Subject-level efficacy analysis*

Results from the subject-level analysis (with PR as covariate) demonstrated a higher mean total efficacy score at day 42 for the 70 mg/kg group than the placebo group and met the prespecified *P* value (2.93 vs 1.93, *P* value = 0.143). Trofinetide did not demonstrate efficacy at the 35 mg/kg dose level (*P* value = 0.502).

*Permutation test*

On the basis of the actual study data and performed under the assumption of no difference between trofinetide and placebo, the overall positive results were observed by chance alone in 45 of 1000 iterations. This finding indicates a false-positive rate for successful outcome in this study of 0.045 ($P = 0.045$).

## Discussion

The study was successful in achieving its primary outcome demonstrating that trofinetide administered at 35 and 70 mg/kg twice daily to adolescent and adult males with FXS was shown to be generally safe and was well tolerated. It also showed preliminary evidence of efficacy for the 70 mg/kg twice daily group compared with placebo. The study makes two other important contributions to treatment development research for FXS: it describes an analytical approach that should be considered as a viable alternative to traditional approaches for early phase studies, and it provides evidence for the utility of two novel outcome measures that are FXS-specific.

Trofinetide was well tolerated at both dose levels and appeared to be safe. There were no dose- or time-dependent patterns of AEs and no evidence of withdrawal effects. Clinical laboratory tests, EKGs, vital signs, and physical examinations (including fundoscopy and tonsil hypertrophy) showed no trends or distinct safety signals. No deaths or serious AEs were reported during the study, and there were no discontinuations because of AEs during the double-blind treatment period.

The results demonstrated consistent signals of efficacy with respect to the core features of FXS in subjects receiving 70 mg/kg twice daily of trofinetide compared with placebo twice daily. This was demonstrated in the group-level analysis by improvement in the major symptoms of the disorder (FXSRS total score), the most concerning aspects of the disease identified by clinicians (FXSDSC-VAS), and in syndrome-associated maladaptive behaviors (ABC-C$_{FX}$). Similarly, benefit from treatment with trofinetide at the higher dose was evident in the subject-level analysis.

Cohen's *D* analyses (unadjusted for either primary baseline or placebo effect) were supportive, demonstrating consistency in the observed benefit in favor of the 70 mg/kg twice daily group. The mean changes from primary baseline in FXSRS total score, FXSDSC total score, and ABC$_{FX}$ total score when adjusted for PR in the GLM were consistent with the Cohen's *D* effect sizes for these variables when unadjusted for PR. The small to medium effect sizes observed across measures are consistent with those reported for pivotal trials of Food and Drug Administration–approved medications where the effect size is considered with respect to clinical meaningfulness.[34]

Similar symptom areas reflecting major symptoms of the FXS phenotype were captured across the three core measures. Symptom areas where improvement was observed include social avoidance, anxiety, communication, stereotypic and repetitive behaviors, hyperactivity, motor impairments, and sensory sensitivity. Demonstration of improvements across multiple domains of the core phenotype of FXS syndrome was of fundamental importance in conferring syndrome-specific benefit. These results represented

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clinically meaningful changes from the perspective of the clinicians and the caregivers.

The concordant trend analyses in this study represent a novel approach to looking at effects across multiple measures indexing multiple domains of the condition. Although this approach does not use traditional *P* value cutoffs for any single end point, multiple criteria for benefit defined *a priori* must be met to meet the statistical criteria defining overall positive outcome of the study.

The pattern of benefit observed in this study is robust, in that it is seen in three measures in two different efficacy domains (both clinician- and caregiver-completed measures). In addition, the subject-level analysis met predefined criteria for efficacy. In general, the combination of multiple prespecified requirements to demonstrate efficacy made achieving the positive study outcome by chance alone very unlikely. This was demonstrated by the permutation test, which is designed to assess the probability of obtaining the positive outcome by chance alone (false-positive rate). This probability in the present study was low (0.045). As such, the result of the permutation test can be interpreted as an overall *P* value for the findings across all prespecified study success criteria.

This analytical approach is appropriate for a study of this size and type (phase 2 proof-of-concept studies), and given the limited data on assay-sensitive outcome measures in FXS. This analytical approach, although providing multiple criteria to protect against false positives, also provides a balance in guarding against the risk of false negatives in early proof-of-concept studies. The two phase 2 studies of trofinetide in the parallel clinical development program for Rett syndrome are exemplary of this. In the first smaller proof-of-concept study in adolescents and adults with Rett syndrome (study Rett 001), we used this same analytical approach, which showed evidence for a pattern of benefit.[29] The larger phase 2 study in a pediatric population, which followed Rett 001, demonstrated efficacy using traditional statistical methods.[35] Using traditional methods in the smaller first-in-patient-population Rett 001 study would have most likely made it infeasible to observe if there was any pattern of benefit, i.e. “proof of life” with respect to efficacy results, which could have discouraged conducting additional studies (i.e., a false negative). Particularly in rare serious diseases or conditions for which there are no approved therapies and for a drug candidate with a reasonable safety profile, emphasizing avoidance of false-negative results from small proof-of-principle clinical trials is warranted. Analyses such as this may also have a significant value for evaluating therapeutics that target core pathophysiologic phenomena “upstream” of symptoms across the disease state rather than a single molecular target or behavior, particularly when studied in early phase trials in a population that evidences substantial heterogeneity in symptom presentation.

This study also demonstrated the sensitivity to change two outcome measures that are specific to the symptomatology of the FXS phenotype. Identifying measures that can assess treatment outcome, which are specific to the FXS, has been one of the major challenges in conducting treatment trials in FXS.[19,26] The development of FXS-specific assessments for assessing change because of treatment was one of the key recommendations from a National Institutes of Health think-tank on improving outcome measures for FXS treatment trials.[19,26]

This study had certain limitations. First, study duration was short and the sample size was relatively small. That said, the downward trajectory of the key outcome measures suggests a potential for trofinetide treatment to provide clinically meaningful improvement in core FXS symptoms with continued treatment. Second, as there are no gold-standard measures to assess core symptoms of FXS, two novel scales were developed for this study that were used as core efficacy measures. Although the measures were developed based on well-accepted procedures (i.e., literature review of the natural history of FXS and clinical expert review), there was no precedent for their use in this population before this study. As such, there were no existing data at the time of the study start on the measures' psychometric properties or to guide decisions with regard to establishing criteria for clinically meaningful change. Moreover, although individuals with FXS manifest a similar set of symptoms reflective of the condition, there is variability in the degree to which they are manifested in any one individual. As a proof-of-concept study, results of this study, including the impact of PR on end point improvement, need to be confirmed in larger studies.

Despite these limitations, the results from this phase 2 study provide evidence of safety and preliminary evidence of efficacy of trofinetide treatment in FXS. These efficacy findings are notable given the short length of the treatment duration (28 days), as well as the degree of disability and symptom severity in these adolescent and adult subjects. This study indicates the potential viability of trofinetide as a treatment for FXS, a serious, debilitating, and lifelong condition for which there are currently no available therapies that address its core features. It provides evidence to support further study of trofinetide as a treatment for this population including studies of the pediatric population. The study also identified three high-quality syndrome-specific efficacy measures that may be suitable for use in clinical trials.

## Ethical Approval

The Neu-2566-FXS-001 study was approved by the Institutional Review Boards at each of the participating centers and for the study centrally by Western Institutional Review Board.

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Author contributions: J.P.H., L.G., and N.E.J. conceptualized and designed the study. M.S. participated in the design and conceptualization of the study, including the selection and development of the clinical rating scales. E.B.-K. assisted with the conceptualization and design of the study and selection of outcome measures. J.P.H. led the coordination and implementation of the study. N.E.J. and L.G. participated in the coordination and implementation of the study. E.B.-K. and N.T. assisted with the implementation of study. R.H., A.K., C.A.E., and S.H provided feedback on the protocol or outcome measures. E.B.-K., N.T., R.H., A.K., C.A.E., S.H., and the FXS-001 investigators enrolled subjects and collected study data. A.Y. and G.S. provided statistical advice and conducted the analysis of

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the safety and efficacy data. J.P.H., L.G., and N.E.J. participated in the analysis. J.P.H., L.G., N.E.J., E.B.-K., N.T., R.H., A.K., C.A.E., and S.H. interpreted the data. J.P.H., L.G., N.E.J., E.B.-K., N.T., R.H., A.K., C.A.E., S.H., M.S., A.Y., and G.S. critically reviewed and revised the manuscript. All named authors read and approved the final manuscript.

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## Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pediatrneurol.2020.04.019.

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