Science Translational Medicine
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*Sci Transl Med* **3**, 64ra1 (2011);
DOI: 10.1126/scitranslmed.3001708

FRAGILE X SYNDROME

# Epigenetic Modification of the *FMR1* Gene in Fragile X Syndrome Is Associated with Differential Response to the mGluR5 Antagonist AFQ056

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**Fragile X syndrome (FXS) is an X-linked condition associated with intellectual disability and behavioral problems. It is caused by expansion of a CGG repeat in the 5′ untranslated region of the fragile X mental retardation 1 (*FMR1*) gene. This mutation is associated with hypermethylation at the *FMR1* promoter and resultant transcriptional silencing. *FMR1* silencing has many consequences, including up-regulation of metabotropic glutamate receptor 5 (mGluR5)–mediated signaling. mGluR5 receptor antagonists have shown promise in preclinical FXS models and in one small open-label study of FXS. We examined whether a receptor subtype–selective inhibitor of mGluR5, AFQ056, improves the behavioral symptoms of FXS in a randomized, double-blind, two-treatment, two-period, crossover study of 30 male FXS patients aged 18 to 35 years. We detected no significant effects of treatment on the primary outcome measure, the Aberrant Behavior Checklist–Community Edition (ABC-C) score, at day 19 or 20 of treatment. In an exploratory analysis, however, seven patients with full *FMR1* promoter methylation and no detectable *FMR1* messenger RNA improved, as measured with the ABC-C, significantly more after AFQ056 treatment than with placebo ($P < 0.001$). We detected no response in 18 patients with partial promoter methylation. Twenty-four patients experienced an adverse event, which was mostly mild to moderately severe fatigue or headache. If confirmed in larger and longer-term studies, these results suggest that blockade of the mGluR5 receptor in patients with full methylation at the *FMR1* promoter may show improvement in the behavioral attributes of FXS.**

## INTRODUCTION

Fragile X syndrome (FXS) is an X-linked genetic condition associated with intellectual disability and behavioral problems including anxiety, aggression, hyperactivity, impulsivity, shyness, attention deficit disorder, and autism (*1*). It is caused by expansion of a CGG trinucleotide repeat in the 5′ untranslated region of the fragile X mental retardation 1 (*FMR1*) gene. This mutation is associated with hypermethylation at the *FMR1* promoter and consequent transcriptional silencing (*2*–*5*). The *FMR1* protein (FMRP) is a cytoplasmic RNA binding protein known to repress the translation of messenger RNAs (mRNAs) at synapses (*6*). It has been suggested that in the absence of FMRP, loss of repression of metabotropic glutamate receptor 5 (mGluR5)–mediated pathways results in the behavioral and cognitive impairments associated with FXS (*7*).

Recent studies in animal models of FXS have suggested that many aspects of the FXS phenotype, including behavioral abnormalities, cognitive deficits, and altered dendritic spines, may be attributable to excessive signaling by mGluR5, a group I mGluR. Genetic down-regulation of mGluR5 expression by crossing *Fmr1* knockout mice with heterozygous *Grm5* knockout mice rescues many of the FXS phenotypes, with the exception of macroorchidism (*8*). Consequently, selective mGluR5 antagonists may offer effective treatment for the symptoms of FXS. These compounds have been available as research tools for nearly a decade, and the prototype of the class, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), has been tested extensively in animal models of FXS. MPEP rescues the most robust central nervous system phenotypes in *Fmr1* knockout mice, namely, hyperactivity and audiogenic seizure susceptibility (*9*). Cognitive and neuroanatomical phenotypes were rescued in a fruit fly model (*10*), neurite branching and craniofacial abnormalities were rescued in a zebrafish model (*11*).

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Numerous animal studies and preclinical research suggest that mGluR5 antagonists may have therapeutic utility in the treatment of a variety of human conditions (*12*). Although no mGluR5 antagonist has yet received regulatory approval, there have been published human studies with fenobam, an anxiolytic agent found to be a selective mGluR5 antagonist (*13*). Investigational trials of fenobam in non-FXS populations showed it to be a modestly effective anxiolytic agent with a good safety profile (*14*), and one small open-label study in adults with FXS observed no significant adverse events and suggested the potential for beneficial clinical effects after a single dose of fenobam in 12 patients (*15*). Several mGluR5 antagonists are in development for FXS.

Here, we aimed to test whether therapeutic blockade of mGluR5 by AFQ056, a subtype-selective inhibitor of mGluR5, can improve behavioral symptoms in male adults with FXS, using a range of scales assessing behavior and social functioning. The primary efficacy assessment was the Aberrant Behavior Checklist–Community Edition (ABC-C) score (*16*), a checklist of 58 items that uses caregiver input to assess problem behaviors of children and adults with developmental disabilities. The secondary efficacy assessments included the Clinical Global Impression (CGI) scale (*17*), Vineland Adaptive Behavior Scale (VABS) (*18*), Repetitive Behavior Scale–Revised (RBS-R) (*19*), Visual Analogue Scale (VAS) of behavior, and the Social Responsiveness Scale–Adult Research Version (SRS) questionnaire (*20*). We also aimed to assess the safety and tolerability of AFQ056 in patients with FXS.

## RESULTS

A total of 16 and 14 patients were randomly assigned to receive either AFQ056 then placebo or placebo then AFQ056, respectively. Baseline demographics and patient characteristics were comparable between the two treatment groups (Fig. 1). All 30 patients completed their treatment period with AFQ056 and were included in the primary analysis. One patient discontinued the study because of a serious drug-unrelated adverse event. All other patients followed and completed the planned titration schedules.

### Effect of AFQ056 treatment on behavioral symptoms

In the primary efficacy analysis, no significant treatment differences were detected between AFQ056 and placebo groups in the change from baseline to day 19 or 20 ABC-C score [treatment difference (90% confidence interval) of −2.10 (−8.26 to 4.06), $P = 0.573$]. However, the RBS-R, a secondary efficacy outcome measure, exhibited a significant treatment difference in this population (Table 1; $P = 0.046$).

### Effect of *FMR1* promoter methylation and mRNA expression on efficacy

FXS is caused by methylation at the *FMR1* promoter and reduced or absent transcription of the *FMR1* gene. Upon consent, whole-blood samples were collected from the patients to assess both the DNA methylation status of the *FMR1* promoter and the level of *FMR1* mRNA. Blood samples were collected from 26 of 30 (87%) patients, and a total of 26 DNA and 24 RNA samples were successfully extracted. Both a methylation-specific polymerase chain reaction (PCR) (MSP) assay and bisulfite sequencing (7 to 13 clones per patient) were used to distinguish between full and partial methylation at the *FMR1* promoter. The term "full methylation" was used when both techniques detected only methylated DNA at the *FMR1* promoter, and "partial methylation" was used when both methylated and unmethylated DNA were detected. There were no significant differences in the pattern of methylated CpG sites between the methylated clones sequenced in patients with full and partial methylation. The methylation status of one patient was undetermined because of discrepancies between the two techniques. Seven of 25 (28%) patients were shown to have full methylation at the promoter by both MSP and bisulfite sequencing. For these patients,

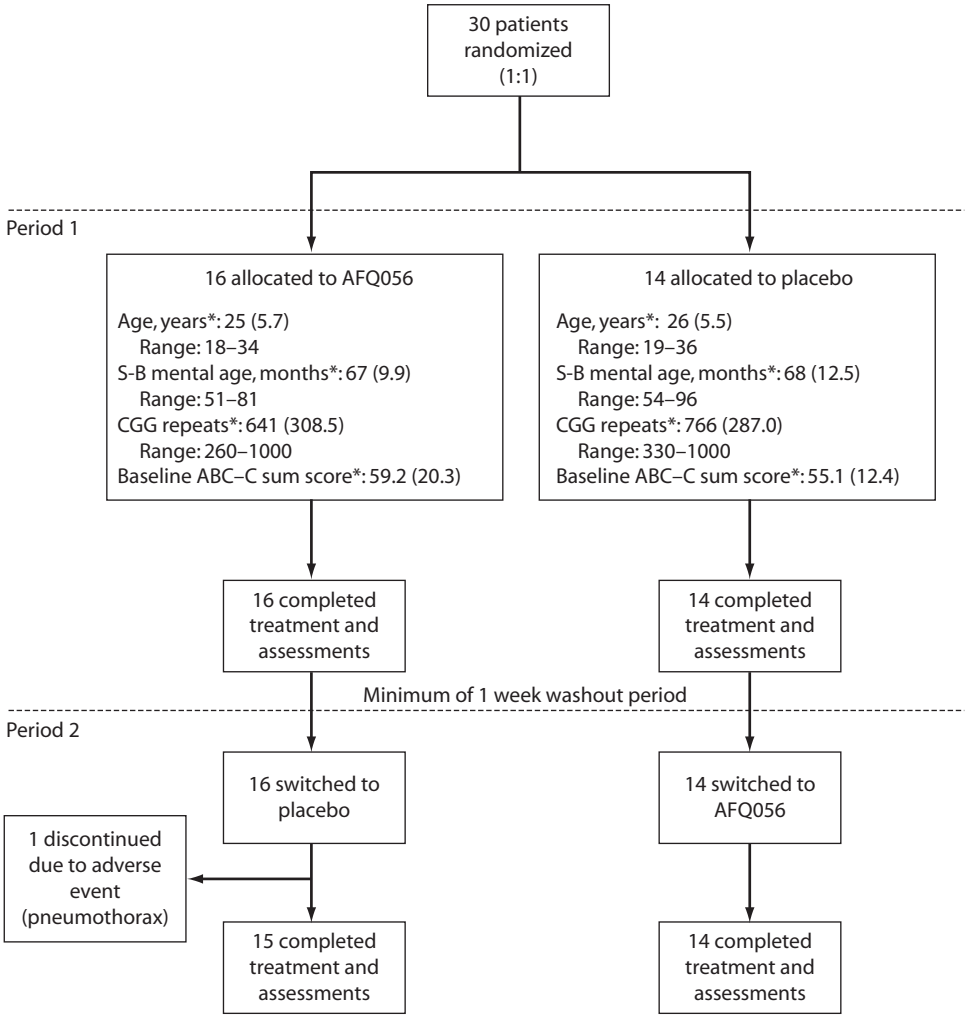


**Fig. 1.** The flow of patients through the study and the baseline population characteristics. *, mean (SD); S-B, Stanford-Binet test; ABC-C, Aberrant Behavior Checklist–Community Edition.

100% of the sequenced clones were methylated. The remaining 18 of 25 (72%) patients had partial methylation at the *FMR1* promoter. Assessment of mRNA levels by quantitative real-time reverse transcription PCR (qRT-PCR) showed that 7 of 24 (29%) patients had no detectable *FMR1* mRNA in their blood, whereas the remaining 17 of 24 (71%) patients had various levels of *FMR1* mRNA, including 1 patient with no detectable expression and 2 patients with expression levels within the range of healthy volunteers (Fig. 2, A and B). Such high *FMR1* mRNA levels in individuals with FXS have been reported previously (*21*). All patients lacking detectable *FMR1* mRNA in the blood had full *FMR1* promoter methylation (Fig. 2, A and B).

The study population was divided into two subpopulations according to *FMR1* promoter methylation status to investigate whether DNA methylation at this locus could be used to identify responders to AFQ056 treatment. Of the seven patients with full *FMR1* promoter methylation, one discontinued from the study during period 2. Therefore, seven patients provided efficacy data for the period of AFQ056 treatment and six for the placebo period. The two subpopulations were demographically and phenotypically similar. The baseline ABC-C, RBS-R, and SRS scores were slightly higher and the VABS score was slightly lower in the subpopulation with full *FMR1* promoter methylation compared with the subpopulation with partial methylation, but the differences were not significant (Table 2). Only the score on the irritability subscale was significantly different between the two populations, with the subpopulation with partial methylation at the *FMR1* promoter being less irritable ($P < 0.05$).

In the efficacy analysis, the subpopulation with a fully methylated *FMR1* promoter showed a significant treatment effect of AFQ056 versus placebo at day 19 or 20 on the ABC-C (Table 3; $P < 0.001$). Individually, all of these patients showed improved behavior on the ABC-C between baseline and day 19 or 20 in their AFQ056 treatment period (Fig. 3). Analysis of the ABC-C subscales in this subpopulation with full methylation at the *FMR1* promoter shows significant improvement on stereotypic behavior, hyperactivity, and inappropriate speech with AFQ056 treatment versus placebo (Table 3; all $P < 0.05$). Significant improvements with AFQ056 treatment were also detected on the CGI Improvement (CGI-I) scale, CGI efficacy index, RBS-R, SRS, and VAS, but not on the VABS (Table 3; all $P < 0.05$). According to the subscales of the RBS-R, AFQ056 showed improvement over placebo on stereotypic behavior and restricted interests (Table 3; all $P < 0.05$).

In the subpopulation with a partially methylated *FMR1* promoter, no significant differences between the treatments were detected on the ABC-C score or the ABC-C subscales (Table 3). Individual patients showed a variety of responses to AFQ056 in this subpopulation, with no detectable treatment-specific pattern (Fig. 3). No significant improvements with AFQ056 versus placebo were detected on any of the secondary outcome measures (Table 3).

### Safety and tolerability of AFQ056

Twenty-four of 30 (80%) patients experienced at least one adverse event in this study, most of which were mild to moderate in severity. Fatigue was the most frequently reported event, occurring in four patients during the up-titration phase of both AFQ056 and placebo treatment and in three patients (one of these patients also reported fatigue in the AFQ056 up-titration phase) during the high-dose AFQ056 phase. Four patients receiving AFQ056 reported headache, and this was the only other adverse event reported by more than two individual patients

**Table 1.** Mean treatment differences between AFQ056 and placebo on the secondary outcome measures at day 19 or 20 in the whole FXS patient population. CI, confidence interval; CGI, Clinical Global Improvement; VABS, Vineland Adaptive Behavior Scale; RBS-R, Repetitive Behavior Scale–Revised; SRS, Social Responsiveness Scale–Adult Research Version; VAS, Visual Analogue Scale. A decrease in CGI-I, RBS-R, and SRS scores indicates improvement. An increase in CGI efficacy index, VABS, and VAS scores indicates improvement. Descriptive statistics for KITAP and PPVT-R scores have not been included because the data were highly variable and no trends were observed.

| | Difference* (90% CI) AFQ056 − Placebo | *P* |
|---|---|---|
| CGI-I | 0.01 (−0.38 to 0.41) | 0.955 |
| CGI efficacy index | −0.01 (−0.41 to 0.39) | 0.974 |
| VABS | 0.82 (−5.07 to 6.72) | 0.814 |
| RBS-R | −3.81 (−6.91 to −0.70) | 0.046 |
| Stereotypic behavior | −1.26 (−2.03 to −0.48) | 0.010 |
| Self-injurious behavior | −0.37 (−0.85 to 0.11) | 0.201 |
| Compulsive behavior | −0.55 (−1.19 to 0.10) | 0.163 |
| Ritualistic behavior | −0.56 (−1.31 to 0.18) | 0.211 |
| Sameness behavior | −0.56 (−1.86 to 0.74) | 0.470 |
| Restricted interests | −0.66 (−1.12 to −0.19) | 0.022 |
| SRS | −1.14 (−7.71 to 5.43) | 0.773 |
| VAS | 5.18 (−3.89 to 14.25) | 0.345 |

*Difference in least-squares means between AFQ056 and placebo, adjusted for baseline covariate.

**Table 2.** Mean baseline ABC-C, VABS, RBS-R, and SRS scores by *FMR1* promoter methylation status. Values are means (SD). ABC-C, Aberrant Behavior Checklist–Community Edition. A decrease in ABC-C, RBS-R, and SRS scores indicates improvement. An increase in the VABS score indicates improvement.

| Baseline score | *FMR1* methylation status: Full (*n* = 7) | *FMR1* methylation status: Partial (*n* = 18) | *P** | *P*† |
|---|---|---|---|---|
| ABC-C | 67.29 (15.02) | 56.61 (16.09) | 0.16 | 0.14 |
| Irritability | 11.57 (4.65) | 6.83 (5.68) | 0.025 | 0.06 |
| Lethargy | 19.71 (11.44) | 21.06 (7.99) | 0.49 | 0.74 |
| Stereotype | 9.71 (4.27) | 6.78 (4.63) | 0.18 | 0.16 |
| Hyperactivity | 16.57 (4.96) | 13.56 (8.05) | 0.22 | 0.37 |
| Inappropriate speech | 9.71 (1.50) | 8.39 (2.79) | 0.39 | 0.25 |
| VABS | 384.86 (60.02) | 391.72 (45.87) | 0.90 | 0.76 |
| RBS-R | 34.29 (13.76) | 26.17 (17.35) | 0.17 | 0.28 |
| SRS | 95.57 (24.58) | 88.82 (24.26) | 0.59 | 0.54 |

*Nonparametric analysis of variance (ANOVA). †ANOVA.

(Table 4). Across both treatment groups, five patients had abnormal laboratory values that were reported as adverse events (one had hyperlipasemia, one had increased hepatic enzymes, one had increased blood creatinine phosphokinase, and two had hyperamylasemia and hyperlipasemia), and a number of patients had high blood pressure and high pulse rates throughout the study, with no obvious relationship to treatment. There was one serious adverse event in the study, which was not suspected to be related to the study drug—the patient experienced a severe pneumothorax and was hospitalized after their first dose of placebo, 15 days after their last dose of AFQ056. There were no deaths during the study.

## DISCUSSION

We present the results of a double-blind, placebo-controlled study evaluating a selective mGluR5 antagonist in FXS. The primary outcome measure (ABC-C score) of this crossover study showed no statistically significant treatment effects of AFQ056 on behavior. A marginally significant treatment effect of AFQ056 was detected on the RBS-R ($P = 0.046$), but not on any of the other secondary outcome measures.

An exploratory analysis of the study data suggested that the response to AFQ056 treatment may be predicted by the methylation status of the *FMR1* promoter. In this analysis, patients with a fully methylated *FMR1*

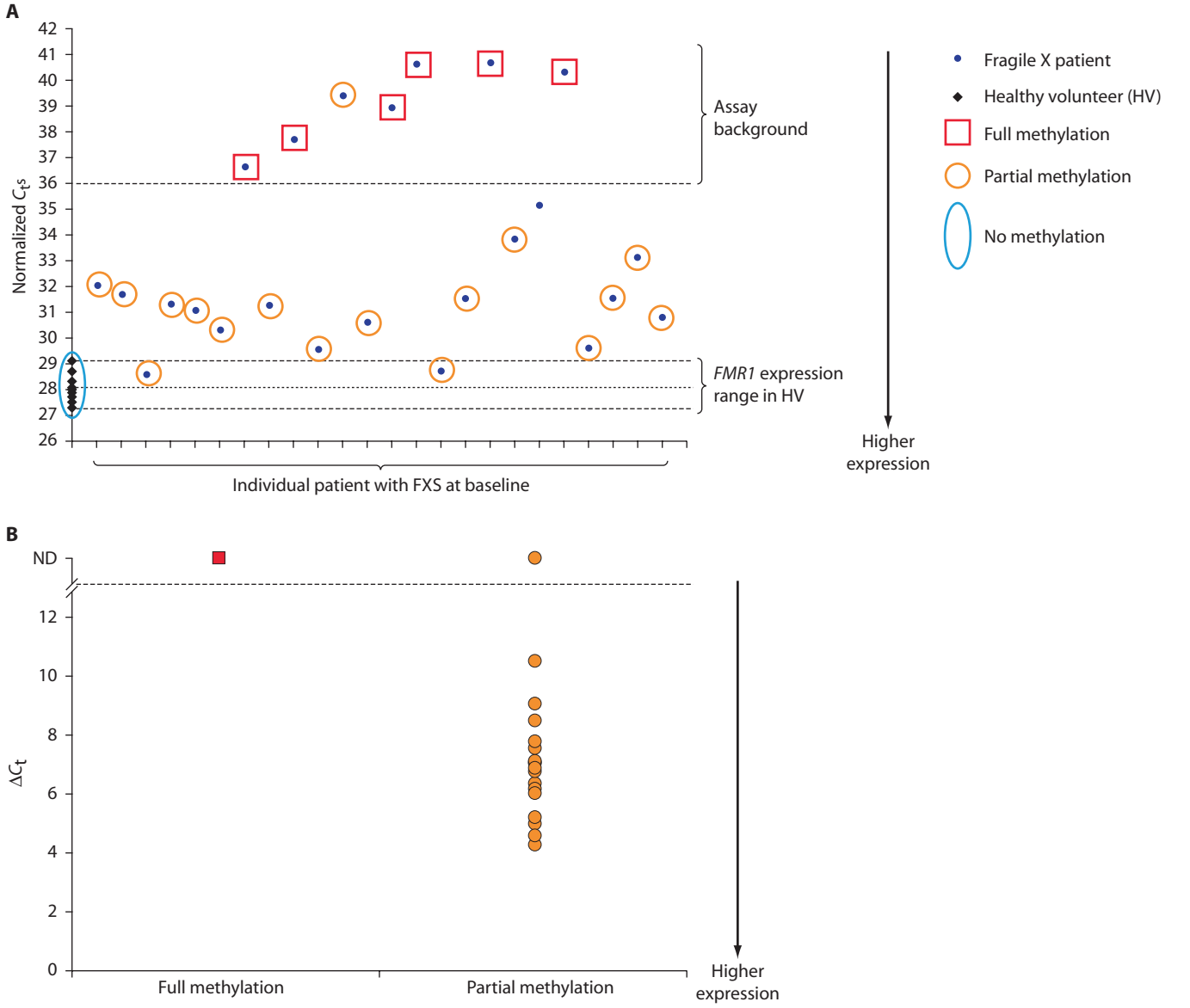


**Fig. 2.** *FMR1* mRNA expression levels at baseline, as assessed by qRT-PCR for the 24 patients analyzed. (**A**) Normalized number of qRT-PCR cycles required to reach a threshold level of DNA ($C_t$) for each patient. The corresponding methylation status at the *FMR1* promoter is also shown for the 24 patients with an *FMR1* mRNA assessment. Of the two patients with an assessment of *FMR1* promoter methylation status but no assessment of *FMR1* mRNA expression, one patient had full methylation and one patient had partial methylation at the *FMR1* promoter. (**B**) $\Delta C_t$* for each patient according to methylation status at the *FMR1* promoter. *, normalized *FMR1* $C_t$ – mean UBC and GAPDH $C_t$; ND, no *FMR1* mRNA detected (normalized $C_t \geq 36$).

promoter and no detectable *FMR1* mRNA in peripheral blood showed statistically significant effects of AFQ056 treatment for all primary and secondary outcome measures except VABS, whereas patients with partial methylation did not show any significant improvements with AFQ056 treatment when compared to placebo. The observed treatment effect of AFQ056 on the RBS-R in the whole population may have reached significance because of the positive treatment response of patients with a fully methylated *FMR1* promoter on the stereotypic behavior and restricted interests subscales. These results suggest that AFQ056 treatment may alleviate behavioral symptoms of FXS, particularly stereotypic behavior, hyperactivity, inappropriate speech, and restricted interests, and also improve autistic behaviors, in the subpopulation of FXS patients with full methylation at the *FMR1* promoter. This positive response to inhibition of mGluR5 function by AFQ056 supports the hypothesis that hyperstimulation of mGluR5-mediated activity in the absence of *FMR1* transcription contributes to the FXS phenotype (*7*).

The only previous study of an mGluR5 antagonist in FXS was an open-label, single-dose, phase I study of fenobam in 12 subjects with FXS. Although this trial was not designed to demonstrate efficacy, the investigators observed calmed behavior within 1 hour of dosing in 9 of 12 subjects, and improvements in prepulse inhibition in 6 subjects (*15*).

Several patients with partial *FMR1* methylation did show improvement on the ABC-C measure of behavior with AFQ056 treatment. The variation in treatment response among the partially methylated patients could potentially be explained by variation in *FMR1* mRNA and FMRP expression, which, according to the mGluR theory of FXS (*7*), would affect the degree of mGluR5 hyperactivation. Hence, to achieve a treatment response in patients with partial methylation of the *FMR1* promoter and less hyperactivation of mGluR5, it may be necessary to reduce the dosage of AFQ056. In support of this hypothesis, the baseline scores on the ABC-C, RBS-R, SRS, and VABS suggest that FXS symptoms were more severe in the subpopulation with full than with partial *FMR1* promoter methylation, although these differences were not statistically significant. However, no significant correlation was detected between *FMR1* mRNA levels and treatment response on the ABC-C ($R^2$ = 0.17). This was possibly due to variability in translation of *FMR1* mRNA into protein, with greater reductions in FMRP production at longer CGG repeat lengths. We could not explore this relationship because we did not measure FMRP levels. Tissue-specific variation in *FMR1* methylation status in patients with partial *FMR1* promoter methylation could also account for the variation in treatment response, as could the large placebo effect observed in some individuals. Indeed, it is known that placebo effect is often high in psychopharmacological studies in patients affected with autism or intellectual retardation (*22*), and may be more pronounced in crossover studies. FXS patients may be particularly sensitive because the nucleus accumbens, a region expressing high levels of mGluR5 and where this receptor plays an impor-

**Table 3.** Treatment differences on the ABC-C and the secondary efficacy variables between AFQ056 and placebo from baseline to day 19 or 20 by *FMR1* promoter methylation status. A decrease in ABC-C, CGI-I, RBS-R, and SRS scores indicates improvement. An increase in CGI efficacy index, VABS, and VAS scores indicates improvement. Descriptive statistics for KITAP and PPVT-R scores have not been included because the data were highly variable and no trends were observed.

| | Subpopulation with full methylation at *FMR1* promoter (*n* = 7) | | Subpopulation with partial methylation at *FMR1* promoter (*n* = 18) | |
|---|---|---|---|---|
| | **Difference* (90% CI)** | ***P*** | **Difference* (90% CI)** | ***P*** |
| ABC-C | −27.82 (−39.05 to −16.59) | <0.001 | 3.10 (−5.61 to 11.82) | 0.554 |
| Irritability | −2.66 (−5.37 to 0.05) | 0.106 | −1.15 (−3.45 to 1.16) | 0.410 |
| Lethargy | −5.53 (−10.87 to −0.18) | 0.090 | 2.66 (−0.81 to 6.13) | 0.206 |
| Stereotypic behavior | −5.06 (−8.66 to −1.46) | 0.027 | 0.78 (−0.70 to 2.25) | 0.383 |
| Hyperactivity | −8.55 (−12.27 to −4.84) | <0.001 | −0.21 (−2.85 to 2.43) | 0.894 |
| Inappropriate speech | −4.31 (−6.26 to −2.36) | 0.001 | 0.81 (−0.80 to 2.41) | 0.403 |
| CGI-I | −1.78 (−2.34 to −1.22) | <0.001 | 0.58 (0.04–1.11) | 0.079 |
| CGI efficacy index | 1.76 (1.13–2.39) | <0.001 | −0.43 (−0.96 to 0.11) | 0.193 |
| VABS | 2.02 (−16.84 to 20.88) | 0.769 | 2.03 (−1.52 to 5.58) | 0.333 |
| RBS-R sum score | −9.81 (−16.57 to −3.05) | 0.038 | −0.81 (−5.06 to 3.43) | 0.747 |
| Stereotypic behavior | −4.13 (−6.47 to −1.79) | 0.017 | −0.31 (−1.24 to 0.62) | 0.573 |
| Self-injurious behavior | −0.60 (−1.62 to 0.43) | 0.323 | −0.30 (−0.94 to 0.34) | 0.429 |
| Compulsive behavior | −1.27 (−2.88 to 0.35) | 0.165 | −0.35 (−1.31 to 0.60) | 0.540 |
| Ritualistic behavior | −1.92 (−4.12 to 0.28) | 0.135 | 0.162 (−0.84 to 1.16) | 0.786 |
| Sameness behavior | −1.30 (−3.05 to 0.46) | 0.208 | 0.09 (−1.86 to 2.05) | 0.937 |
| Restricted interests | −1.32 (−2.17 to −0.48) | 0.016 | −0.36 (−0.96 to 0.24) | 0.316 |
| SRS | −17.91 (−30.04 to −5.77) | 0.031 | 3.22 (−6.54 to 12.99) | 0.582 |
| VAS | 31.84 (14.01–49.67) | 0.006 | −4.15 (−16.73 to 8.44) | 0.584 |

*Difference in least-squares means between AFQ056 and placebo, adjusted for baseline covariate.

tant physiological role (*23*), is the main structure of reward expectation and placebo response (*24*).

Here, methylation of the *FMR1* promoter in whole blood was assessed with an MSP assay of bisulfite-treated DNA and sequencing of bisulfite-treated DNA clones. The sensitivity of these techniques is high compared with the standard methylation-specific Southern blot. This difference in techniques may explain the relatively high observed occurrence of partial *FMR1* promoter methylation in a population in which all of the patients carry the full mutation. In addition, we cannot exclude ascertainment bias toward higher-functioning patients, and thus also toward individuals with partial *FMR1* promoter methylation, given the rigorous study schedule and the procedures involved. The detection of partial methylation at the *FMR1* promoter may suggest the presence of a mosaic of premutation (between 55 and 200 CGG repeats without methylation at the *FMR1* promoter and usually few to no symptoms of FXS) and full-mutation (more than 200 CGG repeats) alleles. Because large premutations (100 to 200 CGG repeats) have been associated with above-normal levels of *FMR1* mRNA, but reduced FMRP (*21*), the normal levels of *FMR1* mRNA detected in two patients could be explained by a mosaic of premutation and full-mutation alleles. Partial *FMR1* promoter methylation could also be explained by distinct *FMR1* promoter methylation status in different tissues after somatic expansion of the CGG repeats in the early stages of embryonic development. Investigation into the tissue-specific pattern of *FMR1* promoter methylation and expression may shed more light on the functional consequences of *FMR1* dysregulation on disease phenotypes and drug response and may offer additional opportunities for discovering other indicators of treatment response.

AFQ056 was reasonably well tolerated in this population, with all patients completing the up-titration, reaching the target dosage, and completing the AFQ056 period of treatment. Adverse events were reported by 80% of the patients in the study, with these events mainly mild or moderate in severity, and most commonly fatigue and headache. Fatigue was reported in both AFQ056 and placebo treatment groups, whereas headache was only reported during AFQ056 treatment. Only one patient experienced a serious adverse event, and because this occurred while he was in the placebo treatment period, the event was not suspected to be related to the study drug.

This study was designed to investigate the effects of AFQ056 treatment on aberrant behaviors in patients with FXS and the safety and tolerability of this drug in this population. A two-treatment, two-period, crossover design was used to allow intraindividual comparisons and hence reduce the number of patients required to achieve satisfactory statistical power. To reduce interpatient variability, we recruited only men with the full FXS mutation to the study. These men were aged between 18 and 35 years, because AFQ056 pharmacokinetic data are only available for adults and because there are few available data on FXS patients older than 35 years. The treatment regimen in this study used AFQ056 doses shown to have an acceptable safety and tolerability profile in healthy subjects. Because this was the first experience of AFQ056 in patients with FXS, a slow titration dosing regimen was used. Because AFQ056 plasma concentrations were expected to reach a steady state within 3 to 4 days, 4-day intervals between dose escalations were considered sufficient to assess the safety and tolerability of each dose. The 28-day treatment periods were chosen on the basis of the available AFQ056 toxicology data, and the 1-week washout period was considered sufficient to avoid carryover effects because complete elimination of AFQ056 was expected after ~3.5 days.

This study is limited by the small sample size of 30 patients, the crossover design, and the short time scale. This is particularly relevant for the exploratory analysis comparing patients with full and partial *FMR1* promoter methylation, where only a small number of patients with full *FMR1* promoter methylation were shown to respond significantly to AFQ056 treatment. The levels of *FMR1* mRNA were assessed, but assessment of the levels of FMRP in each patient may have aided analysis of the results. It seems likely that patients with full methylation at the *FMR1* promoter and no detectable *FMR1* mRNA do not express FMRP, but knowing the levels of FMRP expression in patients with partial *FMR1* promoter methylation may have helped explain the variable treatment response in these patients. Larger, longer-term, prospective trials are required to confirm the findings of this study, to test the long-term efficacy of AFQ056 on behavior, and to study any effects on the cognitive deficits associated with FXS. If these results are positive, then future studies will be needed to test safety and efficacy in children with FXS, because the greatest benefits from mGluR5 inhibition may be derived during development.

We have shown that the selective inhibition of mGluR5 by AFQ056 can provide a significant effect on behavioral problems in a subpopulation of patients with FXS. If confirmed in future studies, these results suggest that AFQ056 may provide valuable improvement in behavioral symptoms of FXS and that this improvement is predicted by full methylation at the *FMR1* promoter. Why subjects lacking *FMR1* expression are better responders to the AFQ056 treatment remains an open ques-

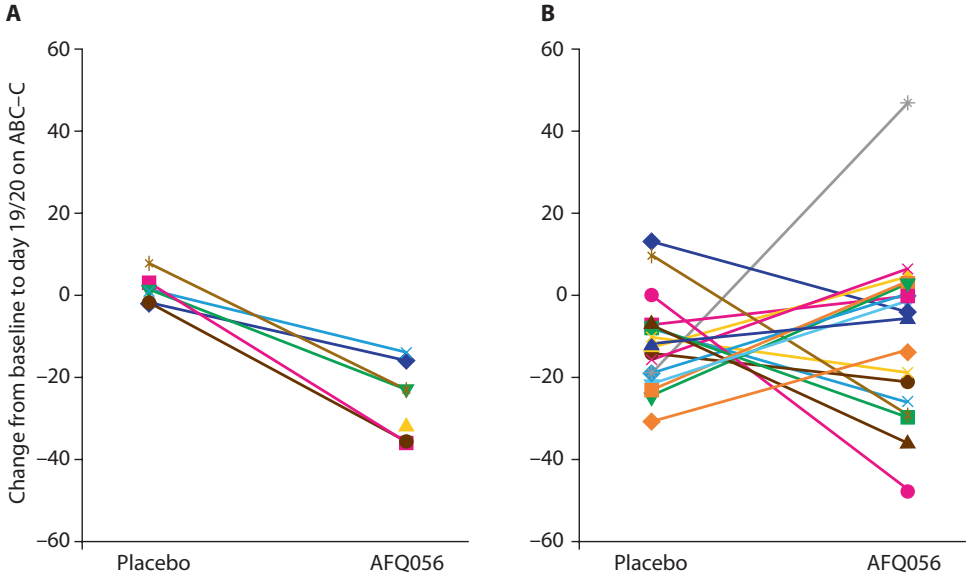


**Fig. 3.** (**A** and **B**) A comparison of the effect of AFQ056 and placebo treatments on the change from baseline to day 19 or 20 on the ABC-C score in individual patients with (A) full methylation at the *FMR1* promoter and (B) partial methylation at the *FMR1* promoter. A decrease in ABC-C score indicates an improvement in behavioral symptoms.

**Table 4.** Number of adverse events (preferred term) reported by the safety population during the up-titration, high-dose, and down-titration AFQ056 and placebo treatment phases. An adverse event was defined as one that was reported by at least two subjects in either treatment group. The safety population was all patients who received the study drug with at least one post-baseline safety assessment. Up-titration, days 1 to 8; high dose, days 9 to 20; down-titration, days 21 to 28.

| | AFQ056 (*n* = 30) | | | Placebo (*n* = 30) | | |
|---|---|---|---|---|---|---|
| | Up-titration | High dose | Down-titration | Up-titration | High dose | Down-titration |
| Patients with adverse events | 9 (30.0%) | 14 (46.7%) | 9 (30.0%) | 9 (30.0%) | 6 (20.0%) | 7 (23.3%) |
| Fatigue | 4 (13.3%) | 3 (10.0%) | 0 | 4 (13.3%) | 0 | 1 (3.3%) |
| Temperature intolerance | 1 (3.3%) | 1 (3.3%) | 1 (3.3%) | 0 | 0 | 1 (3.3%) |
| Headache | 2 (6.7%) | 1 (3.3%) | 1 (3.3%) | 0 | 0 | 0 |
| Affect lability | 1 (3.3%) | 1 (3.3%) | 0 | 0 | 1 (3.3%) | 1 (3.3%) |
| Diarrhea | 1 (3.3%) | 0 | 1 (3.3%) | 0 | 1 (3.3%) | 0 |
| Emotional distress | 0 | 1 (3.3%) | 0 | 1 (3.3%) | 1 (3.3%) | 0 |
| Oral herpes | 0 | 1 (3.3%) | 1 (3.3%) | 0 | 0 | 0 |
| Pancreatic enzymes increased | 0 | 1 (3.3%) | 1 (3.3%) | 0 | 0 | 0 |
| Food craving | 0 | 1 (3.3%) | 1 (3.3%) | 0 | 0 | 0 |

tion. One possible answer is that full methylation at the *FMR1* promoter may reflect the activity, or lack thereof, of other genes whose protein products interact with AFQ056.

## MATERIALS AND METHODS

### Subjects

Those eligible to participate in the study were nonsmoking men, aged between 18 and 35 years with a diagnosis of FXS (more than 200 CGG repeats or a positive cytogenetic test and a family history of FXS). Patients also had to have a mental age of at least 48 months on the Stanford-Binet (S-B) test (*25*), a CGI-S (*17*) score greater than 4, and a score greater than 20 on the ABC-C (*16*) scale. Any patients receiving psychotropic and/or anticonvulsant therapy must have been on a stable regimen for at least 4 weeks before randomization.

Patients were to be excluded from the study if they had a family history of prolonged QT interval syndrome, or a medical history of clinically significant electrocardiogram abnormalities, autonomic dysfunction, and bronchospastic disease. Other exclusion criteria included a diagnosis of schizophrenia; history or presence of psychosis, confusional states, or repeated hallucinations; history of seizures in the past 5 years without any treatment, or in the past 2 years if on stable anticonvulsant therapy; history of clinically significant drug allergy or atopic allergy; participation in a clinical trial within 4 weeks of drug administration; significant illness within 2 weeks of drug administration; donation or loss of 400 ml of blood within 8 weeks of drug administration; and use of potent CYP3A4 inhibitors within 4 weeks of drug administration.

### Study design

This was a three-site, randomized, double-blind, placebo-controlled, two-treatment, two-period, crossover study of AFQ056 in patients with FXS. The study was initiated in June 2008 and completed in February 2009. Patients were randomly assigned to receive either AFQ056 treatment (period 1) followed by placebo (period 2), or placebo (period 1) followed by AFQ056 treatment (period 2). The two treatment periods were separated by a washout period of at least 1 week.

Patients were assigned a randomization number by Novartis Drug Supply Management with a validated system. The unblinded pharmacist at each site then numbered and dispensed the correct study medications according to their treatment allocation cards. To preserve the blinding of study personnel and patients, we made the AFQ056 and placebo capsules identical and supplied them in identical packaging.

Study medication was administered in the morning and evening with about 12 hours between doses. According to the AFQ056 titration schedule, patients were to receive 50 mg twice daily on days 1 to 4, 100 mg twice daily on days 5 to 8, 150 mg twice daily on days 9 to 20, 100 mg twice daily on days 21 to 24, and 50 mg twice daily on days 25 to 28.

There were several protocol deviations during the study. One patient missed his evening 100-mg dose of AFQ056 on day 5 in period 1, and another took 225 and 150 mg of AFQ056 in the morning and evening, respectively, of day 10 in period 1 (total of 375 mg/day instead of 300 mg/day). Five other patients had dosing errors while receiving placebo.

### Assessments

The primary efficacy assessment was the ABC-C (*16*) sum score, which uses caregiver input to assess the problem behaviors of children and adults with developmental disabilities at home. The checklist contains 58 items, each scored from 0 to 3, with a higher score indicating more severe behavioral problems.

Secondary efficacy assessments were used to detect global changes in symptoms and behavioral changes. CGIs were captured with the CGI (*17*) scale, which includes a baseline severity of illness (CGI-S) score, a seven-point scale assessing global improvement (CGI-I), and a ratio of the side effects and therapeutic effects of treatment (efficacy index). Aberrant behaviors were assessed with the VABS (*18*), RBS-R (*19*), and VAS. The VABS score uses caregiver input to assess personal and social functioning, and the RBS-R assesses repetitive behavior across six domains (stereotypic behavior, self-injurious behavior, compulsive behavior, ritualistic behavior, sameness behavior, and restricted interests).

The severity of autistic spectrum conditions was assessed with caregiver responses to the SRS (*20*) questionnaire. The KITAP computerized test battery was used to assess attentional performance, and the Peabody Picture Vocabulary Test–Revised (PPVT-R) (*26*) was used to measure receptive vocabulary and verbal ability.

Efficacy assessments were to be performed by the same person for the duration of the study. These assessments were performed at the study center except on days 8, 12, and 28 when they were performed by the investigator on a telephone call during a home visit by the investigator's deputy or a study nurse. The ABC-C was performed at days −2 (screening) or −1 (baseline), 8, 19 or 20, 28, and at study completion; the CGI on days −2 or −1, 4, 8, and 28; the VABS on days −1 or 1, 19 or 20, and at study completion; the RBS-R and SRS on days −2 or −1, 19 or 20, 28, and at study completion; the VAS on days −1 or −2, 8, 12, 19 or 20, 28, and at study completion; and the KITAP test battery and PPVT-R on days −1 or 1, 19 or 20, and at study completion.

Safety assessments included collection of all adverse event and serious adverse event reports, standard clinical laboratory evaluations, electrocardiograms, and regular assessment of physical condition and vital signs.

### Statistical analyses

The sample size for this study was calculated with a two-sided paired *t* test and data from an open-label study of lithium in FXS patients (*27*). Here, the mean baseline value of 60 points on the ABC-C was reduced by 30% to 42 points after lithium treatment, with an intraindividual SD of 16.7 points. A supposed reduction of 5% under placebo would yield a treatment effect of 15 points. If at day 19 or 20 the true treatment effect of AFQ056 was 15 points and the SD of the intraindividual differences (AFQ056 minus placebo) was 24 points, then 24 patients would be sufficient to achieve 90% power in the primary analysis. Assuming a dropout rate of 20%, 30 patients were to be recruited to obtain at least 24 complete data sets.

The primary analysis tested the null hypothesis that there were no treatment differences on the ABC-C between AFQ056 and placebo at day 19 or 20 to the two-sided level of 10%. All patients who received at least one dose of study drug or placebo and had at least one post-baseline assessment of the primary efficacy variable were included in the primary analysis. A longitudinal mixed-effects model was fitted to the ABC-C sum score, which included fixed-effect terms for period, baseline within period (continuous covariate), day within period, treatment, day-by-treatment interaction, random effects for subject, and subject-by-period interaction. All random effects and the residual error were assumed to be independent. A sequence effect and/or a period × time interaction effect were only included if deemed appropriate. No multiplicity adjustments were performed. Similar analyses were performed on the secondary variables, although for the CGI analysis the CGI-S score was used as a covariate, and for the VABS analysis, time effects were excluded from the model because there was only one post-baseline assessment. Furthermore, the different domains in the KITAP test battery and the PPVT-R were evaluated in a descriptive manner.

### *FMR1* methylation and mRNA expression

Whole-blood samples were collected from patients with consenting guardians to investigate the relationship between *FMR1* methylation and mRNA expression with AFQ056 efficacy. Genomic DNA and total RNA were extracted from whole blood according to the instructions from Gentra Systems Inc. and Qiagen, respectively. Control DNA samples were purchased from Coriell Institute.

The methylation status of the *FMR1* promoter was tested with an MSP assay. Control and patient DNA samples were treated with bisulfite (Qiagen), and MSP was performed with the CpG WIZ Fragile X Amplification Kit from Chemicon. Bisulfite-treated DNA samples were also sequenced with primers designed to amplify a 196–base pair fragment of the *FMR1* promoter: 5′-CCACTGAGTGCACCTCTGCA-GAAATGG-3′ and 5′-CTCTCTCTTCAAGTGGCCTGGGA-3′. The amplified promoter fragment was cloned with the TA kit from Invitrogen, and 7 to 13 clones per patient were sequenced with an ABI3730 XL DNA sequencer (Applied Biosystems). *FMR1* mRNA expression was measured by Taqman qRT-PCR. The primers and probe were designed by Applied Biosystems, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitin C (UBC) were used to adjust for inter-sample variability.

The patients were stratified into two subpopulations according to their *FMR1* promoter methylation status and mRNA expression. In an exploratory analysis, the longitudinal mixed-effects model described above was used to assess the efficacy of AFQ056 relative to placebo in these two subpopulations. No multiplicity adjustments were performed.

### Ethics

The study protocol was reviewed by the Independent Ethics Committee or Institutional Review Board for each center, and the study was conducted according to the ethical principles of the Declaration of Helsinki. According to the guidelines of the European Clinical Trials Directive (EC 2001/20), the patients included in this study belong to the category of incapacitated adults. Therefore, informed consent for their participation was obtained from their legal guardians. To ensure patient safety and the early detection of any worsening of FXS symptoms, a Data Safety Management Board reviewed unblinded safety and tolerability data throughout the study.

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28. **Acknowledgments:** We are indebted to the patients, their families, and volunteers from the Swiss and French "Fragile X le Goëland," "Xraordinaire," and "le Cristal" associations for their involvement in the study. We thank C. Cornu, C. Vigouroux, B. Ravis, S. Courette, G. Melot, and B. Kassai from the Clinical Investigation Center of Lyon for their collaboration; A. Cheylus (L2C2, Lyon) for data processing and interpretation; and N. Isidor and the Service de Génétique Médicale for coordinating and providing the infrastructure to perform the study. We also acknowledge J. Cabantous, A. Lebec, and G. Cipriani for monitoring the clinical trial and B. Dowling and J. Fernandez for their contributions as Medical Data Managers. We would also like to acknowledge the contributions of the Novartis laboratory scientists who performed the genetic and methylation analyses: J. Decker, X. Zhao, K. Rose, L. Manzella, M. Letzkus, A. Holger Brachat, F. Staedtler, and E. Leroy. L. Redrup of Alpha-plus Medical Communications Ltd. (UK) provided editorial and writing support, which was sponsored by Novartis Pharma AG. **Funding:** Supported by Novartis Pharma AG, Basel, Switzerland. **Author contributions:** S.J., A.C., V.d.P., M.G.T., G.N., F.C., N.H., D.M., J.S.B., K.D., and A.B. were involved in data collection, clinical interpretation, and revision of the manuscript. Y.H., C.P., and J.M. were involved in study design, statistical analyses, data interpretation, and revision of the manuscript. E.B.-K., R.J.H., F.J.R., and K.C. were involved in the design and implementation of the study and were members of the Data Safety Management Board. G. Bussy, F.G., T.H., A.F., J.B., G. Bilbe, D.J., and B.G.-M. were involved in study design, data interpretation, and revision of the manuscript. The study sponsor was involved in the design and planning of the study and provided the study drugs. All authors approved the submitted version of the manuscript. **Competing interests:** S.J., G.N., E.B.-K., F.J.R., and K.C. are or have been paid consultants for Novartis. Y.H., C.P., J.M., F.G., T.H., A.F., J.B., G. Bilbe, D.J., and B.G.-M. are employees of Novartis Pharma AG. C.P., J.M., and F.G. hold stock in Novartis Pharma AG. R.J.H. has received funding from Novartis, Roche, Seaside Therapeutics, Forest, Neuropharm, Curemark, and Johnson and Johnson for clinical trials. Novartis has a patent on AFQ056 and has filed a patent regarding the main findings of this paper, that is, predictive markers for clinical response to mGluR antagonists in patients with FXS. **Accession numbers:** The clinical trial is registered in clinicaltrials.gov with the identifier NCT00718341.

Submitted 15 September 2010
Accepted 10 December 2010
Published 5 January 2011
10.1126/scitranslmed.3001708

**Citation:** S. Jacquemont, A. Curie, V. des Portes, M. G. Torrioli, E. Berry-Kravis, R. J. Hagerman, F. J. Ramos, K. Cornish, Y. He, C. Paulding, G. Neri, F. Chen, N. Hadjikhani, D. Martinet, J. Meyer, J. S. Beckmann, K. Delange, A. Brun, G. Bussy, F. Gasparini, T. Hilse, A. Floesser, J. Branson, G. Bilbe, D. Johns, B. Gomez-Mancilla, Epigenetic modification of the *FMR1* gene in fragile X syndrome is associated with differential response to the mGluR5 antagonist AFQ056. *Sci. Transl. Med.* **3**, 64ra1 (2011).