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A phase 1, randomized double-blind, placebo controlled trial to evaluate safety and efficacy of epigallocatechin-3-gallate and cognitive training in adults with Fragile X syndrome

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A phase 1, randomized double-blind, placebo controlled trial to evaluate safety and efficacy of epigallocatechin-3-gallate and cognitive training in adults with Fragile X syndrome

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Abstract

Background & Aims:

Despite the wide spectrum of experimental compounds tested in clinical trials, there is still no proven pharmacological treatment available for Fragile-X syndrome (FXS), since several targeted clinical trials with high expectations of success have failed to demonstrate significant improvements. Here we tested epigallocatechin-3-gallate (EGCG) as a treatment option for ameliorating core cognitive and behavioral features in FXS.

Methods

We conducted preclinical studies in *Fmr1* knockout mice (*Fmr1*–/y) using novel object-recognition memory paradigm upon acute EGCG (10 mg/kg) administration. Furthermore we conducted a double-blind placebo-controlled phase I clinical trial (TESFXS; NCT01855971). Twenty-seven subjects with FXS (18-55 years) were administered of EGCG (5-7 mg/kg/day) combined with cognitive training (CT) during 3 months with 3 months of follow-up after treatment discontinuation.

Results

Preclinical studies showed an improvement in memory in the novel object recognition paradigm. We found that FXS patients receiving EGCG+CT significantly improved cognition (visual episodic memory) and functional competence (ABAS II-Home Living skills) in everyday life compared to subjects receiving Placebo+CT.

Conclusions

Phase 2 clinical trials in larger groups of subjects are necessary to establish the therapeutic potential of EGCG for the improvement of cognition and daily life competences in FXS.

Keywords

61 Fragile-X syndrome, Epigallocatechin gallate, Cognition, Functionality.

Introduction

Fragile-X syndrome (FXS) (OMIM, 300624) is the most common (1/4000 live births) cause of intellectual disability and autism of genetic origin. It is a trinucleotide repeat disorder caused by a CGG repeat expansion at the 5′-end of the *FMR1* gene, which hypermethylation results in transcriptional silencing and loss of expression of Fragile-X mental retardation protein (FMRP). Brain alterations observed in FXS patients are linked to low concentrations of FMRP [1], a RNA binding protein that plays a pivotal role in synaptic function. Despite the wide spectrum of experimental compounds tested in clinical trials (i.e. selective antagonist of 5-$HT2_B$ serotonin receptor, metabotropic glutamate receptor 5 (mGluR5) antagonist, or $GABA_B$ receptor agonists) [2], there is no pharmacological treatment available for ameliorating cognitive and behavioral features in FXS.

We previously showed that epigallocatechin-3-gallate (EGCG), the major catechin in green tea leaves, elicited beneficial effects in intellectual disability. Specifically, in Down syndrome mouse models and patients, EGCG administered alone [3] or in combination with cognitive training, improved cognition and brain functional connectivity [4]. In fact, flavonoid-rich foods can beneficially influence normal cognitive function [5,6] and slow down cognitive decline in non-pathological aging. Mounting evidence suggests that Down syndrome and FXS share common alterations in signaling pathways relevant for neural plasticity and learning and memory processes, such as PI3K, mTOR and ERK1/2 [7,8], that are targeted by EGCG [9,10]. We thus reasoned that EGCG could also rescue the cognitive alteration in FXS.

We first performed a preclinical study in *Fmr1* knockout mice (*Fmr1*−/y) to explore the possible effects of EGCG on cognitive function in an FXS model, and determine the more adequate dosage. Upon positive preclinical results, we performed a Phase 1 randomized, double-blind, placebo-controlled 6 months clinical trial to assess the safety and preliminarily clinical efficacy of EGCG. Based on our previous clinical trial in Down syndrome patients [4], in this

exploratory trial, we compared the effectiveness of EGCG plus cognitive training (CT) with placebo plus CT in an adult population with FXS.

Materials and Methods

1. Preclinical pharmacological studies

Animals

Fmr1 knockout mice (FVB.129P2-Pde6b+ Tyrc−chFmr1tm1Cgr/J) and WT mice (FVB.129P2-Pde6b+ Tyrc−ch/AntJ) on a FVB background were purchased from The Jackson Laboratory and crossed to obtain Fmr1−/y and WT littermates. All experimental mice were bred in the Barcelona Biomedical Research Park (PRBB) Animal Facility. Mice were housed four per cage in a temperature (21°C ± 1°C) and humidity (55% ± 10%) controlled environment. Food and water were available *ad libitum*. Behavioral experiments were performed on 12-16 week old mice during the light phase of a 12h light-dark cycle (lights on at 8 a.m.), after mice were handled for 1 week. All animal procedures were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB) and followed standard ethical guidelines (European Communities Directive 86/60-EEC).

Drugs and treatments

Green tea extract (GTE) nutritional supplement containing 45% of EGCG (Life Extension Decaffeinated Mega Green Tea Extract; http://www.lifeextension.com/vitamins-supplements/item00954/mega-green-tea-extract-decaffeinated), was dissolved in water and administered per oral route in a volume of 10 mL per kg body weight (50, 25 or 10 mg/kg of GTE, equivalent to 22.5, 11.25 and 4.5 mg/kg of EGCG, respectively).

Novel object-recognition memory test

The novel object-recognition memory was assayed as described previously [11]. Briefly, on day one, mice were habituated for 10 minutes to an empty V-shaped maze (V-maze). The next day mice were reintroduced in the V-maze for 10 minutes where two identical objects had been positioned, one at the end of each arm. After the training period mice were randomly assigned to one of the dosages of EGCG (4.5, 11.25, or 22.5 mg/kg) or water groups, and placed back to their home-cage. Novel object-recognition memory was assessed 24h after training. Mice were reintroduced for 10 minutes in the V-maze where the object explored the day before (familiar) and a novel object was located at the end of the arms. The exploration time for both objects (familiar, F, and novel, N) was computed (tF y tN) by experienced observers blind to the experimental groups, and used to define a discrimination index (DI) following the formula: DI=(tN-tF)/tN+tF). DI values >0.3 indicate mouse discrimination between F and N objects, while DI<0.15 indicate lack of discrimination between objects [12].

Statistical analysis

Results are reported as the mean ± S.E.M. Statistical comparisons (Student's t-test between two groups or one-way analysis of variance (ANOVA) for multiple group comparisons) using the Statistica Software. Comparisons were considered statistically significant when $P < 0.05$.

2. Clinical trial

We performed a randomized, placebo-controlled, phase I, 6-month clinical trial (TESXF study; NCT01855971) at the Hospital del Mar Medical Research Institute (IMIM) of Barcelona aimed at investigating if cognitive training (CT) combined with a green tea extract nutritional supplement containing 45% of EGCG (5-7 mg/kg/day) could improve cognitive performance and adaptive functionality compared to CT combined with placebo in adults with FXS. The trial was approved by the local ethical committee (CEIC Parc de Salut Mar, ref. 2012/4781/I), and conducted according to the Declaration of Helsinki and Spanish guidelines and regulations regarding data privacy.

Eligible participants were recruited from the Catalan Association of Fragile-X Syndrome, and two health centers specialized in intellectual disabilities (Parc Hospitalari Martí i Julià, Salt, and Villablanca Serveis Assistencials, Institut d´Investigació Sanitaria Pere Virgili, Reus). All enrolled participants clinically diagnosed as FXS patients had a reliable parent or caregiver ensuring administration of the medication and CT, and completing all visits. We initially planned to include adults of both genders with FXS, aged 18 to 45 years, but due to recruitment difficulties we finally extended the inclusion criteria to 55 years with any of the *FMR1* genetic variations (full mutation, premutation or mosaic). As the molecular diagnosis for FXS was not available in several subjects, we had to confirm clinical diagnosis. Subjects with neurological disease other than FXS, relevant medical disease, unstable co-morbid mental disorder or under any treatment that could interfere with cognitive function were excluded from the study. Other exclusion criteria were: (i) having suffered from any major illness or undergoing major surgery in the last 3 months before the study; (ii) new medication in the month preceding the study; (iii) ingestion of vitamin or catechin supplements or non-steroidal anti-inflammatory drugs (NSAIDs) in the 2 weeks preceding the study; (iv) gastrointestinal, hepatic, renal or any other problems that may alter absorption, distribution, metabolism, or excretion of the drug.

Participants, parents and/or legal guardians were informed of the protocol and gave their written informed consent. Rules for early termination of the study were predefined and included the observation of serious adverse events. The trial was analyzed by the intention-to-treat (ITT) approach to provide unbiased comparisons among the treatment groups. Concerning missing data, an inverse probability weighting method was used, so that complete observations were weighted by the inverse of the estimated probabilities of being observed.

Study enrollment, randomization, and retention were held from June 2013 to July 2015. Initially thirty-one patients were randomized but molecular diagnoses of four of them did not confirm FXS and had to be excluded (3 belonging to the placebo group and one to the EGCG

group). Therefore, twenty-seven patients (61% of those initially contacted, n=44) were randomly assigned to EGCG+CT or Placebo+CT group, using a random-number table and were included in the intention to treat analysis (12 in Placebo+CT and 15 in EGCG+CT group). One participant from the EGCG+CT and one from the Placebo+CT group dropped out from the trial. Both were evaluated at 3 months after randomization, and the subject receiving EGCG+CT was also evaluated at 6 months as the trial (both cases were included in statistical analyses, in one case the whole evaluation at 6 months is missing while in the other case the biochemical and the neurophysiological variables are unavailable; see CONSORT diagram, Fig. 2). Neuropsychological data from these participants were included in the analyses. The demographics were similar in the groups receiving EGCG+CT and Placebo+CT (Table 1).

Regarding intellectual disability level (intellectual quotient, IQ), the distribution of individuals with severe (IQ ≤ 40) and moderate to mild (IQ ≥ 40) intellectual disability (Kaufman Brief Intelligence Test) was different between groups. A higher proportion of individuals with moderate intellectual disability was concentrated in the Placebo+CT group, but even so, the mean IQ in the EGCG+CT group is almost in the cut-off of moderate disability level. Treatment allocation and randomization was performed by the Hospital del Mar Pharmacy Department (Table 1) that also was in charge of concealment under a sealed opaque envelope, randomization sequence generation and supplementation of labeled packs (packs with identical appearance and size zero blue opaque capsules for EGCG and placebo). All members of the research team, the statistician, participants with FXS and families and/or guardians were blind to the allocation to either treatment arm. The double-blind condition was maintained until the end of the follow-up and data analysis.

Design

The trial design included one month of placebo (run-in period), 3 months of EGCG+CT or Placebo+CT treatment and 3 months follow up after pharmacological treatment discontinuation and under CT alone. We administered a nutritional GTE supplement

188 containing 45% of EGCG (Life Extension Decaffeinated Mega Green Tea Extract;
189 http://www.lifeextension.com/vitamins-supplements/item00954/mega-green-tea-extract-
190 decaffeinated) or placebo (rice flour). Capsules were prepared ad hoc for the present clinical
191 trial. Each capsule contained 200 mg of EGCG. All participants were administered two
192 capsules of EGCG (400 mg; equivalent to 5-7 mg/kg) or placebo, one during lunch and
193 another during dinner. All participants underwent CT during the entire intervention period
194 (months 1 to 6 after the run-in period). We provided access to an easy-to-use cognitive
195 software package (FesKits; www.feskits.com/) connected to a telematics platform that
196 includes a wide range of training programs and exercises (more than 5.000) training all
197 cognitive domains. The subjects were specifically trained in memory, executive functions,
198 language and attention processes. However, memory training was preponderant over the
199 other cognitive capacities, comprising 50% of each training session. The duration of each
200 session was recorded to register user compliance. A neuropsychologist monitored the
201 performance of each subject and we used an algorithm to adapt the difficulty of the task to
202 learning progression. During the one-month run-in enrolment period, a neuropsychologist
203 tutored each subject (and their family) to familiarize with the CT telematics platform. All
204 participants of EGCG and placebo groups were instructed to perform three sessions of 30 to
205 50 minutes per week. Patients underwent four evaluations: at baseline, after the placebo run-
206 in, at three months after EGCG/placebo treatment initiation, and three months after
207 EGCG/placebo treatment discontinuation (Figure S1).

208 *Primary and secondary outcome measurements*

209 Primary outcome measures were defined as the changes in cognitive and functional scores,
210 assessed by means of TESFX battery, from baseline to 3 months after treatment
211 randomization and 3 months after EGCG or placebo discontinuation (see Supplementary
212 materials for description of TESFX battery). Secondary outcome measures comprised the
213 changes from baseline to 3 months after treatment randomization and 3 months after EGCG

or placebo discontinuation of: 1) neurophysiological parameters: pre-pulse inhibition (PPI); 2) efficacy biomarkers: changes from baseline in PI3K/mTOR and ERK phosphorylation in human lymphocytes; 3) body composition (bioelectrical impedance); 4) treatment compliance and 5) safety evaluation that included reported adverse events/serious adverse events (AEs/SAEs), vital signs, physical and neurological examinations, electrocardiogram, and standard hematology, clinical chemistry assessments, and urinalysis. Safety laboratory parameters included transaminases, gamma glutamyl transferase, alkaline phosphatase, lipid profile, and urea.

Neuropsychology

We used a customized neuropsychological battery (TESFX), which includes measures of attention, psychomotor speed, memory, executive functions, language, adaptive and aberrant behavior, and quality of life and sleep (see Supplementary materials). The TESFX battery was developed for FXS clinical trials, based on the TESDAD battery for Down syndrome individuals (NCT01394796) [13]. All neuropsychological outcomes were assessed at baseline, 3 months after treatment randomization, and 3 months after EGCG or placebo discontinuation.

Briefly, attention was assessed with reaction time and span capacity measures using the Simple Reaction Time task (SRT, CANTAB), psychomotor speed with the Motor Screening Test (MOT, CANTAB), visual episodic memory and learning using the Paired Associates Learning (PAL, CANTAB) and the Pattern Recognition Memory test (PRM, CANTAB), and verbal episodic memory using the Cued Recall Test (CRT). For executive functioning, we assessed fractioned components of verbal fluency, working memory, planning, mental flexibility, and inhibitory control. Verbal fluency was measured using the semantic fluency word generation task (participants were asked to generate as many words as possible in 1 min belonging to the specified category of animals). Working memory for visual and verbal information was assessed with the Spatial Span recall (SSP, CANTAB) and the Digit Span

recall tests from the Wechsler Adult Intelligence Scale-III (WAIS-III), respectively. Planning capacity was measured using the Tower of London - Drexel University (ToLDx) and mental flexibility with the Weigl Color-Form Sort Test. The Cats and Dogs Test was used to assess response inhibition. Finally, measures of expressive and receptive language were obtained with the Boston Naming Test and the Token Test, respectively.

We used adult versions of the selected cognitive tests with the exception of four more difficult tests in which we used adapted versions for intellectual disability: the CRT (verbal episodic memory), the Cats and Dogs Test (inhibitory control), and the Weigl Color-Form Sort Test (mental flexibility); and a child version: the ToLDX (planning ability), in all cases to avoid floor effects.

Everyday life functionality was assessed with questionnaires for the following domains: adaptive behavior, quality of life, quality of sleep, and problematic behaviors. Measures of adaptive behavior were obtained with the adult version of the Adaptive Behavior Assessment System-Second Edition (ABAS-II). Quality of life was assessed with the 'parents and guardians' version of the Kidscreen-27. Quality of sleep was assessed with the Pittsburgh Sleep Quality Index (PSQI) and problematic behaviors were assessed with the Aberrant Behavior Checklist-C (ABC-C).

Neurophysiological parameters

We used prepulse inhibition (PPI) of acoustic startle responses (ASR) to evaluate neurophysiological changes in sensory processing and inhibitory control of brain information. Participants in the PPI sub-study (31 individuals), underwent three sessions (at baseline, and at 3 and 6 months after treatment initiation). ASR in the orbicularis oculi muscle were recorded bipolary from the right eye with surface cup electrodes located 2cm apart, edge to edge, in the muscle belly, as close to the margin of the lower lid as possible, and the lateral electrode in the external cantus. A ground electrode was placed on the ipsilateral mastoid.

Electromyographic (EMG) signals were rectified and amplified with a band pass of 20Hz to 350Hz. Auditory stimulus were delivered after 1-2 minutes of resting time period, by discharging a magnetic coil of a Medtronic stimulator (MagPro R30) over a metallic platform. The discharge induces a brief loud click, of approximately 130dB intensity. For the acoustic prepulse stimulus the coil was discharged at an intensity that was clearly audible but did not elicited consistent EMG responses. The prepulse preceded the startle stimulus by 50, 100, 150, 200, 500, and 1000 msec. We administered 3 trials for each interinterval stimulus with an intertrial interval that varied from trial to trial with a range of 20 to 50 seconds. If there was voluntary EMG activity, trials were rejected prior to data analysis. Participants with no EMG response following the startle stimulus were considered as non-responders. For trials with response onset between 20 to 100 msec, peak amplitude of the rectified EMG was measured. We calculated: (i) the amplitude and latency of the startle response to pulse alone trials and (ii) the PPI, calculated as the percent decrement in the startle amplitude in the presence of the prepulse compared with the amplitude without the prepulse: 100 X [(response amplitude in the startle stimulus alone trials - response amplitude in the prepulse trials)/response amplitude in the startle stimulus alone trials)]. Average PPI was calculated for each interstimulus interval. When responses were absent, they were given the value of 0 for calculation of the mean amplitude.

Biochemical biomarker analysis

Hepatic enzymes (AST-serum glutamic oxaloacetic transaminase, ALT-serum glutamic pyruvate transaminase, γ-Glutamyl Transferase), lipid profile and oxidation (LDL-low density lipoprotein, HDL-high density lipoprotein, cholesterol, triglycerides, and oxidized-LDL), urea, and alkaline phosphatase were evaluated in study participants. We also evaluated the effect of EGCG on the phosphorylation of the PI3K/mTOR and ERK signaling pathway in human blood lymphocytes, as possible markers of treatment efficacy.

289

290 **Statistical analysis**

291 A description of the baseline characteristics of all study participants included in the analysis is
292 provided using mean and standard deviation for quantitative variables and absolute and
293 relative frequencies for qualitative variables. In the case of the IQ, the median and the range
294 are presented given that IQ is a left-censored variable with lower limit of 40.

295 The data of the preclinical study corresponding to the comparison of the *Fmr1* knockout and
296 the WT mice were analyzed by means of the t-test and F-test.

297 The analyses from the clinical trial were performed on data from the modified intention-to-treat
298 population. To analyze the changes from baseline in scores of primary and secondary
299 outcomes, including all tests scores, plasma biomarkers, and neurophysiology variables, after
300 three and six months, respectively, ANCOVA models were used. These models included
301 treatment, IQ, baseline scores, and the number of training sessions during the respective
302 study period as independent variables. The effect size measure of all the regression models
303 fitted was the adjusted mean difference between both treatments with respect to changes from
304 baseline. This value is an estimation of the expected difference of the changes from baseline
305 between two persons that receive different treatments but have the same values in the
306 remaining variables (IQ, baseline score, number of training sessions). The statistical analyses
307 were performed with the statistical software package R (The R Foundation for Statistical
308 Computing), version 3.2. Statistical significance was set at 0·05.

309 **Results**

310 ***Preclinical studies***

311 ***EGCG improves novel object-recognition memory in the Fmr1 knockout mouse***

312 Fmr1–/y mice showed a marked cognitive deficit in the novel object-recognition memory test
313 (Student's t-test, t=6.002, P<0.001, df=12) (Fig. 1A), similar to that described previously

[12,14]. Acute administration of GTE containing EGCG (p.o.) after the training phase improved novel object-recognition memory in Fmr1–/y mice compared to Fmr1–/y littermates receiving saline (treatment effect: $F(1,19)=5.496$, $p<0.01$) (Fig. 1B). Notably, the dose of 4.5 mg/kg EGCG was as effective as the doses of 11.25 mg/kg or 22.5 mg/kg (Fig. 1B), pointing to the possibility of using low doses.

Clinical trial

Subjects, disposition, and dosing

Forty males and four females with ages in the 18-55 years range were assessed for eligibility for inclusion for the study. From these, thirteen did not enter the study due to exclusion criteria or lack of motivation to participate. As discussed earlier four additional subjects were excluded from the study because they did not met FXS molecular diagnosis criteria. Subject disposition and the composition of the populations are shown in the CONSORT diagram (Fig. 2). The proportion of individuals that completed all procedures was very similar in both groups. Among the 27 subjects included in the analyses, there were two dropouts (7.4%) during the study period: one subject from the Placebo+CT group, who left the study before the 6 months assessment session due to lack of motivation to continue; one subject from the EGCG+CT group, who left the study before the 6 months assessment session due to lack of motivation to participate in the neurophysiological exploration and the biochemical analysis, but agreed to complete the neuropsychological assessment and therefore these data have been included in the analyses. Thus, twenty-seven subjects (Table 1) were included in the final analysis.

Pre-specified efficacy analyses

The primary outcome was the change from baseline to 3 months in cognitive and functional components of the TESFX neuropsychology battery (Data for all the tests are summarized in Table S1).

Effects of combined treatment with EGCG or placebo and cognitive training (CT) after 3 months of intervention

We found statistically significant effects of EGCG+CT treatment on cognitive measures of visual episodic memory, but marginal non-significant effects on executive functions.

After three months of treatment, EGCG+CT treated individuals showed better performance on memory measures of the paired associate visual memory task from the CANTAB battery (PAL) compared to Placebo+CT with larger changes from baseline in the memory score in the first trial (Adjusted mean difference (AMD): 6.87; 95%-CI: [2.68,11.05]; p=0·003 Fig. 3A and Table S1), and in the number of stages completed (AMD: 1.72; [0.20,3.24]; p=0·029), and a larger decrease of errors per total number of trials (AMD: -63.62; [-110.59,-16.66]; p=0·011; Fig. 3B and Table S1). These results indicate a benefit on visual memory associated to EGCG+CT treatment. EGCG+CT treated individuals also showed a higher performance in planning capacity (Tower of London; TOLDX) that did not reach statistical significance. EGCG+CT group showed a larger reduction in the total number of moves for solving the items of the planning task (AMD: -19.42; [-39.38,0.54]; p=0·056) compared to the placebo group.

Regarding the impact of treatment on functional abilities, significant positive effects of EGCG+CT intervention were shown on adaptive behavior. EGCG+CT treated individuals showed an improvement in the ABAS-II home living score compared to baseline (AMD: 10.8; [2.05,19.55]; p=0·018) (Figure 3C and Table S1), whereas Placebo+CT group showed a mild decrease. No significant improvement was detected in the other skill areas: communication abilities, community use, functional academics, health and safety, leisure, self-care, self-direction, social interaction, and working/labor skills), or in the total ABAS score. No statistically significant improvement of Placebo+CT group over EGCG+CT treated individuals was detected.

6 months follow-up upon cessation of EGCG/placebo administration

During months 3 to 6, we discontinued EGCG and placebo, but both groups continued receiving CT. At 6 months, consistent positive effects persisted on visual episodic memory (CANTAB battery PAL) although to a lesser extent than at 3 months of treatment. A slight improvent in adaptive behavior and new beneficial effects emerged on attention, quality of life and, marginally, on executive functions in the EGCG+CT group. Regarding visual episodic memory, patients in the EGCG+CT group showed a significantly better performance in the paired associates visual learning task (CANTAB battery PAL), upon EGCG discontinuation, achieving a larger reduction of errors per number of trials (AMD: -47.80; [-94.00, -1.61]; p=0.043) compared to Placebo+CT. In the simple reaction attention task, the group treated with EGCG+CT showed a statistically significant larger reduction in latency and a larger increase in the number of correct trials compared to Placebo+CT (SRT mean latency: -251.15; [-396·19, -106.11]; p=0·003; SRT total correct trials: 19.12; [0.28, 37.96]; p=0·047), although for both parameters the beneficial effect were slightly lower than at 3 months. Positive effects emerged on executive functions regarding mental flexibility, inhibition and working memory. Although not reaching statistical significance, a larger increase of the total score in the mental flexibility Weigl test (AMD: 1.32; [-0.01, 2.65]; p=0·051), a decrease in the number of errors in the inhibition Cats and Dogs test (AMD: -3.12; [-5.52,-0.73] p=0.055), and an increase in the retention of the reverse span length in verbal working memory in the Digit span test, with (AMD: 0.81; [-0.03, 1.65]; p=0·058) were observed.

In the functional domain, the statistically significant differences with respect to the changes from baseline in adaptive behavior achieved in the ABAS-II home living subscale score during EGCG+CT treatment persisted after 3 months discontinuation (AMD: 10.62; [1.98,19.26]; p=0·019) (Table S1). In addition, in the EGCG+CT group we observed larger differences from baseline in the quality of life scores in the Kidscreen-27 regarding physical wellbeing (AMD: 7.76; [0.17,15.35]; p=0·045) and parent relationship (AMD: 6.63; [0.59,12.66]; p=0·033). No

389 statistically significant improvement of Placebo+CT group was observed in the functional
390 domain compared to EGCG+CT group.

391 *Cognitive training (CT) compliance*

392 Compliance with CT was considered poor in both treatment groups although the number of CT
393 sessions was different. After 3 months, both groups showed a similar number of training
394 sessions accounting for about 2 weekly sessions on average. The mean number of sessions
395 performed by Placebo+CT group was 23.5 ± 23.1, whereas EGCG+CT group performed 21.7
396 ± 15.7. Compliance with the cognitive stimulation program in the last 3 months after
397 EGCC/placebo discontinuation improved in the Placebo+CT group but not in the EGCG+CT
398 group. After 6 months, the mean number of sessions performed by Placebo+CT group was
399 36.7 ± 41.6 sessions, whereas the EGCG+CT group accounted for 28.1 ± 24.8.

400 *Neurophysiology*

401 From the 31 individuals that participated in the neurophysiology study, two were considered
402 non-responders. One individual of the Placebo+CT group dropped out the study after the 3
403 months. The data of four individuals were not considered of sufficient quality for the analysis in
404 one the measurements points. The amplitude of acoustic startle responses and the
405 percentage of prepulse inhibition (PPI) were analyzed in both groups at each interstimulus
406 interval (ISI), at baseline, after 3 months of treatment or placebo and after 3 months treatment
407 discontinuation. At baseline, the average percentage of PPI amplitude for the ISI of 50 msec.
408 was 25,9% (SD: 43,8) for EGCG+CT group and 37,8%±51,6 for Placebo+CT group,
409 41,4%±53,5 and 49,8% ±35,4 for the ISI of 100 msec; 35,5%±61 and 57%±42 for the ISI of
410 150 msec; 33,7%±50,5 and 56,9%±37,1 for the ISI of 200 msec. and 9,52%±59,1 and
411 45,3%±33,2 for the ISI of 500 msec. Differences from baseline, after 3 and 6 months were
412 calculated for both groups and adjusted for baseline values, cognitive stimulation and the IQ
413 KBIT score. The percentage of PPI increase at 3 and 6 months for each. Only for ISI of 150

msec a marginal tendency to significance (Eff.-32.39; p-value=0.07) was observed on the estimation differences between treatments after 6 months.

Safety

All adverse events occurring in more than one subject are listed in Table S2. Eighteen adverse events were reported throughout the clinical trial by thirteen patients that were equally distributed between both groups. From these, 16 adverse events were considered mild non-serious (12 in the EGCG+CT group and 6 in the Placebo+CT group). Two serious adverse events were reported by the same individual of the EGCG+CT group, who was hospitalized for a gallstone, which required cholecystectomy in a second hospitalization. This subject already reported pain episodes that could be related to gallstone prior to entering the study. This adverse effect was not considered related to EGCG. There were no withdrawals related to drug tolerability. No significant differences were detected for the safety biomarkers explored (see Table S3) Therefore, we can conclude that EGCG compound is safe and well tolerated in FXS young adults at the dose administered in our study.

Discussion

The aim of the present study was to investigate the safety and potential benefits of EGCG for improving cognition and everyday life functionality in FXS. Our study showed that EGCG is safe and active on cognitive function. In young adult *Fmr1* knockout mice (*Fmr1–/y)*, acute administration of three different doses of EGCG rescued object recognition memory deficits detected in non-treated *Fmr1–/y* mice. In our clinical trial, EGCG combined with cognitive training (CT) improved performance on cognitive measures of visual associative memory and had a positive impact on home living adaptive skills after a short treatment period of 3 months compared to Placebo+CT. However, no beneficial effects were detected in executive functioning. The amelioration in home living adaptive skills imply increased self-autonomy for taking care of personal possessions and performing routine household tasks such as

preparing meals, tiding up, cleaning, and using domestic equipment, allowing a higher daily competence in EGCG+CT treated individuals. After discontinuing the EGCG treatment, while continuing CT, sustained effects persisted on episodic memory and home living adaptive skills, and new significant improvements emerged in the EGCG-CT group on attention, which were accompanied by a significant improvement of quality of life perception on measures of physical wellbeing and parent relationship. In addition, marginal non-significant beneficial effects were observed on executive functions. This wide spectrum of gains was not observed in the Placebo+CT group, suggesting that the combination of EGCG and CT is most effective.

We speculate that the effects of EGCG could be related to an improvement in hippocampal functional networks, since in *Fmr1–/y* mouse, EGCG rescued hippocampal-dependent memory deficits (object recognition) and the main effect in patients was an improvement of immediate visual episodic memory sensitive to hippocampal dysfunction. These results, along with our previous clinical and preclinical cognitive and neuroimaging studies in Down syndrome [4], suggest that EGCG is probably acting upon brain distributed hippocampal-prefrontal functional networks supporting memory, and attention [3,4]. Noteworthy, a short-term period of 3 months under EGCG+CT combined intervention was sufficient for inducing significant functional changes in everyday life in our sample of FXS individuals. As previously observed in Down syndrome, cognitive and functional gains remained very stable and new emerged after 6 months related to EGCG+CT combined intervention. These results could be interpreted as CT maintenance after ceasing EGCG administration in the last 3 months would be contributing to sustain EGCG effects and may suggest that CT is an effective co-adjuvant for enhancing or sustaining EGCG effects. More studies are required to explore this notion.

We also aimed at detecting possible changes in neurophysiological parameters. FXS patients have reduced prepulse inhibition (PPI) than normal subjects, indicating impairment of sensorimotor gating [15]. We obtained similar results at baseline, but we did not detect significant group differences in PPI from baseline to 3 or 6 months. The heterogeneity within

the neurophysiological behavior of the FXS population, the large PPI impairment and the low number of subjects that finally were analyzed could explain this lack of positive results. Larger sample sizes in future studies are necessary to find differences between treatment groups. Regarding the possible mechanism of action of EGCG, we wanted to assess whether EGCG had an impact on mTOR phosphorylation cascade that could explain the positive effects on cognition [16,17]. EGCG has been proven to inhibit mTOR and reduce phosphorylation of downstream Akt [18]. However, we could not observe a homogenous increase in phosphorylation in basal conditions or a consistent decrease in phosphorylation after 3 months of treatment. Thus, we cannot confirm whether EGCG is having an effect on mTOR phosphorylation.

EGCG+CT did not produce detectable adverse effects. Severe adverse events were only reported in two occasions (same subject) associated to a premorbid medical condition and not to EGCG. As such, we conclude that EGCG is safe and well tolerated in FXS young adults at the dose administered in our study.

The study had some limitations. Our study was exploratory and with short-term administration, and thus phase 2 trials with a larger population and longer follow up periods under treatment in individuals with FXS will be needed to confirm the present results. Second, due to the large number of tests done in the framework of the regression models, the family-wise error rate exceeded 0.05. To protect against type II errors, no corrections for multiple comparisons were applied. Third, further studies are needed to rule out the possibility that any of the significant results was a type I error. Fourth, cognitive training differences in both treatment conditions after 6 months may have biased our results, given the higher attrition observed in the Placebo+CT against EGCG+CT. Even so, positive effects were detected in the former group. Fifth, the fact that there is still no consensus gold standard for testing cognitive changes in FXS despite on-going efforts [19], does not allow validating our findings, which is an important

caveat when interpreting the results of this study. However, valid, reliable, standardized, sensitive tests to mild cognitive changes where used, fact that allows replicating, comparing and validating our findings in future clinical trials. Sixth, it could be stated that sex was not included as a key variable for the randomization process nor as a predictor in the statistical analyses. The proportion of women was small, so we chose to balance and adjust for IQ to maintain the statistical power of our analyses with our reduced sample size. Thus, we do not expect that sex may have contributed to bias our results.

Conclusion

This Phase I study provides support to the benefits of using EGCG combined with CT as a promising therapeutic intervention for improving cognition, functionality in everyday life in adults with FXS, in the absence of substantial adverse effects.

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Statement of Authorship

1. Rafael de la Torre PI of the study and wrote the manuscript
2. Susana de Sola designed neuropsychological battery, explored subjects, wrote the manuscript
3. Magí Farré, pharmacologist participated in study design and subjects medical follow-up
4. Laura Xicota, biologist performed biomarkers analyses, edited the manuscript
5. Aida Cuenca-Royo neuropsychologist, explored subjects, edited the manuscript
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20. Mara Dierssen, Co-PI, edited the manuscript

Conflict of Interest Statement and Funding sources

Researchers declare no conflicts of interest.

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None of the funders or companies collaborating in the study had a role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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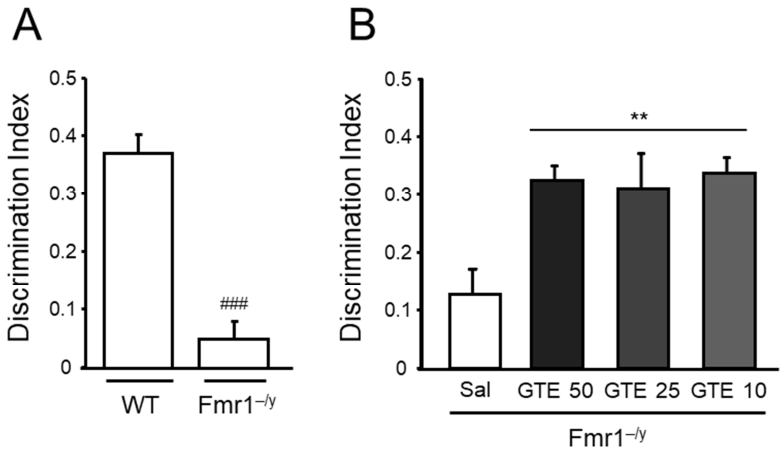
Figure and Tables Legends

Figure 1. The cognitive deficit in the novel object-recognition memory test of Fmr1–/y mice is sensitive to acute GTE administration. A) Fmr1–/y mice show a significant impairment in the novel object-recognition memory test compared to WT littermates. B) An acute administration

of decreasing concentrations of GTE (22.5, 11.25 or 4.5 mg/kg, p.o.) was enough to significantly improve the memory performance of Fmr1–/y mice assessed 24 h later. Data are expressed as mean ± s.e.m. ###P< 0.001 (compared to WT); **P<0.01 (compared to saline group).



668 **Figure 2. Consort diagram showing the flow of participants throughout the clinical trial**

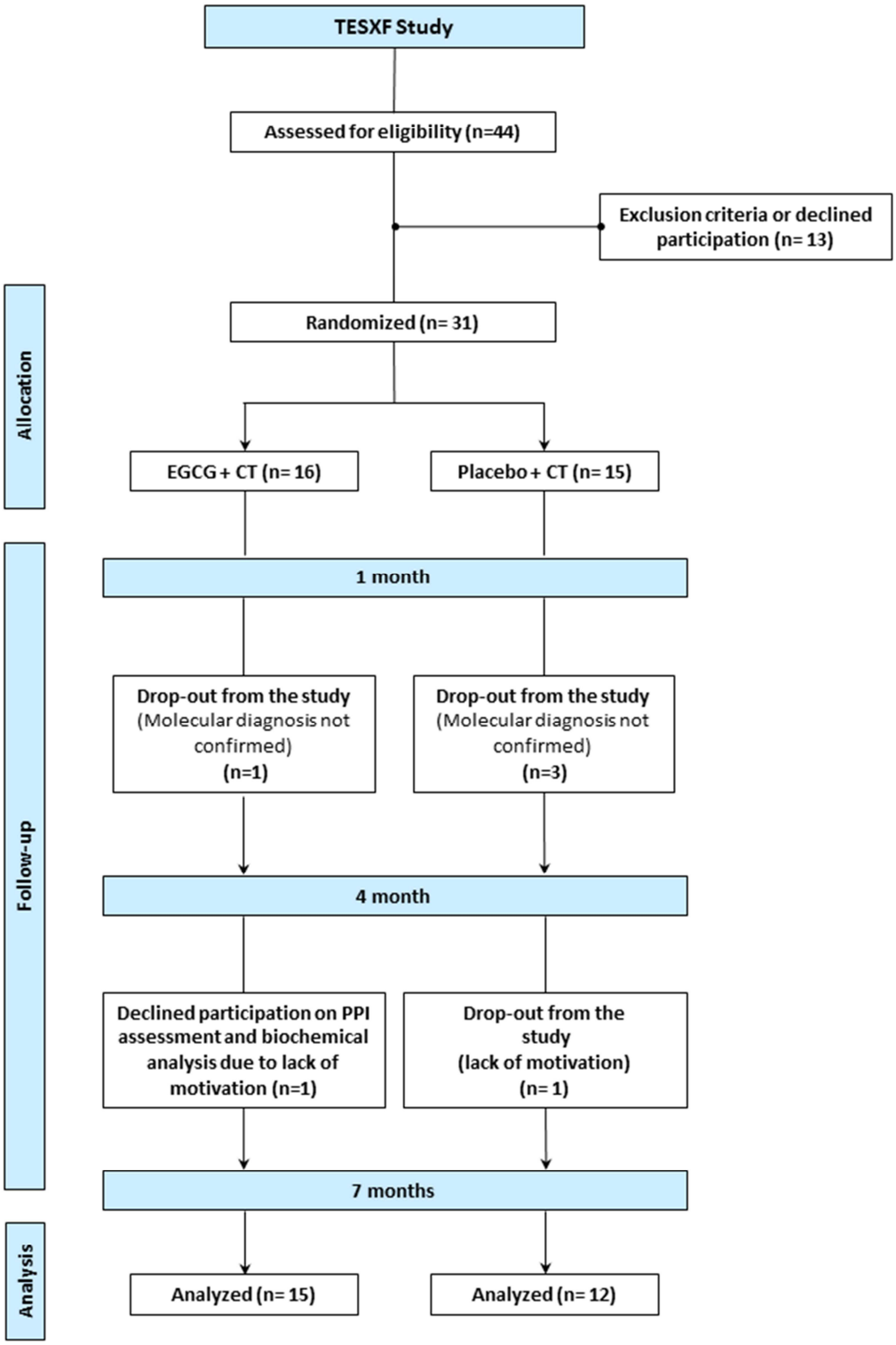


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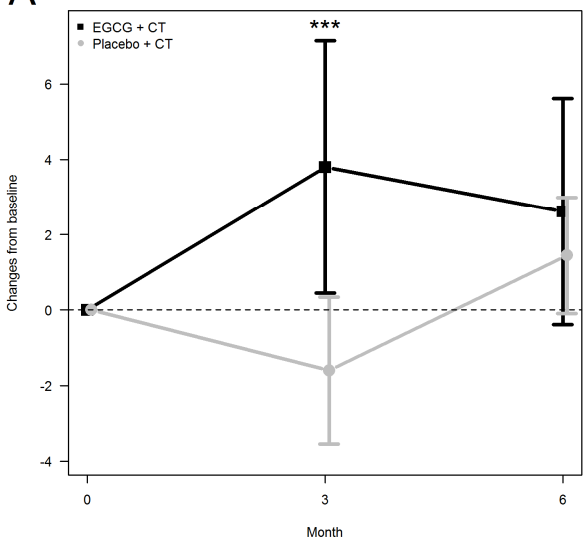
Figure 3. Effects of combined treatment with EGCG+CT and Placebo+CT on neurocognitive performance and adaptive behavior. Data correspond to treatment effects at 3months, and after a washout period of 3 months (mean differences from baseline and 95% confidence intervals). (A) Effects of EGCG+CT vs. Placebo+CT over time in the response of Paired Associates Learning memory task for first trial memory score, (B) Paired Associates Learning memory task for total errors, and (C) and in adaptive behavior in the ABAS II-Home Living skills score.

A



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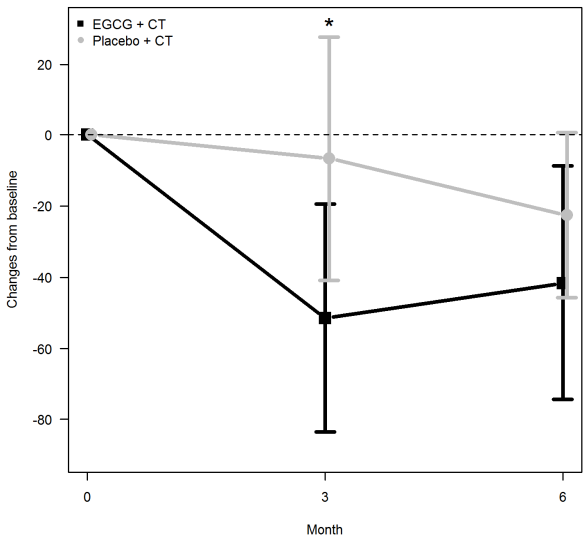
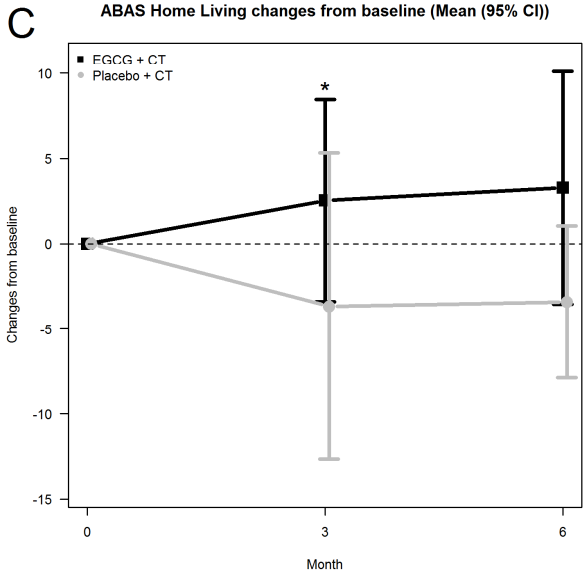


C



677

678 **Table 1. Sociodemographic characteristics and clinical parameters at baseline**

679

Participants characteristics	Placebo+CT n= 12	EGCG+CT n=15
Gender[1]		
Male	11 (91.7%)	12 (80%)
Female	1 (8.3%)	3 (20%)
Age[2]	39.5 (8.2)	32.9 (10)
FMR1[1]		
Full mutation	10 (83.3%)	11 (73.3%)
Mosaicism	2 (16.7%)	4 (26.7%)
BMI[2]	26.5 (3.2)	27.6 (4.3)
IQ[3]	41.5 (40 – 60)	55 (40 – 88)
Intellectual disability level[1]		
Mild	2 (16.7%)	8 (53.3%)
Moderate	10 (83.3%)	7 (46.7%)

680

681 Placebo+CT: Placebo + Cognitive Training group and EGCG+CT: EGCG + Cognitive Training group.

682 BMI: body mass index; IQ: intelligence quotient

683 (1) Number of subjects and (percentage), (2) Mean and (standard deviation), (3) Median and (range).

684