

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

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37 **Abstract**

38 Background & Aims:

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

44 Methods

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

51 Results

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

56 Conclusions

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

59

60 **Keywords**

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

62 **Introduction**

63 Fragile-X syndrome (FXS) (OMIM, 300624) is the most common (1/4000 live births) cause of
64 intellectual disability and autism of genetic origin. It is a trinucleotide repeat disorder caused
65 by a CGG repeat expansion at the 5'-end of the *FMR1* gene, which hypermethylation results in
66 transcriptional silencing and loss of expression of Fragile-X mental retardation protein
67 (FMRP). Brain alterations observed in FXS patients are linked to low concentrations of FMRP
68 [1], a RNA binding protein that plays a pivotal role in synaptic function. Despite the wide
69 spectrum of experimental compounds tested in clinical trials (i.e. selective antagonist of 5-
70 HT_{2B} serotonin receptor, metabotropic glutamate receptor 5 (mGluR5) antagonist, or GABA_B
71 receptor agonists) [2], there is no pharmacological treatment available for ameliorating
72 cognitive and behavioral features in FXS.

73 We previously showed that epigallocatechin-3-gallate (EGCG), the major catechin in green tea
74 leaves, elicited beneficial effects in intellectual disability. Specifically, in Down syndrome
75 mouse models and patients, EGCG administered alone [3] or in combination with cognitive
76 training, improved cognition and brain functional connectivity [4]. In fact, flavonoid-rich foods
77 can beneficially influence normal cognitive function [5,6] and slow down cognitive decline in
78 non-pathological aging. Mounting evidence suggests that Down syndrome and FXS share
79 common alterations in signaling pathways relevant for neural plasticity and learning and
80 memory processes, such as PI3K, mTOR and ERK1/2 [7,8], that are targeted by EGCG
81 [9,10]. We thus reasoned that EGCG could also rescue the cognitive alteration in FXS.

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

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

89

90 **Materials and Methods**

91 **1. Preclinical pharmacological studies**

92 *Animals*

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

104 *Drugs and treatments*

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

110 *Novel object-recognition memory test*

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

123 *Statistical analysis*

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

127 **2. Clinical trial**

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

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

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

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

162 group). Therefore, twenty-seven patients (61% of those initially contacted, n=44) were
163 randomly assigned to EGCG+CT or Placebo+CT group, using a random-number table and
164 were included in the intention to treat analysis (12 in Placebo+CT and 15 in EGCG+CT
165 group). One participant from the EGCG+CT and one from the Placebo+CT group dropped out
166 from the trial. Both were evaluated at 3 months after randomization, and the subject receiving
167 EGCG+CT was also evaluated at 6 months as the trial (both cases were included in statistical
168 analyses, in one case the whole evaluation at 6 months is missing while in the other case the
169 biochemical and the neurophysiological variables are unavailable; see CONSORT diagram,
170 Fig. 2). Neuropsychological data from these participants were included in the analyses. The
171 demographics were similar in the groups receiving EGCG+CT and Placebo+CT (Table 1).
172 Regarding intellectual disability level (intellectual quotient, IQ), the distribution of individuals
173 with severe ($IQ \leq 40$) and moderate to mild ($IQ \geq 40$) intellectual disability (Kaufman Brief
174 Intelligence Test) was different between groups. A higher proportion of individuals with
175 moderate intellectual disability was concentrated in the Placebo+CT group, but even so, the
176 mean IQ in the EGCG+CT group is almost in the cut-off of moderate disability level. Treatment
177 allocation and randomization was performed by the Hospital del Mar Pharmacy Department
178 (Table 1) that also was in charge of concealment under a sealed opaque envelope,
179 randomization sequence generation and supplementation of labeled packs (packs with
180 identical appearance and size zero blue opaque capsules for EGCG and placebo). All
181 members of the research team, the statistician, participants with FXS and families and/or
182 guardians were blind to the allocation to either treatment arm. The double-blind condition was
183 maintained until the end of the follow-up and data analysis.

184 **Design**

185 The trial design included one month of placebo (run-in period), 3 months of EGCG+CT or
186 Placebo+CT treatment and 3 months follow up after pharmacological treatment
187 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-](http://www.lifeextension.com/vitamins-supplements/item00954/mega-green-tea-extract-decaffeinated)
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

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

222 Neuropsychology

223 We used a customized neuropsychological battery (TESFX), which includes measures of
224 attention, psychomotor speed, memory, executive functions, language, adaptive and aberrant
225 behavior, and quality of life and sleep (see Supplementary materials). The TESFX battery was
226 developed for FXS clinical trials, based on the TESDAD battery for Down syndrome
227 individuals (NCT01394796) [13]. All neuropsychological outcomes were assessed at baseline,
228 3 months after treatment randomization, and 3 months after EGCG or placebo discontinuation.

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

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

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

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

256 Neurophysiological parameters

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

264 Electromyographic (EMG) signals were rectified and amplified with a band pass of 20Hz to
265 350Hz. Auditory stimulus were delivered after 1-2 minutes of resting time period, by
266 discharging a magnetic coil of a Medtronic stimulator (MagPro R30) over a metallic platform.
267 The discharge induces a brief loud click, of approximately 130dB intensity. For the acoustic
268 prepulse stimulus the coil was discharged at an intensity that was clearly audible but did not
269 elicited consistent EMG responses. The prepulse preceded the startle stimulus by 50, 100,
270 150, 200, 500, and 1000 msec. We administered 3 trials for each interinterval stimulus with an
271 intertrial interval that varied from trial to trial with a range of 20 to 50 seconds. If there was
272 voluntary EMG activity, trials were rejected prior to data analysis. Participants with no EMG
273 response following the startle stimulus were considered as non-responders. For trials with
274 response onset between 20 to 100 msec, peak amplitude of the rectified EMG was measured.
275 We calculated: (i) the amplitude and latency of the startle response to pulse alone trials and
276 (ii) the PPI, calculated as the percent decrement in the startle amplitude in the presence of the
277 prepulse compared with the amplitude without the prepulse: $100 \times [(response\ amplitude\ in\ the\ startle\ stimulus\ alone\ trials - response\ amplitude\ in\ the\ prepulse\ trials) / response\ amplitude\ in\ the\ startle\ stimulus\ alone\ trials]$. Average PPI was calculated for each interstimulus interval.
279 When responses were absent, they were given the value of 0 for calculation of the mean
280 amplitude.
281

282 Biochemical biomarker analysis

283 Hepatic enzymes (AST-serum glutamic oxaloacetic transaminase, ALT-serum glutamic
284 pyruvate transaminase, γ -Glutamyl Transferase), lipid profile and oxidation (LDL-low density
285 lipoprotein, HDL-high density lipoprotein, cholesterol, triglycerides, and oxidized-LDL), urea,
286 and alkaline phosphatase were evaluated in study participants. We also evaluated the effect
287 of EGCG on the phosphorylation of the PI3K/mTOR and ERK signaling pathway in human
288 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*^{-/-} 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

314 [12,14]. Acute administration of GTE containing EGCG (p.o.) after the training phase improved
315 novel object-recognition memory in *Fmr1*^{-/y} mice compared to *Fmr1*^{-/y} littermates receiving
316 saline (treatment effect: $F(1,19)=5.496$, $p<0.01$) (Fig. 1B). Notably, the dose of 4.5 mg/kg
317 EGCG was as effective as the doses of 11.25 mg/kg or 22.5 mg/kg (Fig. 1B), pointing to the
318 possibility of using low doses.

319 ***Clinical trial***

320 ***Subjects, disposition, and dosing***

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

334 ***Pre-specified efficacy analyses***

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

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

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

342 After three months of treatment, EGCG+CT treated individuals showed better performance on
343 memory measures of the paired associate visual memory task from the CANTAB battery
344 (PAL) compared to Placebo+CT with larger changes from baseline in the memory score in the
345 first trial (Adjusted mean difference (AMD): 6.87; 95%-CI: [2.68,11.05]; $p=0.003$ Fig. 3A and
346 Table S1), and in the number of stages completed (AMD: 1.72; [0.20,3.24]; $p=0.029$), and a
347 larger decrease of errors per total number of trials (AMD: -63.62; [-110.59,-16.66]; $p=0.011$;
348 Fig. 3B and Table S1). These results indicate a benefit on visual memory associated to
349 EGCG+CT treatment. EGCG+CT treated individuals also showed a higher performance in
350 planning capacity (Tower of London; TOLDX) that did not reach statistical significance.
351 EGCG+CT group showed a larger reduction in the total number of moves for solving the items
352 of the planning task (AMD: -19.42; [-39.38,0.54]; $p=0.056$) compared to the placebo group.

353 Regarding the impact of treatment on functional abilities, significant positive effects of
354 EGCG+CT intervention were shown on adaptive behavior. EGCG+CT treated individuals
355 showed an improvement in the ABAS-II home living score compared to baseline (AMD: 10.8;
356 [2.05,19.55]; $p=0.018$) (Figure 3C and Table S1), whereas Placebo+CT group showed a mild
357 decrease. No significant improvement was detected in the other skill areas: communication
358 abilities, community use, functional academics, health and safety, leisure, self-care, self-
359 direction, social interaction, and working/labor skills), or in the total ABAS score. No
360 statistically significant improvement of Placebo+CT group over EGCG+CT treated individuals
361 was detected.

362

363 *6 months follow-up upon cessation of EGCG/placebo administration*

364 During months 3 to 6, we discontinued EGCG and placebo, but both groups continued
365 receiving CT. At 6 months, consistent positive effects persisted on visual episodic memory
366 (CANTAB battery PAL) although to a lesser extent than at 3 months of treatment. A slight
367 improvement in adaptive behavior and new beneficial effects emerged on attention, quality of life
368 and, marginally, on executive functions in the EGCG+CT group. Regarding visual episodic
369 memory, patients in the EGCG+CT group showed a significantly better performance in the
370 paired associates visual learning task (CANTAB battery PAL), upon EGCG discontinuation,
371 achieving a larger reduction of errors per number of trials (AMD: -47.80; [-94.00, -1.61];
372 $p=0.043$) compared to Placebo+CT. In the simple reaction attention task, the group treated
373 with EGCG+CT showed a statistically significant larger reduction in latency and a larger
374 increase in the number of correct trials compared to Placebo+CT (SRT mean latency: -251.15;
375 [-396.19, -106.11]; $p=0.003$; SRT total correct trials: 19.12; [0.28, 37.96]; $p=0.047$), although
376 for both parameters the beneficial effect were slightly lower than at 3 months. Positive effects
377 emerged on executive functions regarding mental flexibility, inhibition and working memory.
378 Although not reaching statistical significance, a larger increase of the total score in the mental
379 flexibility Weigl test (AMD: 1.32; [-0.01, 2.65]; $p=0.051$), a decrease in the number of errors in
380 the inhibition Cats and Dogs test (AMD: -3.12; [-5.52,-0.73] $p=0.055$), and an increase in the
381 retention of the reverse span length in verbal working memory in the Digit span test, with
382 (AMD: 0.81; [-0.03, 1.65]; $p=0.058$) were observed.

383 In the functional domain, the statistically significant differences with respect to the changes
384 from baseline in adaptive behavior achieved in the ABAS-II home living subscale score during
385 EGCG+CT treatment persisted after 3 months discontinuation (AMD: 10.62; [1.98,19.26];
386 $p=0.019$) (Table S1). In addition, in the EGCG+CT group we observed larger differences from
387 baseline in the quality of life scores in the Kidscreen-27 regarding physical wellbeing (AMD:
388 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 $37,8\% \pm 51,6$ for Placebo+CT group,
409 $41,4\% \pm 53,5$ and $49,8\% \pm 35,4$ for the ISI of 100 msec; $35,5\% \pm 61$ and $57\% \pm 42$ for the ISI of
410 150 msec; $33,7\% \pm 50,5$ and $56,9\% \pm 37,1$ for the ISI of 200 msec. and $9,52\% \pm 59,1$ and
411 $45,3\% \pm 33,2$ 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

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

416 **Safety**

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

428

429 **Discussion**

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

440 preparing meals, tidying up, cleaning, and using domestic equipment, allowing a higher daily
441 competence in EGCG+CT treated individuals. After discontinuing the EGCG treatment, while
442 continuing CT, sustained effects persisted on episodic memory and home living adaptive
443 skills, and new significant improvements emerged in the EGCG-CT group on attention, which
444 were accompanied by a significant improvement of quality of life perception on measures of
445 physical wellbeing and parent relationship. In addition, marginal non-significant beneficial
446 effects were observed on executive functions. This wide spectrum of gains was not observed
447 in the Placebo+CT group, suggesting that the combination of EGCG and CT is most effective.
448 We speculate that the effects of EGCG could be related to an improvement in hippocampal
449 functional networks, since in *Fmr1*-/- mouse, EGCG rescued hippocampal-dependent
450 memory deficits (object recognition) and the main effect in patients was an improvement of
451 immediate visual episodic memory sensitive to hippocampal dysfunction. These results, along
452 with our previous clinical and preclinical cognitive and neuroimaging studies in Down
453 syndrome [4], suggest that EGCG is probably acting upon brain distributed hippocampal-
454 prefrontal functional networks supporting memory, and attention [3,4]. Noteworthy, a short-
455 term period of 3 months under EGCG+CT combined intervention was sufficient for inducing
456 significant functional changes in everyday life in our sample of FXS individuals. As previously
457 observed in Down syndrome, cognitive and functional gains remained very stable and new
458 emerged after 6 months related to EGCG+CT combined intervention. These results could be
459 interpreted as CT maintenance after ceasing EGCG administration in the last 3 months would
460 be contributing to sustain EGCG effects and may suggest that CT is an effective co-adjuvant
461 for enhancing or sustaining EGCG effects. More studies are required to explore this notion.
462 We also aimed at detecting possible changes in neurophysiological parameters. FXS patients
463 have reduced prepulse inhibition (PPI) than normal subjects, indicating impairment of
464 sensorimotor gating [15]. We obtained similar results at baseline, but we did not detect
465 significant group differences in PPI from baseline to 3 or 6 months. The heterogeneity within

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

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

480

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

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

499

500 **Conclusion**

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

504

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556

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

580

581 **Conflict of Interest Statement and Funding sources**

582 Researchers declare no conflicts of interest.

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589

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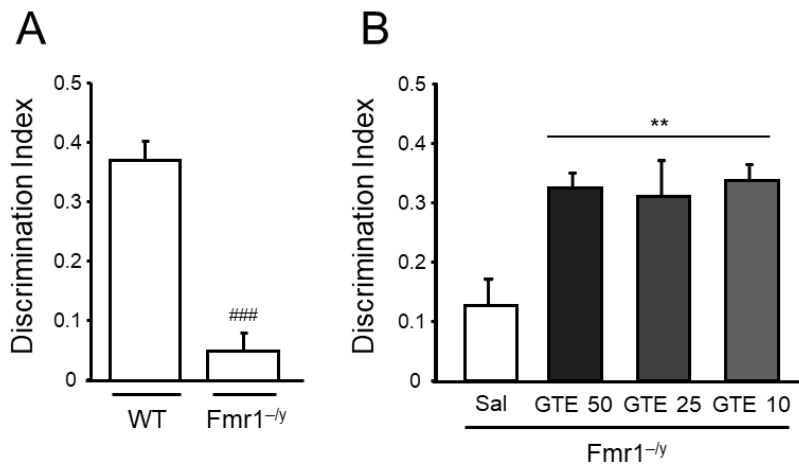
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657

658 **Figure and Tables Legends**

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

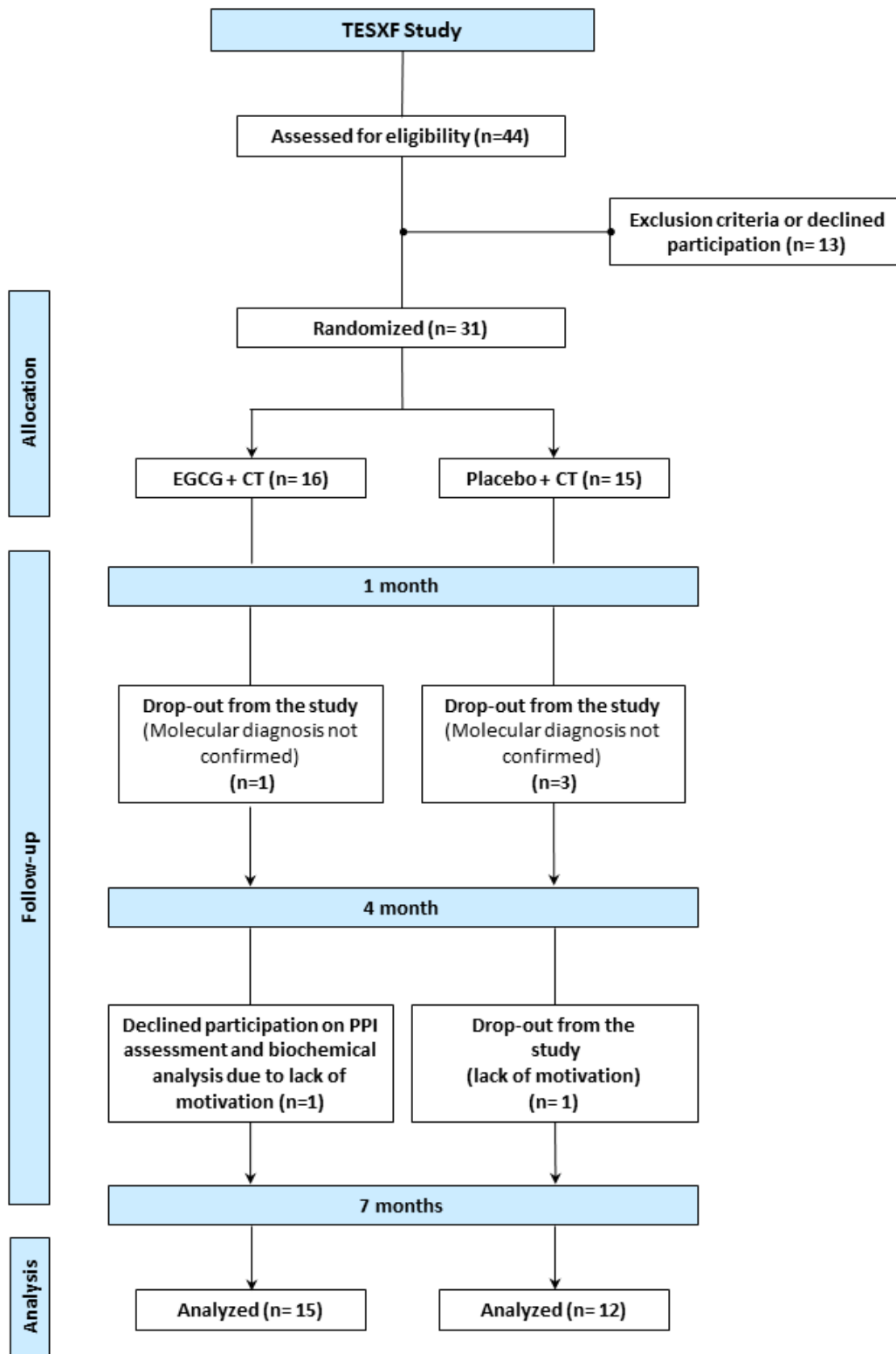
662 of decreasing concentrations of GTE (22.5, 11.25 or 4.5 mg/kg, p.o.) was enough to
663 significantly improve the memory performance of Fmr1^{-/-} mice assessed 24 h later. Data are
664 expressed as mean ± s.e.m. ###P< 0.001 (compared to WT); **P<0.01 (compared to saline
665 group).



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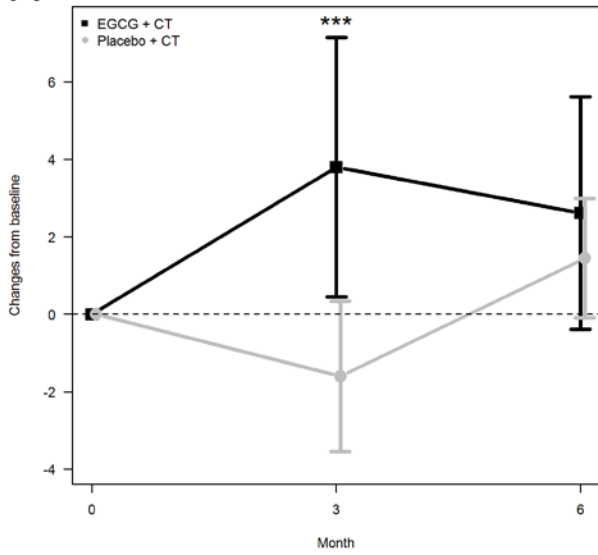
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668 **Figure 2. Consort diagram showing the flow of participants throughout the clinical trial**

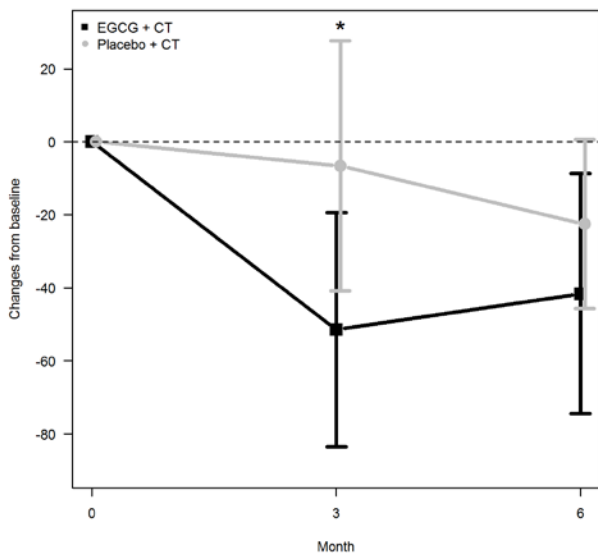


670 **Figure 3. Effects of combined treatment with EGCG+CT and Placebo+CT on**
671 **neurocognitive performance and adaptive behavior.** Data correspond to treatment effects
672 at 3months, and after a washout period of 3 months (mean differences from baseline and 95%
673 confidence intervals). (A) Effects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

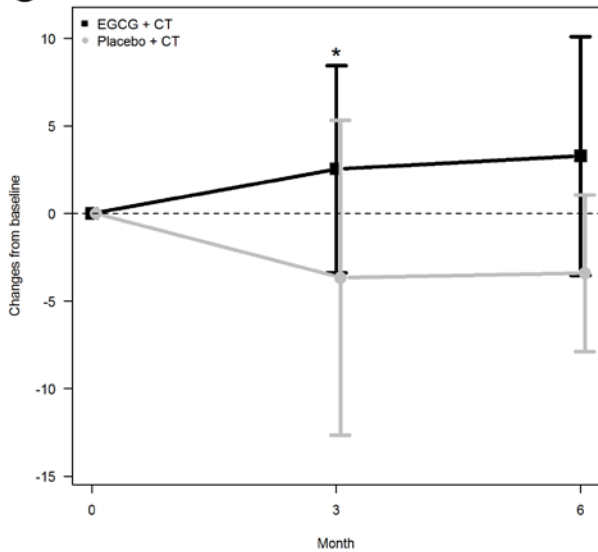
A PAL 1st Trial Memory Score changes from baseline (Mean (95% CI))



B PAL Total Errors Adjusted changes from baseline (Mean (95% CI))



C ABAS Home Living changes from baseline (Mean (95% CI))



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

679

Participants characteristics	Placebo+CT n= 12	EGCG+CT n=15
Gender¹		
Male	11 (91.7%)	12 (80%)
Female	1 (8.3%)	3 (20%)
Age²	39.5 (8.2)	32.9 (10)
FMR1¹		
Full mutation	10 (83.3%)	11 (73.3%)
Mosaicism	2 (16.7%)	4 (26.7%)
BMI²	26.5 (3.2)	27.6 (4.3)
IQ³	41.5 (40 – 60)	55 (40 – 88)
Intellectual disability level¹		
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