1   **Epigallocatechin-3-Gallate Improves Facial Dysmorphology Associated with Down Syndrome**

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25   **Key Words**: Trisomy 21, EGCG, facial development, Procrustes, Euclidean Distance Matrix Analysis,

26   morphometrics, photogrammetry, mouse model

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32

33   **Abstract**

34   In Down syndrome (DS), the overall genetic imbalance caused by trisomy of chromosome 21 leads to a

35   complex pleiotropic phenotype that involves a recognizable set of facial traits. Several studies have

36   shown the potential of epigallocatechin-3-gallate (EGCG), a green tea flavanol, as a therapeutic tool for

37   alleviating different developmental alterations associated with DS, such as cognitive impairment, skull

38   dysmorphologies, and skeletal deficiencies. Here we provide for the first time experimental and clinical

39   evidence of the potential benefits of EGCG treatment to facial morphology. Our results showed that

40   mouse models treated with low dose of EGCG during pre- and postnatal development improved facial

41   dysmorphology. However, the same treatment at high dose produced disparate facial morphology

42   changes with an extremely wide and abnormal range of variation. Our observational study in humans

43   revealed that EGCG treatment since early in development is associated with intermediate facial

44   phenotypes and significant facial improvement scores. Overall, our findings suggest a potential

45   beneficial effect of ECGC on facial development, which requires further research to pinpoint the optimal

46   dosages of EGCG that reliably improve DS phenotypes. Current evidence warns against the non-

47   prescribed intake of this supplement as a health-promoting measure.

48

49   **Introduction**

50   The face, despite being a variable phenotype with high levels of plasticity and remodeling throughout

51   the life of an individual, requires the fine orchestration of multiple morphogenetic events during early

52   development and subsequent growth[1]. Changes to this long-term program can impair growth and bone

53   remodeling and result in facial shapes that fall beyond the typical range of facial morphological

54   variation[2]. Facial dysmorphologies are often associated with congenital and neurodevelopmental

55   disorders. Since brain and face are intimately integrated[3], genetic and/or environmental insults in early

56   embryogenesis can alter the development of both structures. A major genetic alteration results from

57   aneuploidies, which involve the presence of an abnormal number of whole or part of a chromosome[4].

58   Trisomy 21 (Ts21), referred to as Down syndrome (DS) (OMIM 190685), is a complex pleiotropic genetic

59   disorder that is the most common genetic cause of intellectual disability and is associated with a

60   characteristic facies. The specific facial features of DS (i.e., midfacial flatness, oblique palpebral fissures,

61   mandibular hypoplasia, and facial asymmetry), may cause facial stigmata and compromise quality of

62   life[5].

63   Besides altering neural[6] and facial development[7], the overall genetic imbalance resulting from the

64   overexpression of hundreds of chromosome 21 genes perturbs the morphogenesis and growth of many

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65   other body systems and organs, such as immune[8] and gastrointestinal[9] systems, as well as the heart[10]

66   and the skeleton[11]. The complex and widespread phenotypic outcomes of DS are thus the result of

67   combined effects of genes altering specific tissues[12], as well as the integrated effect of genes altering

68   common signaling pathways simultaneously in different tissues during development. This genetic

69   complexity, combined with large degrees of developmental and phenotypic variability in individuals with

70   DS, represents a serious challenge to any attempt to alleviate the alterations caused by Ts21.

71   Nevertheless, recent studies have shown that epigallocatechin-3-gallate (EGCG), a green tea flavanol, is

72   a promising therapeutic tool for individuals with DS[13–17]. Clinical studies indicate that EGCG doses

73   equivalent to a daily intake of eight cups of green tea show efficacy in improving adaptive functionality

74   and cognitive deficits associated with DS[13,16]. Similar effects have been found in a study using DS mouse

75   models[13]. EGCG is a well-known kinase inhibitor[18], but the optimal dosage, onset and duration of the

76   EGCG treatment in DS remain to be elucidated[19].

77   The potential of EGCG as an effective treatment for DS relies, at least in part, on its ability to inhibit the

78   gain of function of the dual specificity tyrosine-phosphorylation-regulated kinase 1A (*DYRK1A)*[20], which

79   is one of several chromosome 21 genes triplicated by Ts21 and at dosage imbalance in individuals with

80   DS that map to the so-called Down syndrome critical region (DSCR)[21]. Traditionally, the activity of

81   *DYRK1A* has been associated with neural development[22], but in recent years new roles for *DYRK1A* have

82   been identified for development of the face[23], the skeleton[14,15], and the heart[24]. Interestingly, recent

83   studies in mice have reported that EGCG may alleviate some skull[17] and skeletal alterations[14,15] in DS

84   mouse models.

85   Given the potential of EGCG as an effective treatment to minimize many of the alterations present in

86   DS[13,15–17], we here focused on assessing the effect of EGCG on the development of the face. We started

87   examining the effect of a standardized EGCG treatment on facial skeletal development using the well-

88   established *Ts65Dn* DS mouse model. We also performed an observational study to investigate for the

89   first time whether EGCG treatment also affects facial dysmorphology in children with DS.

90

91   *Experimental effect of EGCG treatment on facial shape in DS mouse model*

92   We explored the potential of EGCG prenatal treatment to prevent the facial malformations associated

93   with DS, assessing facial morphology in trisomic (TS) and euploid (wild-type or WT) mice after randomly

94   treating half of the litters either with a high (30 mg/kg/day) or a low (9 mg/kg/day) EGCG dose from

95   embryonic (E) day 9 to post-natal (P) day 29 (see Table SI_1 for sample composition and Materials and

96   Methods for more details on mouse models and treatment conditions). We compared mouse facial

97   shape using the 3D coordinates of 12 facial landmarks registered on micro-computed tomographic (μCT)

98   images of the craniofacial skeleton of the mice (Fig. SI_1 and Table SI_2).

99   Results of a Procrustes-landmark[25] based shape analysis showed that, despite slight morphospace

100   overlap, untreated WT and TS mice showed different facial shapes (Fig. 1). However, the effect of the

101   EGCG treatment varied greatly depending on the dose. Both WT and TS mice treated with the high EGCG

102   dose presented large ranges of morphological variation, spanning through the whole morphospace (Fig.

103   1). Regardless of their genotype, mice presented disparate facial phenotypes: some mice, both WT and

104   TS, fell within the typical range of variation of WT untreated mice, whereas others fell on the opposite

105   extreme of variation, associated with unique morphology and severe facial phenotypes (Fig. 1). These

106   results indicated that, at high dosages, the effect of EGCG on facial shape is highly variable and

107   potentially detrimental in some cases.

108   On the contrary, mice treated with the low EGCG dose showed moderate ranges of variation and both

109   WT and TS treated mice presented WT-like facial shapes, different from TS facial shapes (Fig. 1). WT

110   mice treated at low EGCG doses did not show any effect of the treatment on facial shape, whereas 80%

111   of the TS mice treated with low EGCG doses fell within the range of variation of untreated WT mice (Fig.

112   1). This strongly supports a predictable normalization of global facial shape upon low dose EGCG

113   treatment.

114   To provide further evidence about the effects of EGCG on murine facial morphology at a more localized

115   level, we developed a novel approach to estimate facial improvement scores (FISs) and assess if the

116   EGCG treatment significantly changes the number of facial traits that differentiate the euploid and the

117   trisomic facial phenotypes for each of the EGCG dosages. This approach is based on the results of

118   Euclidean Distance Matrix Analyses (EDMA)[26], a technique that calculates all unique linear distances

119   between the facial landmarks for each individual and performs pairwise shape comparisons between

120   groups. To estimate FIS scores, we first ran an EDMA to detect how many linear distances were

121   significantly different between WT and TS untreated mice. Then, we ran another EDMA between WT

122   mice and TS mice treated with EGCG, and by comparing the results from both EDMAs we estimated the

123   corresponding FIS (see formula in Materials and Methods). A FIS score close to 0 represents no effect of

124   the EGCG treatment, as the number of significant facial differences between WT and TS mice remains

125   equal with or without EGCG treatment. A positive FIS score represents a normalization of the facial

126   phenotype, as it is achieved by a reduction of the facial shape differences between WT and TS mice after

127   treatment. A negative FIS score indicates a detrimental effect of the treatment, in which the EGCG

128   treatment exacerbates the severity of the DS facial traits, increasing the number of facial shape

129   differences between WT and TS mice. Finally, to confirm that the patterns revealed by the EDMA

130   analyses were not due to random chance alone, we tested the statistical significance of the results

131   performing simulation tests. We performed a subsampling approach in which we generated random

132   pseudo-subsamples with increasing number of DS treated cases, and for each simulation we

133   recomputed all the EDMA analyses and estimated the associated FIS scores (see Materials & Methods

134   for further details).

135   The histograms in Fig. 2 compare the distribution of randomly generated FIS scores with the FIS

136   obtained using the experimental data. Although the tests did not reach statistical significance because of

137   large degrees of variation, the results of the simulation analyses tended to confirm our previous results:

138   a negative effect of the high EGCG dose, but a positive effect of the low dose. The high dose EGCG

139   treatment was associated with a negative FIS, as the number of facial differences between WT and TS

140   mice increased when more TS treated mice were included in the simulated groups, and thus suggesting

141   a detrimental effect of the high dose EGCG treatment (Fig. 2). On the contrary, the low dose EGCG

142   treatment was associated with a positive FIS (Fig. 2), as the number of facial differences decreased with

143   increasing number of TS treated mice, suggesting a normalization of the facial phenotype at low EGCG

144   doses.

145

146   ***Effect of EGCG treatment on facial shape of children with DS***

147   The findings based on the experimental DS mouse models led us to explore whether EGCC treatment

148   can also modulate facial development in humans. To this aim, and given that some parents are

149   administering over-the-counter green tea extracts to their children because these are commercially

150   available as dietary supplements, we were able to perform the first observational study ever to

151   investigate whether EGCG treatment improves facial dysmorphology in children with DS. Our

152   investigation is a cross-sectional study, on a cohort of individuals with DS that started green tea

153   consumption on the initiative of their parents encouraged by the pro-cognitive effects of EGCG in DS.

154   The participants in our study did not follow a systematic EGCG usage pattern, and thus had not

155   homogeneous or standardized dosage, onset, and duration of treatment. Yet, assessing whether there is

156   an effect of EGCG treatment on DS facial development may provide valuable evidence to determine

157   whether EGCG treatment can alleviate facial dysmorphologies in DS.

158   To assess the effect of EGCG supplementation on facial development of children with DS, we first

159   acquired 3D facial images of children with DS, children with DS that have received EGCG treatment,

160   mosaic cases with quantified percentages of trisomic cells (50-80%), and euploid individuals (see Table

5

161   S1_3 for sample composition and Table SI_4 for cohort and EGCG treatment details). Individuals were

162   categorized into three age groups (0-3 yrs., 4-12 yrs., 13-18 yrs.) to determine whether EGCG treatment

163   could have differing effects depending of the stage of development and onset of the treatment. Hence,

164   facial shape was evaluated within each age group using multivariate geometric morphometric methods.

165   We first assessed the overall face shape differences between euploid children and children with DS, with

166   particular focus on children with DS that had received EGCG supplementation. Our results, based on

167   Procrustes analyses of a configuration of 21 landmarks located throughout the face (Fig. SI_1), showed

168   almost complete separation between euploid children and those with Ts21 in all three age groups (Fig.

169   3), with slight to moderate overlap depending on the specific age range. Overall, facial phenotypes

170   associated with DS included flat and wider faces, oblique palpebral fissures, flat nasal bridges, wider

171   noses, and thicker lips. Facial phenotypes associated with the euploid condition presented slender and

172   slightly convex faces, with horizontal palpebral fissures, narrow noses and thin lips. The position in the

173   morphospace of the DS cases treated with EGCG varied greatly in the different age groups. Depending

174   on the age of initiation of the treatment, DS cases treated with EGCG showed facial phenotypes that fell

175   within the ranges of variation of either the DS or the euploid groups (Fig. 3).

176   In the age group including teenagers from 13 to 18 years old, the participants that had taken the EGCG

177   treatment completely overlapped with other individuals with DS that never received a treatment. Thus,

178   the facial shapes of teenagers with DS were similar regardless of the EGCG treatment, suggesting that

179   EGCG did not have any effect on facial shape when the treatment started late in adolescence (Fig. 3a).

180   In the age group from 4 to 12 years old, the two DS treated cases fell in an intermediate morphospace

181   position, one within the euploid range of variation and the other within the overlapping area between

182   the euploid and the DS range of variation. This result showed a tendency of the children with DS treated

183   with EGCG to display milder facial phenotypes. The child receiving the lower dose fell closer to the

184   euploids, but no conclusive evidence can be drawn from these results due to the small sample size and

185   the substantial differences in the dosage/length of EGCG treatment (Fig. 3b).

186   Finally, in the age group of youngest children (from 0 to 3 years old), we observed the most prominent

187   effect of EGCG treatment: most children with DS who had taken EGCG since early in development

188   presented a moderate facial phenotype (Fig. 3c). Six out of seven EGCG treated cases fell in an

189   intermediate morphospace position between the euploid and trisomic samples. Three fell in the range

190   of variation of children with DS showing mild facial phenotypes closer to euploid morphospace, and

191   three fell within the range of variation of the euploid subsample (Fig. 1c). Only one child with DS treated

192   with EGCG did not follow this tendency and was positioned within the range of variation defined by

193   untreated DS cases (Fig. 3c), but this difference could not be significantly associated with any of the

194   conditions of the treatment.

195   The position of three mosaic DS individuals within the range of variation of euploid children correlated

196   with the degree of mosaicism. The mosaic case with the lowest reported percentage of trisomic cells

197   (50%) fell in the middle of the range of variation of euploid children, whereas the other two mosaic

198   cases with higher percentage of trisomic cells (75 and 80%) showed a more typical DS phenotype, but

199   still overlapped with euploid children and DS cases with less marked DS facial traits (Fig. 3c). Our results

200   thus provide evidence that reduced degree of genetic imbalance, resulting either from mosaic trisomy

201   or EGCG treatment, is associated with milder facial phenotypes and reduced dysmorphology.

202   Next, we used EDMA to detect statistically significant local facial shape differences and to pinpoint

203   exactly where EGCG may influence facial shape. We estimated the baseline comparison of DS and

204   euploid samples, and then assessed whether the result for each linear distance was significantly

205   different when the comparison only included children with DS that had been treated with EGCG. A

206   potential beneficial effect of EGCG would be revealed by a decrease in the number of significantly

207   different facial measurements between euploid and trisomic children treated with EGCG relative to the

208   baseline comparison of the euploid children and untreated DS cases.

209   When assessing facial differences in the 13-18 year age group, our results indicated only a small

210   reduction in the number of significant linear distances. A total of 61% of linear measures significantly

211   differed between the baseline comparison of DS and euploid samples (Fig. 4a), while 53% were still

212   significantly different between euploid and treated samples (Fig. 4b). When comparing untreated

213   children with DS and those treated with EGCG, only a minimal number of significant differences were

214   found (5%), indicating overall similarity between untreated and treated children with DS (Fig. 4c, d).

215   A similar pattern was detected in the 4-12 year age group. EGCG treatment did not produce a

216   substantial change in the number of facial differences between euploid and trisomic children. The

217   baseline comparison of DS and euploid cases estimated that 59% of facial measurements were

218   significantly different in this age-range (Fig. 4e), and this percentage was only reduced to 46% when

219   comparing EGCG treated individuals with DS to the euploid sample (Fig. 4f). However, we detected that

220   the number of significant shape differences between treated and untreated children with DS was larger

221   in the 4-12 age group (Fig. 4g) than in the 13-18 age group (Fig. 4c). This result could be suggesting an

222   incipient positive effect of the EGCG treatment that was however not sufficient to rescue the facial

223   phenotype entirely (Fig. 4g, h).

224 Finally, the analysis of the youngest age group of 0-3 year olds revealed the most significant effect. The

225 EDMA comparison between euploid and DS children showed that 57% of linear distances were

226 significantly different (Fig. 4i). However, only 25% of linear distances remained significant when young

227 children with DS treated with EGCG were compared to the euploid sample (Fig. 4j). This indicated an

228 overall improvement of more than 50% of total facial measurements relative to the baseline

229 comparison. A significantly large beneficial EGCG treatment effect is thus suggested, especially on those

230 facial traits that appear as significantly different when comparing EGCG treated and untreated children

231 with DS (Fig. 4k, l). The local EDMA morphological assessments indicated that various dimensions of the

232 nose, philtrum, cheeks and midface were improved after EGCG treatment, with the strongest effect

233 occurring in the age group of 0-3 year olds, but with less of an effect in older age groups.

234 To verify the results suggesting that EGCG improved facial development in young individuals with DS,

235 summary scores of facial improvement (FISs) were estimated and tested using the random simulation

236 approach. The FIS score quantified whether EGCG treatment decreased or increased the number of

237 shape differences identified by the EDMA between children with DS and euploid children. Results

238 showed that in older children (from 13 to 18 and from 4 to 12 years old), the FIS score was positive but

239 low, indicating only a minor relative improvement of facial shape (Fig. 5). The simulation tests indicated,

240 however, that the effect of EGCG at these later stages of development was not significantly different

241 from what would be obtained from a random distribution (Fig. 5). In contrast, the results at the earliest

242 stage of development showed a large positive effect. In 0 to 3 year old children with DS treated with

243 EGCG, the facial improvement was high (FIS=58%) and significant. The simulation tests showed a clear

244 tendency towards increasing the FIS as the number of DS treated cases included in the simulations

245 increased (Fig. 5). Since there were only three random simulations that produced higher FIS scores, our

246 results demonstrate that the effect of EGCG treatment on facial shape at young age is highly significant

247 (*P-value*=0.008) and beneficial.

248

249 **Discussion**

250 No approved intervention exists for the clinical amelioration of individuals with DS. However,

251 accumulating evidence is revealing that modulation of a complex regulatory circuit controlling neural,

252 facial and skeletal development by EGCG kinase activity inhibition can improve several aspects of the DS

253 phenotype[21,24]. Previous research has suggested that EGCG enhances cognitive function in individuals

254 with DS[13,16], as well as cranial morphology[17] and long-bone skeletal architecture[14,15] in DS mouse models.

255 Our study extends the evidence for potential benefits of the EGCG treatment to facial morphology with

256 new experimental data on dosage effects, and provides, for the first time, clinical evidence of EGCG

257 effects in an observational study.

258

259 **Effects of EGCG treatment depend on the dose in mouse experimental data**

260 Our analyses using *Ts65Dn* mouse model (Fig. 1) suggested that administration of EGCG at low doses

261 from E9 to P29 improved facial dysmorphology. However, the same treatment at high dose produced

262 disparate facial morphology changes with an extremely wide and abnormal range of variation (Figs. 1

263 and 2). Since some mice receiving the high EGCG dose had relatively normal facial morphology, while

264 others exhibited different and unique dysmorphology (Fig. 1), the bimodal distribution of these results

265 may be suggestive of a threshold effect of EGCG that alters developmental canalization and reaction

266 norms by shifting craniofacial development to a different developmental pathway with a separate

267 phenotypic fate[27]. The unpredictable facial morphologies resulting from high EGCG doses, affecting both

268 wild type and trisomic mice, require further investigation to confirm the potential therapeutic window

269 of EGCG to prevent and alleviate facial dysmorphologies associated with DS.

270 Ours results agree with previous research using DS mouse model experiments indicating that the

271 potential rescuing effect of EGCG treatment depends on several factors, such as dosage, onset and

272 duration of the treatment, as well as method of delivery and composition of EGCG-containing

273 supplements[19]. De la Torre et al. (2014) showed that 1 month of 2-3mg of EGCG per day can rescue

274 cognitive deficits in 3-month old adult *Ts65Dn* mice (trisomic background including *Dyrk1a* triplication),

275 and transgenic *TgDyrk1a* mice (disomic background including *Dyrk1a* triplication). This result concurred

276 with the effect observed in humans, where the combined intervention of EGCG treatment and cognitive

277 training seemed to ameliorate memory and executive deficits in young adults with DS after taking oral

278 doses of 9 mg/kg/day of EGCG for 3-12 months[13,16]. However, *Ts65Dn* mice treated with ~10, ~20, or

279 ~50mg/kg/day of EGCG starting at P24 for 3- or 7-weeks failed to show cognitive improvements[28].

280 Regarding the effect of EGCG on skeletal traits, a recent study indicated that EGCG treatment of 200

281 mg/kg/day on gestational days 7 and 8 of pregnant *Ts65Dn* DS mouse models rescued some cranial vault

282 morphologies measured two months later in 6-week old offspring[17]. Research on limb long bones has

283 shown that specific conditions of EGCG treatment can lead to correction of skeletal deficits associated

284 with DS. For instance, when 3 week old *Ts65Dn* mice were treated with ~9 mg/kg/day of EGCG for 3

285 weeks beginning in early adolescence, they exhibited increased femoral bone mineral density relative to

286 untreated trisomic mice and trabecular microarchitecture (*i.e.,* percent trabecular bone volume,

287 trabecular number and thickness) was rescued to euploid levels[15]. However, *Ts65Dn* mice treated with

288 ~50mg/kg/day for 7 weeks starting at P24 exhibited detrimental effects on femoral bone structure and

289 mechanical properties, such as reductions in cortical area and thickness, structural strength (yield force),

290 stiffness, and ultimate force[19]. Further experimentation with mouse models is thus critical to explain the

291 discordant effects of different conditions of EGCG treatment.

292

293 ***Potential of EGCG for facial bone remodeling***

294 In the observational study in humans, our morphometric assessment of facial shape revealed the

295 strongest and most beneficial effect for EGCG in the youngest human age group, 0-3 years, when most

296 craniofacial growth occurs (Fig. 4). Only younger children with DS treated with EGCG since their first

297 months of life up to 3 years presented with facial phenotypes that fell close to or within the euploid

298 range of variation (Fig. 3c). It is during these first years of life when the skull and the face exhibit high

299 growth velocities associated with relatively large size and shape change[29], likely driven by rapid brain

300 expansion and masticatory changes as offspring are weaned to semi-solid and solid foods. After facial

301 shape maturation is reached by 15-16 years of age[29], facial shape is maintained fairly constant and bone

302 remodeling only occurs at a lower rate. This period coincides with our oldest age group (13 to 18 years),

303 in which no effect of the EGCG treatment was observed, probably because faces have already attained

304 their final size and shape (Figs. 3a, 4a-d) and facial plasticity is minimal.

305 Since soft-tissues of the face are built upon the bony scaffold of the skull[30], and because we found soft-

306 tissue morphology improvement in humans and facial skeleton improvement in mouse models using a

307 low dose of EGCG, we suspect that EGCG has a potential bone-remodeling effect during early

308 development and subsequent growth. It has been previously shown that EGCG supplementation can

309 normalize many skeletal deficits. EGCG is known to modulate bone metabolism[31], bone formation[32,33],

310 and bone resorption[34], potentially by hindering osteoclastogenesis[34] and inducing osteoblast

311 differentiation[35–39]. However, as we found in our experimental analyses on the effect of EGCG on facial

312 shape (Figs. 1 and 2), the specific effects greatly depend on the dosage: high doses of EGCG may

313 suppress bone formation and exacerbate skeletal defects, whereas moderate doses of EGCG may

314 enhance bone formation and attenuate birth defects[40,41].

315 We hypothesize that the distinctive faces associated with DS may result from poor craniofacial growth.

316 As individuals with DS show overall impairment of growth and bone formation, displaying shortened

317 limbs, low bone mineral content and density, reduced bone strength[42] and remodeling[43], as well as

318 osteopenia and osteoporosis that worsens with age[44], deficiencies in remodeling and relocation of

319 craniofacial bones as the face grows during the first years of development[45] may explain the typical

320 facial traits associated with DS. Increased bone resorption, reduced bone deposition, and/or lack of
321 proper relocation may cause midfacial hypoplasia and the altered shape and position of the maxilla, the
322 mandible, and nasal and orbital cavities, which are the bones with higher predisposition to resorption[45]
323 and are indeed the most affected by Ts21.
324 Despite all the caveats of an observational study based on a limited sample size, including children from
325 different ages, sexes, and geographic regions and the fact that they did not received the same doses of
326 EGCG or had the same length of treatment, the simulation tests consistently detected a signal indicating
327 that EGCG treatment since early in development is associated with intermediate facial phenotypes and
328 significant facial improvement scores (FISs) (Fig. 5). The findings of our observational study underline the
329 need to reassess the effects of EGCG on facial morphology in a clinical trial, to statistically assess and
330 control the different sources of morphological variation in larger samples of individuals following a
331 systematic treatment.
332

Modulating a multisystem circuit to minimize developmental alterations in DS

334 The pleiotropic effects of EGCG are likely mediated by a complex regulatory circuit controlling neural,
335 facial, cardiac and skeletal development. EGCG likely inhibits the activity of several kinases across
336 different organism tissues[19,46], including DYRK1A, a protein that is encoded by one of the genes that has
337 emerged to play a critical role in DS. *DYRK1A* is expressed in a gene-dosage dependent manner within a
338 critical region of chromosome 21, and both under- or overexpression of *DYRK1A* lead to neural
339 dysfunction[21] as well as to brain, craniofacial and skeletal dysmorphologies[17]. The available evidence
340 suggests that *DYRK1A* participates in a regulatory circuit of neural, skeletal, as well as cardiac
341 development, probably mediated through varying control of the Nuclear factor of activated T-cells
342 (NFAT) signaling pathway, a critical regulator of vertebrate development and organogenesis[24].
343 The whole circuit is clearly altered in DS by the 1.5-fold increase in gene dosage due to Ts21. Although
344 all the genes and signaling pathways participating in this circuit are yet to be discovered, research on
345 *DYRK1A* has led to the discovery that a multitude of DS phenotypes can be modulated through inhibition
346 of kinase activity by EGCG. However, the potential rescuing effect of EGCG treatment on multiple
347 systems depends on several factors, such as dosage, onset and duration of the treatment, as well as
348 method of delivery and composition of EGCG-containing supplements. Hence, additional research is
349 needed to pinpoint the optimal dose and time window for EGCG administration, minimizing possible
350 detrimental and side effects, and maximizing benefits to cognitive function and biological growth,
351 including skeletal and facial development. This research is critical since EGCG is already commercially

352 accessible and a growing number of people are consuming EGCG as a dietary supplement with no

353 medical prescription or governmental regulation.

354

355 **Conclusion**

356 Here we demonstrate the potential of EGCG to alleviate facial dysmorphology in DS, likely through a

357 bone-remodeling effect. The results presented here strongly suggest that supplementation with green

358 tea extracts that contain EGCG during early growth and development improves facial dysmorphology of

359 human children with DS. The experimental studies of *Ts65Dn* mice confirm these effects at low EGCG

360 dose, which corresponds to the one used in individuals with DS[13,16]. However, the appearance of

361 potentially detrimental effects in mouse models when high dose of EGCG treatment are used,

362 emphasizes the importance of identifying dosages of EGCG that reliably improve DS phenotypes as a

363 whole, linking the effects to actions of EGCG in specific targets of the brain, the face and craniofacial

364 bones. Current evidence warns against the generalized, non-prescribed intake of this supplement as a

365 health-promoting measure[47], as non-tested doses of EGCG can have detrimental effects on both trisomic

366 and euploid conditions.

367

368 **Methods**

369 **Mouse experiments**

370 We performed two carefully controlled experiments using the *Ts65Dn* mouse model and assessed the

371 effect of two doses of EGCG treatment on the facial shape of adult mice.

372

373 **Animals.** For each experiment we established a breeding colony composed of 4 WT males and 6 *Ts65Dn*

374 females (refs. 001875 and 001924, the Jackson Laboratory Bar Harbor, ME, USA). Mice were housed at

375 the animal facility of KU Leuven in standard individually ventilated cages (40 cm long x 25 cm wide x 20

376 cm high) under a 12h light/dark schedule in controlled environmental conditions of humidity (50% -

377 70%) and temperature (22 ± 2ºC) with food and water supplied *ad libitum*. Experimental protocols were

378 in compliance with all local, national and European regulations and authorized by the Animal Ethics

379 Committee of the KU Leuven (ECD approval number P004/2016).

380

381 **Treatment.** In the first experiment, mice were treated with a high dose of EGCG (30 mg/kg/day), and in

382 the second experiment they received a lower dose (9 mg/kg/day). Since EGCG crosses the placenta and

383 reaches the embryo[48], the treatment was administered prenatally at embryonic day 9 (E9) to optimize

384   treatment efficiency. We chose this time point because this is when the face starts developing and

385   *Dyrk1A* begins to be expressed in mouse embryos. EGCG treatment continued over the duration of the

386   experiment, from E9 until adulthood at P29. During gestation and lactation, EGCG was delivered to dams

387   dissolved in water at a concentration of 0.326 mg/L in the high dose experiment and 0.09 mg/L in the

388   low dose experiment. After weaning at postnatal day 21 and until postnatal day 29, EGCG dissolved in

389   water at the same concentrations was made available to young mice that drank *ad libitum*. The EGCG

390   solution was prepared freshly from a green tea extract [Mega Green Tea Extract, Life Extension, USA;

391   EGCG content of 326.25 mg per capsule] every 3 days. The dosage received was thus approximately 30

392   mg/kg/day in the high dose experiment, and 9 mg/kg/day in the low dose experiment for an adult

393   mouse, taking into account that, on average, mice weigh 20 g and drink 2 ml of water per day.

394

395   **Morphometric craniofacial study.** We used a total of 55 mice from eight litters. We administered EGCG

396   prenatal treatment to 50% of pregnant dams, chosen randomly, to produce approximately the same

397   number of EGCG treated and non-treated mice. Mice were genotyped by quantitative PCR from ear snip

398   samples and were distributed in four groups according to their genotype and treatment: Euploid (WT),

399   Euploid treated with EGCG (WT+EGCG), trisomic *Ts65Dn* (TS) and trisomic *Ts65Dn* treated with EGCG

400   (TS+EGCG). See Table SI_1 for details on sample composition.

401   We acquired whole-body micro computed tomography scans of the mice at postnatal day 29 using a

402   dedicated fast low-dose *in vivo* μCT scanner (SkyScan 1278, Bruker Micro-CT, Kontich, Belgium) at high

403   resolution (50 μm isotropic). Free-breathing mice were scanned under isoflurane anesthesia (~1-2% in

404   oxygen), while physiological functions were constantly monitored and maintained via a feedback loop

405   (temperature, breathing, visual inspection via camera). Scans were reconstructed using the software

406   NRecon v1.6.10.4, whereas skull visualization and landmarking were performed by a single observer

407   using the Amira 6.3 software (Visualization Sciences Group, FEI). Measurement error was within the

408   range of previous studies[49]. We recorded the three-dimensional coordinates of a set of 12 facial

409   anatomical landmarks (Fig. SI_1 and Table SI_2), to capture the shape differences between WT and TS

410   mice and to assess whether the EGCG treatment has an effect on facial development.

411

412   **Patient cohort and recruitment.**

413   **Facial image acquisition and anatomical landmark collection.** The human sample includes 288

414   European and North American children from 0 to 18 years old recruited for photographic sessions at

415   schools, research centers, and DS family congresses between 2009 and 2017 (Table SI_3 and SI_4). To

13

416   photograph the children and to obtain relevant clinical information we obtained informed consent from

417   their parents or legal tutors (CEIC-Parc de Salut MAR, Nº 2012/4849/I; UCF IRB approval SBE-15-11524).

418   Human faces were digitalized using non-invasive photogrammetry techniques. The facial images of each

419   individual were acquired in an upright position with neutral facial expression using either a 3dMD Face

420   system (3dMD, Atlanta, GA) or self-built photographic rigs, as previously described (Starbuck et al. 2017,

421   González et al 2018). Each system acquired 5-10 images simultaneously of an individual's face and used

422   photogrammetry to merge separate two-dimensional images into a single three-dimensional model that

423   accurately represents facial shape. The software used for obtaining the 3D reconstructions was 3dMD

424   (3dMD, Atlanta, GA), PhotoModeler (2015) version 1.1.1546 (Eos Systems, Inc. Canada), and AgiSoft

425   PhotoScan Standard (2016) version 1.3.2. All data was collected with high precision and accuracy based

426   in the same principles of stereo photogrammetry, thereby avoiding potential bias due to the use of

427   different photographic set-ups and software. Scale differences from the different acquisition systems

428   were accounted for in the subsequent analyses by transforming pixel sizes to their corresponding mm

429   measures.

430   We collected 3D coordinates of 21 facial landmarks from each individual's 3D image following published

431   protocols[7]. Landmarks correspond to homologous biologically meaningful points with precise anatomical

432   definitions that can be reliably registered across all individuals (Fig. SI_1 and Table SI_5). Measurement

433   error was within the range of previous studies (0.26 mm along the x-dimension, 0.30 mm along the y-

434   dimension, and 0.31 mm along the z-dimension)[50] and thus considered negligible. Following inspection

435   of landmark coordinates for gross error, anatomical landmark coordinates were used to conduct

436   morphometric analyses.

437

438   **Morphometric analyses**

439   We compared facial shape using different geometric morphometric methods.

440

441   **Procrustes method.** First, we used a General Procrustes Analysis (GPA)[25] to assess the facial phenotype

442   from a global perspective, considering facial shape as a whole. Using this method, the 3D spatial

443   relationship between landmarks defining facial shape was always preserved throughout the analysis,

444   and facial shape was analyzed as a whole 3D facial shape landmark configuration. Within the GPA,

445   landmark facial shape configurations of all individuals were superimposed by shifting them to a common

446   position, rotating, and scaling them to a standard size until a best fit of corresponding landmarks was

447   achieved. Although the resulting Procrustes coordinates were scale-free, the shape data still contained a

14

448   component of size-related shape variation due to allometry[51], which refers to organism shape change

449   relative to body size changes that occur from growth during development. Since our human dataset

450   included children of different ages, we removed the allometric effects by performing a multivariate

451   regression of shape on age within each age group[51]. The Procrustes residuals from this regression were

452   the input for further statistical analysis.

453   Principal Component Analysis (PCA) was used to explore facial morphological variation[25] and to test

454   whether individuals grouped according to their genotype and/or treatment. PCA is a multivariate

455   technique that performs an orthogonal decomposition of the data and transforms variance covariance

456   matrices into a smaller number of uncorrelated variables called Principal Components (PCs), which

457   successively account for the largest amount of variation in the data. Each individual is then scored for

458   every PC, and individuals are plotted using these scores along the morphospace defined by the principal

459   axes as an ordination method to visualize patterns of shape variation associated with individual axes.

460   The distribution of groups throughout the morphospace along different PC axes reveals similarities or

461   differences in facial shape and variation among groups. Importantly, this ordination method allowed us

462   to assess whether individuals with DS treated with EGCG present an intermediate facial phenotype

463   relative to untreated individuals with DS and euploid controls along the PC axes explaining the most

464   variation across groups.

465

466   **EDMA method.** EDMA is a powerful morphometric method for assessing local differences between

467   samples because it allows researchers to pinpoint exactly which linear measurements significantly differ

468   between pairwise sample contrasts and to compare patterns of significant differences across samples[26].

469   In the human sample, using EDMA two-sample shape contrast analyses based on a non-parametric

470   bootstrap (10,000 resamples), a total of 210 unique facial measurements were calculated from

471   Procrustes-based allometrically corrected shape coordinates and evaluated for each age grouping (0-3

472   yrs., 4-12 yrs., 13-18 yrs.) of the DS, DS treated with EGCG, and euploid groups. Local EDMA results,

473   based on confidence interval testing ($\alpha = 0.10$), revealed unique morphological patterns of variation for

474   each two-sample comparison by evaluating the number of statistically significant linear distances in each

475   comparison and by plotting significant linear distances upon facial figures to pinpoint specific local shape

476   differences.

477

478   **Facial improvement score (FIS).** Using the results of the EDMA analyses we estimated a relative facial

479   improvement as the following percentage:

480
$$\frac{\% \text{ significant distances (DS vs EU)} - \% \text{ significant distances (DS + EGCG vs EU)}}{\% \text{ significant distances (DS vs EU)}} \times 100$$

481

482 To verify the statistical validity of the patterns revealed by the EDMA analyses, we ran simulation tests.

483 We performed a subsampling approach in which we generated random pseudo-subsamples with

484 increasing number of DS treated cases. For each age group, the whole sample was randomly divided into

485 groups of N individuals (where N is the number of EGCG treated individuals with DS in the original

486 sample) following a controlled random subsampling approach that created pseudo sub-samples

487 containing a known number (namely M) of treated individuals. Thus, a series of random pseudo sub-

488 samples containing from M=0 to M=N treated individuals were created. We computed a FIS score

489 corresponding to each simulation and analyzed the FIS score histogram of each random group. Finally,

490 we computed an aggregated FIS score histogram of all groups and compared the distribution of random

491 FIS scores with the observed FIS score. The *P-value* of the comparison was computed as the ratio

492 between the number of simulations that provided a higher FIS score than the observed FIS score and the

493 total number of simulations and was used to assess statistical significance ($\alpha = 0.05$).

494

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610

Acknowledgements

623

Author contributions

625   JMS, MD, GVV and NM-A conceived the project. JS, MD, GVV and NM-A contributed with experimental
626   mouse models; SL, JA and JW conducted experiments and scanning; JMS, RdT, MD and NM-A recruited
627   participants for observational study; JMS, SL, RG, AS, AG, XS and NM-A collected and analyzed the data.
628   NM-A and JMS wrote the paper and all authors critically reviewed the manuscript.

629

21

630   **Figure legends**

631

632   **Figure 1**. **Principal Component Analyses based on the Procrustes-based landmark analysis of the global**

633   **facial shape in DS mouse models treated at high and low EGCG dose.** Scatterplot of PC1 and PC2 axes

634   with the corresponding percentage of total morphological variation explained is displayed. Ellipses

635   represent the 70% confidence interval within each group of mice.

636

637   **Figure 2**. **Simulation tests based on facial improvement scores (FIS) in DS mouse models that received**

638   **an experimental prenatal EGCG treatment.** FIS is a summary value that was computed by contrasting

639   the number of significant differences in facial traits between 1) wildtype mice and TS mice not treated

640   with EGCG and 2) wildtype mice and TS mice treated with EGCG. Histograms representing the simulation

641   results for each random group are provided separately as well as aggregately. Each group contains an

642   increasing number of EGCG treated mice. The red line shows the FIS score obtained with the observed

643   EGCG treated mice. *P-value*s are provided for each group.

644   **Figure 3**. **Principal Component Analyses based on the Procrustes-based landmark analysis of the global**

645   **facial shape at each age group.** a) Children from 13 to 18 years old, b) Children from 4 to 12 years old, c)

646   Children from 0 to 3 years old. Scatterplots of PC1 and PC2 axes with the corresponding percentage of

647   total morphological variation explained are displayed for each analysis, along with anterior facial

648   morphings associated with the extreme negative and positive values of PC1. Orange solid line represents

649   facial phenotype associated with DS, whereas blue solid line represents facial phenotype associated with

650   euploid condition. Both shapes are compared to average facial shape, represented by dashed grey line.

651   Convex hulls represent the ranges of variation within each group of children.

652

653   **Figure 4**. **Localized facial shape comparisons based on EDMA analyses at each age group.** Black solid

654   lines represent linear facial measurements that are significantly different in the two groups being

655   compared. First row: children with Down syndrome (DS) *versus* euploid children (EU); second row:

656   children with Down syndrome treated with EGCG (DS+EGCG) *versus* euploid children (EU), third row:

657   EGCG treated (DS+EGCG) *versus* non treated children with Down syndrome (DS), last row: Venn

658   diagrams representing number of significant differences that are common or unique in each comparison

659   for each age group. Specifically, the circles in the Venn diagrams indicate the number of unique

660   significant differences DS *vs* EU (left), the number of common significant differences in DS+EGCG and DS

661   (intersection), and the number of unique significant differences DS+EGCG *vs* EU (right).

22

662

663   **Figure 5**. **Simulation tests based on facial improvement scores (FIS) at each age group.** FIS is computed

664   by contrasting the number of significant differences in facial traits between 1) euploid children and

665   children with DS not treated with EGCG and 2) euploid children and children with DS that have been

666   treated with EGCG. Histograms representing the simulation results for each random group are provided

667   separately as well as aggregately. Each group contains an increasing number of EGCG treated DS cases,
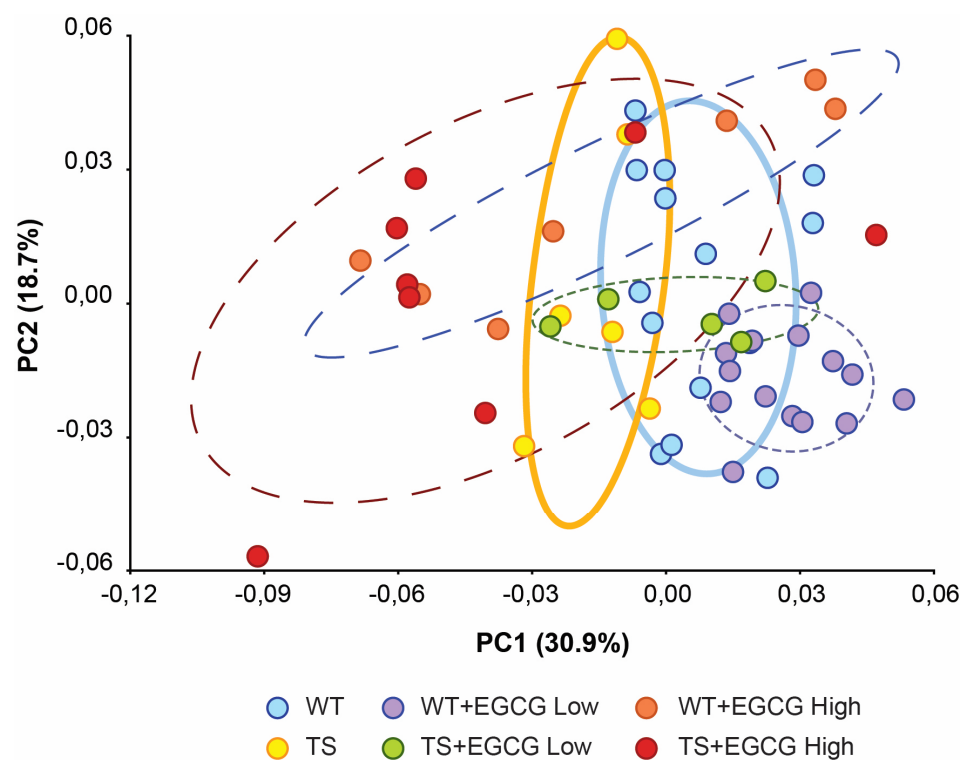
668   from 0 to 6. The red line shows the FIS score obtained with the observed including 7 EGCG treated DS

669   cases. *P-values* are provided for each group, statistical significant comparisons are marked with **.

670

671

672 **FIGURE 1**

673
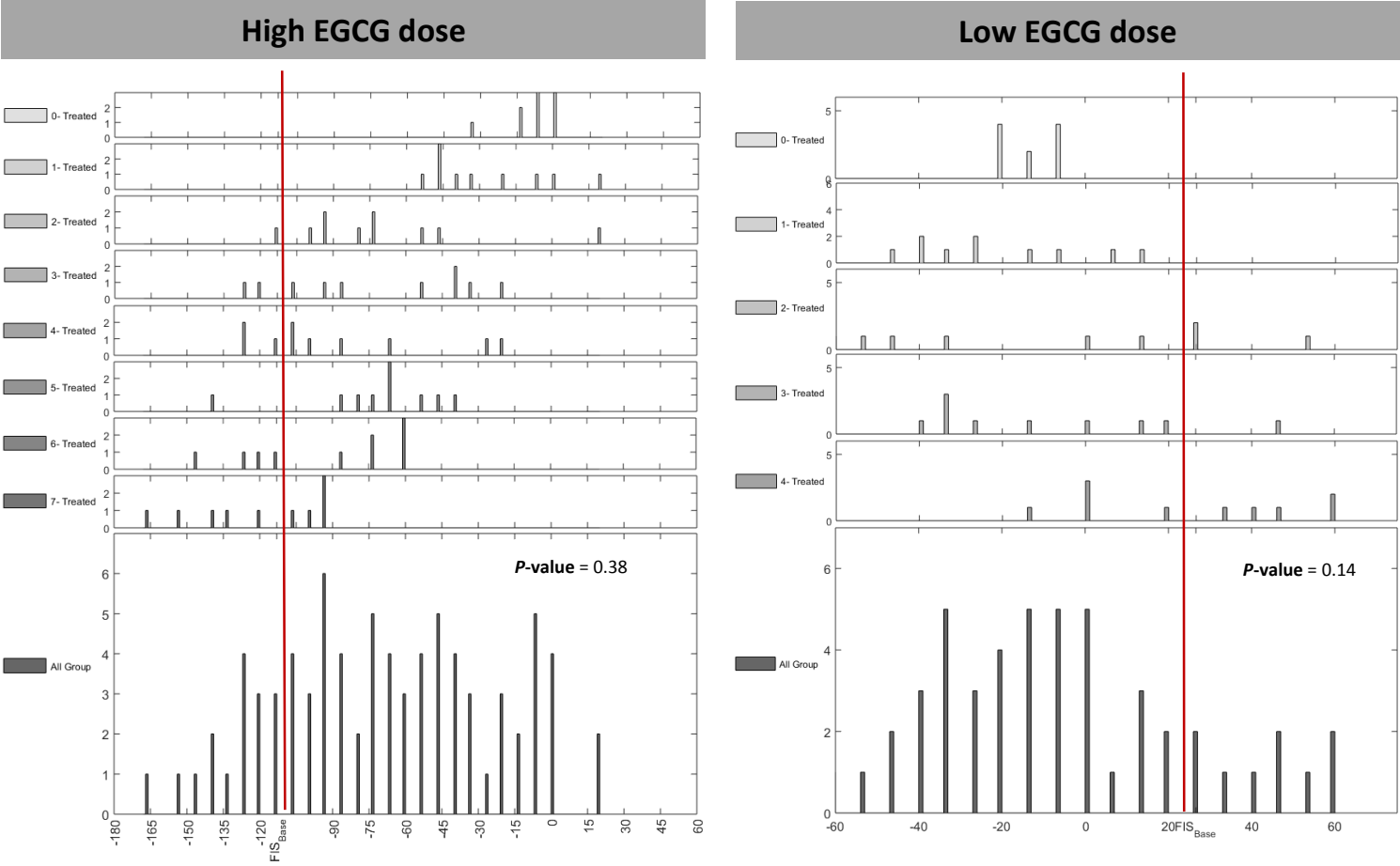
674   **FIGURE 2**
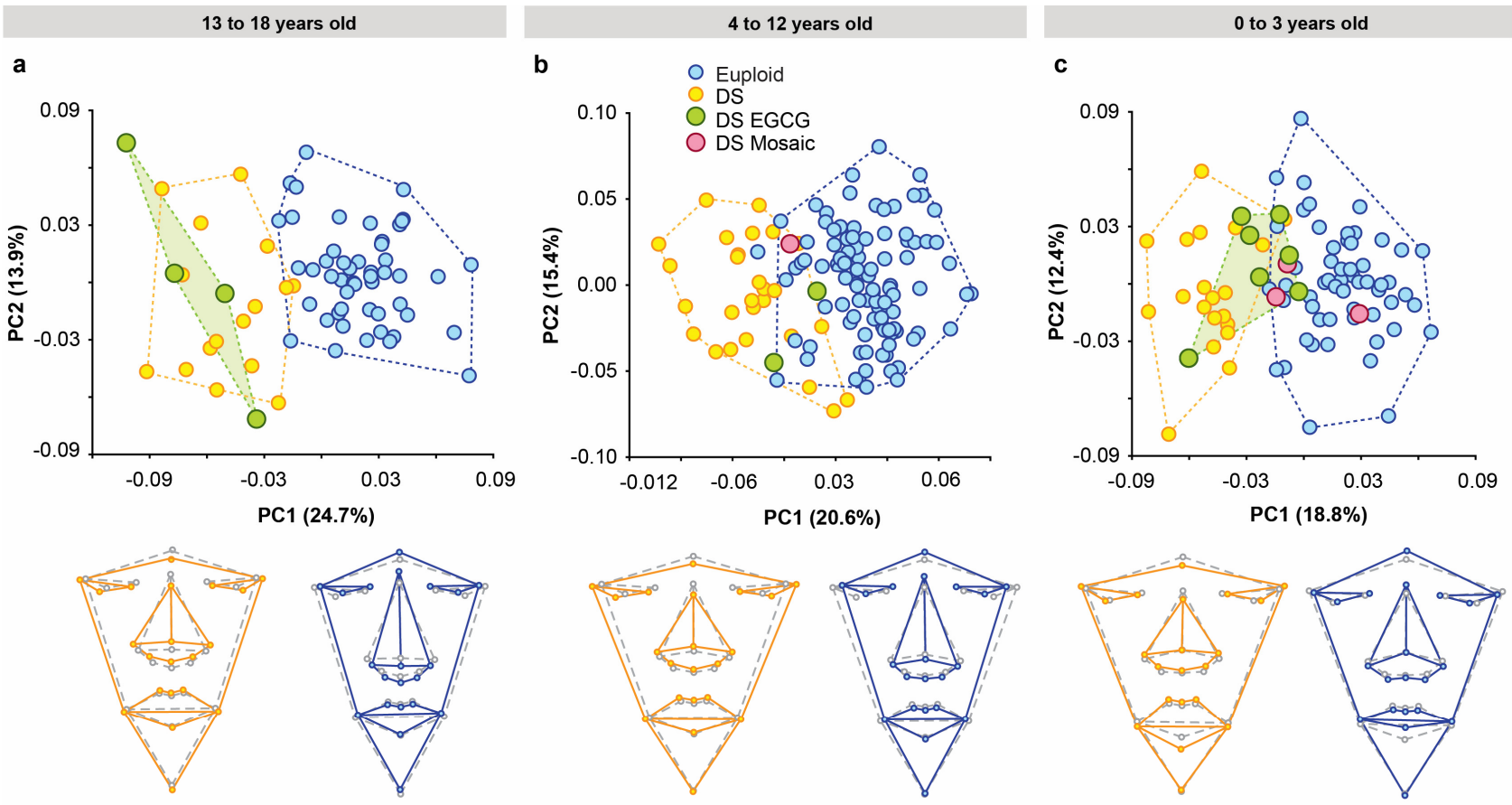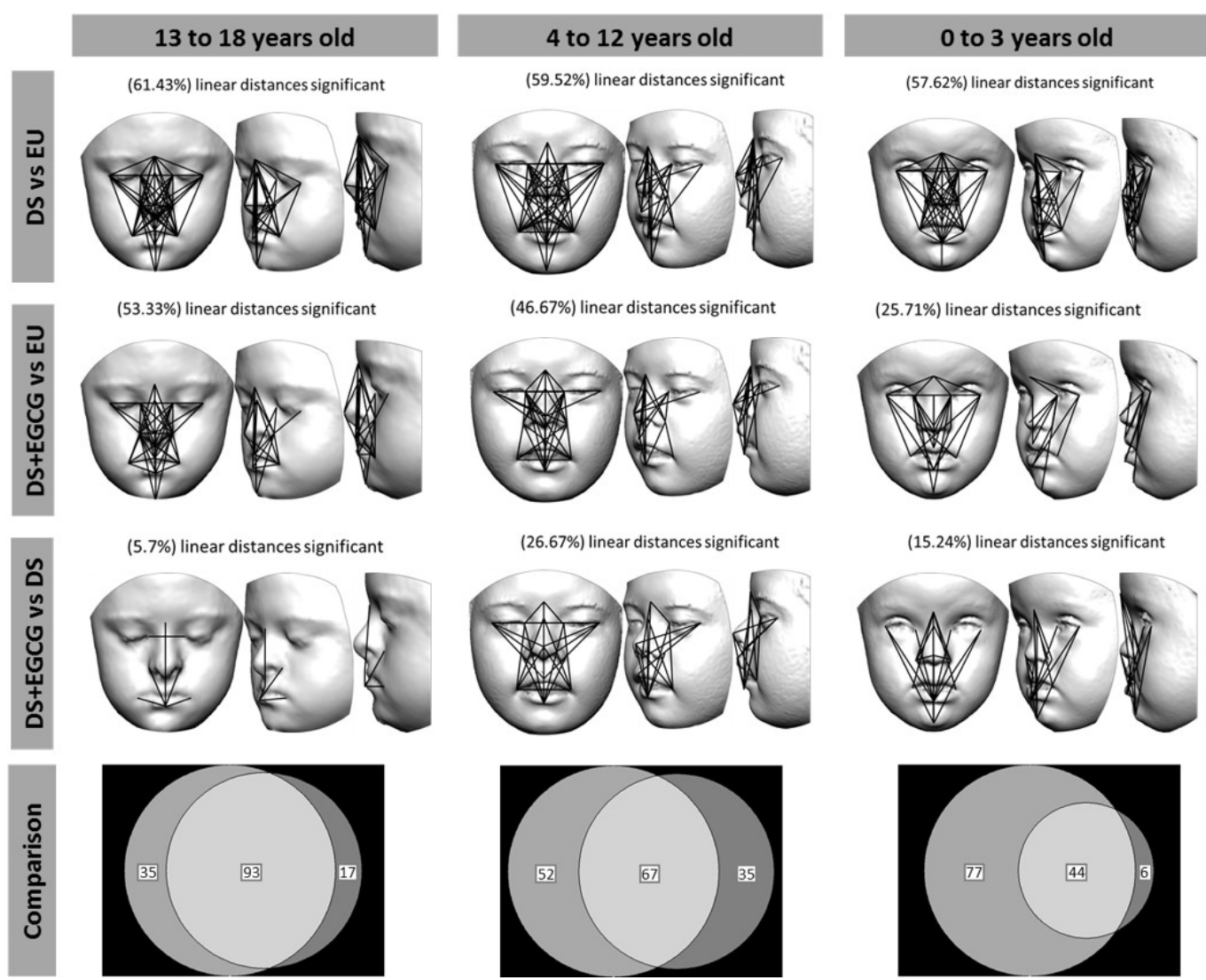
675

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25

678   **FIGURE 3**

679

680

681

	13 to 18 years old	4 to 12 years old	0 to 3 years old
DS vs EU	(61.43%) linear distances significant	(59.52%) linear distances significant	(57.62%) linear distances significant
DS+EGCG vs EU	(53.33%) linear distances significant	(46.67%) linear distances significant	(25.71%) linear distances significant
DS+EGCG vs DS	(5.7%) linear distances significant	(26.67%) linear distances significant	(15.24%) linear distances significant
Comparison	35 93 17	52 67 35	77 44 6

684

FIGURE 5