

1 **Title page**

2 **Manuscript Title:** The impact of mosaicism in preimplantation genetic diagnosis (PGD):
3 Approaches to PGD for dominant disorders in couples without family history.

4 **Running Head:** PGD for dominant disorders without family history.

5 **Authors:**

6 **First and corresponding author:** Roy Pascal Naja*, Preimplantation Genetics Group, Institute
7 for Women's Health, University College London, London, UK. Address: 86-96 cheries mews,
8 WC1E 6HX, London, UK. Telephone: +447923574300. Email: r.naja@ucl.ac.uk.

9 **Second author:** Seema Dhanjal*, Preimplantation Genetics Group, Institute for Women's
10 Health, University College London, London, UK.

11 **Third author:** Alpesh Doshi, the Centre for Reproductive and Genetic Health, London, UK.

12 **Fourth author:** Paul Serhal, the Centre for Reproductive and Genetic Health, London, UK.

13 **Fifth author:** Joy Delhanty, Preimplantation Genetics Group, Institute for Women's Health,
14 University College London, London, UK.

15 **Last author:** Sioban B. SenGupta, Preimplantation Genetics Group, Institute for Women's
16 Health, University College London, London, UK.

17 *These authors contributed equally to this work

18 **Word, figure and table count:** Word count: 3235. One table. Three figures.

19 **Financial Disclosure:** None declared.

20 **Conflict of Interest:** The authors of this manuscript declare “No conflict of interest”.

21 **What’s already known about this topic:**

- 22
- 23 • Preimplantation genetic diagnosis (PGD) is a reproductive option available to patients
24 that are carrier of an autosomal dominant disorder and seeking to have an “unaffected”
child.
 - 25 • The transmission risk where the patient is a mosaic with no family history for the
26 disorder can deviate from the mendelian 50% which can lead to confusion at the level of
27 diagnosis.

28 **What’s does this study add:**

- 29 • Careful pre-clinical analysis and follow up studies on embryos in some cases of
30 autosomal dominant disorders has detected mosaicism for the causative mutation.
- 31 • Knowledge that the patient undergoing PGD for an autosomal dominant disorder with no
32 family history is a potential mosaic for the causative mutation will render the diagnosis
33 more robust, thereby, reducing the risk of misdiagnosis.

34 **Ethics statement:** PGD for the disorders presented in this study is licenced by the Human
35 Fertilisation & Embryology Authority (HFEA). The IVF clinic (Centre for Reproductive and
36 Genetic Health) providing the embryonic samples has an HFEA licence to conduct PGD. All
37 patient consent forms were obtained for any post PGD/PGD follow-up studies on untransferred
38 embryos covered by the HFEA licence. No additional ethical approval was required for this
39 study and the authors declare that no ethics was breached.

40 **Abstract**

41 Objectives

42 Mosaicism in certain dominant disorders may result in a “non-Mendelian” transmission for the
43 causative mutation. Preimplantation genetic diagnosis (PGD) is available for patients with
44 inherited disorders to achieve an unaffected pregnancy. We present our experience for two
45 female patients with different dominantly inherited autosomal disorders; neurofibromatosis type
46 1 (NF1) and tuberous sclerosis complex type 2 (TSC2).

47 Methods

48 PGD protocol development was carried out using single cells from the patients. PGD was carried
49 out on polar bodies and different embryonic cells.

50 Results

51 Protocol development for NF1 using lymphocytes from the patient suggested mosaicism for the
52 mutation. This was supported further by quantitative fluorescent-PCR performed on genomic
53 DNA. During PGD, polar bodies and blastomeres lacked the mutation that probably was absent

54 or present at very low levels in the patient's germline. Single lymphocyte analysis during
55 protocol development for TSC2 did not indicate mosaicism, however, analysis of single buccal
56 cells and multiple embryo biopsies across two consecutive IVF/PGD cycles confirmed
57 gonosomal mosaicism.

58 Conclusions

59 The trend in PGD is for blastocyst biopsy followed by whole genome amplification, eliminating
60 single cell analysis. In the case of certain dominantly inherited disorders, pre-PGD single cell
61 analysis is beneficial to identify potential mosaicism that ensures robust protocols.

62 **Introduction**

63 Mosaicism is a condition where an individual has two (or more) genetically distinct cell types.
64 Mosaicism may be limited to somal tissues (somatic mosaicism), or the gonads (gonadal
65 mosaicism) or present in both (gonosomal mosaicism). The precise timing of post-zygotic
66 mutations that occur during development determines the distribution of mutant cells in the
67 individual. Mutations that occur before the primordial germ cell (PGC) differentiation, before
68 ~15 mitotic divisions, can be present in both somatic and germ tissues¹. Mutations that occur
69 after PGC differentiation will only be present in either the somatic or germline lineages. For
70 autosomally inherited disorders an affected individual who has no previous family history has a
71 *de novo* mutation that arose in the individual themselves as a post-zygotic event or in the germ
72 cells of a parent of the individual. Depending upon when the *de novo* mutation occurred, the
73 individual will have a transmission risk ranging from 0-50%; therefore careful counselling is
74 necessary when considering reproductive options.

75 Neurofibromatosis type 1 (OMIM# 162200) is a neurocutaneous disorder with autosomal
76 dominant inheritance, complete penetrance and variable expressivity². It is caused by mutations
77 in the *NF1* gene located on 17q11.2 and encodes the neurofibromin protein, a negative regulator
78 of Ras GTPases³. The *NF1* gene has a very high mutation rate⁴; approximately 50% of all
79 patients with NF1 have no family history of the disease⁵. A high rate of sporadic mutations
80 resulting in mosaic occurrence may explain a milder clinical phenotype known as segmental
81 NF1⁶. Germline mosaicism in NF1 has been reported in cases where affected children with a
82 characterised mutation were born to healthy parents not showing the mutation in their
83 lymphocytes⁷.

84 Tuberous sclerosis complex type 2 (TSC2; OMIM# 191092) is also a neurocutaneous
85 multisystem disorder with autosomal dominant inheritance and variable expressivity⁸. It is
86 caused by mutations in *TSC2* located on the short arm of chromosome 16 (16p13.3) and encodes
87 tuberin⁹. Molecular genetic studies have shown that approximately 65% of all patients with
88 *TSC2* have no family history of the disease¹⁰. Mosaicism leading to a variable clinical phenotype
89 in *TSC2* has been described⁹ and germline mosaicism has been reported¹¹.

90 Preimplantation genetic diagnosis (PGD) is a reproductive option for couples which, unlike
91 prenatal diagnosis, allow genetic analysis prior to establishment of a pregnancy¹³. The couple
92 undergoes assisted reproduction so that the embryos are produced by *in vitro* fertilisation (IVF).
93 Embryo biopsy is usually performed at the cleavage stage where 1-2 blastomeres are removed or
94 at the blastocyst stage where clumps comprising of 3-5 cells are removed for genetic testing.
95 Embryos without the causative mutation are transferred to the uterus so that if a pregnancy
96 ensues the fetus is expected to be unaffected for the indication being tested.

97 Here we present two cases of PGD where mosaicism for the causative mutation was detected.

98 In the first case, mosaicism of an *NF1* mutation in a female patient was identified in lymphocytes
99 during development of a single-cell PCR protocol prior to PGD. This was supported by analysis
100 of polar bodies and blastomeres during PGD.

101 In the second case, mosaicism for a mutation in *TSC2* was suspected in a female patient during
102 the first cycle of PGD on blastomeres. This was confirmed from further analysis of buccal cells
103 from the patient, in addition to the analysis of blastomeres, trophoctoderm cells and whole
104 untransferred embryos of a second cycle of PGD.

105 **Materials and methods**

106 **Patient description**

107 NF1

108 The patient was a female aged 29 diagnosed with NF1. The referral clinical genetics report
109 indicated a heterozygous seven base pair (7bp) duplication in the *NF1* gene which caused a
110 frameshift in the subsequent coding sequence. No family history of NF1 was reported indicating
111 a *de novo* mutation. Based on a 50% transmission risk of the causative mutation, the patient was
112 referred to our centre for PGD.

113 TSC2

114 The patient was a female aged 31 diagnosed with TSC at the age of 21. The referral genetics
115 report indicated that she was heterozygous for a substitution mutation C>T resulting in a stop
116 codon and leading to a premature termination of the protein. No family history of TSC2 was
117 reported indicating a *de novo* mutation.

118 **Genomic DNA extraction, lymphocyte separation and buccal epithelial cell preparation**

119 Genomic DNA was extracted from peripheral blood collected in tubes containing EDTA from
120 the patient couples and female patients' relatives using the QIAamp DNA Blood Maxi Kit
121 (QIAGEN, Manchester, UK).

122 Lymphocyte separation was performed on peripheral blood collected in tubes containing lithium
123 heparin from the patient couples using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little
124 Chalfont, UK).

125 Buccal epithelial cells were collected from the patients with a buccal swab, and resuspended in
126 1x PBS (Invitrogen, Paisley, UK).

127 **Single cell isolation**

128 Single cell isolation was performed on the separated lymphocytes and buccal epithelial cells
129 under an inverted microscope using a 0.2-mm polycarbonate microcapillary (Biohit, Cheshire,
130 UK). Single cells were washed three times in 1x PBS drops, which contained 0.1% polyvinyl
131 alcohol (Sigma, Dorset, UK). The cells were then transferred to RNase-, DNase-free 0.2-ml PCR
132 tubes containing alkaline lysis buffer (50mM dithiothreitol, 200mM NaOH) and stored at -20°C
133 until cell lysis at 65°C for 10 min and further amplification.

134 **Confirmation of mutational status**

135 DNA from the affected female and her partner for both the NF1 and TSC2 cases were analysed
136 by Sanger sequencing to confirm the presence of the reported mutation. DNA from the parents of
137 the affected female with NF1 and from one parent and one sibling from the patient with TSC2
138 was available. These samples were used to establish the female haplotypes at linked STR
139 markers for the *NF1* and *TSC2* genes respectively.

140 **PCR protocols and fragment analysis**

141 The assessment of informativity and haplotyping for linked microsatellite polymorphic markers
142 was performed on genomic DNA from the couples and family members using PCR with
143 fluorescently labelled primers (Eurogentec, Liège, Belgium). Fragment analysis was performed
144 on ABI 3130xl (Life Technologies, Dorset, UK) and the data was analysed using the
145 Genemapper analysis software v3.5 (PE Applied Biosystems, Warrington, UK).

146 NF1

147 Multiplex fluorescent PCR for simultaneous direct mutation detection and linkage analysis was
148 carried out using the following: 1) Primers for the mutation locus encompassing the 7bp
149 duplication and 2) Linked polymorphic STR markers ivs27AC28.4, D17S1166, NF1int29
150 intragenic and centromeric, and D17S1800 0.26Mb telomeric to the 7bp duplication in the *NF1*
151 gene. The detection of a 250bp PCR product at the mutation locus indicated the presence of the
152 of the 7bp duplication within the *NF1* gene whereas presence of a 243bp fragment identified the
153 normal allele.

154 TSC2

155 Multiplex fluorescent PCR for simultaneous direct mutation detection and linkage analysis was
156 carried out using the following 1) Primers for the mutation locus encompassing the C>T
157 mutation, 2) Linked polymorphic STR markers D16S3024 0.44Mb telomeric, D16S664 0.26Mb
158 and D16S663 0.36Mb centromeric to the C>T mutation. A second round PCR with the mutation
159 locus primers and subsequent minisequencing was used to detect the mutant T allele.
160 Minisequencing was performed using the SNaPshot® Multiplex Kit (Life Technologies).

161 **Optimisation of single cell protocols**

162 To optimise the protocols, single cells were tested under different PCR conditions to achieve an
163 amplification efficiency of $\geq 95\%$ and an allele-drop-out (ADO) rate of $\leq 5\%$ at each locus.

164 Efficiency of the marker was defined as the proportion of cells tested that resulted in amplified
165 product at the mutation locus. ADO was defined as the proportion of cells known to be
166 heterozygous that appeared to be homozygous at a specific locus. This included changing the
167 annealing temperature of the protocols and concentration of the primers for the mutation loci. In
168 addition, two rounds of PCR were carried out such that the mutation locus was split into a
169 singleplex PCR and the remaining STR markers into a quadruplex (in the case of NF1) and
170 triplex (in the case of TSC2) for the second round of amplification. The optimised multiplex
171 PCR protocol was performed on more than 30 lymphocytes (in the case of NF1) and 50
172 lymphocytes (in the case of TSC2) from the female affected partner. Both protocols used the
173 QIAGEN Multiplex PCR Kit (QIAGEN) at an annealing temperature of 60°C for 40 cycles.
174 Singleplex PCR for the different mutation markers were also performed using the QIAGEN Taq
175 PCR Master Mix Kit (QIAGEN).

176 **Quantitative fluorescent-PCR (QF-PCR)**

177 Quantitative fluorescent PCR was performed on duplicate genomic DNA samples from the
178 patient with NF1 and her partner with the optimised multiplex protocol for a reduced number of
179 cycles; 20 and 25. QF-PCR was also performed using a singleplex protocol with the mutation
180 primers for the same number of reduced cycles. The QF-PCR was repeated on genomic DNA
181 extracted from the peripheral blood of the patient collected seven months after the first sample.
182 Quantification was performed after measuring the peak areas of the normalized mutant and
183 normal alleles for the mutation locus.

184 **PGD/IVF treatment**

185 The IVF treatment was conducted at the Centre for Reproductive and Genetic Health (CRGH).
186 The CRGH has a license to conduct PGD from the Human Fertilisation & Embryology Authority

187 (HFEA). Both NF1 and TSC2 are disorders licensed by the HFEA. All consent forms were
188 obtained from the patients regarding PGD and the use of results/findings in research.

189 Following controlled ovarian stimulation human chorionic gonadotropin was administered and
190 oocytes were collected transvaginally 37 h later.

191 For the NF1 case, oocytes were denuded and the first polar body (PB1) was biopsied and then
192 intra-cytoplasmic sperm injection (ICSI) was performed. The second polar body (PB2) was
193 biopsied post ICSI. At cleavage stage two blastomeres were removed from each embryo that had
194 more than six cells and one blastomere when the embryo had six cells or fewer.

195 For the TSC2 case, oocytes were denuded and subjected to ICSI for each of two PGD cycles. A
196 cleavage stage biopsy was carried out as described above. A trophectoderm biopsy was also
197 performed on all embryos that developed into blastocysts. All untransferred embryos from both
198 cycles were collected for follow-up/confirmation analysis.

199 All biopsied samples and collected embryos were placed in separate tubes containing alkaline
200 lysis buffer. The tubes were stored at -80°C before the cell lysis step and subsequent analysis
201 was performed at the UCL Centre for PGD.

202 **Results**

203 **NF1**

204 **Single-cell multiplex protocol**

205 To determine the efficiency of diagnosis prior to clinical application, > 30 single lymphocytes
206 from the affected female were tested using the optimised multiplex PCR protocol, with the
207 acceptance criteria of $\geq 95\%$ for amplification efficiency and $\leq 5\%$ for ADO at each locus. Even
208 after many modifications to the multiplex PCR protocol, we observed an unusually high apparent

209 ADO (29%) for the mutation locus only (Table 1). This high apparent drop-out was solely for the
210 mutant allele and not the normal one (Figure 1 A, B, C).

211 The high apparent ADO was observed for both lymphocyte (Table 1) and buccal epithelial cells
212 (data not shown) of the female patient. The ADO rates of all other markers were well within the
213 acceptance criteria. In addition, singleplex PCR for the mutation locus on either cell type also
214 showed high apparent ADO of the mutant allele for the affected female patient (data not shown).

215 **Quantitative fluorescent-PCR**

216 To determine if mosaicism was the cause of the high apparent drop-out observed for the mutant
217 allele, QF-PCR was performed. Results obtained from duplicate female genomic DNA samples
218 showed that the mean mutant peak area was considerably lower compared to that of the normal
219 at two different PCR cycles (mean ratio = 0.46 at 20 cycles and 0.655 at 25 cycles). In addition,
220 the allele peak areas of the other heterozygous microsatellite markers present in the multiplex
221 were of comparable size (Figure 1 D). The QF-PCR was repeated on genomic DNA extracted
222 from the peripheral blood of the patient at another time point with similar results (data not
223 shown).

224 **PGD strategy**

225 Polar body 1, PB2 and two blastomeres were biopsied sequentially from each embryo. This
226 approach of multiple biopsies was undertaken to ensure a robust diagnosis in spite of the
227 suspected mosaicism for the *NFI* mutation in the female patient.

228 **Embryo diagnosis**

229 Nineteen embryos (designated 1 to 19) were biopsied and a total of 74 cells were collected. All
230 but nine cells (12%) gave results. Seven embryos (3, 4, 7, 8, 9, 11 and 17) gave complete results
231 from PB1, PB2 and two blastomeres. These embryos were used to determine the parental

232 haplotype and deduce the chromosomal segregation at meiosis (Figure 2). Embryo 19 (not
233 included in Figure 2) showed external contamination therefore no diagnosis could be reached.
234 The mutation was not detected in any of the 63 cells that had successfully amplified and that
235 inherited either homologue of maternal chromosome 17.
236 The female had an embryo transfer that ensued in a pregnancy and a live birth of an unaffected
237 baby. Also, ten embryos that had reached the blastocyst stage were cryopreserved.

238 **TSC2**

239 **Single-cell multiplex protocol**

240 With the same acceptance criteria described before for efficiency and ADO, > 50 single
241 lymphocytes from the affected female patient were tested using the optimised multiplex PCR
242 protocol. All tested markers were within the acceptable ranges except for that of the mutation
243 locus which was slightly out of range for ADO (5.9% > 5%) despite many modifications to the
244 PCR multiplex protocol and subsequent minisequencing reaction. However, this slightly high
245 apparent ADO rate did not alert any suspicion of mosaicism at this pre-clinical single cell
246 analysis stage.

247 **Embryo diagnoses at PGD and follow-up**

248 **Cycle one of PGD**

249 Six embryos (designated 1 to 6) were biopsied at cleavage stage with a total of 12 blastomeres
250 taken and analysed. Figure 3 shows the expected transmission of the parental haplotypes to the
251 embryos with the exception of embryo 2 that showed only one paternal chromosome.
252 Minisequencing did not detect the mutation in any of the cells from embryos that inherited either
253 homologue of maternal chromosome 16. In order to rule out errors due to technical problems, a
254 rebiopsy was performed at day 4 post-fertilisation for embryos 1, 3 and 6. Embryo 4 had

255 arrested at this stage and the whole embryo was tubed. Analysis was performed on these samples
256 and minisequencing confirmed the results obtained before. Embryo 5 was a hatching blastocyst
257 on day 6 that was rebiopsied and cryopreserved. The remaining whole embryos (1, 3 and 6)
258 which had arrested and the trophoctoderm biopsy from Embryo 5 were analysed by both
259 minisequencing and Sanger sequencing. The results reconfirmed the diagnosis obtained from
260 previous analyses. Figure 3 shows the sequential mutation detection results obtained for embryo
261 4 as compared to the patient couple.

262 The optimised PGD protocol was then performed on buccal epithelial cells isolated from the
263 female patient. A high apparent ADO rate (12.5%) was observed for the mutant allele hinting
264 that the female patient might be mosaic for the mutation in her buccal epithelial cell lineage.

265 Cycle two of PGD

266 The patient was counselled and opted for a second cycle of PGD. Five embryos (designated 1 to
267 5) were biopsied at cleavage stage with a total of nine blastomeres taken and analysed. Figure 3
268 shows the transmission of the parental haplotypes to the embryos with the exception of embryo 5
269 that showed only one paternal chromosome. Minisequencing detected the presence of the mutant
270 allele T in Embryo 3, but not in Embryo 4, which had the same haplotype at the linked markers
271 indicating that the female patient was mosaic in her germline for the *TSC2* mutation. This finding
272 of germline/gonadal mosaicism was supported by the results obtained from the first cycle of
273 PGD where the mutation was not detected in any of the embryonic samples having inherited
274 either maternal haplotype. Having detected both somatic (buccal cells) and germline mosaicism
275 we suggest that the female patient had gonosomal mosaicism for the mutation. Embryo 2 from
276 the second cycle was transferred because it did not have the mutation and also had the other
277 maternal homologue of chromosome 16 compared to embryo 3. The remaining untransferred

278 embryos were reanalysed by both minisequencing and Sanger sequencing confirming the results
279 obtained in PGD (data not shown).

280 **Discussion**

281 For the NF1 case, the single cell analysis of lymphocytes performed prior to PGD indicated that
282 the female patient was mosaic for the mutation at least in her lymphoblastic lineage. This was
283 supported by QF-PCR performed on her genomic DNA where there was a decrease in the peak
284 area for the mutant allele compared to the normal one. Results from all PBs and embryos did not
285 detect the mutation identified in lymphocytes from the patient indicating that her germline is
286 either mosaic or lacking the mutation.

287 For the TSC2 case, the single cell analysis performed prior to PGD using lymphocytes did not
288 show a clear indication of mosaicism for the mutation. PGD in the first cycle showed absence of
289 the mutation in spite of each maternal homologue of chromosome 16 being separately
290 represented in the embryos. Testing of buccal epithelial single cells showed a high apparent
291 ADO rate (12.5%) for the mutation and the analyses of untransferred embryos obtained from
292 cycle one and two altogether indicated gonosomal mosaicism in this individual.

293 Both NF1 and TSC are frequent autosomal dominant disorders. Up to 50% of patients with NF1⁵⁻
294 ⁶ and 65 % of patients with TSC¹⁰ have *de novo* mutations with no family history for the disease.
295 This can result in germline mosaicism resulting in < 50% transmission risk, which is a major
296 consideration that should be taken into account in diagnostic settings such as PGD. Clinically, it
297 can be difficult to identify mosaicism for a mutation especially in the case of genetic diseases
298 with variable expressivity.

299 Here we report mosaicism for a mutation in two disorders that are commonly considered for
300 PGD. Somatic mosaicism was identified in one situation (NF1) through routine single cell

301 analysis conducted prior to PGD. This led to analysis of PBs and blastomeres in the clinical
302 cycle to ensure that the protocol was sufficiently robust to give a conclusive diagnosis when
303 there was evidence of mosaicism. In another situation (TSC2), both somatic and germline
304 mosaicism for the causative mutation was identified through extensive embryo analysis and
305 follow-up studies, which enabled appropriate counselling of the patient prior to embryo transfer.
306 PGD for dominantly inherited disorders where there is no family history relies on detection of
307 the mutation in embryos. Once the phase of the mutation is established with the haplotype at
308 linked markers the diagnosis is not only dependent on the mutation locus alone. When
309 mosaicism is identified in somatic tissues, testing of many single sperm will identify germline
310 mosaicism in males. In females however it is not possible to predict whether there is mosaicism
311 or if the mutation is present in the germline.

312 Recently the blastocyst has become the preferred stage of biopsy although fewer embryos reach
313 this stage of development¹⁴. ADO is low in trophectoderm samples derived from a blastocyst
314 biopsy and single cell analysis prior to PGD is minimal with no protocol optimisation. In spite of
315 the lower ADO rate in trophectoderm samples a conclusive diagnosis may be difficult in *de novo*
316 cases when there are few embryos and it is uncertain if germline mosaicism for the causative
317 mutation is present. For this reason and for cases of autosomal dominant disorders that show
318 mosaicism, single cell analysis prior to PGD should include the cells of different lineages to
319 identify mosaicism in order to minimise inconclusive results at PGD. Making a diagnosis using
320 independent tests such as polar body analysis together with blastomere or trophectoderm analysis
321 reduces the risk of misdiagnosis in these cases.

322

323 **References**

324

- 325 1 Campbell IM, Shaw CA, Stankiewicz P, Lupski JR. Somatic mosaicism: implications for
326 disease and transmission genetics. *Trends Genet* 2015; e-pub ahead of print 21 April
327 doi;10.1016/j.tig.2015.03.013.
328
- 329 2 Clementi M, Barbujani G, Turolla L, Tenconi R. Neurofibromatosis-1:a maximum
330 likelihood estimation of mutation rate. *Hum Genet* 1990;84:116–118.
331
- 332 3 Wallace MR, Marchuk DA, Andersen LB, *et al.* Type 1 neurofibromatosis gene:
333 identification of a large transcript disrupted in three NF1 patients. *Science* 1990;249:181–
334 186.
335
- 336 4 Huson SM, Compston DA, Clark P, Harper PS. A genetic study of von Recklinghausen
337 neurofibromatosis in south east Wales. I. Prevalence, fitness, mutation rate, and effect of
338 parental transmission on severity. *J Med Genet* 1989;26:704–711.
339
- 340 5 Kehrer-Sawatzki H, Cooper DN. Mosaicism in sporadic neurofibromatosis type 1:
341 variations on a theme common to other hereditary cancer syndromes? *Med Genet* 2008;e-pub
342 ahead of print 29 May 2008;doi: 10.1136/jmg.2008.059329.
343
- 344 6 Ruggieri M, Huson SM. The clinical and diagnostic implications of mosaicism in the
345 neurofibromatoses. *Neurology* 2001;56:1433–1443.
346
- 347 7 Trevisson E, Forzan M, Salviati L, Clementi M. Neurofibromatosis type 1 in two siblings
348 due to maternal germline mosaicism. *Clin Genet* 2014;e-pub ahead of print 27 May
349 2013;doi:10.1111/cge.12177.
350
- 351 8 Curatolo P, Bombardieri R, Jozwiak S. Tuberous sclerosis. *Lancet* 2008;372:657–68.
352 Narayanan V. Tuberous sclerosis complex: Genetics to pathogenesis. *Pediatric Neurology*
353 2003;29:404–409.
354
- 355 9 Barron RP, Kainulainen VT, Forrest CR, *et al.* Tuberous sclerosis: Clinopathologic features
356 and review of the literature. *Journal of Cranio–Maxillofacial Surgery* 2002;300:361–366.
357
358
- 359 10 Altarescu G1, Beeri R, Eldar-Geva T, *et al.* PGD for germline mosaicism. *Reprod Biomed*
360 *Online* 2012;e-pub ahead of print 20 July 2012;doi:10.1016/j.rbmo.2012.07.003.
361
- 362 11 Merker VL, Murphy TP, Hughes JB, *et al.* Outcomes of preimplantation genetic diagnosis
363 in neurofibromatosis type 1. *Fertil Steril* 2015;e-pub ahead of print 31 December 2014;doi:
364 10.1016/j.fertnstert.2014.11.021.
365
- 366 12 Sermon K, Van Steirteghem A and Liebaers I. Preimplantation genetic diagnosis. *Lancet*
367 2004;363:1633–1641.
368
- 369 13 Scott KL, Hong KH, Scott RJ. Selecting the optimal time to perform biopsy for
370 preimplantation genetic testing. *Fertil Steril* 2013;100:608–614.

371
372 14 Chang LJ, Huang CC, Tsai YY, *et al.* Blastocyst biopsy and vitrification are effective for
373 preimplantation genetic diagnosis of monogenic diseases. *Hum Reprod* 2013;28:1435–1444.
374

375

376

377

378

379 **Captions to table and figures**

380

381 Table 1. Results of the optimised pre-PGD single cell PCR tests of the female patient with NF1
382 showing an unusually high level of apparent ADO (29%) for the NF1 mutation locus. The
383 apparent drop-out at the mutation locus was only for the mutant allele and not the normal one.
384 The patient was not heterozygous at the D17S1166 locus and thus % ADO was not applicable
385 (N/A).

386 Figure 1. Electropherograms of the optimised multiplex PCR protocol performed on single
387 lymphocytes (A, B, C) and QF-PCR at 20 cycles performed on genomic DNA (D) from the
388 patient couple. (A) Lymphocyte from the affected female showing both mutant and normal
389 alleles. (B) Lymphocyte from the affected female showing an apparent drop-out of the mutant
390 allele. (C) Lymphocyte from the male partner showing only the normal allele. (D) A decrease in
391 the mutant allele peak area relative to the normal one for the NF1 mutation locus is observed; for
392 comparison, the peak areas of both alleles for STR marker D17S1800 are shown to be of similar
393 size.

394 Figure 2. The transmission of the parental haplotypes on chromosome 17 to the embryos based
395 on results obtained for the linked STR polymorphic markers IVS27AC28.4, D17S1166,
396 NF1int29 centromeric and D17S1800 telomeric to the mutation locus. The mutant *NF1* allele

397 (250bp) was not detected in any of the biopsied cells. Embryos were colour-coded and the
398 proposed segregation results are shown. Embryos 1, 2 and 15 showed homologous
399 recombination in meiosis I. There was a non-disjunction event at meiosis II for Embryo 5,
400 whereas, for Embryo 14 premature segregation of sister chromatids in meiosis I may have
401 occurred. Errors in meiosis I or II appear to have occurred in embryos 10 and 16. resulting in a
402 possible trisomy of chromosome 17 in embryo 16. Each of the two blastomeres from embryo 12
403 showed a different and only one parental haplotype suggestive of 'embryonic mosaicism' for
404 chromosome 17. E: embryo, PB1: polar body 1, PB2: polar body 2, B: blastomere, NR: no result,
405 ADO: allele-drop-out.

406 Figure 3. The transmission of the parental haplotypes on chromosome 16 to the embryos for
407 PGD cycles one and two of the patient couple with TSC2. This was based on results obtained for
408 the linked STR polymorphic markers D16S3024 telomeric and D16S664, D16S663 centromeric
409 to the *TSC2* C>T mutation. Compared to the affected female and normal male partners, the
410 mutant T allele was not detected in any of the biopsied cells in cycle one of PGD when analysed
411 by Sanger sequencing and minisequencing (left and right electropherograms respectively),
412 shown here only for embryo 4. Minisequencing in cycle two of PGD detected the mutant T allele
413 in embryo 3, which was used to establish the phase with the linked STR markers. SS: Sanger
414 sequencing, MS: minisequencing, E: embryo, B: blastomere.

415

416

417

418

419

420

421

422

423

424 Table 1.

	IVS27AC28.4	D17S1166	NF1int29	Mutation locus	D17S1800
Efficiency (%)	32/32 (100)	32/32 (100)	32/32 (100)	31/32 (97)	32/32 (100)
Apparent ADO (%)	1/32 (3)	N/A	0/32 (0)	9/31 (29)	0/32 (0)

425

426

427

428

429

430

431

432

433

434

435

436

437

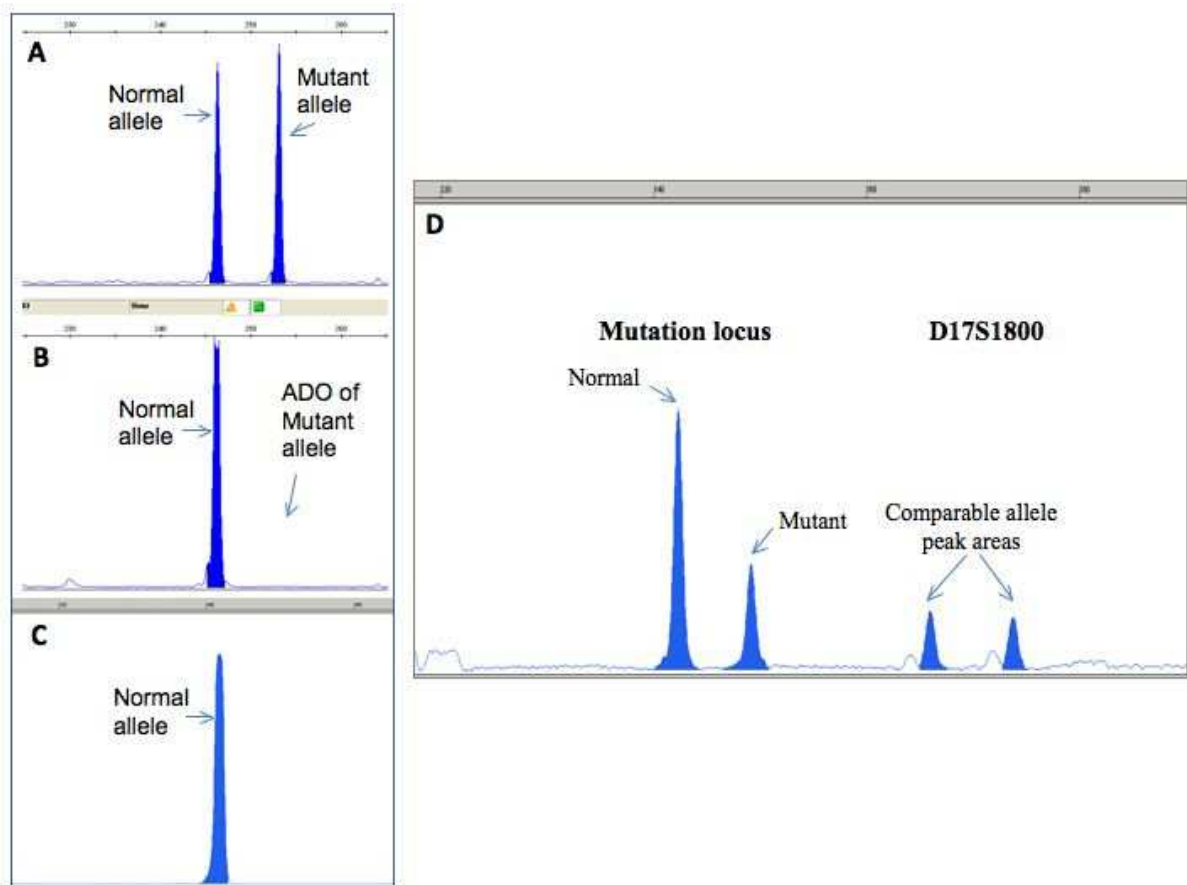
438

439

440

441

442 Figure 1.



443

444

445

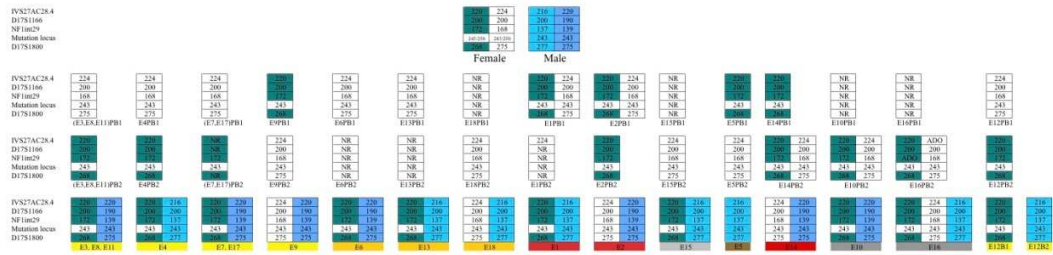
446

447

448

449

450 Figure 2.



451

452

453

454

455

456

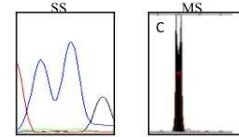
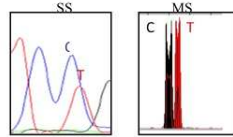
457

458 Figure 3.

D16S3024
C-T
D16S664
D16S663

225	225
C-T	C-T
214	214
112	112

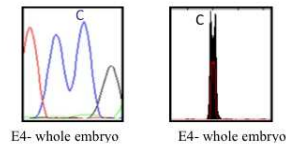
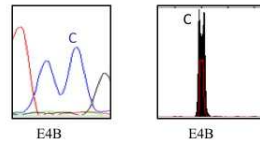
231	231
C	C
218	220
128	124



D16S3024
C-T
D16S664
D16S663

Cycle one

225	231	225	231	231	225	231
C	C	C	C	C	C	C
218	218	214	218	218	214	220
128	128	112	128	128	112	124
E6	E5	E3, E4	E2	E1		



Cycle two

225	231	225	ADO	225	231	225	231	225
C	C	C	C	T	C	C	C	C
ADO	220	214	220	218	220	ADO	218	218
NR	NR	112	124	121	124	128	128	128
E1	E2	E3	E4	E5				

