

Supplemental Material and Methods

1

Family 1

2

3 Trio Exome Sequencing (ES) was performed and analyzed at the University Hospital of Lyon. Library
4 preparation was performed with the Medexome kit (Roche) following manufacturers' instructions.
5 Paired-end 2x150 sequencing was performed on a NextSeq500 instrument (Illumina). Genomic
6 alignment against the hg19/GRCh37 assembly and variant calling were, respectively, done with BWA-
7 MEM v.0.7.12 (Li and Durbin, 2009) and GATK HaplotypeCaller v.3.4 (Broad Institute, Boston, MA, USA)
8 while QC were evaluated using DeCovA (Dimassi et al., 2015). Only highly confident variants were kept
9 for analysis (total depth >9; alternative allele depth >4; no strand bias; mosaicism >10%). Rare variants
10 were considered as having a frequency below 1% in gnomAD database.

Family 2

11

12 Trio exome Sequencing was performed at the University of Edimburg and analyzed at Lille University
13 Hospital. Library preparation, targeting exons and flanking introns (+/10pb), was performed with the
14 SureSelect XTHSV2 kit (Agilent Technologies, Santa Clara, CA, USA). The indexed library was subjected
15 to paired-end sequencing on a Hiseq400 (Illumina, San Diego, CA, USA). Sequence reads were then
16 aligned to the human reference genome (GRCh38/hg38) and genetic variants were identified and
17 annotated using Dragen (Illumina, San Diego, CA, USA) and in-house programs.

Family 3

18

19 Exome sequencing was performed in index-parent trio. Genomic DNA was captured using Agilent in-
20 solution enrichment methodology (SureSelect) with biotinylated probes library (Human All Exon v5-
21 50Mb, Agilent), followed by paired-end sequencing on Illumina HiSEQ 2000. The bioinformatics analysis
22 of sequencing data is based on the Illumina pipeline (CASAVA1.8.2) and was performed by IntegraGen
23 (Evry, France). Sanger validation was performed to confirm the compound heterozygous variants in
24 the *XRCC4* gene in proband, and the biparental inheritance in parents. Chromosomal breakage analysis

25 after Mitomycine-C treatment on metaphasic chromosomes was performed following national
26 protocol for Fanconi anemia diagnosis (1).

27 **Family 4**

28 DNA isolation from EDTA blood was carried out following standard protocols. Samples were subjected
29 to a targeted multigene panel analysis of 119 microcephaly-associated genes. For library preparation,
30 SureSelect™ QXT target enrichment kit (Agilent) with enzymatic fragmentation was used following
31 manufacturer's protocol (Agilent). Libraries were sequenced on an Illumina NextSeq 550 (Illumina, San
32 Diego, CA), and Sequence Pilot (jsi medical systems GmbH) software was used to align sequences to a
33 human reference genome (hg19) and for single nucleotide variant (SNV) calling. Parental samples were
34 not available.

35 **Family 5**

36 Trio Exome Sequencing was performed using an adapted GATK-based approach and following a
37 previously described protocol (2).

38 **Family 6**

39 Fetal growth was measured by ultrasound and assessed using the curves of Robinson et al. for crown-
40 rump length (3) and Verburg et al. for head circumference, femur length, biparietal diameter and
41 abdominal circumference (4). A prenatal Trio Exome Sequencing was performed on DNA extracted
42 from chorion villus biopsy and parental blood samples, using a 4700 genes virtual panel for variant
43 prioritization in multiple congenital anomalies, following a previously described protocol (5).

44

45

46 **Supplemental Results: clinical reports**

47 **Family 1**

48 The first patient (P1, **Figure 1A-C**), a 13-year-old boy, was the first of three siblings born to parents
49 from eastern Europe, who are not known to be related, with no relevant family history. His antenatal
50 history is unknown, but he was born at 39 weeks of gestation (WG), hypotrophic with a weight of 1500
51 g (-4 standard deviation SD), a height of 36 cm (-7.5 SD) and an occipitofrontal circumference (OFC) of
52 30 cm (-4 SD). His growth then proved steady, with a height and weight of around -5 SD, but his OFC
53 gradually decreased. At age 10, he weighed 13.9 kg (-5.5 SD), measured 107 cm (-5 SD) and had an OFC
54 of 43 cm (-7 SD). Facial features included sloping forehead, deep set eyes, low-set, posteriorly rotated
55 ears with hypoplastic lobes, beaked nose with sharp nasal root and broad tip, low columella, short
56 philtrum, and a pointed chin giving a triangular face shape. He has clinodactyly of the 5th fingers. He
57 has undergone surgery for bilateral cryptorchidism. He also presented a dermatofibrosarcoma
58 protuberans (Darrier-Ferrand sarcoma) of the left foot at 5 years, treated by surgical resection with
59 poor healing. He had several bouts of bronchitis, ear infections and gastro-enteritis, as well as episodes
60 of asthma that required treatment. He had teeth extraction because of cavities, but no abnormalities
61 in the shape of his teeth have been reported. His hair and nails are normal. Early motor acquisitions
62 were considered normal: he walked at 14 months and could ride a bicycle at 5 years of age. Language
63 was delayed, with first words appearing before 20 months. At 11 years, he said only a few words
64 without associating them. Reading and writing were not acquired. Neurological examination reported
65 brisk reflexes since 3 years old, and action tremor since the age of 10 years, impairing fine motor skills.
66 At 12 years, polykinetic reflexes was reported in all four limbs, with bilateral ankle clonus, but no
67 reported muscle weakness. Brain MRI was normal. At the last examination, at the age of 13, weight
68 was 16.8 kg (-5 SD), height was 121.5 cm (-6 SD) and OFC was 43.5 cm (-8 SD). It was noted that the
69 child walked on tiptoes with hollow feet. In addition, pigmentary anomalies with hyperpigmented
70 areas located around the scar on the foot, but also on the lower limbs and the front of the wrists were
71 noted for the first time. He has no hearing impairment.

72 The second patient (P2, **Figure 1D-F**), young sister of P1, presented with an intra-uterine growth
73 retardation (IUGR) at 28 WG observed on antenatal ultrasound. She was born at 35+3 WG, hypotrophic

74 with a weight of 1280g (-3.3 SD), a height of 36 cm (-6 SD) and an OFC of 26.5 cm (-4.5 SD). Her statural
75 growth was regular, but she showed post-natal OFC inflection. At age 9, she weighed 9.5 kg (-5 SD),
76 measured 98 cm (-6.3 SD) and had an OFC of 40 cm (-8 SD). The facial features were similar to her
77 brother and was additionally noted broad thumbs with distal implantation. Early motor development
78 was normal, with walking acquired at 12 months, but she presented language delay, with first words
79 pronounced at 3 years and a few words without association nor sentences at 10 years. Neurological
80 examination showed brisk and slightly diffused reflexes in the lower limbs since 3 years of age. At the
81 last examination, at 12, weight was 11.5 kg (-6 SD), height was 104 cm (-7.5 SD) and OFC was 40.2 cm
82 (-10 SD).

83 Because of their cognitive difficulties and low level of autonomy, both siblings attend school in a
84 medicalized educational institute. For both, cardiac, abdominal and brain imaging (MRI for patient 1
85 and scanner in patient 2) revealed no anatomical abnormalities apart from the known microcephaly.
86 Blood count was overall normal, apart from discrete lymphopenia in P1 and P2 at 6 and 5 years
87 respectively. Despite the absence of recurrent infections, a mild lymphopenia was observed in P1 and
88 P2 (1.38 and 1.37 giga/L at 6 and 5 years respectively [normal values for age (N): 2-8]), prompting
89 immunophenotyping. Both had mainly CD4+ T-cell lymphopenia (446 and 528/ μ L at 6 and 5 years
90 respectively [N: 700-2200], then 365 and 288/ μ L at 13 and 12 years [N: 530-1300]) with a particularly
91 marked deficit in the naive CD4+ CD45RA+ subpopulation, as well as B-cell lymphopenia (146 and
92 443/ μ L at 6 and 5 years respectively [N: 390-1400], then 62 and 24/ μ L at 13 and 12 years [N: 110-570]).
93 CD8+ and NK cells count was normal. A slight weight deficit in immunoglobulins was observed only in
94 P2 at the age of 12 (IgG 5.99 g/L [N: 7.10-15.60], IgA 0.71 g/L [N: 0.65-3.56], IgM g/L 0.48 [N: 0.66-
95 2.50]). Thus, this immune deficiency, although subclinical, appears to be progressive in P2, but not in
96 P1 (**Table S2**). IGF-1, IGFBP3, and TSH/T4L were normal for both. P2 showed mild dyslipidemia and
97 prediabetes at 12 years. Array-CGH and karyotype were normal in P2, with no increased chromosomal
98 breakage after in vitro mitomycin-C treatment. Analysis of a microcephaly NGS panel was performed
99 in 2016 in P1, but did not find any explanatory cause since *XRCC4* was not yet included among the 36

100 genes studied. The diagnosis was made 2021 by quatuor ES analysis, revealing in both patients a
101 homozygous pathogenic variant in *XRCC4*: c.127T>C p.(Trp43Arg), present in each parent at the
102 heterozygous state.

103

104 **Family 2**

105 P3 (**Figure 1H-I**) is a female, third child of non-consanguineous and healthy parents of European origin
106 without relevant familial history. Pregnancy was marked by early proportionate IUGR at 18 WG.
107 Amniotic fluid sampling was performed and showed a normal standard karyotype 46,XX. Delivery was
108 provoked at 32 WG because of growth stagnation. Anatomopathology of the placenta was normal.
109 Birth weight was 1180 g (-2.5 SD), height 37cm (-2.5 SD) and OFC 24 cm (-4 SD), Apgar score 10/10.
110 She presented with respiratory failure requiring non-invasive ventilation the first week. Hearing tests,
111 cardiac, abdominal and renal ultrasounds were normal. At 3 months of age, bilateral glaucoma was
112 diagnosed and surgically repaired. Because of failure to thrive, a gastric tube was placed. She also had
113 gastroesophageal reflux disease, treated by esomeprazole. She presented with global delay in
114 psychomotor acquisitions, sitting at 16 months and saying two words at 2 years. Neurological
115 examination showed peripheral hypertonia and stereotypical hand wringing. Brain MRI performed at
116 4 months was normal. She received specialized education and intensive re-education. From the age of
117 3, she experienced progressive pancytopenia, without adenopathy or hepatosplenomegaly. The blood
118 count at 3 years 6 months showed aregenerative macrocytic anaemia with 9.3 g/dL of haemoglobin
119 (normal range for age: 11.5-12.5), 2.610 G/L erythrocytes (N: 3.9-5.3), a mean corpuscular volume of
120 104 fL (N: 75-87), and 78 G/L reticulocytes (N: 20-120). A moderate thrombopenia was associated with
121 55 G/L platelets (N: 150-400), and a mild leukopenia with 3.38 G/L leukocytes (N: 5.5-15.5) and 0.9 G/L
122 lymphocytes (N: 3.0-10.5), while neutrophil count was normal. B12 and B9 levels were normal.
123 Immunophenotyping was not performed. Peripheral blood smear examination showed dacrocytes,
124 suggestive of extramedullar dyshaematopoiesis as seen in myelofibrosis. However, the myelogram

125 initially showed a bone marrow of normal appearance and cell abundance, with no abnormalities
126 suggestive of myelodysplastic syndrome. She was first treated symptomatically with folic acid, then
127 pancytopenia progressively worsened towards bone marrow aplasia, and required iterative red blood
128 cells transfusions every month, with preventive cotrimoxazole as anti-infective treatment. At 4 years,
129 she presented with two episodes suggestive of generalized convulsive seizure, tonic-clonic then tonic,
130 with loss of consciousness in both episodes. The electroencephalogram, performed on the day of the
131 second episode, showed polyspike-waves and slow waves, predominantly bi-frontal, increased during
132 sleep. A treatment with Levetiracetam was introduced, followed by Vigabatrin because of
133 haematological disorders, and she no longer had seizures. At 5 years, walking was not achieved, she
134 only produced few words and was still fed with gastric tube with almost no oral feeding. She showed
135 MPD with a weight of 8,18 kg (-7 SD), height 80 cm (-7 SD) and OFC 37.5 cm (-9 SD). Morphological
136 features included micrognathia, prominent nasal bridge and small teeth. She died at 5 in a context of
137 fever and hemoptysis, with no identified triggering factor. Autopsy was declined by the family. Trio ES
138 revealed pathogenic compound heterozygous biallelic variants in *XRCC4* gene, the c.25delC variant
139 resulting in the p.(His9ThrfsTer8) frameshift inherited from the mother and the c.482G>A
140 p.(Arg161Gln) missense variant inherited from the father.

141

142 **Family 3**

143 The fourth patient (P4, **Figure 1J-Q**) is a 22-year-old male, third child of unrelated parents, with no
144 family history. During pregnancy, ultrasounds at 12 WG revealed IUGR and suspected left renal
145 agenesis. At birth, after labor induction at 37 WG, the parameters were below the normal range for
146 gestational age with a weight of 2200 g (-2.1 SD), length of 44 cm (-2.2 SD) and OFC of 28.5 cm (-4 SD).
147 The neonatal period was marked by feeding difficulties, requiring nasogastric tube at 4 months, then
148 gastrostomy tube during 2 years. Abdominal ultrasounds confirmed a left renal hypoplasia with normal
149 renal function. Cardiac ultrasounds were normal. Skeletal radiographs showed delayed bone age with

150 slender long bones. Brain MRI showed a slight lateral ventricular dilatation and pituitary hypoplasia
151 with a relatively frontal lobe atrophy at 4-year-old. The patient presented repeated episodes of
152 hypothermia that required multiple hospitalizations during infancy, evocative of impaired
153 thermoregulation. He also had bilateral cryptorchidism, operated during childhood. Dental
154 examination revealed a delay in the primary dentition loss and oligodontia with eight missing teeth.
155 He also presented with astigmatism and hyperopia. At 12-year-old, the patient developed type 2
156 diabetes, currently stabilized with Metformine treatment. A hypogonadotropic hypogonadism was
157 diagnosed at 16 years old. Excision of a mandibular osteoid osteoma was performed aged 20. Biological
158 testings at 19 years old revealed hypertriglyceridemia (3.81 mmol/L [N: 0.60 – 1.70]) and
159 dyscholesterolemia (total cholesterol 4.92 mmol/L [N: 3.9 – 5.7]; HDL 0.90 mmol/L [N: 1.0 – 2.0]; VLDL
160 1.73 mmol/L [N: 0.25 – 0.78]; LDL 2.29 mmol/L [N: 1 – 4.15]). Dyslipidemia rapidly worsened at 21
161 years: serum was lactescent, triglyceride reached a maximum value of 10.07 mmol/L, with
162 hypercholesterolemia (total cholesterol 6.44 mmol/L; HDL 0.72 mmol/L; VLDL 2.01 mmol/L; LDL 3.16
163 mmol/L). It was complicated by a progressive hepatic steatosis, diagnosed on abdominal ultrasounds,
164 without hepatic fibrosis (hepatic elastography by FibroScan of 6.3 KPa for a standard of 3.1 to 6.9 Kpa)
165 and liver function tests were normal, with gamma-GT and transaminases within normal ranges. The
166 patient benefited from combined therapy with Atorvastatin, Ezetimibe and Dapagliflozin since the age
167 of 21 and his lipid profile is currently normalized at 23 (triglycerides 1.59 mmol/L; total cholesterol 2.60
168 mmol/L; HDL 0.97 mmol/L; VLDL 0.72 mmol/L; LDL 0.91 mmol/L). Regarding his neurodevelopment,
169 early milestones were normal with first words at 15 months and good interactions, contrasting with
170 the severe microcephaly. However, from 7 years of age, he had some learning difficulties, dyspraxia
171 and dyslexia, that required specific education. In adulthood, a disabled worker status was obtained.
172 On last clinical examination, aged 22, weight was at 37 kg (-3.96 SD), height at 146 cm (-4.87 SD), and
173 OFC at 47.5 cm (-7.5 SD). Dermatological inspection revealed some pigmented and depigmented
174 cutaneous spots macules on the skin. No abnormalities of the blood count were observed in this
175 patient. Karyotype and array-CGH were normal, 46,XY. Interestingly, adjunction of Mitomycine-C on

176 metaphase chromosomes showed a significant increase in chromosome breaks number (on 24% of
177 metaphases), while this number was normal in spontaneous conditions. No radial pattern nor
178 chromosomal rearrangement was observed. Trio ES revealed compound heterozygous pathogenic
179 variants in *XRCC4*: c.25delC p.(His9ThrfsTer8) inherited from the mother and c.823C>T p.(Arg275Ter),
180 inherited from the father.

181

182 **Family 4**

183 Patient P5 (**Figure 1R-X**) is a daughter from a family with no known medical history nor known
184 consanguinity. The pregnancy was marked by oligohydramnios, and low weight for gestational age,
185 but there was no pregnancy follow-up. She was born at 34 WG with a weight of 1425 g (-1.95 SD), a
186 height of 37 cm (-2.97 SD) and an OFC of 28 cm (-2.18 SD). She had severe bilateral pes equinovarus
187 with short Achilles tendons, probably secondary to oligohydramnios, and was treated with foot
188 abduction orthosis. Hypotonia was noted as early as 6 months of age, followed by a global
189 developmental delay: she pronounced her first words at 19 months and her first sentences after 2
190 years. She was sitting at 6 months but did not walk until 2.5 years-old, although the pes equinovarus
191 probably hampered motor acquisitions. She had intellectual disability, stereotypic hand wringing,
192 needed routines and help on all activities of everyday life. She presented severe feeding difficulties
193 since birth, requiring nasogastric feeding until 7 months of age, then gastrostomy-tube feeding. At 11
194 years, she could drink some water but no oral feeding was possible. Regarding initial investigations,
195 brain imaging, abdominal and cardiac ultrasound revealed no abnormalities. At 18 months,
196 radiographs showed osteopenia with a severely delayed bone age. A biological assessment at age 9
197 revealed prediabetes with hyperinsulinemia. At age 11, she weighed 17.9 kg (-4.5 SD), was 105.5 cm
198 tall (-6.7 SD), had an OFC of 39 cm (-10 SD). A hypergonadotropic hypogonadism was diagnosed, (FSH
199 71.3 mIU/ml [N: 2.1-11.1], LH 41.3 mIU/ml [N <11.9 mIU/ml], Estradiol 26 pg/ml [N: 30-400]),
200 associated with hypertriglyceridemia (triglyceride 2.57 g/L [N <1.5 g/L], total cholesterol 1.65 g/L [N

201 <1.90 g/L], HDL cholesterol 0.45 g/L [N > 0.48 g/L], LDL cholesterol 0.98 g/L [N < 1.16 g/L]). Clinical
202 examination showed low set ears without superior crus of antihelix, thin upper lip, short palpebral
203 fissures, brachymesophalangy of the fifth finger, acanthosis nigricans on the neck and armpits, and
204 hyperhydrosis of the hands. Karyotype and array-CGH were normal, 46,XX. A multigene panel
205 sequencing revealed two pathogenic truncating variants in *XRCC4*, c.25delC p.(His9ThrfsTer8) and
206 c.673C>T p.(Arg225Ter). Segregation study was not possible due to the impossibility to obtain parental
207 blood samples.

208

209 **Family 5**

210 Patient P6 is a 11-month boy, born to unrelated parents with no family history. During pregnancy, a
211 harmonious IUGR was noted at 20 WG. Labour was induced at 37+3 WG due to ongoing concerns with
212 his growth. He was born healthy, with a weight of 2230g (-2.5 SD), a height of 48 cm (-0.5 SD), and an
213 OFC of 30.5 cm (-2.5 SD). A sacral dimple was noted with borderline low termination of the spinal cord
214 at spinal ultrasound, with no indication for surgery. Growth retardation became more pronounced and
215 at 11 months, he weighed 5.8 kg (-3.8 SD), was 67 cm tall (-3.7 SD) and had an OFC of 40.4 cm (-4.4
216 SD). He has mild global developmental delay with predominant difficulties in expressive speech, and
217 at 4 years he still cannot count beyond 3. Biological investigations, including full blood count, thyroid,
218 renal and liver function tests, CKs, glucose, lactate, acylcarnitine profile, homocysteine and
219 mucopolysaccharidosis screen, were all normal. Immunophenotyping was not performed. Brain MRI
220 showed no abnormalities, and genetic testing for Silver-Russel syndrome was negative. Trio ES
221 revealed two pathogenic compound heterozygous variants in *XRCC4* (NM_003401): the c.673C>T
222 p.(Arg225Ter) inherited from the father, and c.37G>T p.(Glu13Ter) inherited from the mother.

223

224 **Family 6**

225 Patient P7 was a male fetus, fifth pregnancy from unrelated parents. They already had two healthy
226 children and 2 early miscarriages (at 7 and 8.5 WG respectively). This pregnancy resulted from
227 ovulation induction due to polycystic ovary syndrome. There was no other relevant family history. At
228 12+2 WG, the fetus had an increased nuchal translucency of 4.9 mm, a growth restriction around -3.3
229 SD for crown-rump length (48.6 mm) and -5 SD for head circumference (50.5 mm). A week later, the
230 nuchal translucency was normalized (1.1 mm) but growth was still delayed. Non-invasive prenatal
231 testing for aneuploidy was normal. At 14 WG, the proportionate early IUGR was confirmed. The fetus
232 had a crown-rump length of 63.1 mm (-4.2 SD) and a head circumference of 69.4 mm (-6 SD). Biparietal
233 diameter (19.3 mm), abdominal circumference (58.2 mm) and femoral length (8.9 mm) were also <-
234 2.3 SD. A chorion villus biopsy was done. QF-PCR and SNP-array on fetal DNA were normal. Because of
235 the recurrent miscarriages, chromosomal analysis was performed for parents and showed normal
236 karyotypes. Trio ES identified pathogenic compound heterozygous mutations in *XRCC4* (NM_003401):
237 c.25delC p.(His9Thrfs*8) inherited from the mother and c.613C>T p.(Arg205*), inherited from the
238 father. Pregnancy was terminated at 16+2 WG and autopsy was declined by the parents.

239

240 **Supplemental Table S1** : See Excel Sheet

241 Detailed clinical and molecular data for patients newly and previously described.

242

243 **Supplemental Table S2** : See Excel Sheet

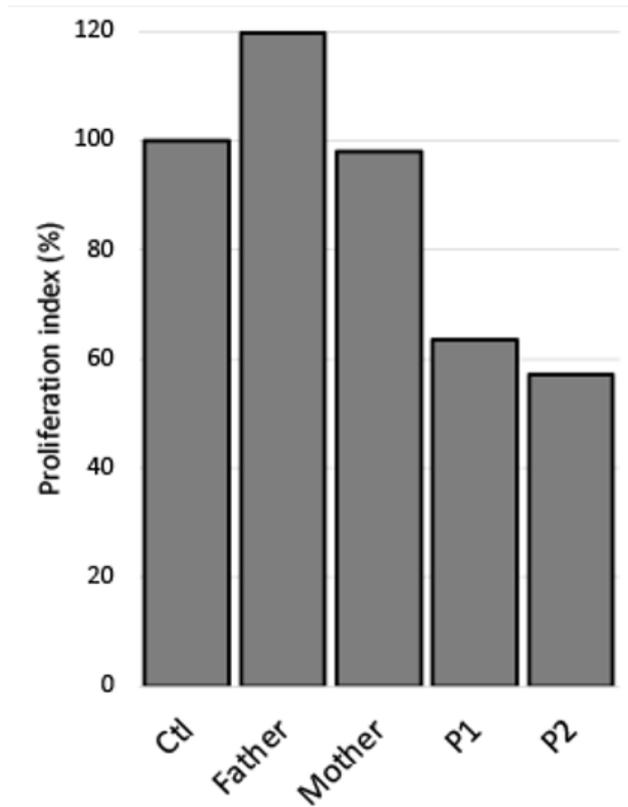
244 Immunological abnormalities in patients P1 and P2.

245 Values below the minimum threshold are shown in bold.

246

247 **Supplemental Figure 1**: T cell Activation proliferation as determined by cell counts at 6 days of

248 culture. Results are expressed the proliferation relative to a healthy control sample.



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250

251 **Web Resources**

252 GnomAD v4.0.0 : <https://gnomad.broadinstitute.org/>

253 CADDv1.6 : <https://cadd.gs.washington.edu/>

254 ProteinPaint : <https://proteinpaint.stjude.org/>

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256 **Supplemental References**

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