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► **To cite this version:**

Agathe Hercent, Edouard Amar, Alexander Valent, Stéphanie Belloc, Xavier Ferraretto, et al.. Various Genital and Reproductive Phenotypes in 46,XX/46,XY Chimeras. *Sexual Development*, 2020, 13 (5-6), pp.271-277. 10.1159/000510532 . inserm-03798540

HAL Id: inserm-03798540

<https://inserm.hal.science/inserm-03798540v1>

Submitted on 28 Jul 2023

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Various genital and reproductive phenotypes in 46,XX/46,XY chimeras

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33 Short title:

34 Human chimeras in reproductive pathology

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44 Key words: Human chimeras / Male infertility / XX testicular DSD / Sex development / Ovotestis

45 **Abstract:**

46 Tetragametic chimeras are due to the fusion of two different zygotes after fertilization. When
47 occurring between embryos of different chromosomal sex, the phenotype ranges from fertile
48 individuals to infertile patients and even to patients with variations in sex development. Here we
49 report three new cases of XX/XY chimeras, one in a young boy carrying an abnormal gonad which
50 turned out to be an ovary and two in phenotypically normal infertile men, one of whom had been
51 diagnosed previously as a XX-SRY negative male. These cases highlight the importance of
52 combining several cytogenetic and molecular techniques on different tissues for a proper diagnosis
53 and an appropriate prognosis.

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58 Introduction:

59 Chimera and chimerism are defined by the coexistence of two whole distinct genomes within the
60 same organism. They differ from mosaicism where the organism contains only one genome where
61 a mutation or a chromosomal event occurred during the first mitotic divisions of the zygote, leading
62 to two different cellular populations.

63 Chimerism is confined to one tissue or to one compartment. It can be temporary in the course of
64 blood transfusion or definitive as the consequence of allografting in patients receiving an organ or
65 bone marrow cells from a HLA-compatible donor. Chimerism can also be the result of
66 hematopoietic stem cell travel from an embryo to another during dizygotic or even monozygotic
67 twin pregnancy (Bourthoumieu et al., 2005, 2006). This type of chimerism must be distinguished
68 from microchimerism, a normal process during every pregnancy which is due to the bi-directional
69 trafficking of fetal and maternal cells through placenta. The persistence of fetal cells in pregnant
70 women, and even in mothers a long time after the birth of their child, predispose them to develop
71 various autoimmune diseases, particularly multiple sclerosis (Aractingi et al., 1998; Nelson, 2002;
72 Bloch et al., 2011)

73 In humans, chimeras generally occur after the fusion of two different zygotes, thus resulting in a
74 single but dizygotic embryo named tetragametic chimera (Wenk, 2018). *In vitro* fertilization, and
75 the replacement of more than one embryo, has been suspected to increase this risk of fusion
76 (Simon-Bouy et al., 2003). In some rare cases, accidental fertilization of a second polar body (Chen
77 et al. 2005) or parthenogenetic development of a zygote can lead to the formation of a chimera
78 (Strain et al, 1995, Kaise-Roger et al. 2006, Conlin et al., 2010).

79 Whether a chimera results from the fusion of two zygotes with the same (XX/XX or XY/XY) or
80 different (XX/XY) chromosomal genders, and depending on the repartition of each genome within
81 the different tissues, the phenotype range from strictly typical and fertile male or female individuals
82 (Bromilow and Duguid, 1989) to infertile patients (Laursen et al., 2018, Choi et al., 2019) and even
83 to a complete absence of sexual differentiation (Sugawara et al., 2012). Here we report three new

84 cases of XX/XY chimeras in male patients who have been investigated by using conventional and
85 molecular cytogenetic or genetic techniques. These cases raise the possibility that some XX-*SRY*
86 negative males, some infertile patients or some children with a variation in sex development could
87 be in fact chimeras.

88

89 **Patients**

90 Patients 1 and 2 and the parents of patient 3 gave their written informed consent and signed for
91 genetic analyses performed according to the rules of the Agence de la Biomédecine (France)
92 according to the French legislation.

93 Patient 1

94 Patient 1 was a 26 years old male consulting for infertility. Clinical examination showed a normal
95 penis with two intrascrotal testes measuring 10 mL on both sides.

96 Endocrine analysis showed normal inhibin B (86 pg/ml), FSH (4,7 IU/l) and testosterone 4ng/ml)
97 levels.

98 Karyotype analysis was initially performed from blood lymphocytes using standard techniques and
99 R and G banding. It showed an homogenous 46,XX chromosomal female constitution confirmed by
100 FISH using chromosome X and Y centromeric probes on 400 interphase nuclei and by FISH with a
101 probe coding for the *SRY* gene which showed that this gene was not present on the X chromosomes.
102 SNP-array analysis (OmniExpress-24, 715K, Illumina) showed a normal 46, XX profile without
103 any deletion or duplication, especially upstream from the *SOX9* gene (Benko et al., 2011). The total
104 lack of Y chromosome in peripheral blood was also confirmed by PCR analysis which showed the
105 absence of *SRY* and *ZFY* sequences as well as the total lack of AZFa, b and c regions (Figure 1).
106 Therefore, this patient was initially diagnosed as a XX *SRY*-negative male. However, sperm
107 analysis revealed a cryptozoospermia at the first examination and then a severe oligozoospermia
108 (20 000 spermatozoa/ml) which allowed the cryopreservation of sperm samples. Because such a
109 result is theoretically impossible in XX testicular DSD (Disorders/Differences of Sex

110 Development), Y-positive cells were sought in another tissue than blood and, indeed, FISH analysis
111 with chromosome X and Y centromeric probes revealed 53% of XY cells on a buccal cell smear,
112 mixed with XX cells (Figure 1). Unfortunately, genomic DNA extraction from buccal cells failed.
113 Sperm FISH analysis using chromosomes X, Y and 18 centromeric probes was then performed on a
114 sample of ejaculated sperm cells (n=50). It showed a normal ratio 1:1 between X and Y bearing
115 spermatozoa thus indicating that these gametes were produced from normal XY meiotic cells.

116

117 Patient 2

118 Patient 2 was a 35 years old male consulting for infertility. Clinical examination showed a normal
119 penis with two intrascrotal testes measuring 3.5 ml on the left and 8 ml on the right. The left testis
120 also presented a stage 3 varicocele. Sperm analysis revealed a non-obstructive azoospermia and
121 endocrine analysis showed decreased inhibin (18pg/ml) and increased FSH (16 IU/l) levels and a
122 normal testosterone level (3.68 ng/ml).

123 Karyotype study was performed from blood lymphocytes using standard techniques and R and G
124 banding. It showed the presence of two different cell lines: one with a normal 46,XY male
125 karyotype in 75% of the cells analyzed and one with a normal 46,XX female karyotype in the
126 others. Molecular analysis of the Y chromosome using PCR was normal indicating the presence of
127 the *SRY* gene as well as the lack of deletion of the AZFa, b and c regions. The two populations were
128 also present in the same proportions (75% XY, 25% XX), on cells from a buccal swab analyzed by
129 FISH using probes coding for the X and for the Y centromeres. A SNP-array analysis
130 (OmniExpress-24, 715K, Illumina) was performed on DNA extracted from blood lymphocytes. The
131 high number of SNPs on this type of array (more than 700 000) allowed identifying 9 distinct allele
132 frequencies confirming the presence of 4 different haplotypes in a mosaic state (Figure 2).

133 This patient underwent a testicular biopsy which revealed a normal testicular architecture associated
134 with Leydig cell hyperplasia in the right gonad. Sperm cells were successfully retrieved and frozen.
135 Sperm FISH analysis using chromosomes X, Y and 18 centromeric probes was performed on the

136 supernatant obtained after biopsy shredding. It showed a normal ratio 1:1 between X and Y bearing
137 spermatozoa thus indicating, like in patient 1, that these gametes were produced from normal XY
138 meiotic cells (Figure 1). FISH study of paraffin embedded sections using the same probes showed a
139 majority of XY cells in both gonads with around 80% of Y-positive nuclei and 20% of XX cells.
140 Most of these XX cells were found in the interstitial compartment of the testis and in the peritubular
141 sheath of seminiferous tubules. However, some of them were also found within the seminiferous
142 tubules (Figure 1).

143 The couple underwent *in vitro* fertilization after intra-cytoplasmic sperm injection which resulted in
144 a singleton pregnancy and in the birth of a healthy boy.

145

146 Patient 3

147 He was a 9 years old boy referred for clinical and biological follow-up after left gonadectomy. It
148 was performed at the age of six years in the course of the chirurgical cure of unilateral
149 cryptorchidism because of the abnormal aspect of the gonad. Histological examination of this latter
150 showed a gonad corresponding to an ovary with primordial follicles surrounded by a canal structure
151 evoking Mullerian remains like a hemi-uterus. Sections of tubes corresponding either to a
152 hypoplastic epididymis or to Wolffian vestigial structures were also described. No testicular tissue
153 was observed.

154 At the time of his evaluation, clinical examination showed a well-developed boy weighing 29.2kgs
155 (+0.5 SD) and measuring 1.28m. Left testis was lacking and the right one was in scrotal position,
156 measuring 15x10mm. Penis was 45mm long. Achromic spots were noticed on the whole body.
157 Chromosomal analysis on blood cells after R and G banding revealed a 46,XX female karyotype in
158 76% of cells and a 46,XY male karyotype in 24%. These percentages were slightly different on
159 cells from a buccal swab (60% XX, 40% XY) analyzed by FISH with chromosome X and Y
160 centromeric probes. SNP-array analysis (Humanomniexpress-24 715K, Illumina) performed on

161 DNA from blood cells confirmed the diagnosis of tetragametic chimera by visualizing nine different
162 allele frequencies and estimating the rate of XY cells to 10-15%.
163 FISH study of paraffin embedded sections of the removed gonad using the same probes showed
164 percentages of XY ranging from 4% to 90% according to the type of histological structure (4% in
165 the ciliated epithelium vs 90% in the epididymis-like tubes) (Figure 3).

166

167 **Discussion:**

168 As for the diagnosis and the evaluation of mosaicism, chromosomal analysis by FISH is a powerful
169 and sensitive method for detecting and measuring chimera. It allows a cell by cell identification of
170 the chromosomal content as opposed to molecular techniques which give a result as a whole. In the
171 3 patients we analyzed, FISH was able to give a precise evaluation of the XY/XX cell ratio and,
172 when coupled to morphological study on histological tissue sections, to correlate this ratio to the
173 nature of the tissue or the cells like mature gametes in patients 1 and 2. On the contrary, the very
174 high sensitivity of PCR allowed us to assert that patient 1 had a homogenous XX chromosomal
175 constitution in blood cells with absolutely no Y chromosome material. In patient 2, PCR indicated
176 also that his Y chromosome had a normal structure, without any molecular deletion. Therefore, we
177 preconize that patients suspect to carry a chromosomal mosaicism or to be a chimera should be
178 studied by using both cytogenetic, including FISH, and molecular techniques. Such a procedure has
179 been particularly useful in patient 1 who was initially considered as a 46,XX-*SRY* negative male.
180 The fact that he presented with cryptozoospermia led us to question this diagnosis and to perform a
181 FISH analysis on another tissue than blood. A smear of cells from a buccal swab appeared to be the
182 simplest and non-invasive technique for exploring a chromosomal mosaicism or a chimera by FISH
183 and, in his case, it allowed to correct the diagnosis. If azoospermic, this patient certainly would not
184 have been evaluated by this technique. This raises the question of the diagnostic procedure in XX-
185 *SRY* negative males in whom molecular investigations fail to find a cause such as a molecular
186 duplication upstream from *SOX9* gene (Benko et al, 2011) or a mutation in the *WNT4/RSPO1* gene

187 pathway (Micali et al., 2005). Therefore, we recommend that every patient in this situation should
188 have a complementary examination of another tissue than blood, by FISH or PCR, in order to detect
189 a possible XX/XY chimera.

190 In our patients, FISH was also applied on testicular cells and mature sperm cells when available. In
191 patients 1 and 2, it revealed a normal 1:1 ratio between X and Y bearing spermatozoa confirming
192 that spermatogenesis is possible only from XY spermatogonia and that meiotic segregation of sex
193 chromosomes at meiosis is normal, even in the presence of a variable contingent of XX cells. To
194 our knowledge, only one study described the evaluation of testicular cells by FISH in a
195 46,XX/46,XY true hermaphrodite patient (Sugawara et al., 2012) but the authors presented their
196 results as a whole without discriminating spermatozoa from other testicular cells. Our results are of
197 importance since they suggest strongly that spermatozoa from XX/XY males are chromosomally
198 normal and can be used by *in vitro* fertilization to give birth to a normal offspring, as it was the case
199 in patient 2.

200 Finding XX cells within seminiferous tubules raises the question of their origin. Since the
201 differentiation of supporting cells into Sertoli cells in the primitive gonad needs the expression of
202 the *Sry* gene in mouse (Ungewitter and Yao, 2013), XX cells in a human adult seminiferous tubule,
203 as found in patient 2, are unlikely to be Sertoli cells. Therefore, they might be germ cells which
204 would be unable to initiate the meiotic process although it remains to be elucidated.

205 In patient 3, who was still a child, FISH was only possible on paraffin-embedded sections from the
206 unilateral gonadectomy that he underwent at the age of six. However, results were particularly
207 interesting since they showed that most of the XY cells were localized in the male-differentiated
208 parts of the gonad, i.e. those resembling to epididymis-like tubes, 90% vs 4% in the ciliated
209 structures probably deriving from a uterine horn (Figure 3). This observation highlights the
210 usefulness of FISH performed on histological sections which can superimpose genetic data to tissue
211 or cellular structures.

212 Comparative genomic hybridization on DNA-arrays (CGH-array) is a very sensitive method for the
213 detection of chromosomal imbalances but is unable to diagnose balanced structural chromosomal
214 rearrangements. When performed with SNP-arrays, it allows determining the genotype of the
215 patients by analyzing the status of each DNA marker designed as the B-allele frequency (BAF):
216 homozygous (AA or BB) or heterozygous (AB), leading to the visualization of three dot plots on
217 the graph. Therefore, SNP-array can detect losses of heterozygosity of entire chromosomal regions,
218 as seen in consanguineous patients, as well as uniparental isodisomy or heterodisomy, when
219 parental DNAs are available, in patients presenting with a genetic disease due to parental genomic
220 imprinting (Conlin et al., 2010; Kearney et al, 2011). Moreover, SNP-array is also able to diagnose
221 a maternal contamination in fetal cell samples by revealing more than two different haplotypes.
222 Indeed, a 50/50 mix of two different DNAs would lead to six recognizable haplotypes (AA/AA,
223 AA/AB or AB/AA, AA/BB or BB/AA, AB/AB, AB/BB or BB/AB and BB/BB). The fact that
224 DNAs are in different proportions reaches this number to nine and the frequency of each haplotype
225 gives the level of contaminating DNA. In chimeras due to the fusion of two embryos, the situation
226 is stackable to that seen in the contamination of a DNA sample by an external one. In patient 1,
227 SNP-array performed on blood cells was normal since he had an homogenous 46,XX constitution in
228 this tissue and DNA from a buccal swab was not available. In patient 2 and 3, SNP-array profile
229 revealed nine dot plots confirming that both chimeras had a tetragametic origin.

230 Another explanation to the homogenous XX karyotype in patient 1 could be a chimerism secondary
231 to blood exchange with a female fetus during pregnancy, as already described (Sudik et al., 2001;
232 Johannsen et al., 2003). To our knowledge, in patient 1, pregnancy was not twin. This information
233 should be systematically sought in case of discordance between blood karyotype and phenotype in
234 order to avoid confusion between chimerism and disorder/difference of sex development.

235 In conclusion, these three reports illustrate the genital phenotype of XX/XY chimera in man when
236 abnormal. They highlights situations where the search for chimera, or chimerism, should be
237 performed i) in 46,XX-SRY negative males in whom known molecular causes have been excluded

238 ii) in men presenting with idiopathic non-obstructive infertility iii) in children with a
239 difference/disorder of sex development without cytogenetic or molecular cause identified.
240 Diagnosis of chimera should be made on different tissues by conventional and molecular (FISH)
241 techniques completed by a SNP-array study in order to identify the causal mechanism.

242

243 Author's roles:

244 AH, AV, SCB, MP and HM made the study and wrote the paper. EA and SB realized the clinical
245 and biological care of patient 1. XF, JFH and RBB realized the clinical and biological care of
246 patient 2. CA, ACT, IGV and FD realized the clinical and biological care of patient 3.

247 Statement of Ethics: Patients or parents of patients gave their written informed consent for the
248 genetic analyses performed in this study. The research was conducted ethically in accordance with
249 the World Medical Association Declaration of Helsinki.

250 Funding: There was no source of funding for this study.

251 Conflict of interest: The authors have no conflicts of interest to declare.

252

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316

317 **Legends**

318 Figure 1:

319 Patient 1: A) FISH on blood cells with probes coding for the chromosome X centromere and for the
320 *SRY* gene showed a homogenous XX profile and the lack of *SRY* at the tip of one of the X
321 chromosomes. B) PCR on blood cell DNA with primers specific of the AZF regions of the Y
322 chromosome. Patient's DNA (lane 4) gave a negative result as for female DNA (lane 5) when
323 compared to male patients (lane 2 and 3). C) SNP-array on blood cell DNA revealed a normal X
324 chromosome ratio when compared to XX DNA (bottom). B-allele frequency (top) showed only
325 three dot plots indicating the existence of two different haplotypes. D) FISH on buccal cells with
326 probes coding for the chromosomes X (green) and Y (red) centromeres found 53% of XY cells.

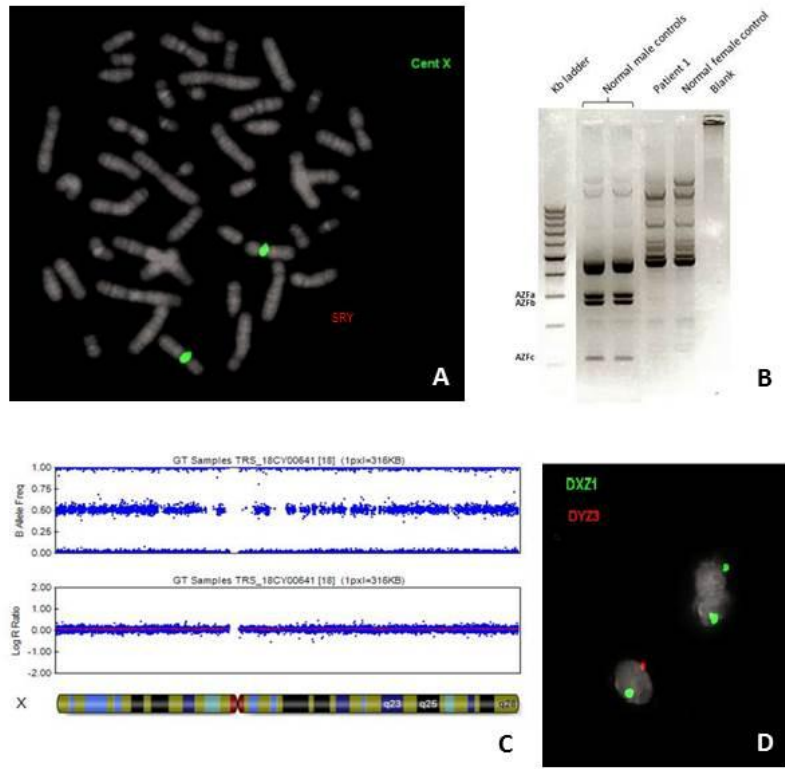
327 Figure 2:

328 Patient 2: A and B) FISH on buccal cells (A) and blood cells (B) with probes coding for the
329 chromosomes X (green) and Y (red) centromeres showed 25% of XX cells and 75% of XY cells. C)
330 SNP-array on blood cell DNA. The B-allele frequency revealed nine different haplotypes indicating
331 the existence of a tetragametic chimera. D) FISH on the supernatant of the testicular biopsy with
332 probes coding for the chromosomes X (green) and Y (red) centromeres showed both XX and XY
333 cells (fine arrows) mixed with haploid X or Y positive cells with a smaller nucleus (large arrows).
334 E) FISH on paraffin embedded sections with probes coding for the chromosomes X (green) and Y
335 (red) centromeres showing the presence of XX cells in the peritubular sheath of seminiferous
336 tubules. F) On the same sections, rare XX cells are also found within seminiferous tubules. The
337 peritubular sheath is visible on the right as a faint green fluorescence containing an elongate
338 nucleus.

339 Figure 3:

340 Patient 3: FISH on paraffin embedded sections of the gonadectomy with probes coding for the
341 chromosomes X (green) and Y (red) centromeres. Most nuclei in the epithelium surrounding the

- 342 tubules corresponding to a hypoplastic epididymis were XY (A) while most cells in the rest of the
- 343 gonad corresponding to an ovary and to tubules evoking Mullerian remains were XX (B).

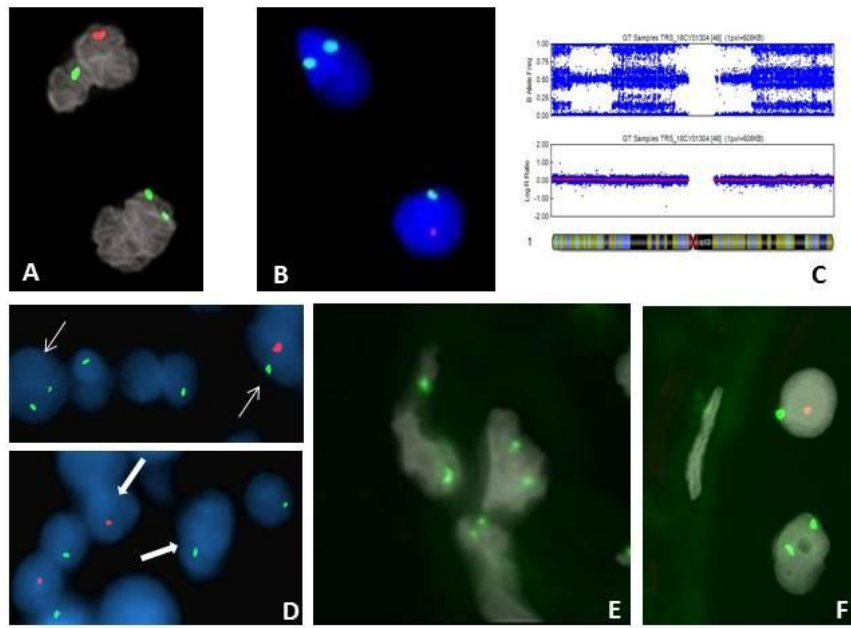


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Figure 1

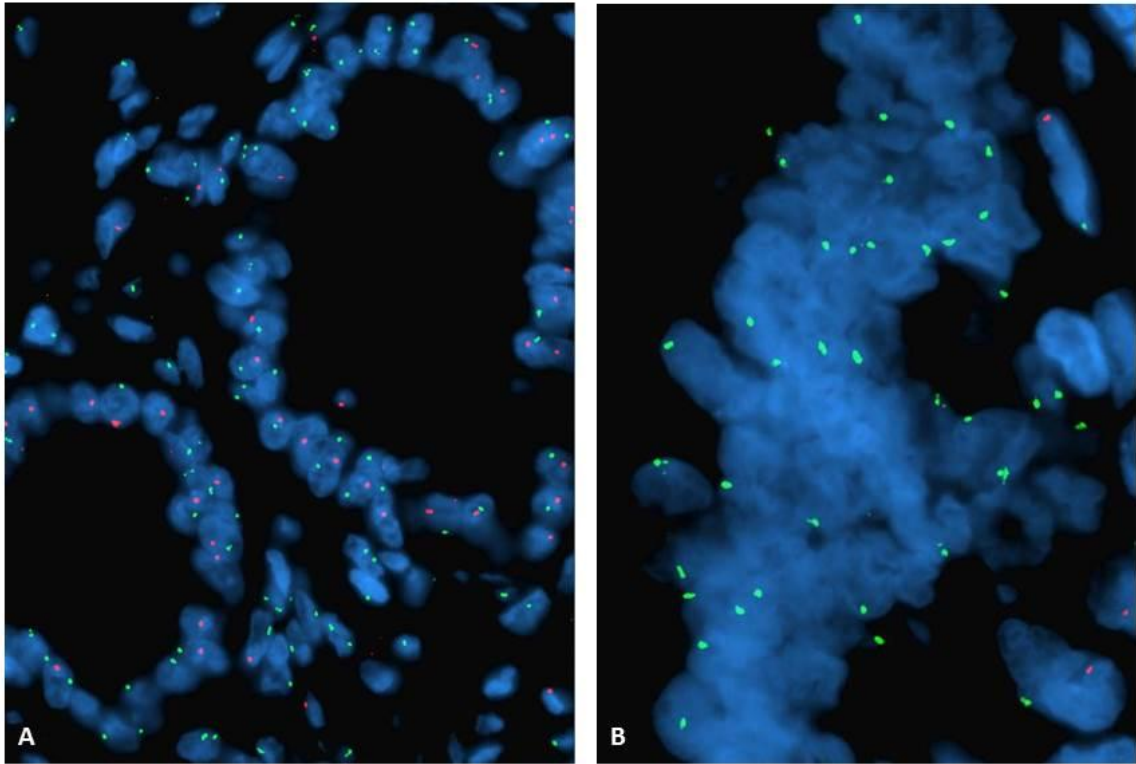


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Figure 2



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Figure 3