

34 **Abstract**

35

36 **Objective:** To characterize ovarian follicles of girls and young women with Turner syndrome
37 (TS) who underwent ovarian tissue cryopreservation (OTC).

38 **Design:** Retrospective case-control study.

39 **Setting:** University hospital.

40 **Patients/Animals:** 15 girls and young women with TS aged 5-22 years at OTC were included
41 together with 42 control girls and young women aged 1-25 years who underwent OTC due to
42 cancer.

43 **Intervention(s):** None

44 **Main Outcome Measure(s):** Follicle density (follicles/mm³), morphology and health were
45 assessed in ovarian cortex biopsies from TS patients and compared to control ovaries. Hormone
46 concentrations were measured in blood samples and follicle fluid samples. Immature cumulus
47 oocyte complexes (COCs) were obtained and matured *in vitro*.

48 **Results:** Follicles were found in 60% (9/15) of the biopsies from TS ovaries. In 78% (7/9) of
49 the ovaries with follicles, the follicle density was within the 95% CI of the control group. There
50 was a high rate of abnormal follicle morphology. 6 follicle specific proteins were expressed
51 similarly in TS and control ovaries. However, markers of apoptosis and zona pellucida were
52 found to be abnormal in TS. TS follicle fluid from small antral follicles had lower
53 concentrations of estrogen and testosterone and higher concentrations of AMH than controls
54 ($p=0.036$, 0.001 , 0.005 , respectively). 31 COCs were collected from one patient and cultured
55 for 48 hours *in vitro*, resulting in 5 MII oocytes (maturation rate 16%, degeneration rate 19%).

56 **Conclusion:** The benefits of OTC may be limited to a highly selected group of TS mosaic
57 patients in whom a sizeable pool of normal follicles is present at OTC.

58 **Keywords:** Turner syndrome, follicle density, ovarian tissue cryopreservation

59 **Introduction**

60 Turner syndrome (TS) is caused by the absence of one of the two X chromosomes in all cells
61 or a proportion of cells, affecting approximately 1:2000 Caucasian girls (1). The most common
62 karyotype is 45,X (47%), followed by different mosaicisms, most commonly 45,X/46,XX
63 (17%). The main reproductive effect of TS is Primary Ovarian Insufficiency (POI) (2,3).
64 Although menarche occurs spontaneously in 15-30% of TS girls the prevalence of natural
65 pregnancy is as little as 2-7% (2-6).

66

67 Two previous studies have reported the presence of ovarian follicles in 15/47 (7) and in 8/9 (8)
68 adolescent TS patients. Mosaicism and spontaneous menarche were predictive for the presence
69 of follicles, consistent with pregnancy being most prevalent in women with mosaic TS (2,5),
70 and ovarian tissue cryopreservation (OTC) has been suggested as an option for fertility
71 preservation (7). Predictors for the presence of ovaries without follicles in TS girls include
72 karyotype 45,X, low serum AMH, high serum FSH, and absent menarche or puberty (7,8).
73 Oocyte donation is the only way for TS patients with POI to conceive, but pregnancies carry a
74 substantial risk to mother and fetus (6,9,10).

75 The aim of this study was to characterize the number and morphology of follicles in girls and
76 young women with TS, who underwent OTC. We have also evaluated follicle and oocyte
77 function to assess the potential for future fertility restoration in order to evaluate whether or not
78 to perform OTC in this group of patients.

79

80 **Material and Methods**

81 *Patients and ovarian tissue cryopreservation*

82 A total of 15 girls and young women with TS aged 5.0-22.4 years (mean age 15.4 years) were
83 included together with 42 control girls and young women aged 1.5-25.5 years (mean age 15.2
84 years). The control group were referred to the Danish program for fertility preservation by OTC
85 because of a cancer diagnosis and have not received any gonadotoxic treatment before OTC.
86 Patients from both groups were only included if an ovarian cortex biopsy was spared for
87 histology in connection with OTC. All patients underwent OTC between the years 2002 and
88 2016. Follicle densities in the control girls with cancer below the age of 18 years has previously
89 been published (11). One additional ovarian biopsy from a girl with Fanconi anemia was
90 included for immunohistochemical (IHC) staining. During the preparation of ovarian tissue,
91 two patients presented with visible antral follicles on the ovarian surface from which follicle

92 fluid from a total of 8 small antral follicles was collected. In one of these patients additionally
93 a total of 31 cumulus oocyte complexes (COCs) were obtained from the medulla tissue. Patient
94 characteristics are given in Table 1; there were 7 in the Danish cohort, 5 from UK and 3 from
95 Australia. All controls were age matched patients having OTC for conditions other than TS in
96 Denmark. The ovarian cortex was prepared as previously described for slow-freezing (12,13)
97 and stored in liquid nitrogen. Additionally, one small piece of cortex ($\leq 2 \times 2 \times 1$ mm) was
98 obtained for histological examination. The OTC schemes were approved by the ethics
99 committee of Copenhagen and Frederiksberg (H-2-2001-044) and Lothian Health (ref
100 06/S1103/26). The storage and collection of patient data were approved by the Ministry of
101 Health (J. no. 30-1372) and by the Danish authorities to comply with European Union tissue
102 directives. All participants, or parents for younger patients, gave informed consent in writing.

103

104 *Tissue processing*

105 Tissues from Edinburgh (subjects 1, 3, 9, 13, and 14) were fixed in 10% neutral buffered
106 formalin (NBF); tissues from Melbourne (subjects 7, 8, and 11) were fixed in 4%
107 paraformaldehyde, whereas the remaining tissues from Copenhagen were fixed in Bouin's
108 solution. In Edinburgh and Melbourne 5 or 6 μm sections of paraffin embedded human ovarian
109 cortex were prepared, de-waxed and stained with haematoxylin and eosin for estimation of
110 follicle density (14). In Copenhagen, 30 μm sections were stained with periodic-acid Schiff and
111 Mayer's reagents for further estimation of follicle density, 5 μm sections were processed for
112 IHC staining.

113

114 *Immunohistochemical staining*

115 Sections were de-paraffinated in xylene, rehydrated in ethanol followed by antigen retrieval in
116 either 10 mM sodium citrate, pH 6 or 10 mM Tris, pH 9. Retrieval was not required for zona
117 pellucida protein 1 and 2 (ZP 1 and ZP 2) (15). Endogenous activity was inhibited using 1.5%
118 peroxidase, followed by inhibition of nonspecific binding with 1% bovine serum albumin
119 (BSA) (Sigma Aldrich, Copenhagen, Denmark). Sections were incubated with primary
120 antibodies overnight at 4°C except for ZP protein antibodies, which were incubated at 37°C for
121 1 hour; details of antibodies and conditions are given in Supp. Table 1. Secondary antibody
122 used was rabbit-anti-mouse-HRP (Dako, Glostrup, Denmark) and visualised with 3,3'-
123 diaminobenzidine tetrahydrochloride (DAB+ Substrate Chromogen System, Dako). Both
124 Universal negative control serum® (BioCare Medical, CA, USA) and antibody dilution buffer

125 was used in place of primary antibody as negative controls and showed no staining (Supp. Fig.
126 1). An Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, North Ryde,
127 Australia) detecting apoptosis terminal deoxynucleotidyl transferase mediated dUTP Nick End
128 Labeling (TUNEL) was also included.

129

130 *Follicle density*

131 Two methods were used to estimate the non-growing follicle density in the ovarian cortex. In
132 Copenhagen, the follicle density was estimated in 30 μm section using a mathematical model
133 described by Schmidt and colleagues (16). In brief, this model was based on the fraction of
134 sections, the mean primordial follicle diameter, and a correction factor (α) to account for the
135 possibility of counting the same follicle more than once. Since the mean diameter of a
136 primordial follicle is 44 μm (17) and the sections were 30 μm , there was a possibility to count
137 the same follicle two or three times (16). In Edinburgh and Melbourne follicle density was
138 measured in 5 μm sections by McLaughlin and colleagues (14). In brief, all tissues sections
139 were examined for the presence of follicles. To avoid overcounting, follicles were only assessed
140 when the nucleolus was observed. The follicle density was determined by dividing the total
141 number of follicles in the biopsy by the volume of tissue analyzed. To evaluate if the two
142 methods of data collection were comparable a predictive model was used (14), which combine
143 an age-related normative model for follicle population in the human ovary (18) and an age-
144 related normative model for the volume of the human ovary (19). Comparison of data obtained
145 by the two methods used shows good agreement using the predictive model (14).

146

147 *Hormone assays*

148 Follicle fluid was aspirated using a 29 gauge syringe from small antral follicles (< 7.0 mm in
149 diameter) during preparation of ovarian tissue for cryopreservation. Estradiol, testosterone,
150 AMH, and inhibin-B concentrations were measured in follicle fluid (after appropriate dilution)
151 using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Estradiol and
152 testosterone were measured with Nova Tech ELISA assays (DNOV003, DNOV002, Aalborg,
153 Denmark), AMH and inhibin-B were measured with the UltraSensitive AMH/MIS and inhibin-
154 B ELISA kit (AL-105, AL-107, Ansh Labs, Webster, TX, USA) (20).

155

156 *Western blot*

157 Western blot analyses were performed according to the manufacturer's instructions (Invitrogen
158 provided by Thermo Fischer, Hvidovre, Denmark). In brief, follicle fluid proteins were
159 separated on a NuPAGE 4-12% Bis-Tris mini gel, the proteins were subsequently blotted to a
160 PVDF membrane (Thermo Fischer). The membrane was blocked in 5% skimmed milk and
161 incubated with primary AMH antibody (AMH 39/48, Ansh Labs, TX, USA) overnight at 4°C
162 and subsequently with secondary horseradish peroxidase-conjugated goat-anti-mouse antibody
163 (Sigma Aldrich, Brøndby, Denmark) for 1 hour at room temperature. Signal was detected with
164 Pierce™ SuperSignal West Femto Substrate (Termo Fisher) and visualized with the DNR
165 MicroChemi 4.2 bio-imaging system. We have previously validated the specificity of the AMH
166 antibody used by blocking with surplus recombinant AMH, whereafter all AMH related bands
167 disappeared (21).

168

169 *In vitro maturation of COCs*

170 Immature COCs from small antral follicles were collected from the surplus medulla tissue from
171 one patient with mosaic TS and cultured in IVM medium (Origio, Måløv, Denmark)
172 supplemented with FSH and LH and overlaid with liquid paraffin at 37°C in 5% CO₂ humidified
173 incubator for 48 hours as previously described (22). Cumulus cells were removed after 48 hours
174 and the developmental stage of the denuded oocytes was evaluated using an inverted
175 microscope and classified as either: 'GV', with a distinct germinal vesicle; 'MI', no germinal
176 vesicle and no polar body; 'MII', one polar body extruded.

177

178 *Statistics*

179 Data on follicle density from the Danish girls below the age of 18 years have previously been
180 published (11). An adjusted model including the new data has more normally distributed
181 residuals (84% of a perfect Gaussian distribution), and hence is expected to have lower
182 generalization error when assessing densities from subjects not used to derive the model.
183 Statistical analysis was performed using GraphPad Prism 6.07 program (GraphPad Software,
184 Inc., CA, USA) and Microsoft Excel version 14.6, with linear regression to evaluate follicle
185 density against age and Mann-Whitney *U*-test to compare the hormone concentrations in
186 follicular fluid. Significance was defined as $p < 0.05$.

187

188

189

190 **Results**

191 A total of 15 girls and young women with TS aged 5.0-22.4 years old were included in this
192 study. Three cases were postpubertal (i.e. >18 years old). Moreover, 11 of the 15 cases were
193 diagnosed with mosaic TS.

194

195 *Follicle density*

196 Follicles were found in 60% (9/15) of the biopsies from TS ovaries - 8 girls with a mosaic
197 karyotype and one 45,X, who was aged only 5 years and the youngest in the series (Table 1).
198 No follicles were found in 2 patients with menopausal FSH levels (ages 14 and 17), in 2 with
199 mildly elevated FSH levels (aged 17 and 22), nor in a further 2 patients (aged 8 and 14) with
200 undetectable AMH levels. All patients with follicles had a detectable AMH level and/or FSH
201 <10I U/L (except one on whom no hormone data were available).

202 While follicle density was below age-matched mean for most TS patients, it was within the
203 95% CI for controls in 78% (7/9) of the cases, in 22% (2/9) it was below the 95% CI (Fig. 1).
204 Including both TS patients with and without follicles (n=15), no correlation between follicle
205 density and age was found ($p>0.1$). When follicle densities from the present study were
206 combined with previously published TS data including both patients with and without follicles
207 (n=23) (8), no correlation between follicle density and age was found ($p>0.1$) (Fig. 1). The
208 cortex tissues were fixed in either 4% paraformaldehyde or Bouins solution before histological
209 examination and theoretically this difference in fixation may impact the follicle densities,
210 however we find this very unlikely.

211

212 *Follicle morphology and immunohistochemistry (IHC)*

213 Most (6/9) ovaries showed a high rate of abnormal follicle morphology. The follicle
214 morphology of three subjects are illustrated in Fig. 2. The major abnormalities observed in
215 primordial follicles were misshapen, vacuolated oocytes and an incomplete layer of granulosa
216 cells surrounding the oocyte (Fig. 2A) leading to irregular oocyte shape and partial lack of
217 connection to the basal lamina and stromal cells. This also manifested in di-oocytic follicles
218 (Fig. 2B). In many follicles the granulosa cells were swollen and did not have the flattened
219 appearance normally observed in primordial follicles. Nuclear material within some oocytes
220 was diffuse or pale suggesting an absence of the germinal vesicle membrane (Fig. 2A). Empty
221 and degenerating follicles were often seen (Fig. 2A,B,D). Granulosa cell invasion of the oocyte
222 of primary follicles were occasionally seen (Fig. 2E) together with shrunken granulosa cells

223 and contracted ooplasm (Fig. 2F). In some subjects, normal morphology was detected in the
224 majority of follicles (Fig 2G,H,I), as in control ovarian tissue.

225 The presence of 6 granulosa cell or oocyte specific proteins (20,23) were detected by IHC in
226 three TS ovaries (subjects 1, 6 and 12) and one age-matched control (Supp. Fig. 2). TUNEL
227 staining (subject 8) showed some areas with healthy follicles (Supp. Fig. 3C), however there
228 were other areas of poor stromal integrity with most follicles having only an occasional healthy
229 granulosa cell and evidence of apoptosis in the oocyte (Supp. Fig. 3D). High levels of ZP 1 and
230 2 staining were observed within the oocytes and scattered throughout the cortical stromal tissue,
231 which may indicate residual ZP proteins from eliminated follicles and has been observed
232 previously following xenografting of normal ovarian cortex (Gook, unpublished) (Supp. Fig
233 3A,B). Normal very low levels of ZP 1 expression was detected in 43% (83/193) of the
234 primordial follicles and was elevated in 57% (110/193), which suggests atresia. Normal ZP 2
235 expression was detected in 23% (45/199) of the follicles. The proportion of morphologically
236 normal follicles was estimated to be 7% from TUNEL staining.

237

238 *Hormone concentrations in follicle fluid*

239 Hormone concentrations were measured in 8 follicles obtained from two girls with mosaic TS
240 (subject 4 and 12): none of the other girls had small antral follicles that could be aspirated. The
241 mean diameter \pm SEM of the follicles was 5.0 mm \pm 0.4 (range: 3.4-6.7 mm) and the mean
242 concentrations in follicle fluids were: estradiol 19 \pm 9 nmol/L; testosterone 132 \pm 23 nmol/L;
243 AMH 2,941 \pm 587 ng/ml; and inhibin-B 81 \pm 15 ng/ml, respectively (Supp. Table 2; Fig. 3A).
244 The concentrations of estrogen and testosterone in follicular fluid from girls with TS were
245 significantly lower and AMH higher than the concentrations in follicular fluid from size-
246 matched (3.4-5.9 mm) follicles from age-matched controls (24) ($p=0.036$, 0.001 , 0.005 ,
247 respectively; Supp. Table 2). No differences in inhibin-B concentrations between TS and
248 normal follicle fluids were found ($p>0.1$). All 6 follicle fluids from subject 12 was analysed
249 with western blot detecting for AMH and compared to 6 follicle fluids obtained from control
250 size matched follicles and no differences in AMH processing was detected (Fig. 3B).

251

252 *In vitro maturation of oocytes*

253 COCs (n=31) were cultured from one mosaic girl (subject 12). After 48 hours of culture, 6 had
254 degenerated, 13 remain at the germinal vesicle (GV) stage, while 12 had resumed meiosis (7
255 at MI and 5 at the MII stage), resulting in a maturation rate of 16% and degeneration rate of

256 19%. Although this outcome is only from one patient, this is a lower maturation rate and higher
257 degeneration rate than that previously reported by our group for COC from young women (<20
258 years) with normal ovaries (55%, 4% respectively) (22).

259

260 **Discussion**

261 To our knowledge, this study is the first to characterize follicles and explore the IVM potential
262 of oocytes from girls and young women with TS in comparison to age-matched controls.
263 Follicles were detected in the ovarian cortex in 9 of 15 patients with TS, and the presence of
264 follicles was associated with detectable serum AMH and normal FSH levels. Follicle density
265 was within the 95% CI of normal age-matched girls and young women in 7 of these 9 patients.
266 TS patients originated in Denmark, UK, and Australia, whereas all control patients were
267 Danish, and we cannot rule out that the evaluated ovarian parameters would have been different
268 in a cohort originated in UK or Australia, though we find it unlikely. All except one had mosaic
269 TS, confirming observations from Borgström and colleagues (7) and consistent with women
270 with this karyotype having a higher chance of conceiving (2,5,7,9). While follicles were
271 detected in the youngest patient included, a 5-year-old with 45X karyotype, primordial follicle
272 morphology was abnormal, with most follicles having an incomplete granulosa cell layer,
273 oocyte vacuoles or collapse, or absent oocyte (empty follicles).

274

275 The diversity of follicle morphology between patients further complicated the prediction of who
276 may benefit from OTC. In some cases, follicle morphology was similar to normal whereas in
277 others, a high proportion and range of abnormalities were seen. This is supported by increased
278 expression of ZP proteins (normally very low in healthy primordial follicles and elevated in
279 atretic follicles (25)) and DNA fragmentation detected in one TS patient. This may indicate
280 limited potential for later fertility in this TS patient. Empty follicles have been observed in
281 human ovarian cortex cultured in the presence of an inhibitor of mTOR (26) and were detected
282 in ovaries analysed in all 3 centres negating any effect of different fixation methods. However,
283 all 6 glycoproteins related to oocyte growth and follicle health that were evaluated were
284 detected, suggesting that at least a proportion of TS follicles may be normal and functional.

285

286 From one 18-year-old girl with TS, immature COCs were aspirated and matured *in vitro*. This
287 demonstrates that TS oocytes can develop to the MII stage and may possess fertility potential.
288 This is consistent with a case study that reported oocyte retrieval and maturation to MII in a

289 young woman with mosaic TS (27), with 65% of the oocytes obtained having a normal
290 karyotype. While the karyotype was not assessed here, these findings suggest that immature
291 oocytes can be collected from the medulla tissue in connection with OTC and that these oocytes
292 could be an additional source for fertility preservation in mosaic TS, although the maturation
293 rate appeared lower than with oocytes from normal women.

294

295 We also identified that hormone concentrations in follicle fluid from small antral follicles from
296 TS ovaries were strikingly different from the concentrations found in size-matched normal
297 follicles, with low concentrations of estradiol and testosterone, and markedly higher AMH.
298 Inhibin-B concentrations appeared normal. The low testosterone concentration may reflect
299 abnormal theca cell function, and may impact follicle development including granulosa cell
300 proliferation, which is reflected in the low estradiol concentrations. AMH is predominantly
301 present during follicle development until follicular selection for dominance (20). There is a
302 strong negative relationship between follicle fluid AMH and estradiol in normal women (28),
303 which appears to be exaggerated in TS. These high AMH concentrations together with very low
304 steroid concentration in TS follicles may reflect abnormal function of the somatic cells of the
305 follicle, and additionally through impairment of the regulation of folliculogenesis, contribute to
306 the accelerated follicle loss in TS confirmed here. Western blot was used to evaluate the
307 processing of AMH in TS follicle fluids and non TS (normal) follicle fluids from size matched
308 follicles. No difference in AMH processing was detected between TS and normal, suggesting
309 that the processing of AMH in TS patients are similar to normal.

310

311 Although it is now well recognized that transplanted frozen/thawed ovarian tissue can restore
312 fertility no one has, to our knowledge, transplanted ovarian tissue to a woman with TS, despite
313 OTC being reported (7–9,27,29,30). Thus, it remains to be demonstrated whether transplanted
314 frozen/thawed ovarian tissue from TS girls/adolscents has the capacity to restore fertility. The
315 rationale behind OTC in TS patients is different from other medical indications like
316 chemotherapy. In case of cancer, the ovarian tissue has been exposed to no or a discrete injury
317 before OTC. This contrast with TS ovarian tissue, which itself has a limited life expectancy and
318 auto-grafted TS tissue will therefore have a limited survivability, why the benefit of OTC in TS
319 patients may be limited. Further, maternal risks, including mortality during pregnancy in TS
320 are very high, largely due to cardiovascular risks (9,10), which has to be taken into account
321 when considering auto-grafting in TS patients. The cardiovascular risks may reflect the

322 underlying connective tissue abnormalities present in TS (31), which may also be relevant to
323 the stromal tissue and somatic cells of the ovary. *In vitro* maturation and surrogacy may also be
324 considered an option for TS patients. A recent review suggested that TS patients should be
325 evaluated in early childhood to allow them to benefit from fertility preservation options (32),
326 which is important to these patients and their parents (33).

327

328 **Conclusions**

329 The present analysis showed that even where follicles are present in girls with mosaic TS, many
330 of these follicles may show abnormalities that are likely to limit their potential for later
331 development and to support fertility. However, normal follicles were also present. Therefore, it
332 appears reasonable to consider OTC for fertility preservation as an option for highly selected
333 adolescent patients with mosaic TS where endocrine assessment does not indicate POI, and if
334 other health issues do not preclude pregnancy. Oocytes from TS medulla tissue may also
335 provide an additional fertility option. However, it is important to note that transplantation of
336 frozen/thawed ovarian tissue has not yet been performed in women with TS, and it remains to
337 be seen whether the procedure can restore fertility.

338

339 **Author's roles**

340 LSM designed the project, wrote the paper, did IHC staining, figures, and tables. KC wrote the
341 paper, measured follicle density, did IHC staining and tables. RAA wrote the paper, recruited
342 patients, responsible for the cryopreservation of ovarian tissue (UK). EET and MMcL analysed
343 ovarian tissue and measured follicle densities (UK). TWK did the statistical analysis. SGK
344 cryopreserved ovarian tissue and did histological analysis (Denmark). DAG wrote the paper,
345 recruited patients, measured follicle density, cultured oocytes, and did IHC staining, responsible
346 for the cryopreservation of ovarian tissue (Australia). EE recruited patients and did the
347 ovariectomies (Denmark). CYA designed the project and wrote the paper.

348

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457 **Figure legends**

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459 **Fig. 1.** Follicle density in normal ovaries (triangles) and in girls with TS (filled circles) plotted
460 against age. Only TS tissues with follicles detected (9/15) are included. Previously published
461 data on follicle density in girls with TS (n=5) are included (8) (open circles).

462 **Fig. 2.** Ovarian morphology in tissue from 3 girls with TS. **(A,B,C)** Subject 8. **(A)** Primordial
463 follicles with missing granulosa cells (arrows), some follicles had diffuse or pale nuclear
464 material suggesting absence of germinal vesicle membrane (arrowheads); **(B)** fused follicles
465 (arrows); **(C)** follicles with normal granulosa cells (arrows), scale bar: 100 μm . **(D,E,F)** Subject
466 1. **(D)** Primordial follicles with collapsed oocytes (arrows) and empty follicle (arrowhead); **(E)**
467 granulosa cell invasion of the oocyte of a primary follicle (arrow); **(F)** primary to secondary
468 transition, shrunken granulosa cells and contracted ooplasm, scale bar: 50 μm . **(G,H,I)** Subject
469 12. **(G, H)** Normal morphology in the majority of primordial follicles with flat granulosa cells
470 (arrows) and primary/intermediate follicles with cuboidal granulosa cells (arrow heads); **(I)**
471 tertiary follicle, scale bar: 50 μm .

472 **Fig. 3.** **(A)** Concentration of AMH and inhibin-B in follicle fluids (FF) (red circles) obtained
473 from two girls with TS aged 13.5 and 17.8 years (subject 4 and 12) and in age-matched controls
474 (grey triangles). AMH concentrations in these TS follicles are extremely high, whereas the
475 inhibin-B concentration is similar to age-matched controls. **(B)** Detection of AMH in AMH
476 standard (Std.) (lane 1), FF from TS patient subject 12 (lane 2-7), and in size matched follicles
477 from different normal women (lane 8-13). Lane 7 was loaded with less FF than the remaining
478 because the sample was used up, which explain the weaker/no bands seen. Arrows indicate
479 AMH cleavage fragments. The blots show no difference in the composition of AMH forms in
480 TS and normal FF.

481 **Supp. Fig. 1.** Negative controls. **(A,B)** TS Mosaic, 14.4 years; **(C,D)** TS Mosaic, 17.8 years;
482 **(E,F)** Control, 10.5 years. **(A,C,E)** Primary antibody preplaced with antibody dilution buffer
483 (1% BSA in PBS). **(B,D,F)** Primary antibody preplaced with Universal Negative Control
484 serum®.

485 **Supp. Fig. 2.** Expression of 6 proteins important for follicular growth: pro-region of Anti-
486 Müllerian hormone (proAMH), growth/differentiation factor 9 (GDF9), bone morphogenetic
487 protein 15 (BMP15), insulin-like growth factor-binding protein 4 (IGF BPB4), pregnancy-

488 associated plasma protein A (PAPP-A), stanniocalcin 2 (STC2) in ovarian cortical tissue from
489 three TS patients aged 5.0, 14.4 and 17.8 years and one control aged 10.5 years.

490 **Supp Fig. 3.** Expression of zona pellucida proteins (ZP 1 and ZP 2) and apoptotic marker
491 (TUNEL) in a TS ovary aged 14.8 years (subject 8). **(A)** ZP 1 protein was detected in a pattern
492 resembling normal follicles (arrows) and in some follicles aberrant staining was observed
493 (arrowhead). **(B)** Low expression of ZP 2 was observed in healthy looking primordial follicles
494 (arrows), while an apparent increased staining was observed in other follicles. **(C)** Stromal cells,
495 oocytes (arrows) and granulosa cells in this area were predominantly TUNEL negative (green
496 staining), though TUNEL was observed in some granulosa cells (arrowhead). **(D)** Single
497 stranded DNA (TUNEL positive) was in other areas observed in some oocytes and granulosa
498 cells (arrows). Cells of the stromal tissue also had evidence of single strand DNA (weak brown
499 staining). Dotted boxes indicate enlarged areas. Scale bar: 100 μm and 50 μm on
500 magnifications.