

Histologic Analysis of Testes from Prepubertal Patients Treated with Chemotherapy Associates Impaired Germ Cell Counts with Cumulative Doses of Cyclophosphamide, Ifosfamide, Cytarabine, and Asparaginase

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1 **Running title:** Gonadotoxic effects of chemotherapy in prepubertal testes

2

3 **Histologic analysis of testes from prepubertal patients treated with chemotherapy associates**

4 **impaired germ cell counts with cumulative doses of Cyclophosphamide, Ifosfamide,**

5 **Cytarabine and Asparaginase**

6

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19

20 ABSTRACT

21 Cryopreservation of immature testicular tissue is an experimental strategy for the preservation
22 of fertility in prepubertal boys that will be subjected to a gonadotoxic onset, as is the case of
23 oncologic patients. Therefore, the objective of this study was to assess the impact of
24 chemotherapeutic treatments on the testicular histologic phenotype in prepubertal patients. A
25 total of 56 testicular tissue samples from pediatric patients between 0 and 16 years old (28
26 with at least one previous chemotherapeutic onset and 28 untreated controls) were
27 histologically analyzed and age-matched compared. At least two 5µm sections from testis per
28 patient separated by a distance of 100 µm were immunostained for the germ cell marker
29 VASA, the spermatogonial markers UTF1, PLZF, UCHL1 and SALL4, the marker for proliferative
30 cells KI67, and the Sertoli cell marker SOX9. The percentage of tubule cross-sections positive
31 for each marker and the number of positive cells per tubule cross-section were determined
32 and association with the cumulative dose received of each chemotherapeutic drug was
33 statistically assessed. Results indicated that alkylating agents cyclophosphamide and
34 ifosfamide, but also the antimetabolite cytarabine and asparaginase were associated with a
35 decreased percentage of positive tubules and a lower number of positive cells per tubule for
36 the analyzed markers. Our results provide new evidences of the potential of chemotherapeutic
37 agents previously considered to have low gonadotoxic effects such as cytarabine and
38 asparaginase to trigger a severe testicular phenotype, hampering the potential success of
39 future fertility restoration in experimental programs of fertility preservation in prepubertal
40 boys.

41

42 **Key words:** Prepubertal patients; Fertility preservation; Testicular tissue; Chemotherapy;
43 Gonadotoxicity.

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45

46 **DECLARATIONS**

47 **Funding:** This work was supported by a private donation of the Celtic Submarí club- Villareal
48 C.F. to Hospital Universitario y Politécnico La Fe intended to promote the scientific research on
49 fertility preservation in child with cancer, and an AES project grant (PI16/00931) conceded by
50 the Instituto de Salud Carlos III.

51 **Conflicts of interest/Competing interests:** There is no conflict of interest to declare.

52 **Ethics approval:** Samples used in this study were recruited at Hospital La Fe in Valencia (Spain)
53 (32 samples), and UZ Brussel in Brussels (Belgium) (36 samples) after the approval by the
54 respective Institutional Review Boards of Hospital La Fe (ref: 2013/0457) and UZ Brussel (ref:
55 2000/149D and 2017/061).

56 **Consent to participate:** Acceptance by parents or legal guardians of the patients of an
57 informed consent.

58 **Consent for publication:** Not applicable.

59 **Availability of data and material:** All authors declare that all data and materials included in
60 this manuscript comply with field standards.

61 **Code availability:** All statistical analyses were performed using R (version 3.5.3) and the R
62 packages glmnet (version 2.0-16), cluster (version 2.0.7-1) and brms (version 2.8.0).

63 **Authors' contributions:** JVM, ENM, AP and MMA conceived this work. MMA and EG provided
64 samples. JVM, TVP and ANG conducted the experiments. DH performed statistical analysis of
65 data. JVM analyzed data and wrote the manuscript. All listed authors revised and approved the
66 manuscript.

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69

70 **INTRODUCTION**

71 Their high mitotic rate makes male germ cells particularly susceptible to injury by cytotoxic
72 drugs commonly employed to treat cancer patients [1, 2]. As a consequence, recent reports
73 indicate that approximately 30% of patients exposed to chemotherapy may be in risk of
74 suffering permanent infertility [3]. Therefore, fertility preservation is indicated for patients
75 that will be subjected to potentially gonadotoxic treatments such as radiotherapy or
76 chemotherapy. However, although sperm banking is the gold standard to preserve fertility in
77 adult men [4], prepubertal boys unable to produce sperm for freezing before starting a
78 gonadotoxic treatment cannot benefit. Nonetheless, numerous studies in animal models
79 indicate that spermatogonial stem cells that reside within the prepubertal testes are able to
80 restore spermatogenesis upon their transplantation back into the testes once the gonadotoxic
81 treatment is finished [5-12]. Based on this, experimental clinical protocols to preserve the
82 fertility of prepubertal boys are focused on the extraction and cryopreservation of a testicular
83 biopsy before their exposure to a potentially gonadotoxic onset [13-22], with the aim of using
84 this tissue to restore the fertility of patients in the future.

85 Since cryopreservation of testicular tissue is an experimental procedure, strict selection criteria
86 of patients is mainly based on their survival prognosis and the estimated gonadotoxic damage
87 of the chemotherapeutic drugs that will receive [16, 23, 24]. In this regard, it is known that
88 especially alkylating drugs such as busulfan and cyclophosphamide, have a severe impact on
89 sperm counts [25]. In a systematic literature review, the International Late Effects of Childhood
90 Cancer Guideline Harmonization Group found evidence for adverse effects of
91 cyclophosphamide, mechlorethamine and procarbazine on spermatogenesis [26]. Although
92 there exist evidences that cyclophosphamide equivalent doses over 4000 mg/m² are linked
93 with azoospermia and oligozoospermia [27], a predictive threshold dose for impaired

94 spermatogenesis has resulted difficult to depict mainly due to the fact that alkylating agents
95 are commonly used in combination with other agents in different chemotherapeutic protocols,
96 which may have an additive adverse effect on spermatogenesis [26]. Moreover, the
97 gonadotoxic effects of many chemotherapeutic drugs are not completely understood and,
98 importantly, their impact in prepubertal patients comes from indirect data extrapolated from
99 studies performed on adult men [16, 25]. In this regard, although recent reports have
100 described how the administration of alkylating drugs can decrease the number of
101 spermatogonia per tubule in prepubertal testicular biopsies as it does with sperm counts in
102 adult men [28, 29], data regarding how other drugs commonly included in chemotherapeutic
103 protocols affect the prepubertal testicular histology is extremely scarce.

104 Therefore, considering that in many cases patients fulfill selection criteria to be offered
105 testicular biopsy for cryopreservation after they have already started chemotherapeutic
106 treatments [30], a better knowledge of the gonadotoxic effects of these drugs in the
107 prepubertal testis is mandatory in order to establish clear criteria and timing to offer them this
108 technique.

109 Based on this background, in this study we aimed to assess the association between the
110 histological phenotype of prepubertal testes from boys selected for fertility preservation and
111 the cumulative dose for each individual chemotherapeutic drug that they have already
112 received before testicular biopsy. For this, we analyzed the expression profile of the germ cell
113 marker VASA [31], the spermatogonial markers UTF1, UCHL1, SALL4 [32] and PLZF [33], the
114 marker for proliferative cells KI67 [34], and the Sertoli cell marker SOX9 [32] in testicular
115 biopsies from patients exposed to chemotherapy, and compared them to the expression of
116 age-matched control biopsies without previous exposure to any gonadotoxic insult. This
117 analysis led us to find that not only alkylating drugs but also previously considered low-
118 gonadotoxic drugs such as the antimetabolite cytarabine and asparaginase, can be associated
119 with a decrease in the number of testicular germ cells.

120

121 **MATERIALS AND METHODS**122 **Sample source:** Samples used in this study were recruited at Hospital La Fe in Valencia (Spain)

123 (32 samples), and UZ Brussel in Brussels (Belgium) (36 samples) after the approval by the

124 respective Institutional Review Boards of Hospital La Fe (ref: 2013/0457) and UZ Brussel (ref:

125 2000/149D and 2017/061) and the consentment of legal guardians of all patients recruited for

126 fertility preservation to the use of samples employed for pathologic diagnostic for research

127 applications. Assessment of the pubertal stage of patients by Tanner stage evaluation was

128 performed in all patients over 10 years old. Despite that in some cases of patients over 14

129 Tanner stage indicated an advanced pubertal maturation, biopsy was performed due to several

130 clinical reasons such as diagnostic purposes (different from this study), severe

131 oligo/azoospermia, and psychologic or ethical impediments to obtain a sperm sample by

132 masturbation or vibrostimulation. Therefore, testicular tissue samples from 68 pediatric

133 patients between 0 and 16 years old that were subjected to a testicular biopsy for diagnostic

134 or fertility preservation purposes were embedded in paraffin. Among recruited samples, 12

135 were discarded due to either leukemic testicular infiltration or bad preservation of tissue

136 histology, resulting in the analysis of a total of 56 samples for this study (Supplemental Table I).

137 **Histological evaluation:** Tissue was fixed in 10% formaldehyde o/n at 4°C, dehydrated,

138 embedded in paraffin and sliced in 5µm sections. Subsequently, deparaffinized slides were

139 subjected to hematoxylin-eosin staining and analyzed by pathologists to determine the overall

140 status of the testicular histology of each patient.

141 **Immunostaining:** Deparaffinized slides were subjected to antigen retrieval by treating them

142 with 10mM citrate buffer pH6 for 20' at 97°C before a blocking step with phosphate buffered

143 saline + 10% normal donkey serum + 1% bovine serum albumin + 0.1% Triton X-100 (all from

144 Sigma-Aldrich) for one hour at room temperature. Incubation of primary antibodies was

145 carried out overnight at 4°C (Supplemental Table II). Secondary Alexa fluor antibodies were
146 incubated for one hour in darkness at room temperature prior to mount the slides with
147 ProLong Gold antifade reagent with DAPI (Life Technologies). Negative controls were
148 performed with unspecific IgGs (data not shown). Slides were visualized using a fluorescence
149 microscope DM2500 (Leica).

150 **Quantitative analysis of testicular histology:** All samples were triple stained with three
151 combinations of markers (UTF1/Ki67/VASA, UCHL1/SALL4/VASA and VIM/SOX9/PLZF). Two
152 triple stained sets of consecutive 5µm serial sections with a depth distance of 100µm in-
153 between were assessed for cell counts. The percentage of tubule cross-sections with at least
154 one positive cell and the number of positive cells per tubule cross-section was assessed for
155 each marker (detailed in Table I and Supplementel Table IV). Incomplete tubule cross-sections
156 were discarded from counts to avoid bias. In order to avoid subjectivity, cell counts were blind
157 and performed by two researchers independently. Therefore, all counts were compared and
158 repeated when discrepancy between researchers was higher than 25%. Finally, the mean of
159 the cell counts for each marker and sample was added to the data matrix for subsequent
160 statistical analysis. Although there exist several morphometric approaches and mathematical
161 corrections that partially solve the issue that cell counts on histologic sections may generate a
162 bias in the estimation of the absolute number of cells within testis, they were not applied to
163 this study since its goal was not to estimate the absolute number of cells but just analyze a
164 representative sample of testicular biopsies.

165 **Statistical analysis:** Data resulting from histological counts were summarized using mean
166 (standard deviation) and median (1st, 3rd quartile) in the case of continuous variables and by
167 relative and absolute frequencies in the case of categorical variables (Tables I and II). Status of
168 the samples from treated patients was summarized using a fuzzy clustering algorithm and
169 assigning membership probabilities for two opposing groups (one with overall lower values for
170 all analyzed markers that was identified as “severely affected group”, and another with overall

171 higher values for all analyzed markers identified as “weakly affected group”). The data set used
172 for performing the fuzzy clustering on the % of VASA+ tubules status was created by estimating
173 the z-score value for each studied marker on each treated patient based on a regression model
174 fitted on the untreated control patients with the studied variable as response and a smooth
175 function of age as predictor. Subsequent association of the classification of treated patients
176 with the different cumulative doses of chemotherapy that they received was assessed using an
177 elastic net penalized logistic regression model. Selection of the penalization parameter lambda
178 was performed by performing 500 repetitions of cross-validation and selecting the optimum
179 lambda value in each of them. Then, the median lambda value was estimated and used as the
180 final penalization factor for the logistic regression model. Finally, a Bayesian logistic regression
181 model was adjusted with the selected variables and 95% credibility intervals for the ORs of
182 each variable were estimated. Additionally, the posterior probability of the effects of each
183 drug being negative regarding the testicular histologic phenotype was also estimated. All
184 statistical analyses were performed using R (version 3.5.3) and the R packages glmnet (version
185 2.0-16), cluster (version 2.0.7-1) and brms (version 2.8.0).

186

187 **RESULTS**

188 **A subgroup of samples from patients that received chemotherapy before the testicular** 189 **biopsy showed a severely affected phenotype**

190 Preliminary histological evaluation of samples identified clear differences between controls
191 without previous chemotherapeutic exposure and some samples from patients exposed to
192 chemotherapy before the testicular biopsy showing a phenotype that may correlate with
193 Sertoli cell only (SCO) syndrome (Figure 1A).

194 Therefore, in order to quantify the histologic phenotype of testicular biopsies, samples were
195 stained with the germ cell marker VASA, the spermatogonial markers UTF1, PLZF, UCHL1 and

196 SALL4, the marker for proliferative cells KI67, and the Sertoli cell marker SOX9 (Figure 1B). For
197 each marker, data regarding the percentage of positive tubule cross-sections (considering a
198 positive tubule when at least one cell within cross-section was positive for the analyzed
199 marker), and the average number of positive cells within tubule cross-sections were collected.
200 Overall, a total of 27678 tubule cross-sections, with an average of 494 tubules per patient,
201 were counted and considered to create the data matrix for statistical analysis (Supplemental
202 Table III).

203 Subsequent fuzzy clustering analysis clearly differentiated between two groups within samples
204 from patients previously exposed to chemotherapy, according to the z-score values of the
205 different studied markers compared to the non-treated group values, showing a sharp
206 difference between a relatively small group of 9 treated patients with higher overall z-score
207 values in all studied variables (weakly affected group) and a larger group of 19 patients with
208 lower overall z-score values in all studied variables (severely affected group) (Figure 2).
209 Remarkably, all variables behaved similarly, so the use of the cluster variable as a marker of
210 the overall status of the treated patients was justified.

211 According to this classification, the graphic representation of an age-matched regression
212 model showing the percentage of positive tubules and the number of positive cells per tubule
213 for the analyzed markers clearly showed how the non treated controls and the weakly affected
214 group behaved similarly, showing higher values for all markers except for the percentage of
215 SOX9 positive tubules, compared to the group of severely affected samples (Figure 3).

216 Therefore, next step was to study if there exists an association between this severe phenotype
217 and the cumulative dose of chemotherapeutic drugs received in order to identify which drugs
218 are associated with gonadotoxicity.

219

220 **Regression model indicates that alkylating drugs and cytarabine exposure are associated**
221 **with a severe testicular histology**

222 A summary of cumulative doses of each chemotherapeutic agent is showed in Table II and
223 Supplemental Table IV. Results of the elastic net logistic regression model identified seven
224 drugs associated with the altered histologic phenotype of testicular biopsies. In agreement
225 with previous studies reporting a decrease in sperm counts from adult survivors of childhood
226 cancer [27], both alkylating agents cyclophosphamide and ifosfamide showed a correlation
227 with a severe phenotype in the histology of prepubertal patients. However, regression analysis
228 led us to identify that the cumulative dose of the antimetabolite cytarabine as well as
229 asparaginase are also associated with a worse histologic phenotype, whereas the
230 topoisomerase inhibitors daunorubicin and idarubicin, and the antimetabolite 6-
231 mercaptopurine seemed to be associated with a better patient status. Coefficients and OR for
232 the adjusted model are provided in Table III.

233 Moreover, in order to understand better the influence of each drug identified by the elastic
234 net model, results from a Bayesian logistic regression model adjusted with the selected
235 variables allowed us to estimate the posterior probability of the effects of each drug being
236 negative regarding the testicular histologic phenotype (Table IV). These results are graphically
237 shown in a heatmap depicting the concentration values of each selected drug on each patient,
238 showing how severely affected samples received higher doses of alkylating agents, cytarabine
239 and asparaginase (Figure 4).

240 Overall, data indicated that cumulative doses of cyclophosphamide of 4036.42 ± 3004.25
241 mg/m^2 , $1415.78 \pm 2093.97 \text{ mg/m}^2$ of ifosfamide, $6503.26 \pm 7310.19 \text{ mg/m}^2$ of cytarabine and
242 $8735.78 \pm 2546.91 \text{ UI/m}^2$ of asparaginase correlate with a severe testicular histologic
243 phenotype. A summary of the data regarding the percentage of positive tubules and the
244 number of positive cells per tubule for each marker, together with the cumulated dose of

245 chemotherapeutic drugs for non-treated controls and the two subgroups of treated samples
246 can be seen in Tables I and II, and in Supplemental Tables III and IV.

247

248 **DISCUSSION**

249 Fertility preservation in prepubertal patients is based on the existence of spermatogonial stem
250 cells within the testes with the ability to restore the fertility of patients subjected to
251 gonadotoxic treatments such as chemotherapy [5-12]. Therefore, it is desirable that
252 cryopreserved testicular tissue remains unexposed to any kind of chemotherapy in order to
253 prevent deleterious effects in the spermatogonial population and maximize the chances to
254 restore the fertility of patients upon transplantation back to their testes. However, in the real
255 clinical routine, many often patients proposed for fertility preservation have already been
256 exposed to chemotherapy [30]. This situation is common in many patients diagnosed with
257 acute lymphoblastic leukemia (ALL), which are usually offered fertility preservation after a
258 relapse of the pathology (Supplemental Table I). In these cases, patients are offered testicular
259 biopsy when their cumulative doses of chemotherapy before the biopsy are considered to
260 have low gonadotoxic effect, according to previous studies in adults that correlate the
261 cumulative dose of alkylating drugs received by patients in terms of Cyclophosphamide
262 Equivalent Dose (CED) with sperm counts [16, 25, 27]. Because of this, the observation of
263 prepubertal testicular biopsies from boys subjected to fertility preservation showing a severe
264 germ cell loss (Figure 1A) was a surprising result.

265 Due to its experimental clinical consideration and strict criteria to be eligible, the proportion of
266 prepubertal patients proposed for fertility preservation is very low. Therefore, although there
267 are some important studies regarding the gonadotoxic effects of chemotherapeutic drugs in
268 this population [27, 35-39], most of them focus in the long term effect of chemotherapy

269 exposure, especially alkylant drugs, on sperm counts once patients reach adulthood instead of
270 the effects in the prepubertal testicular histology.

271 Although there exist in the literature some recent pioneer reports that highlight the dramatic
272 effect of alkylating drugs on the number of germ cells within seminiferous tubules [28, 29], to
273 our knowledge, this is the first report that aims to correlate cumulative doses of different
274 chemotherapeutic drugs with the prepubertal testicular histology. For that, the germ cell
275 marker VASA was chosen as the main indicator of the total number of germ cells within the
276 tissue [29], and employed the percentage of positive tubule cross-sections in control samples
277 without previous exposure to chemotherapy as a template to compare samples with previous
278 chemotherapeutic exposure. As a result, fuzzy clustering analysis revealed a subgroup of 19
279 out of 28 samples from patients previously exposed to chemotherapy that showed a
280 significantly decreased percentage of VASA+ tubules when compared with age-matched
281 controls. When we applied the same analysis for the data regarding the specific
282 spermatogonial markers UTF1, UCHL1, PLZF and SALL4, and the cell proliferation marker KI67,
283 we observed a similar clustering behaviour (Figure 2), indicating that severely affected samples
284 had a lower percentage of tubule cross-sections with proliferating spermatogonia compared
285 with age-matched non-treated controls. Moreover, a similar behaviour was observed
286 regarding the number of positive cells per tubule cross-section for the same markers (Figure
287 2), suggesting that not only a reduction in the percentage of positive tubules was evident in
288 the severely affected group, but also that positive tubules showed an altered histology
289 characterized by a loss of spermatogonia. Importantly, the number of VASA+ cells per tubule
290 cross-section shown by the controls of this study was comparable to the results from a recent
291 meta-analysis where reference values for age-related number of spermatogonia within
292 prepubertal testes was described [40]. This highlights a relatively constant ratio of
293 spermatogonia per tubule until the initiation of puberty, which is accompanied by an increase
294 of this ratio in controls and weakly affected samples, but not in severely affected samples that

295 show lower numbers independently of the age of patients (Figure 3). Interestingly, acute
296 lymphoblastic leukemia (ALL) was the most prevalent diagnosis among treated patients,
297 representing around 50% of cases in both weakly and severely affected groups of patients
298 (Supplemental Table I). However, due to the different moment of recruiting patients for
299 fertility preservation, sometimes after a relapse of the disease, the resulting high variability in
300 the cumulative dose of the different drugs received by patients even when they share a similar
301 diagnostic (Supplemental Tables III and IV) impeded us to associate the testicular phenotype to
302 the pathology.

303 Interestingly, although both untreated controls and weakly affected patients behave similarly
304 according to the fuzzy clustering analysis that discriminated weakly and severely affected
305 patients according to the z-score values of the different studied markers compared to the non-
306 treated group values, we found a slightly higher number of germ cells within tubules in the
307 weakly affected group (Figure 3). Despite the considerable number of patients included in this
308 study (28 untreated controls and 28 treated patients), it is possible that this behaviour can be
309 explained by the sample size bias resulting from the reduced number of weakly affected
310 patients (9 out of 28 treated patients) after the fuzzy clustering analysis. Also, the slightly
311 different age range of the weakly affected group of patients (range from 3 to 15 years),
312 compared with untreated controls (range from 0 to 14 years), explains that this group of
313 patients show a higher number of germ cells since its number trends to increase with age. It is,
314 however, temptative to hypothesize that the slight increase in the number of germ cells in
315 weakly affected patients may be due to a niche homeostasis response to chemotherapy, in the
316 way that the stress induced by the treatment itself may trigger a rapid cell division of surviving
317 cells to replenish the ones that die, as can be suggested by the higher number of Ki67 positive
318 cells found in weakly affected patients (Figure 3). Nevertheless, data resulting from this study
319 is not enough to explain these differences and future studies may be focused in this interesting
320 observation.

321 Once statistics clearly defined a subgroup of severely affected samples, next step was to find
322 the candidate drugs to explain this altered histology. In agreement with previous reports, we
323 found that both alkylating drugs, cyclophosphamide and ifosfamide, were associated with a
324 severe phenotype [27]. However, statistic analysis also identified that cumulative dose of the
325 antimetabolite cytarabine and asparaginase are also associated with this phenotype (Tables III
326 and IV, and Figure 4). The gonadotoxicity of cytarabine has been already reported in animal
327 studies [41]. However, this is the first report on human samples that suggests its cumulative
328 dose as a possible major gonadotoxic drug. On the other hand, there are no previous reports
329 on the gonadotoxicity that cumulative doses of asparaginase can trigger. Nevertheless, since
330 the administration of both cytarabine and asparraginase usually is accompanied by alkylant
331 drugs (Supplemental Table V) in chemotherapeutic protocols for ALL and some types of
332 lymphoma, future studies focused in the possible gonadotoxic effects of cytarabine and
333 asparaginase by themselves should clarify the infertility risk associated with their
334 administration in prepubertal boys.

335 The finding of such associations between cumulative doses of alkylating agents, cytarabine and
336 asparaginase with a severely affected testicular phenotype needed the application of complex
337 Bayesian regression models due to the limited number of patients and the great variability
338 regarding the different chemotherapeutic protocols applied even to patients sharing a similar
339 pathology. As a result, the wide range of cumulative doses of drugs that correlate with a
340 severe phenotype (Table II) makes difficult to determine narrow ranges of dose thresholds of
341 risk. Moreover, due to the same limitations commented above, correlations between
342 phenotype and cumulative drug doses did not include combined effects of the drugs included
343 in the chemotherapeutic protocol received by each patient. Finally, our study focused in the
344 combined effect of cumulative doses of chemotherapeutic agents on the testicular phenotype,
345 but did not considered the time of exposure. Therefore, our results should be considered a

346 pilot study that must be confirmed by further prospective studies with a bigger sample size
347 and homogenization of treatments.

348

349 **CONCLUSIONS**

350 This report manifests our scarce knowledge regarding the gonadotoxic effect of most of
351 chemotherapeutic drugs on the prepubertal testis, highlighting the need of more studies
352 specifically focused on the prepubertal population. Since the preservation of healthy
353 spermatogonial stem cells is mandatory for the success of fertility restoration, a better
354 knowledge of the gonadotoxic effects of chemotherapeutic drugs is necessary to prevent the
355 severe histologic alteration found in many samples that may compromise the future success of
356 fertility restoration. Therefore, the association of the cumulative dose of alkylating agents,
357 cytarabine and asparaginase, and their synergistic effects as well with a severe testicular
358 phenotype should be considered at the moment of selecting patients for fertility preservation
359 in order to prevent the massive germ cell death associated with their administration in the
360 testicular biopsy that they will cryopreserve.

361

362 **AUTHORS' ROLES**

363 JVM, ENM, AP and MMA conceived this work. MMA and EG provided samples. JVM, TVP and
364 ANG conducted the experiments. DH performed statistical analysis of data. JVM analyzed data
365 and wrote the manuscript. All listed authors revised and approved the manuscript.

366

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369 Hospital Universitario y Politécnico La Fe intended to promote the scientific research on

370 fertility preservation in child with cancer, and an AES project grant (PI16/00931) conceded by
371 the Instituto de Salud Carlos III.

372

373 **CONFLICTS OF INTEREST**

374 There is no conflict of interest to declare.

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- 509

510 **FIGURES AND TABLES**

511 **Figure 1. Representative pictures of the testicular histology. (A)** Representative pictures of
512 the testicular histology of an untreated patient with chronic granulomatose disease without
513 any previous chemotherapy and two patients recruited for fertility preservation after a relapse
514 of their respective diseases (ALL and Burkitt lymphoma, respectively). The ALL patient (middle
515 picture) belongs to the weakly affected group of patients, showing a normal testicular
516 histology with seminiferous cross-section filled with both Sertoli cells and spermatogonia,
517 whereas the one with Burkitt lymphoma (third picture) is from the severely affected group,
518 showing a histologic phenotype with a marked germ cell loss compatible with SCO. **(B)**
519 Representative pictures of the co-localization of the selected markers for this study:
520 UTF1/KI67/VASA, UCHL1/SALL4/VASA and VIMENTIN/SOX9/PLZF. With the exception of
521 VIMENTIN that was only employed to facilitate visualization of the histology, the percentage of
522 positive tubules for each marker and the number of positive cells per tubule were quantified
523 for subsequent statistical analysis. Scale bars correspond to 250µm. White arrowheads
524 indicate triple positive cells.

525 **Figure 2. Fuzzy clustering of the z-score values of the different studied variables compared to**
526 **the non-treated group values for each variable.** The heatmap shows a sharp difference
527 between a relatively small group of 9 weakly affected samples with higher overall z-score
528 values in all studied variables, and a larger group of 19 severely affected samples with lower
529 overall z-score values in all studied variables.

530 **Figure 3. Graphic representation of regression models showing the percentage of positive**
531 **tubules and the number of positive cells per tubule for the analyzed markers along the age**
532 **of patients.** Each dot corresponds to one single patient. Data is accompanied by the credibility
533 interval (grey areas) of each regression model for each group of patients.

534 **Figure 4. Heatmap of the concentration values on each patient for the treatments selected**
535 **by the elastic net analysis.** Values have been normalized to z-scores to make variables on
536 different scales comparable. Order of rows has been determined by hierarchical clustering and
537 patients have been ordered by their condition (weakly vs. severely affected).

538 **Table I. Descriptive statistics of the testicular histology for non-treated control patients,**
 539 **weakly affected patients and severely affected patients.**

	Non-treated controls (n=28)		Weakly affected (n=9)		Severely affected (n=19)	
	Mean (SD)	Median (1stQ, 3rd Q)	Mean (SD)	Median (1stQ, 3rd Q)	Mean (SD)	Median (1stQ, 3rd Q)
Age (years)	6.89 (4.54)	8.00 (2.75, 10.25)	7.11 (4.16)	5.00 (5.00, 8.00)	6.94 (4.30)	5.00 (4.00, 10.00)
% of tubules VASA+	60.79 (33.07)	73.95 (35.55, 86.23)	77.90 (30.19)	90.20 (75.21, 92.60)	30.54 (21.54)	33.56 (12.18, 43.45)
No. of VASA+ cells/Tubule	4.85 (3.781)	3.51 (2.56, 6.51)	9.57 (10.42)	5.92 (4.61, 6.82)	2.85 (1.91)	2.23 (1.95, 3.93)
% of tubules UCHL1+	15.11 (19.62)	4.59 (0.72, 28.5)	20.83 (19.17)	14.07 (3.04, 38.46)	3.50 (4.85)	1.39 (0.00, 4.73)
No. UCHL1+ cells/Tubule	1.23 (0.92)	1.23 (0.5, 1.91)	1.71 (0.85)	1.80 (1.25, 2.23)	0.73 (0.76)	0.66 (0.00, 1.24)
% of tubules SALL4+	15.19 (22.32)	5.80 (1.27, 15.2)	19.20 (20.34)	10.86 (5.03, 30.00)	2.36 (3.58)	0.64 (0.00, 2.77)
No. SALL4+ cells/Tubule	1.25 (0.97)	1.30 (0.5, 1.64)	1.51 (0.68)	1.38 (1.11, 1.98)	0.61 (0.69)	0.50 (0.00, 0.95)
% of tubules UTF1+	14.12 (21.65)	6.49 (1.04, 15.15)	30.66 (24.94)	34.28 (1.29, 1.82)	1.32 (1.95)	0.25 (0.00, 2.39)
No. UTF1+ cells/Tubule	1.15 (0.98)	1.03 (0.5, 1.43)	1.69 (0.63)	1.46 (1.29, 1.89)	0.52 (0.57)	0.50 (0.00, 1.00)
% of tubules KI67+	10.84 (15.46)	4.81 (1.67, 12.17)	29.44 (21.23)	19.84 (14.77, 40.16)	4.20 (8.01)	0.25 (0.00, 2.78)
No. KI67+ cells/Tubule	1.06 (0.73)	1.04 (0.53, 1.42)	1.95 (1.08)	1.37 (0.53, 2.08)	0.59 (0.65)	0.50 (0.00, 1.10)
% of tubules PLZF+	39.52 (33.55)	28.18 (9.47, 68.85)	55.62 (36.42)	67.74 (36.09, 82.85)	14.34 (18.72)	10.60 (0.00, 19.83)
No. of PLZF+ cells/Tubule	2.10 (1.74)	1.66 (1.08, 2.48)	2.35 (1.56)	1.64 (1.39, 3.69)	1.07 (1.03)	1.30 (0.00, 1.60)
% of tubules SOX9+	88.96 (17.76)	97.71 (83.03, 100.00)	87.63 (33.07)	100.00 (100.00, 100.00)	95.29 (6.99)	100.00 (89.91, 100.00)
No. of SOX9+ cells/Tubule	17.88 (11.43)	16.20 (8.14, 23.43)	20.90 (15.01)	20.61 (8.95, 31.56)	10.52 (8.35)	7.74 (4.48, 14.44)

540

541 **Table II. Descriptive statistics of the cumulative gonadotoxic dose exposures for non-treated**
 542 **control patients, weakly affected patients and severely affected patients.** Data regarding
 543 non-treated control patients (n=28) is not shown since this group of patients were not exposed
 544 to any chemotherapeutic drug prior to the testicular biopsy and therefore their values are
 545 Mean (SD): 0.00 (0.00); Median (1stQ, 3rd Q): 0.00 (0.00, 0.00).

	Weakly affected (n=9)		Severely affected (n=19)	
	Mean (SD)	Median (1stQ, 3rd Q)	Mean (SD)	Median (1stQ, 3rd Q)
Cyclophosphamide (mg/m ²)	1933.33 (2188.6)	1000.00 (0.00, 3000.00)	4036.42 (3004.25)	4000.00 (2000.00, 5400.00)
Ifosfamide (mg/m ²)	222.22 (666.66)	0.00 (0.00, 0.00)	1415.78 (2093.97)	0.00 (0.00, 2700.00)
Cisplatin (mg/m ²)	0.00 (0.00)	0.00 (0.00, 0.00)	42.42 (101.73)	0.00 (0.00, 0.00)
Carboplatin (mg/m ²)	311.11 (625.38)	0.00 (0.00, 0.00)	738.68 (2593.77)	0.00 (0.00, 0.00)
Etoposide (mg/m ²)	350.00 (500.00)	0.00 (0.00, 800.00)	446.21 (735.72)	0.00 (0.00, 508.00)
Doxorubicin (mg/m ²)	26.66 (52.91)	0.00 (0.00, 0.00)	51.15 (100.45)	0.00 (0.00, 97.50)
Daunorubicin (mg/m ²)	61.66 (67.76)	40.00 (0.00, 120.00)	42.89 (65.51)	0.00 (0.00, 120.00)
Idarubicin (mg/m ²)	5.33 (10.58)	0.00 (0.00, 0.00)	0.00 (0.00)	0.00 (0.00, 0.00)
Mitoxantrone (mg/m ²)	0.00 (0.00)	0.00 (0.00, 0.00)	844.21 (3670.14)	0.00 (0.00, 0.00)
Epirubicin (mg/m ²)	0.00 (0.00)	0.00 (0.00, 0.00)	7.89 (18.73)	0.00 (0.00, 0.00)
Actinomycin (mg/m ²)	0.00 (0.00)	0.00 (0.00, 0.00)	0.11 (0.51)	0.00 (0.00, 0.00)
Methotrexate (mg/m ²)	836.44 (2346.67)	0.00 (0.00, 36.00)	17846.73 (22725.37)	9000.00 (0.00, 27900.00)
Cytarabine (mg/m ²)	1702.22 (2346.67)	0.00 (0.00, 3090.00)	6503.26 (7310.19)	5000.00 (0.00, 10825.00)
6-Mercaptopurine (mg/m ²)	7724.44 (16150.76)	0.00 (0.00, 0.00)	4051.05 (9036.81)	0.00 (0.00, 0.00)
6-Thioguanine (mg/m ²)	148.88 (446.66)	0.00 (0.00, 0.00)	158.94 (389.21)	0.00 (0.00, 0.00)
Fludarabine (mg/m ²)	16.66 (50.00)	0.00 (0.00, 0.00)	0.00 (0.00)	0.00 (0.00, 0.00)
Vincristine (mg/m ²)	5.83 (9.55)	0.00 (0.00, 9.00)	11.10 (12.41)	6.00 (0.00, 19.30)
Vindesine (mg/m ²)	0.33 (1.00)	0.00 (0.00, 0.00)	0.15 (0.68)	0.00 (0.00, 0.00)
Asparaginase (UI/m ²)	2061.11 (6164.60)	0.00 (0.00, 0.00)	8735.78 (25469.91)	0.00 (0.00, 240.00)
Bortezomib (mg/m ²)	0.00 (0.00)	0.00 (0.00, 0.00)	0.70 (1.72)	0.00 (0.00, 0.00)
Dexamethasone (mg/m ²)	80.44 (241.33)	0.00 (0.00, 0.00)	178.15 (307.75)	0.00 (0.00, 183.00)
Prednisolone (mg)	53.22 (145.61)	0.00 (0.00, 0.00)	100.78 (181.74)	0.00 (0.00, 75.00)
Rituximab (mg)	0.00 (0.00)	0.00 (0.00, 0.00)	38.68 (168.62)	0.00 (0.00, 0.00)
Tozilizumab (mg/m ²)	0.00 (0.00)	0.00 (0.00, 0.00)	15.78 (68.82)	0.00 (0.00, 0.00)

546

547 **Table III. Coefficients and OR of the elastic net logistic regression model.** Only the non-zero
 548 coefficients of the elastic net model statistically associated with the shown phenotype of
 549 testicular samples are presented in the table.

	Estimate	OR
(Intercept)	-0.594	0.551
Alkylating agent cyclophosphamide (mg/m ²)	-5.9e-05	0.999
Alkylating agent ifosfamide (mg/m ²)	-8e-06	0.999
Topoisomerase inhibitor daunorubicin (mg/m ²)	0.648	1.912
Topoisomerase inhibitor idarubicin (mg/m ²)	1.395	4.038
Antimetabolite cytarabine (mg/m ²)	-2.8e-05	0.999
Antimetabolite 6-mercaptopurine (mg/m ²)	0.399	1.491
Asparaginase (mg/m ²)	0.383	1.467
lambda	0.117	

550

551 **Table IV. Bayesian logistic regression model adjusted with the chemotherapeutic drugs**
 552 **selected by the elastic net model, 95% credibility intervals for the ORs of each variable and**
 553 **posterior probability of the effect selected drugs in the testicular histologic phenotype.** The
 554 lower the OR, the higher the negative effect of the agent, whereas the higher the posterior
 555 probability, the more evidence that there is a negative association with the testicular
 556 phenotype. Text in bold highlights those drugs with a probability of negative effect greater than
 557 85%.

Variables	Estimate	Std.Error	OR	Lower.95%	Upper.95%	Post. Prob
Intercept	0.167	0.813	1.181	0.239	5.735	
Cyclophosphamide	-0.366	0.303	0.693	0.352	1.177	0.9
Ifosfamide	-1.551	1.273	0.212	0.01	1.387	0.92
Daunorubicin	-0.061	1.536	0.941	0.042	19.986	0.51
Idarubicin	3.219	1.75	25.009	1.493	1361.689	0.01
Cytarabine	-2.701	2.215	0.067	0.001	3.459	0.89
6-mercaptopurine	3.385	1.574	29.515	1.818	842.837	0.01
Asparaginase	-1.46	1.343	0.232	0.009	1.309	0.92

558

559 SUPPLEMENTAL FIGURES AND TABLES

560 Supplemental Table I. Summary of the diagnostic of the 56 patients included in this study.

Diagnostic	Non-treated controls (n=28)	Weakly affected (n=9)	Severely affected (n=19)
Acute lymphoblastic leukemia	0	5	10
Acute myeloid leukemia	1	2	0
Atypical teratoid rhabdoid tumor	1	0	0
B-cell lymphoma	0	0	2
Burkitt lymphoma	0	0	1
Chronic granulomatose disease	2	0	1
Drepanocytosis	7	0	0
Ewing sarcoma	2	0	0
Hodgkin lymphoma	1	0	0
Idiopathic medullary aplasia	1	0	0
Medulloblastoma	3	1	1
Myelodysplastic syndrome	1	0	0
Nasopharyngeal carcinoma	1	0	0
Neuroblastoma	0	1	2
Osteosarcoma	2	0	0
Rhabdomyosarcoma	1	0	0
Severe aplastic anemia	1	0	0
T-cell lymphoma	0	0	1
Thalasemia major	2	0	0
Turner syndrome mosaicism (45,X/46,XY)	1	0	0
Wilms tumor	0	0	1
Wiscott-Aldrich syndrome	1	0	0

561

562 **Supplemental Table II. List of primary antibodies employed in this study.**

Primary antibody	Marker	Reference	Dilution
Goat anti-VASA	Germ cells	R&D Systems, AF2030	1/200
Goat anti-PLZF	Undifferentiated spermatogonia	R&D Systems, AF2944	1/100
Mouse anti-UTF1	Undifferentiated spermatogonia	Millipore, MAB4337	1/100
Mouse anti-UCHL1	Undifferentiated spermatogonia	Bio-Rad, 7863-1004	1/200
Mouse anti-VIMENTIN	Sertoli cells	DAKO, M072529	1/100
Rabbit anti-SALL4	Undifferentiated spermatogonia	Abcam, ab29112	1/500
Rabbit anti-Ki67	Proliferating cells	Abcam, ab16667	1/200
Rabbit anti SOX9	Sertoli cells	Millipore, AB5535	1/500

563

564

565 **Supplemental Table III. Original data matrix regarding the mean of cell counts performed by**
566 **two researchers independently for each marker in testicular samples from untreated and**
567 **treated patients employed for the** fuzzy clustering analysis to identify differences between
568 untreated and treated patients and group treated patients as . Classification of treated
569 patients as weakly or severely affected resulting from fuzzy clustering analysis has been also
570 included in order to facilitate the identification of patients of each group.

571

572 **Supplemental Table IV. Original data matrix regarding the cumulative dose of each drug**
573 **received by patients.** Classification of treated patients as weakly or severely affected resulting
574 from fuzzy clustering analysis has been also included in order to facilitate the identification of
575 patients of each group.

576

577 **Supplemental Table V. Association between the cumulative doses of Cytarabine and**
 578 **Asparaginase with cumulative doses of alkylant drugs in treated patients.**

	Mean (SD)		Median (1stQ, 3rd Q)	
	Cyclophosphamide (mg/m ²)	Ifosfamide (mg/m ²)	Cyclophosphamide (mg/m ²)	Ifosfamide (mg/m ²)
Cytarabine (mg/m²)	4688.67 (2689.09)	1726.67 (2192.35)	5000.00 (2015.00, 6500.00)	0.00 (0.00, 1200.00)
Cytarabine + Asparaginase (UI/m²)	4000.00 (1224.74)	2400.00 (1673.32)	4000.00 (4000.00, 5000.00)	2000.00 (2000.00, 4000.00)
Without Cytarabine nor Aparraginase	2160.18 (2483.15)	272.73 (904.53)	2000.00 (0.00, 1750.00)	0.00 (0.00, 0.00)

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