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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Published in:
Reproductive Sciences

DOI:
10.1007/s43032-020-00357-6

Publication date:
2021

License:
Other

Document Version:
Accepted author manuscript

Running title: Gonadotoxic effects of chemotherapy in prepubertal testes

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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ABSTRACT

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

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

DECLARATIONS

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

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

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

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

Consent for publication: Not applicable.

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

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

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

INTRODUCTION

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

Since cryopreservation of testicular tissue is an experimental procedure, strict selection criteria of patients is mainly based on their survival prognosis and the estimated gonadotoxic damage of the chemotherapeutic drugs that will receive [16, 23, 24]. In this regard, it is known that especially alkylating drugs such as busulfan and cyclophosphamide, have a severe impact on sperm counts [25]. In a systematic literature review, the International Late Effects of Childhood Cancer Guideline Harmonization Group found evidence for adverse effects of cyclophosphamide, mechlorethamine and procarbazine on spermatogenesis [26]. Although there exist evidences that cyclophosphamide equivalent doses over 4000 mg/m^2 are linked with azoospermia and oligozoospermia [27], a predictive threshold dose for impaired

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

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

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

MATERIALS AND METHODS

Sample source: Samples used in this study were recruited at Hospital La Fe in Valencia (Spain) (32 samples), and UZ Brussel in Brussels (Belgium) (36 samples) after the approval by the respective Institutional Review Boards of Hospital La Fe (ref: 2013/0457) and UZ Brussel (ref: 2000/149D and 2017/061) and the consentment of legal guardians of all patients recruited for fertility preservation to the use of samples employed for pathologyc diagnostic for research applications. Assessment of the pubertal stage of patients by Tanner stage evaluation was performed in all patients over 10 years old. Despite that in some cases of patients over 14 Tanner stage indicated an advanced pubertal maturation, biopsy was performed due to several clinical reasons such as diagnostic purposes (different from this study), severe oligo/azoospermia, and psychologic or ethical impediments to obtain a sperm sample by masturbation or vibrostimulation. Therefore, testicular tissue samples from 68 pediatric patients between 0 and 16 years old that were subjected to a testicular biopsy for diagnostic or fertility preservation purposes were embedded in paraffin. Among recruited samples, 12 were discarded due to either leukemic testicular infiltration or bad preservation of tissue histology, resulting in the analysis of a total of 56 samples for this study (Supplemental Table I).

Histological evaluation: Tissue was fixed in 10% formaldehyde o/n at 4ºC, dehydrated, embedded in paraffin and sliced in 5µm sections. Subsequently, deparaffinized slides were subjected to hematoxylin-eosin staining and analyzed by pathologists to determine the overall status of the testicular histology of each patient.

Immunostaining: Deparaffinized slides were subjected to antigen retrieval by treating them with 10mM citrate buffer pH6 for 20’ at 97ºC before a blocking step with phosphate buffered saline + 10% normal donkey serum + 1% bovine serum albumin + 0.1% Triton X-100 (all from Sigma-Aldrich) for one hour at room temperature. Incubation of primary antibodies was

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

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

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

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

RESULTS

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

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

Therefore, in order to quantify the histologic phenotype of testicular biopsies, samples were 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

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

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

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

Overall, data indicated that cumulative doses of cyclophosphamide of 4036.42 +/- 3004.25 mg/m^2, 1415.78 +/- 2093.97 mg/m^2 of ifosfamide, 6503.26+/-7310.19 mg/m^2 of cytarabine and 8735.78 +/- 2546.91 UI/m^2 of asparaginase correlate with a severe testicular histologic phenotype. A summary of the data regarding the percentage of positive tubules and the number of positive cells per tubule for each marker, together with the cumulated dose of

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

DISCUSSION

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

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

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

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

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

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

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

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

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

CONCLUSIONS

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

AUTHORS' ROLES

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

FUNDING

This work was supported by a private donation of the Celtic Submarí club- Villareal C.F. to 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.

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373 **CONFLICTS OF INTEREST**

374 There is no conflict of interest to declare.

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FIGURES AND TABLES

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

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

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

Figure 4. Heatmap of the concentration values on each patient for the treatments selected by the elastic net analysis. Values have been normalized to z-scores to make variables on different scales comparable. Order of rows has been determined by hierarchical clustering and 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

Table II. Descriptive statistics of the cumulative gonadotoxic dose exposures for non-treated control patients, weakly affected patients and severely affected patients. Data regarding non-treated control patients (n=28) is not shown since this group of patients were not exposed to any chemotherapeutic drug prior to the testicular biopsy and therefore their values are 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/m²)	1933.33 (2188.6)	1000.00 (0.00, 3000.00)	4036.42 (3004.25)	4000.00 (2000.00, 5400.00)
Ifosfamide (mg/m²)	222.22 (666.66)	0.00 (0.00, 0.00)	1415.78 (2093.97)	0.00 (0.00, 2700.00)
Cisplatin (mg/m²)	0.00 (0.00)	0.00 (0.00, 0.00)	42.42 (101.73)	0.00 (0.00, 0.00)
Carboplatin (mg/m²)	311.11 (625.38)	0.00 (0.00, 0.00)	738.68 (2593.77)	0.00 (0.00, 0.00)
Etoposide (mg/m²)	350.00 (500.00)	0.00 (0.00, 800.00)	446.21 (735.72)	0.00 (0.00, 508.00)
Doxorubicin (mg/m²)	26.66 (52.91)	0.00 (0.00, 0.00)	51.15 (100.45)	0.00 (0.00, 97.50)
Daunorubicin (mg/m²)	61.66 (67.76)	40.00 (0.00, 120.00)	42.89 (65.51)	0.00 (0.00, 120.00)
Idarubicin (mg/m²)	5.33 (10.58)	0.00 (0.00, 0.00)	0.00 (0.00)	0.00 (0.00, 0.00)
Mitoxantrone (mg/m²)	0.00 (0.00)	0.00 (0.00, 0.00)	844.21 (3670.14)	0.00 (0.00, 0.00)
Epirubimycin (mg/m²)	0.00 (0.00)	0.00 (0.00, 0.00)	7.89 (18.73)	0.00 (0.00, 0.00)
Actinomycin (mg/m²)	0.00 (0.00)	0.00 (0.00, 0.00)	0.11 (0.51)	0.00 (0.00, 0.00)
Methotrexate (mg/m²)	836.44 (2346.67)	0.00 (0.00, 36.00)	17846.73 (22725.37)	9000.00 (0.00, 27900.00)
Cytarabine (mg/m²)	1702.22 (2346.67)	0.00 (0.00, 3090.00)	6503.26 (7310.19)	5000.00 (0.00, 10825.00)
6-Mercaptopurine (mg/m²)	7724.44 (16150.76)	0.00 (0.00, 0.00)	4051.05 (9036.81)	0.00 (0.00, 0.00)
6-Thioguanine (mg/m²)	148.88 (446.66)	0.00 (0.00, 0.00)	158.94 (389.21)	0.00 (0.00, 0.00)
Fludarabine (mg/m²)	16.66 (50.00)	0.00 (0.00, 0.00)	0.00 (0.00)	0.00 (0.00, 0.00)
Vincristine (mg/m²)	5.83 (9.55)	0.00 (0.00, 9.00)	11.10 (12.41)	6.00 (0.00, 19.30)
Vindesine (mg/m²)	0.33 (1.00)	0.00 (0.00, 0.00)	0.15 (0.68)	0.00 (0.00, 0.00)
Asparaginase (UI/m²)	2061.11 (6164.60)	0.00 (0.00, 0.00)	8735.78 (25469.91)	0.00 (0.00, 240.00)
Bortezomib (mg/m²)	0.00 (0.00)	0.00 (0.00, 0.00)	0.70 (1.72)	0.00 (0.00, 0.00)
Dexamethasone (mg/m²)	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/m²)	0.00 (0.00)	0.00 (0.00, 0.00)	15.78 (68.82)	0.00 (0.00, 0.00)

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 highligts 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
Rhabdomiosarcoma	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

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564

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

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 **Asparraginase 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/m^2)	4688.67 (2689.09)	1726.67 (2192.35)	5000.00 (2015.00, 6500.00)	0.00 (0.00, 1200.00)
Cytarabine + Asparaginase (UI/m^2)	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)

579