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Histone H4 acetylation is dysregulated in active seminiferous tubules adjacent to testicular tumors

Running title: Spermatogenic altered H4ac in testicular tumor patients

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Abstract

Study question: Is histone H4 acetylation (H4ac) altered in the seminiferous tubules of patients affected by testicular tumors?

Summary answer: A dramatic dysregulation of H4ac was detected in the seminiferous tubules' cells adjacent to testicular tumors of different etiology and prior to any treatment, while no comparable alterations were observed in patients with disrupted spermatogenesis.

What is known already: Altered H4ac levels have been associated with a variety of testicular pathological conditions. However, no information was so far available regarding potential alterations in the spermatogenic cells adjacent to the neoplasia in testicular tumor patients.

Study design, size, duration: A retrospective analysis using testicular sections from 33 men aged between 21 and 74 years old was performed. Three study groups were defined and subjected to double-blind evaluation: a control group with normal spermatogenesis (n=6), patients with testicular tumors (n=18) and patients with spermatogenic impairments (n=8). One additional sample with normal spermatogenesis was used as a technical internal control in all evaluations.

Participants/materials, setting, methods: Immunohistochemistry against H4ac and, when needed, Placental-like alkaline phosphatase (PLAP) and CD117, was performed on testicular sections. The H4ac H-Score, based on the percentage of detection and signal intensity, was used as the scoring method for statistical analyses. Protein expression data from the Human Protein Atlas were used to compare the expression levels of predicted testicular tumors' secreted proteins with those present in the normal tissue.

Main results and the role of chance: We revealed, for the first time, a drastic disruption of the spermatogenic H4ac pattern in unaffected seminiferous tubule's cells from

different testicular tumor patients prior to any antineoplastic treatment, as compared to controls ($p<0.05$). Since no similar alterations were associated with spermatogenic impairments and the *in silico analysis* revealed proteins potentially secreted by the tumor to the testicular stroma, we propose a potential paracrine effect of the neoplasia as a mechanistic hypothesis for this dysregulation.

Limitations, reasons for caution: Statistical analysis was not performed on the hypospermatogenesis and Leydig cell tumor groups due to limited availability of samples.

Wider implications of the findings: To the best of our knowledge, this is the first report showing an epigenetic alteration in cells from active seminiferous tubules adjacent to tumoral cells in testicular tumor patients. Our results suggest that, despite presenting spermatogenic activity, the global epigenetic dysregulation found in the testicular tumor patients could lead to molecular alterations of the male germ cells. Since testicular tumor is normally diagnosed in men at reproductive age, H4ac alterations might have an impact when these testicular tumor patients express desire for fatherhood.

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Key words: Histone H4 acetylation, immunohistochemistry, spermatogenesis, testicular tumor, epigenetics, epigenetic dysregulation, fertility preservation

Introduction

The spermatogenesis is a tightly regulated cellular process, in which the diploid spermatogonia differentiate into highly specialized haploid cells, the spermatozoa. Histone variants and histone marks play a crucial role in the chromatin dynamics occurring during this process, required to achieve spermatozoa with fertilization potential (de Mateo *et al.*, 2011; Hammoud *et al.*, 2011; Oliva and Ballescà, 2012; Azpiazu *et al.*, 2014; Castillo *et al.*, 2014, 2015; Barrachina *et al.*, 2018a). Specifically in mammals, a hyperacetylation of the core histones occurs during the last stage of the spermatogenesis, the spermiogenesis, leading to the process of chromatin reorganization known as nucleohistone-to-nucleoprotamine (NH-NP) transition (Christensen and Dixon, 1982; Oliva and Mezquita, 1982; Grimes and Henderson, 1984; Oliva *et al.*, 1987; Hazzouri *et al.*, 2000; Oliva and Castillo, 2011). The impact of histone acetylation relies on the specific modification of lysine residues within the N-terminal domains, which neutralizes the positive charge of the histone tails, destabilizes the histone binding to the DNA, affects the nucleosome-nucleosome interactions, modifies chromatin compaction, and acts as an epigenetic mark. The NH-NP transition is a gradual process triggered when a wave of histone H4 hyperacetylation recruits the Bromodomain-containing testis-specific protein (BRDT) (Pivot-Pajot *et al.*, 2003; Gaucher *et al.*, 2012; Goudarzi *et al.*, 2014). Histones are firstly replaced by testis-specific histone variants, subsequently by transition proteins, and ultimately by the small and extremely basic protamines, giving rise to tightly packed nucleoprotamine toroidal structures (Oliva and Dixon, 1991; Kimmins and Sassone-Corsi, 2005; Oliva, 2006; Balhorn, 2007; Oliva and Castillo, 2011; Rathke *et al.*, 2014; Bao and Bedford, 2016; Barrachina *et al.*, 2018b). Of note, when a complete NH-NP transition occurs, a 5-15 % of the histones are retained in the human sperm nucleus, mainly associated with developmental genes and gene promoters, among other specific genomic sites

(Gatewood *et al.*, 1987; Oliva, 2006; Balhorn, 2007; Arpanahi *et al.*, 2009; Hammoud *et al.*, 2009; Castillo *et al.*, 2014, 2015; Torres-Flores and Hernández-Hernández, 2020).

In human testes, histone H4 hyperacetylation has been detected in spermatogonia (SPG), round spermatids (rSPD), elongating spermatids (eSPD), and the supporting Sertoli cells (SC) (Sonnack *et al.*, 2002; Faure *et al.*, 2003; Kleiman *et al.*, 2008). The maximum peak of histone H4 acetylation (H4ac) seems to occur in eSPD, when the NH-NP transition is taking place. This pattern has been found highly conserved among several distant species such as stallion, rat, and mouse, birds, rainbow trout, and the fruit fly (Oliva and Mezquita, 1982; Christensen *et al.*, 1984; Grimes and Henderson, 1984; Meistrich *et al.*, 1992; Wolf *et al.*, 1993; Hazzouri *et al.*, 2000; Ketchum *et al.*, 2018). Interestingly, after a normal spermatogenic process, histone H4ac has been also detected in the remaining nucleosomes of mature human sperm (Gatewood *et al.*, 1990; Paradowska *et al.*, 2012). Published results support that H4ac is involved in the proper development of the spermatogenic process. For instance, altered levels of H4ac have been detected in testicular sections of patients with disrupted spermatogenesis (Sonnack *et al.*, 2002; Faure *et al.*, 2003; Kleiman *et al.*, 2008). Additionally, increased H4ac levels have also been found in Sertoli cells from men with a mixed phenotype of Sertoli cell-only syndrome (SCOS) and testicular tumor. However, due to the absence of germ cells, no potential alterations in the spermatogenesis of testicular tumor men were reported (Faure *et al.*, 2003).

Fertility preservation is common in testicular tumor patients since it is normally diagnosed among the adolescent and young adults (AYA) population, comprising the reproductive age (Saltzman and Cost, 2018). Clinical guidelines state that these patients should be offered semen cryopreservation and pre-treatment fertility assessment, with strong recommendations of discussing sperm banking prior to orchidectomy or radio/chemotherapy treatments, which can damage fertility (Meistrich, 2013; Moody *et al.*, 2018; Oktay *et al.*, 2018; Laguna *et al.*, 2020). However, this option is not always

available in testicular tumor patients presenting male subfertility (Peng *et al.*, 2009; Williams *et al.*, 2009; Fraietta *et al.*, 2010). By pursuing possible fertility preservation options for these patients, less invasive surgical methods have been developed as an alternative to the classical intervention of radical orchiectomy either in the treatment or in the initial evaluation of testicular tumor (Picton *et al.*, 2015). Indeed, techniques such as the testis-sparing surgery (TSS) and the oncological testicular sperm extraction (onco-TESE) coupled with orchiectomy are currently offered (Giannarini *et al.*, 2010; Borghesi *et al.*, 2015; Suzuki *et al.*, 2015; Luján *et al.*, 2016; Tsutsumi *et al.*, 2017; Hamano *et al.*, 2018; Kuroda *et al.*, 2018). However, whether the adjacent testicular tumor affects the epigenetic profile of spermatogenic cells from intact seminiferous tubules has not been explored so far. Nonetheless, recent evidences point out that sperm used for fertility preservation in testicular tumor patients might contain molecular alterations even before the initiation of the antitumor treatment (Dias *et al.*, 2020).

In the present study, we aimed to update the spermatogenic H4ac pattern during human spermatogenesis based on a detailed spermatogenic classification system, as well as to shed new light on the potential alterations of the spermatogenic H4ac pattern in patients affected by different testicular pathologies. To that end, we have evaluated the H4ac levels in the seminiferous tubules of a) patients with normal spermatogenesis, b) testicular tumor patients displaying spermatogenic activity adjacent to the neoplasia, and c) patients with spermatogenic impairments. Since this is the first report assessing H4ac levels in the intact seminiferous tubules adjacent to the tumor tissue, our results provide novel and useful information towards the identification of the potential epigenetic risks underlying the fertility preservation of apparently healthy male germ cells from testicular tumor patients.

Materials and methods

Biological Material

Human testicular biopsies (n=33) were provided by the Departments of Pathology from the Hospital Clínic de Barcelona (Barcelona, Spain) and the Hospital Universitari de Vic (Vic, Spain). All patients signed informed consent in accordance with the Declaration of Helsinki. Samples were used following the appropriate ethical guidelines and Internal Review Board, and the Clinical Research Ethics Committee of the corresponding hospitals approved the biological material storing and processing. Testicular biopsies were obtained from infertile patients with azoospermia or severe oligozoospermia, related or not with spermatogenic defects, and patients with testicular tumors. Three main groups of study were established (Figure 1): 1) Patients with histologically normal spermatogenesis corresponding to the male partners of couples undergoing assisted reproduction study (1 post-vasectomy individual, 2 patients with obstructive azoospermia due to CFTR genotypes, and 3 azoospermic patients) showing all germ cell types and spermatogenic stages (control group; n=6); 2) patients with testicular neoplasia (n=18); and 3) azoospermic or severe oligozoospermic infertile patients with altered spermatogenesis (n=8). The second group was subdivided according to the type of testicular tumor, as follows: seminoma (SEM; n=4), spermatocytic tumor (SPCT; n=3), embryonal carcinoma (EmCA; n=5), teratoma (TER; n=4), and non-malignant Leydig cell tumor (LCT; n=2). The third group was subdivided according to the type of spermatogenic alteration into patients with SCOS (n=3), hypospermatogenesis (Hp; n=2), and spermatogenic arrest (SA; n=3). Within the SA patients, two of them presented spermatocyte (SPC) arrest (one with complete arrest and the other one with partial arrest), and the third one evidenced a partial arrest in rSPD. In addition to the groups above, one sample corresponding to a patient with normal spermatogenesis was used as a technical internal control in all evaluations. A detailed description of the number of subjects and the clinical histological evaluation is shown in Figure 1.

Testicular tissue processing and paraffin-embedding

Samples were fixed prior to paraffin-embedding in either Bouin's fixative or 4 % Formol, depending on the sample size (biopsies or orchiectomy specimens, respectively), for histopathologic evaluation or other procedures. Following the hospital's routine procedures, biopsies were fixed in Bouin solution for 3-4h, and surgical specimens in 4 % Formol for 24-48h (detailed individual information is included in Supplementary Table SI). After fixation, the specimens were processed in a fluid-transfer advanced automatic tissue processor and paraffin-embedded to make the blocks. The tissue was covered in molten paraffin in a mold, and the paraffin cooled down until solidifying. The block was cut into 4 µm-sections using a microtome.

Testicular histopathological evaluation

The histopathological evaluation of testicular sections was exhaustively performed by an anatomopathologist. The haematoxylin-eosin-stained sections from each patient were reviewed to histologically identify spermatogenic defects (Cerilli *et al.*, 2010) and/or testicular tumors, following the World Health Organization (WHO) guidelines (Moch *et al.*, 2016). When needed, tumor diagnosis was confirmed by checking the expression of the appropriate biomarkers. Ultrasound evaluation was monitored to discard the presence of testicular microlithiasis (Leblanc *et al.*, 2018). The presence of either complete or partial spermatogenic activity in the seminiferous tubules adjacent to the tumor tissue was a strict inclusion criterion for the group of patients with testicular tumors. After histopathological confirmation, additional testicular sections from each patient were used for further immunohistochemical analyses.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded testicular sections, as described previously (Barrachina *et al.*, 2019). Testicular tissue samples were randomly grouped in different runs, and an internal positive control was included in each of them to check the different runs' overall intensity. This internal positive control corresponded to a section obtained from an orchitectomy specimen of 4.5 cm showing normal spermatogenesis and no presence of testicular tumor (Supplementary Table SI). Note that due to the size of the surgical material, the internal positive control was fixed in 4% formol and showed the same H4ac pattern as the samples from the control group fixed in Bouin (Figure 2). Therefore, we discarded any potential bias related with the tissue fixation method. The presence of the normal spermatogenic H4ac pattern in the internal positive control was verified in all IHC runs.

Sections were dewaxed in toluene and rehydrated through graded series of ethanol to water with 0.3 % hydrogen peroxide incubation. Subsequently, antigen retrieval was performed with 10 mM sodium citrate (pH 6.0) at 99.5 ºC for 20 min. Slides were then blocked with PBS-5 % skim milk for 30 min at room temperature (RT) and incubated with Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA). The sections were incubated overnight at 4 ºC with the primary antibody. A polyclonal anti-acetyl histone H4 antibody (5 µg/ml; #06-598, Millipore, Billerica, MA, USA), detecting histone H4 acetylated on lysines 5, 8, 12 and 16, was used to analyze the presence of H4ac (Figure 1). Regarding our aim of evaluating global H4 acetylation, we considered using an antibody able to detect several acetylated residues in the same protein sequence rather than an antibody against a single specific residue. However, we also confirmed the H4ac IHC staining using an alternative monoclonal antibody against the specific acetylation of the H4K5 residue (dil 1:100, #PTM-163, PTM BIO LLC, Chicago, IL, USA), obtaining similar results (data not shown). Negative controls without primary antibody were included. Afterward, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) and an avidin-biotin-

peroxidase detection kit (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Colour development was achieved by reaction with diaminobenzidine (DAB, 3,3'-diaminobenzidine tetrahydrochloride) and sections were additionally counterstained with haematoxylin and Periodic Acid-Schiff's (PAS) reagent (Sigma-Aldrich, St. Louis, MO, USA). Sections were dehydrated in ethanol, cleared in toluene, and mounted in Eukitt Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA) to finally be analyzed by a transmission light microscope (Olympus BX50, Olympus, Tokyo, Japan) (Barrachina *et al.*, 2019). For the testicular tumor groups, consecutive histological sections to those used for H4ac staining were used to identify neoplastic areas in SEM (n=2) and EmCA (n=2) patients (Figure 1). Specifically, the routine germ cell tumor markers Placental-like alkaline phosphatase (PLAP) (0.15 µg/µL, PLAP-NB10 clone, #760-2664, F. Hoffmann-La Roche Ltd, Basilea, Switzerland), and CD117 (0.1 µg/µL, CD117-EP10 clone, #790-7061, F. Hoffmann-La Roche Ltd, Basilea, Switzerland), were used (Iczkowski *et al.*, 2008). PLAP and CD117 IHC stainings were performed using Ventana's automated staining platform BenchMark ULTRA IHC/ISH System (Ventana Medical Systems Inc., Oro Valley, AZ, USA), following the manufacturer's recommendations.

Spermatogenic stage classification and IHC evaluation

The classification of testicular cells and spermatogenic stages of the seminiferous tubules sections was based on Muccacia and colleagues' classification of human spermatogenesis (Muciaccia *et al.*, 2013). This classification includes 12 different stages of human spermatogenesis (expressed in Roman numerals), based on the position of the testicular cells along the seminiferous epithelium, the cell morphology, and the acrosome development. For each patient, up to 30 seminiferous tubule sections at different stages were analyzed. In the biopsies from testicular tumor patients, we evaluated the neoplasia *in situ* and the seminiferous tubule sections adjacent to the tumor.

The IHC assessment was achieved by two independent operators using PAS–Haematoxylin staining, blinded to the experimental procedures. The method selected for the H4ac scoring system was the H-score (Fedchenko and Reifenrath, 2014), with minor modifications. Specifically, the H-score was determined by adding the proportion of cells presenting each level of intensity (expressed in percentage) multiplied by the intensity (expressed in ordinal values), as follows:

$$H\text{-}score = \left(\%_{intensity\ 0} \times 0\right) + \left(\%_{intensity\ 1} \times 1\right) + \left(\%_{intensity\ 2} \times 2\right) + [\dots]$$

This scoring method normally comprises a semi-quantitative scale of intensity detection corresponding to ordinal values from 0 to 3. Due to the good signal obtained with the antibody used, we could broaden the intensity scale establishing values from 0 to 6. Thus, 0 corresponded to “no signal”, 1 to “weak”, 2 to “mild-weak”, 3 to “mild”, 4 to “mild-strong”, 5 to “strong” and 6 to “very strong” signals. Because of that, our H-score presents a dynamic range from 0 to 600. A minimum of 30 seminiferous tubular sections were analyzed per subject. Since the intensity of the signal within cells belonging to the same specific cell type was homogeneous along the seminiferous tubule section, the mean intensity of each cell type was calculated and used for H-score determination. Thus, H-score for each testicular cell type analyzed per seminiferous tubule section was provided for each individual patient (Figure 1). The results corresponding to the different sample groups were expressed as the mean H4ac H-score ± standard deviation (SD).

Statistical analysis

Data analysis was conducted in RStudio software version 1.2.5033 (RStudio Team, 2019) and the *car* package (Fox and Weisberg, 2019), and using GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Normal distribution was assessed by Shapiro-Wilk normality test followed by Levene’s homoscedasticity test. According to the distribution of each dataset,

either a multiple Student t-test or Mann-Whitney-U was performed between normal and pathological groups for each testicular cell type, and between consecutive spermatogenic cell types, in order to establish the spermatogenic H4ac pattern. P-values <0.05 were considered statistically significant. Statistical limitations were considered due to the low number of patients in some studied groups, and only groups formed by at least three patients were included in the statistical analysis. Principal component analysis (PCA) was performed considering H4ac H-scores in the different cell types as variables, including patients with complete spermatogenesis (therefore presenting values of H4ac H-score for all variables analyzed) from normal and tumor groups.

In-silico prediction of testicular tumor secreted proteins

Proteins secreted by the testicular tumor were predicted using protein expression data from the Human Protein Atlas version 20.0 (Uhlen *et al.*, 2017). Briefly, the list of proteins expressed in the testicular tumor was retrieved from the Pathology dataset (release date February 6th, 2021) and filtered according to the available set of predicted secreted proteins (https://v20.proteinatlas.org/search/protein_class:Predicted+secreted+proteins). Only those proteins expressed at high and medium levels with enhanced, approved or supported reliability in the majority of testicular tumor patients included in the dataset were considered for further analyses. Subsequently, protein expression data in the normal testicular tissue were collected and compared with the levels of the corresponding predicted testicular tumor secreted proteins. To infer the functional involvement of the predicted secreted proteins, we determined the enrichment on Gene Ontology (GO) Biological Process and Molecular Function annotations enclosed in the Gene Ontology Consortium Database (http://www.geneontology.org/). P-values <0.05 after Bonferroni correction were considered significant. Pathway enrichment analysis

was also conducted using Reactome (Jassal *et al.*, 2020), in which P-values <0.05 after false-discovery rate (FDR) correction were considered significant.

Results

Characterization of the histone H4 acetylation levels in the testicular cells of patients with normal spermatogenesis

Histological sections (testicular biopsies fixed in Bouin's fixative) from individuals displaying normal spermatogenesis (controls) were subjected to IHC analysis after morphological confirmation by an anatomopathologist. The H4ac H-score values for each cell type are shown in Table I (mean value ± SD), while the graphical overview of the spermatogenic H4ac pattern during normal spermatogenesis, together with representative images of the H4ac immunolabeling, are shown in Figure 2. According to the H4ac H-score, SPG displayed intermediate levels of H4ac (212.7 ± 82.5), which decreased to minimum levels at the SPC stage (22.4 ± 41.5, $p<0.01$, Supplementary Figure SI) and were restored after meiosis ($p<0.01$, Figure 2, Supplementary Figure SI, Table I). Specifically, early-stages (stages I to V) round spermatids (rSPD I-V) showed intermediate H4ac levels (263.4 ± 128.0), followed by a non-significative trend of higher levels in later-stages (stages VI to VIII) round spermatids (rSPD VI-VIII; 366.3 ± 106.0), reaching the maximum peak in eSPD (559.5 ± 33.3, $p<0.01$). Finally, the H4ac levels dramatically decreased up to minimum levels in condensed spermatids (cSPD; 22.2 ± 36.8, $p<0.01$). In the non-spermatogenic SC, H4ac was detected with an H-score of 90.7 ± 66.9 (Figure 2, Table I). Of note, the same H4ac spermatogenic pattern was described for the internal technical control fixed with 4% Formol (Figure 2A and C), discarding any variability in the detection of the H4ac levels due to the fixation method (Oosterhuis *et al.*, 2011). Individual H4ac H-scores for each patient included in this study are shown in Supplementary Table SI.

The spermatogenic histone H4 acetylation pattern is dramatically impaired in seminiferous tubules adjacent to testicular tumors

Testicular tumor patients stratified by tumor type into five groups of study were included in the analysis. According to the tumor origin, three groups comprised patients affected by germ cell tumors derived from germ cell neoplasia *in situ* (GCNIS) (SEM, EmCA, and TER), one group included patients affected by a germ cell tumor unrelated to germ cell neoplasia *in situ* (SPCT), and the last one was formed by patients affected by a sex cord-stromal tumor (LCT). Spermatogenic activity in seminiferous tubules adjacent to tumor areas was detected for all neoplasias included in this study (Figure 3, Table I). All the samples showed a global H4ac dysregulation with increased levels in all germ cell types from SPG to rSPD VI-VIII, as well as in SC (Figure 3A, Table I). Consequently, the characteristic gradual increase of H4ac associated with normal spermatogenesis was not detected in this group of patients with testicular tumors (Figure 3A, Supplementary Figure SI). Conversely, eSPD and cSPD displayed a discrete reduction of the H4ac levels. Of note, the most drastic differences were found in the stage of SPC, showing remarkably higher levels of H4ac compared with the minimum levels found in the control group (295.2 ± 198.5 for SEM, 454.2 ± 107.8 for SPCT, 472.1 ± 67.6 for EmCA, 364.5 ± 174.8 for TER, versus 22.4 ± 41.5 for the N group; Table I).

H4ac H-scores of all the cell types analyzed in the LCT group revealed a similar behavior compared with the rest of the groups of patients with testicular tumor. However, the LCT group was not considered for statistical analysis due to the limited number of available samples (Table I). Individually detailed information about the H4ac H-scores is shown in Supplementary Table SI, and representative images of the altered H4ac found in the different subtypes of testicular tumor patients are shown in Figure 3B. Principal component analysis (PCA) supports these findings, with principal components 1 and 2 explaining the 77.98% of the variability of our data (60.89% and 17.09%, respectively),

and separating control and tumor groups in two independent clusters (Figure 3C). Interestingly, all patients affected by a tumor are clustered together independently of the type of tumor, and are characterized by high values of the variables corresponding to early spermatogenesis (SPG, SPC, and rSPD), and somatic SC.

In order to determine whether similar H4ac alterations might be also found in presence of non-tumoral dysregulations, we evaluated the spermatogenic H4ac pattern in testicular tissue from infertile patients with spermatogenic impairments (Supplementary Figure SIIA, Table I). Although the H4ac H-score of all germ cell types associated with the Hp and SA phenotypes tended to be lower than in the control group, the characteristic gradual increase of H4ac from SPG to post-meiotic germ cells observed in the patients with normal spermatogenesis was maintained (Supplementary Figure SIIA). Representative images comparing H4ac immunolabeling between infertile men showing normal spermatogenesis and patients with spermatogenic impairments are shown in Supplementary Figure SIIB-E. Detailed information about the H4ac H-score per individual patient is specified in Supplementary Table SI.

The impaired spermatogenic histone H4 acetylation pattern of testicular tumor patients is not dependent on the presence of seminiferous tubules with pre-invasive germ cell neoplasia *in situ*

We further explored whether H4ac dysregulated levels detected in the different cell types within the seminiferous tubules adjacent to the tumor tissue might be due to the presence of a pre-invasive GCNIS. To that end, we investigated the presence of pre-invasive GCNIS in biopsies from all testicular tumor groups through IHC detection of PLAP and CD117 (Supplementary Figure SIII). No seminiferous tubules containing pre-invasive GCNIS were observed in SPCT, TER, and LCT patients. In contrast, pre-invasive GCNIS was identified in localized areas of the testis biopsy in patients with EmCA and SEM. Considering that the same altered H4ac spermatogenic pattern is observed in patients

with and without pre-invasive GCNIS, and that H4ac levels were thoroughly studied all along the testis biopsy, we can confirm that the global H4ac alteration observed is independent of the presence or not of pre-invasive GCNIS in seminiferous tubules with spermatogenic activity. In order to confirm this fact, we selected a representative SEM patient to analyze H4ac levels in seminiferous tubules with and without pre-invasive GCNIS, evidencing similarly altered H4ac spermatogenic levels (Table II).

The potential secretory effect of the testicular germ cell tumors as a mechanistic hypothesis behind the altered intratubular spermatogenic H4ac pattern

By analyzing the data contained in the Human Protein Atlas Database, 71 proteins were predicted as secreted by testicular germ cell tumors (Figure 4, Supplementary Table SII). The Gene Ontology enrichment analyses revealed an overrepresentation of biological processes and molecular functions related to cell communication and signal transduction ($p<0,05$ after Bonferroni correction; Supplementary Figure SIVA-B). Furthermore, the Reactome analysis resulted in an overrepresentation of metabolic pathways involved in cell signaling (Supplementary Figure SIVC). Of note, pathways involved in immune system signaling through cytokines (interleukins and TNFs), signaling by growth factors, and phosphorylation-mediated signal transduction were also enriched. Some of the proteins predicted to be secreted by the testicular cancer were not detected or were lowly expressed in the healthy testicular tissue (Supplementary Table SII).

Discussion

The role of histone H4 acetylation (H4ac) in nucleosome destabilization and chromatin remodeling during the spermatogenic process is well established and highly conserved among different species (Christensen and Dixon, 1982; Oliva and Mezquita, 1982; Grimes and Henderson, 1984; Hazzouri *et al.*, 2000). In the present study, we reported

that the spermatogenic H4ac pattern during normal human spermatogenesis is in agreement with the results previously reported by others (Sonnack *et al.*, 2002; Faure *et al.*, 2003; Kleiman *et al.*, 2008). As a novelty, we further defined the H4ac levels among different subtypes of post-meiotic cells, such as the early-stages round spermatids (stages I to V), late-stage round spermatids (stages VI to VIII), elongated spermatids (stages IX to XI), and condensed spermatids. We have detected the highest H4ac levels in eSPD, which were found preceded by a gradual increase starting from early-stages rSPD. These results might be reflecting the hyperacetylation wave that triggers the NH-NP transition underwent by the post-meiotic cells during spermiogenesis (Christensen and Dixon, 1982; Oliva and Mezquita, 1982; Grimes and Henderson, 1984; Oliva *et al.*, 1987, 1990; Hazzouri *et al.*, 2000). Similarly, the dramatic drop of H4ac levels detected in cSPD was coherent with the small proportion of histones that remain associated with the sperm genome after completion of the spermiogenesis (Gatewood *et al.*, 1987; Oliva, 2006; Balhorn, 2007; Castillo *et al.*, 2014; Rathke *et al.*, 2014). The normal spermatogenic H4ac pattern was observed in all control biopsies fixed with Bouin's fixative, as well as in the internal technical control from an orchiectomy specimen fixed with 4% formol, discarding any technical bias caused by the fixative used. In our analysis, SPG evidenced intermediate levels of H4ac, which differs from the previously reported high levels of H4ac for this specific cell type (Sonnack *et al.*, 2002; Faure *et al.*, 2003). Our slightly lower values could be explained by the use of different antibodies or due to the refinement of the scoring method applied herein. Indeed, we used an H-score method establishing a much broader dynamic range of intensities of up to six different levels. Anyhow, the detection of H4ac levels in SPG was expected since this modification is physiologically required as part of the replicative and transcriptionally active mitotic state in a normal spermatogenic process (Jan *et al.*, 2017; Hermann *et al.*, 2018). However, this H4ac normal pattern is completely disturbed in the presence of testicular tumor, showing a marked increase in the H4ac levels of both germ and somatic cells in apparently healthy seminiferous tubules adjacent to the tumor. Of note, increased H4ac

levels in SC of SEM and EmCA patients were previously described by Faure *et al*. (2003). However, since the testicular tumor patients selected for their study also presented SCOS, no information was available regarding the spermatogenic cells adjacent to the testicular tumors (Faure *et al.*, 2003). It is interesting to highlight that, in germ cells, altered H4ac levels were mainly detected in early stages of spermatogenesis, while later spermatogenic cell types, such as eSPD and cSPD, displayed slightly lower H4ac levels in the testicular tumor patients compared with the normal spermatogenic pattern. These higher H4ac levels in SPG and SPC, together with the loss of gradual increase of the H4ac levels in post-meiotic cells found in normal spermatogenesis, might lead to a premature nucleosome destabilization and histone displacement, therefore leading to an early loss of H4 hyperacetylation in later stages of the spermatogenesis (Christensen and Dixon, 1982; Oliva and Mezquita, 1982; Grimes and Henderson, 1984; Oliva *et al.*, 1987, 1990; Hazzouri *et al.*, 2000). It has been described by others that chromatin epigenetic impairments during spermatogenesis are linked to decreased H4ac levels in later stages of the spermatogenesis (eSPD) and, consequently, to defective histone retention and chromatin remodeling (Moretti *et al.*, 2017). With that, the observed H4ac alteration during the spermatogenesis in testicular tumor patients could be related with a subsequent defective retention of histone epigenetic marks in mature sperm, potentially leading to the transmission of altered epigenetic modifications and affecting events beyond fertilization.

Even though some EmCA and LCT patients evidenced an arrest at rSPD or eSPD, our results suggest that the effect produced by the tumor itself is stronger than the one produced by the spermatogenic impairment, since their altered pattern is similar to that of tumor patients with complete spermatogenesis. Besides, H4ac staining was positive in all tumoral cells, which is in accordance with previous observations describing that germ cell neoplasia harbors a general abundance of epigenetic marks associated with active chromatin states (Omisanjo *et al.*, 2007; Almstrup *et al.*, 2010; Kristensen *et al.*,

2012; van der Zwan *et al.*, 2014; Rajpert-De Meyts *et al.*, 2015). This could be creating a globally altered environment in the testicular stroma with a potential effect on the seminiferous tubule cells.

Because of the above findings, we further sought to decipher whether the altered spermatogenic H4ac pattern found in the testicular tumor groups was caused by an intrinsic neoplastic state of the seminiferous tubule cells. Theoretically, no direct affectation of the neoplasia in the seminiferous tubule cells of patients with LCT, a tumor without a germ-cell-related origin, might be expected. Indeed, we discarded the presence of pre-invasive germ cell neoplasia in situ (GCNIS) in LCT patients through IHC staining of PLAP and CD117. However, H4ac H-scores of LCT patients seem to indicate an altered spermatogenic H4ac pattern similar to that from other tumor subgroups. Noteworthy, this would point to the tumor environment as responsible for the epigenetic alteration within the seminiferous tubules. In the case of germ cell tumors, the presence of seminiferous tubules with pre-invasive GCNIS was discarded in SPCT and TER groups, but SEM and EmCA groups included patients with tubules presenting pre-invasive CGNIS and spermatogenic activity. Despite that, similar altered H4ac profiles were observed in all tumor groups, and even between patients with or without pre-invasive GCNIS. Besides, our extensive evaluation of the H4ac levels covered all areas of the biopsy. All these facts indicate that the impaired H4ac epigenetic profile was observed independently of the presence of pre-invasive GCNIS in active seminiferous tubules. Indeed, the analysis of seminiferous tubules with and without pre-invasive GCNIS showed a similar H4ac alteration in a representative SEM patient. These striking observations may indicate that cells from apparently normal seminiferous tubules would be affected by the surrounding tumor regardless of their origin.

Taking all this together, we suggest a potential paracrine effect of the testicular tumor on the adjacent seminiferous tubules responsible for the global dysregulation of the H4ac levels. The simple presence of a neoplasia could impair the homeostasis in the testicular

stroma, causing a dysregulation of the interstitial protein levels, finally leading to an impaired cell signaling (Figure 4). We hypothesize that the tumor secretes small proteins or paracrine signals that could impair the intercellular communication causing an altered signal transduction, ending up in a dysregulation of intracellular signaling cascades altering nuclear gene expression in the intratubular cells. In fact, our *in-silico* prediction of the testicular cancer protein secretome, despite being limited to testicular germ cell tumors, suggests that some of the proteins expressed at higher levels in the tumor are not detectable, or are expressed at lower levels, in the healthy testicular tissue, supporting the existence of a potential paracrine disruption of the cell signaling. Indeed, we found an overrepresentation of pathways that could be supporting the paracrine communication between the tumor and seminiferous tubules' cells, such as ligand-receptor pathways, including cytokine signaling and growth factors, as well as phosphorylation-mediated signal transduction. Since infiltration of immune cells is common in testicular tumors and contributes to the tumor microenvironment composition, some cytokines and chemokines that are normally protective could have a negative impact on testicular function and physiology (Loveland *et al.*, 2017). Furthermore, immune cell infiltration is associated with a disruption of the blood-testis barrier in the affected seminiferous tubules, allowing the permeabilization of small molecules (Pérez *et al.*, 2013). Through the binding to surface receptors, the dysregulation of intracellular phosphorylation cascades directly involved in the male germ cell development would carry out changes in gene activity that could be related to changes on H4ac levels. For example, Bone Morphogenetic Protein 6 (BMP6) has been related to the regulation of Hippo signaling, which is known to regulate cell survival and proliferation. Remarkably, some of the proteins involved in this pathway are essential for a proper development of spermatogenesis (Pulkkinen *et al.*, 2020). Also hepatocyte growth factor (HGF) is related to the activation of intracellular signalling cascades through the JAK/STAT pathways and associated kinase activities, one of the major pathways that maintain stem cell activity and identity in the testis niche (Zhang *et al.*, 2018). JAK/STAT proteins have been also

related with epigenetic effectors in the maintenance of the germ cell status (Tarayrah *et al.*, 2015). In addition, histones are phosphorylated in specific residues by kinases, and the crosstalk with other histone post-translational modifications can affect histone acetylation levels. In fact, not only histones, but also histone modifiers (acetyltransferases and deacetylases) are phosphorylated (Baek, 2011). Therefore, H4ac levels could be affected by the crosstalk between kinases involved in phosphorylation cascades and other nuclear cofactors, such as effectors of histone acetylation or other histone marks (Baek, 2011; Moretti *et al.*, 2017). However, the interpretation of our *in silico* prediction is limited by the scarce availability of gene expression data in testicular tumors, which is not sorted by tumour subtypes. Because of that, we would like to emphasize that this mechanistic hypothesis can orientate future research in the field, but has to be experimentally confirmed. Indeed, this common global increase of H4ac levels found in all types of tumors might rely on mechanisms that could vary according to their intrinsic differences in their cellularity and secretory patterns. As detailed in Supplementary Table SI, we have analyzed specimens with variable tumor size representing different proportions of the total quirurgic material, and the increase in the H4ac levels reported in patients affected by testicular tumors are coherent among all of them. However, the reported results correspond to a partial representation of the testicle that always included the tumor (except for patient TER1). To that extent, we could hypothesize this secretory effect in a representative part of the testicle, but without the possibility to check the whole organ. To the best of our practice, within the tissue section, the evaluation of the H4ac levels was performed in seminiferous tubules from different areas, to avoid any bias, and we observed similar patterns regardless the distance to the tumor.

Nowadays, the simple production of spermatozoa or the presence of testicular germ cells are sufficient for fertility preservation before starting the antineoplastic therapy (Meistrich, 2013; Moody *et al.*, 2018; Oktay *et al.*, 2018; Laguna *et al.*, 2020). However, the

molecular evaluation presented in this work evidences that the spermatogenic cells in seminiferous tubules adjacent to the tumor, as well as the potentially resulting spermatozoa, might present an impaired epigenetic state. Therefore, these alterations may affect post-fertilization events or transmit altered epigenetic information to the progeny. In fact, recent evidences have pointed out alterations of the semen quality and mature sperm protein profile from testicular tumor patients before any reprotoxic treatment (Suzuki *et al.*, 2015; Bruno *et al.*, 2018; Dias *et al.*, 2020; Xavier *et al.*, 2021). Testicular microlithiasis, a condition associated with an increased risk of developing testis tumor, is also associated with reduced sperm count (Barbonetti *et al.*, 2019; Anvari Aria *et al.*, 2020). Alterations in sperm DNA methylation have been reported associated with oligozoospermia and some sperm tumor patients (Bruno *et al.*, 2018; Åsenius *et al.*, 2020). Thus, it should be important to be aware of the potential imprinting alterations when sperm banking or testicular tissue cryopreservation are performed before or coupled to the orchiectomy or the tumor treatment (Giannarini *et al.*, 2010; Borghesi *et al.*, 2015; Suzuki *et al.*, 2015; Luján *et al.*, 2016; Tsutsumi *et al.*, 2017; Hamano *et al.*, 2018; Kuroda *et al.*, 2018).

To the best of our knowledge, this is the first report providing evidence of epigenetic alterations of the testicular tissue with spermatogenic activity adjacent to testicular tumors. Despite the relatively low number of patients evaluated and the use of a semi-quantitative technique, a global dysregulation of H4ac was found in the spermatogenic cells of all the different subtypes of testicular tumors assessed. Our findings pinpoint a common behavior in testicular tumor patients that might be considered regarding fertility preservation purposes. Further studies and cohort validations will contribute to determining the magnitude and potential consequences of this epigenetic risk underlying fertility preservation in testicular tumor patients.

Author's roles

F.B., A.I., M.J., J.C., and R.O. participated in the study design. C.M., L.R-C., J.L.B., and J.M.C were involved in the clinical evaluation of the patients. F.B., A.I., A.S-V., and A.G. executed the study. F.B., A.I., M.J., J.C., and R.O. analyzed the results. F.B., A.I., M.J., J.C., and R.O. drafted the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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Data Availability

The data underlying this article are available in the article and in its online supplementary material.

Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table I. Comparative immunohistochemical levels of histone H4 acetylation in the seminiferous tubule sections from each group of patients included in the study. Results are expressed as the mean H-score ± Standard Deviation (SD).

		H4ac H-score (Mean ± SD)						
Group	**Testicular histology**	**SC**	**SPG**	**SPC**	**rSPD I-V**	**rSPD VI-VIII**	**eSPD**	**cSPD**
Infertile patients with normal spermatogenesis (Controls)	Normal spermatogenesis (N)	90.7 ± 66.9	212.7 ± 82.5	22.4 ± 41.5	263.4 ± 128.0	366.3 ± 106.0	559.5 ± 33.3	22.2 ± 36.8
Infertile patients with altered spermatogenesis	Sertoli cell-only syndrome (SCOS)	153.2 ± 81.1	-	-	-	-	-	-
	Hypospermatogenesis (Hp)	55.5 ± 8.9	152.1 ± 21.6	0.3 ± 0.4	84.0 ± 45.8	248.7 ± 257.5	531.4 ± 21.2	50.1 ± 70.9
	Spermatogenic arrest (SA)	34.0 ± 27.1	109.2 ± 64.6	0.6 ± 1.0	112.5 ± 123.5	162.6 ± 17.8	358.5 ± 153.2	14.1 ± 3.8
Testicular tumor patients	Seminoma (SEM)	320.9 ± 126.5*	498.6 ± 91.9**	295.2 ± 198.5*	472.4 ± 109.5	500.1 ± 115.4	578.0 ± 25.9	2.1 ± 4.2
	Embryonal carcinoma (EmCA)	411.5 ± 109.3**	491.4 ± 6.3**	472.1 ± 67.6**	520.2 ± 52.9**	506.0 ± 75.3*	512.4 ± 134.9	3.8 ± 6.6
	Teratoma (TER)	336.3 ± 96.0**	429.6 ± 54.3**	364.5 ± 174.8*	495.0 ± 87.5*	459.5 ± 63.7*	459.5 ± 124.4	0.0 ± 0.0
	Spermatocytic Tumor (SPCT)	426.8 ± 39.0*	473.0 ± 42.3*	454.2 ± 107.8*	475.0 ± 141.9	465.4 ± 175.9	325.0 ± 129.7*	2.8 ± 4.8
	Leydig cell tumor (LCT)	381.0 ± 40.7	477.6 ± 24.6	470.1 ± 113.3	516.6 ± 77.2	502.8	279.0	0.0

SC: Sertoli cells; SPG: spermatogonia; SPC: spermatocytes, rSPD I-V: early-stages (I-V) round spermatids; rSPD VI-VIII: late-stages (VI-VIII) round spermatids; eSPD: elongating spermatids, cSPD: condensing spermatids. * $p<0.05$, ** $p<0.01$, resulting from the comparison with the control group with normal spermatogenesis (N). Note that the Hp and LCT groups were not considered for statistical analysis due to the limited sample availability.

Table II. Comparative histone H4 acetylation levels seminiferous tubules with and without pre-invasive germ cell neoplasia *in situ* (GCNIS) from a seminoma patient

(SEM3). Immunohistochemistry results are expressed through the H4ac H-score. The number of tubular sections showing each of the analyzed cell types is indicated in brackets. H-Score mean values are shown for the control group (N).

	H4ac H-score				
Testicular histology	**SPG**	**SPC**	**rSPD**	**eSPD**	**cSPD**
N group (mean value)	212.7	22.4	314.9	559.5	22.2
Seminiferous tubules without pre-invasive GCNIS	376.8 (30)	210.0 (30)	342.6 (21)	533.4 (9)	19.2 (21)
Seminiferous tubules with pre-invasive GCNIS	446.4 (30)	199.8 (29)	337.8 (21)	600.0 (11)	0.0 (17)

SPG: spermatogonia; SPC: spermatocytes, rSPD: round spermatids; eSPD: elongating spermatids, cSPD: condensing spermatids.

Figure legends

Figure 1. **Overall strategy used for the characterization and statistical comparison of the spermatogenic H4ac pattern between the different groups of study through the immunohistochemical H4ac H-Score.** The study groups and types of patients are indicated at the top of the figure. See Supplementary table SI for detailed individual information of each sample included in the present study. The methodological approach and the main derived conclusion are shown in the middle and bottom of the figure respectively.

Figure 2. Histone H4 acetylation (H4ac) levels in patients with normal spermatogenesis. **A** Immunohistochemistry (IHC) results according to the analyzed testicular cell type. Mean H4ac H-Scores are shown with error bars corresponding to the standard deviation. The progress of the spermatogenic H4ac pattern during normal spermatogenesis (using Bouin's fixative) and the internal technical control (using fixation with 4% formol) are represented with a soft line (in gray and orange, respectively). **B** Microscopy images of seminiferous tubule sections from a biopsy of a patient with normal

spermatogenesis fixed with Bouin's fixative. Representative images show the different spermatogenic stages with positive H4ac IHC detection (brown colour). **C** Microscopy images of seminiferous tubule sections from the internal technical control displaying normal spermatogenesis, which was obtained from an orchiectomy specimen and fixed with 4% Formol. Representative images show the different spermatogenic stages with positive H4ac IHC detection (brown colour). Note that, regardless the fixative used, a similar staining pattern was obtained in B and C. Tissues were counterstained with PAS-haematoxylin. Scale bars = 10 µm. SC, Sertoli cells; SPG, spermatogonia; SPC, spermatocytes, rSPD I-V, early-stages (I-V) round spermatids; rSPD VI-VIII, late-stages (VI-VIII) round spermatids; eSPD, elongating spermatids, cSPD, condensing spermatids.

Figure 3. IHC analyses of H4ac on testicular biopsies from patients with testicular tumor. A H4ac H-scores (mean value ± SD) for each testicular cell type analyzed in controls and testicular tumor patients. The pattern of H4ac in normal spermatogenesis (N) and the internal technical control are represented with a soft line (in gray and orange, respectively). **B** Representative images of H4ac IHC detection (brown colour) in seminiferous tubule sections from testicular biopsies of patients with normal spermatogenesis (Normal), Teratoma (TER), Seminoma (SEM), Embryonal carcinoma (EmCA), Leydig cell tumor (LCT), and Spermatocytic tumor (SPCT). Increased H4ac levels are observed in the seminiferous tubules' cells from testicular tumor patients compared with the control group (normal spermatogenesis). Tissues were counterstained with PAS-haematoxylin. Scale bars = 125 µm (main images, left), 25 µm (magnifications, right). **C** Principal component analysis (PCA) of controls ("Normal", red dots) and patients affected by a tumor ("Tumor", blue dots). Principal component 1 (Comp.1, x axis) and principal component 2 (Comp.2, y axis) explain 60.89% and 17.09% of the variability of our data, respectively. Grey arrows scaled to the origin indicate the strength (loadings) of each variable. The tumor group, independently of the type of tumor, presents high H4ac H-scores in early spermatogenic stages and somatic

Sertoli cells. Individual patients are shown as listed in Supplementary Table 1, and only patients with complete spermatogenesis were considered for the analysis. PC1 = 0.45SC + 0.43SPG + 0.454SPC + 0.444rSPD(I-V) + 0.378rSPD(VI-VIII) - 0.216eSPD – 0.143cSPD. PC2 = 0.224SC – 0.318rSPD(I-V) - 0.442rSPD(VI-VIII) - 0.291eSPD – 0.75cSPD. *p<0.05, **p<0.01. SC, Sertoli cell; SPG, Spermatogonia; SPC, Spermatocyte; rSPD I-V, early-stages (I-V) round spermatids; rSPD VI-VIII, late-stages (VI-VIII) round spermatids; eSPD, elongating spermatids; cSPD, condensing spermatids.

Figure 4. Pipeline of the explanatory mechanistic hypothesis behind the altered H4ac levels found in the testicular cancer patients. Pathology data and predicted secreted proteins from the Human Protein Atlas (http://v20.proteinatlas.org) allowed us to identify 71 potentially secreted testicular cancer proteins with putative paracrine effects associated with signaling pathways involving cytokines and growth factors activating signal transduction through phosphorylation cascades. A schematic representation of the testicular tissue in both non-tumor (left side) and tumor (right side) conditions is shown. In non-cancerous conditions, normal epigenetic regulation of the spermatogenic process is carried out in the healthy seminiferous tubules, leading to a normal gene expression pattern and a proper NH-NP transition. In presence of testicular cancer, the homeostatic state would be potentially impaired due to the secretion of tumoral paracrine factors, leading to an increase of the H4ac levels in the different cell types. The altered H4ac levels could be also promoted by an epigenetic crosstalk of other potentially altered histone marks and through their effectors. The global epigenetic dysregulation during the spermatogenesis would give rise to altered gene expression patterns and a disrupted chromatin remodeling, resulting in an altered epigenetic cargo of the germ cells that would be further used for fertility preservation purposes. NH-NP, nucleohistone to nucleoprotamine transition; H4ac, histone H4 acetylation.

Supplementary Figure SI. Comparative IHC spermatogenic H4ac pattern in patients with normal spermatogenesis and patients affected by testicular tumor. The H4ac pattern described in patients with normal spermatogenesis (upper left), characterized by the significant changes observed along the spermatogenesis, is lost in the spermatogenesis of patients affected by the different types of testicular tumors. H4ac H-scores (mean value ± SD) are shown for each testicular cell type analyzed in controls and testicular tumor patients. *p<0.05, **p<0.01. SEM, Seminoma; EmCA, Embryonal carcinoma; TER, Teratoma; SPCT, Spermatocytic tumor; LCT, Leydig cell tumor; SC, Sertoli cell; SPG, Spermatogonia; SPC, Spermatocyte; rSPD, round spermatids; eSPD, elongating spermatids; cSPD, condensing spermatids.

Supplementary Figure SII. IHC analyses of H4ac on testicular biopsies from patients with spermatogenic impairments. A H4ac H-Scores (mean value ± SD) corresponding to each testicular cell type analyzed in the groups of patients with normal (N) and altered spermatogenesis (Hypospermatogenesis, Hp; Spermatogenic arrest, SA; and Sertoli cell-only Syndrome, SCOS). The progress of the spermatogenic H4ac pattern in normal spermatogenesis (N) and the internal technical control are represented with a soft line (in gray and orange, respectively). **B-E** Representative images of H4ac IHC detection (brown colour) in seminiferous tubule sections from testicular biopsies of patients with normal and altered spermatogenesis: Normal spermatogenesis (**B**), Hypospermatogenesis (**C**), Spermatogenic arrest (**D),** and Sertoli cell-only Syndrome (**E**). No significant differences were found compared to the control group. The spermatogenic cells from patients with spermatogenic impairments (in Hp and SA, when available) displayed the gradual increase in the H4ac levels observed in patients with normal spermatogenesis. Tissues were counterstained with PAS-haematoxylin. Scale bars = 100 µm. SC, Sertoli cell; SPG, Spermatogonia; SPC, Spermatocyte; rSPD I-V, early-stages (I-V) round spermatids; rSPD VI-VIII, late-stages (VI-VIII) round spermatids; eSPD, elongating spermatids; cSPD, condensing spermatids.

Supplementary Figure SIII. The impaired spermatogenic histone H4 acetylation pattern of testicular tumor patients is not dependent on the presence of seminiferous tubules with pre-invasive germ cell neoplasia *in situ* (GCNIS). Consecutive testicular sections were immunohistochemically stained with H4ac, PLAP, and CD117 in patients with seminoma (SEM), embryonal carcinoma (EmCA), teratoma (TER), spermatocytic tumor (SPCT), and Leydig cell tumor (LCT). PLAP and CD117 stainings allowed to discern the seminiferous tubules containing pre-invasive GCNIS, evidencing a positive pattern in SEM and EmCA. The increased H4ac levels in the seminiferous tubules observed in all groups of testicular cancer patients is independent of the presence or not of pre-invasive GCNIS. Tissues were counterstained with PAS-haematoxylin. Scale bars = 200 µm.

Supplementary Figure SIV. Implications of the potential tumor's paracrine effect on the healthy testicular tissue. Representation of a selection of enriched Gene Ontology (GO) annotations for biological processes (**A**) and molecular functions (**B**) identified in the predicted subset of secreted testicular tumor proteins ($p<0,05$ after Bonferroni correction). The number of proteins identified corresponding to each GO annotation is represented in the X axis. **C** Selection of significantly enriched pathways associated with the testicular tumor predicted secretome after Reactome analysis ($p<0,05$ after FDR correction). The horizontal axis represents the $-\log_{10}$(p-value) for each pathway.

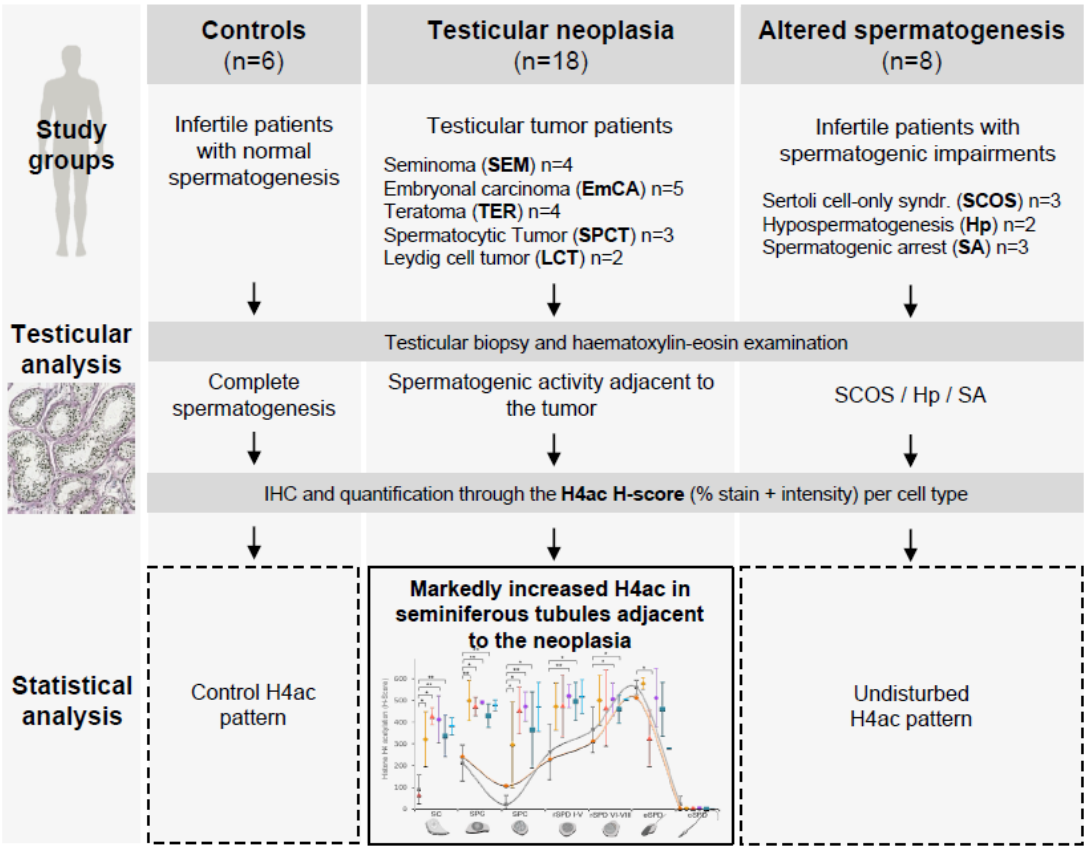
Controls
(n=6)
Testicular neoplasia
(n=18)
Altered spermatogenesis
(n=8)
Study groups
Infertile patients with normal spermatogenesis
Testicular tumor patients
Seminoma (SEM) n=4
Embryonal carcinoma (EmCA) n=5
Teratoma (TER) n=4
Spermatocytic Tumor (SPCT) n=3
Leydig cell tumor (LCT) n=2
Infertile patients with spermatogenic impairments
Sertoli cell-only syndr. (SCOS) n=3
Hypospermatogenesis (Hp) n=2
Spermatogenic arrest (SA) n=3
Testicular analysis
Testicular biopsy and haematoxylin-eosin examination
Complete spermatogenesis
Spermatogenic activity adjacent to the tumor
SCOS / Hp / SA
IHC and quantification through the H4ac H-score (% stain + intensity) per cell type
Statistical analysis
Control H4ac pattern
Markedly increased H4ac in seminiferous tubules adjacent to the neoplasia
Undisturbed H4ac pattern

Figure 1

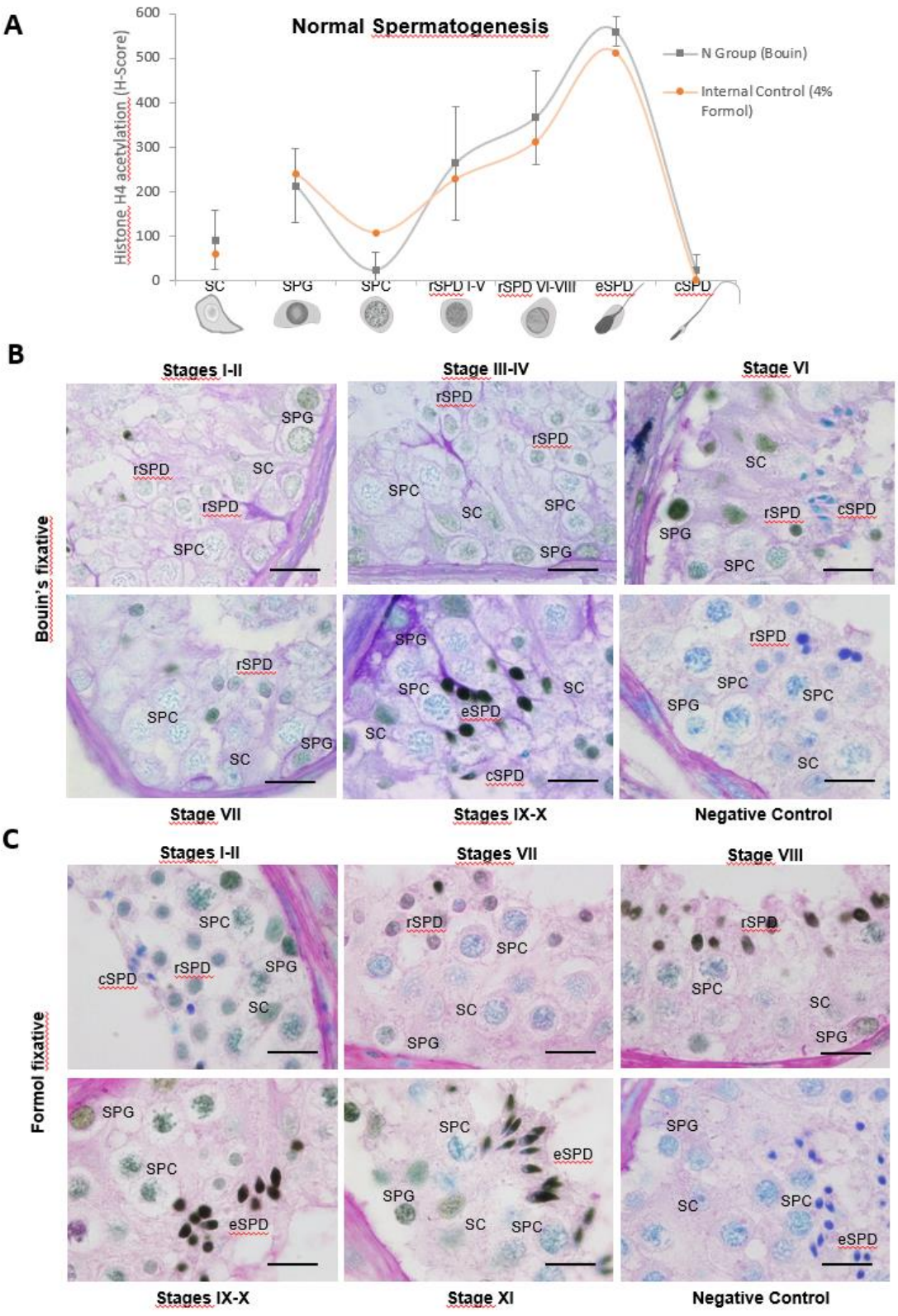
A
Normal Spermatogenesis
Histone H4 acetylation (H-Score)
600
500
400
300
200
100
0
SC
SPG
SPC
rSPD I-V
rSPD VI-VIII
eSPD
cSPD
N Group (Bouin)
Internal Control (4% Formol)
B
Bouin's fixative
Stages I-II
Stage III-IV
Stage VI
Stage VII
Stages IX-X
Negative Control
C
Formol fixative
Stages I-II
Stages VII
Stage VIII
Stages IX-X
Stage XI
Negative Control

Figure 2

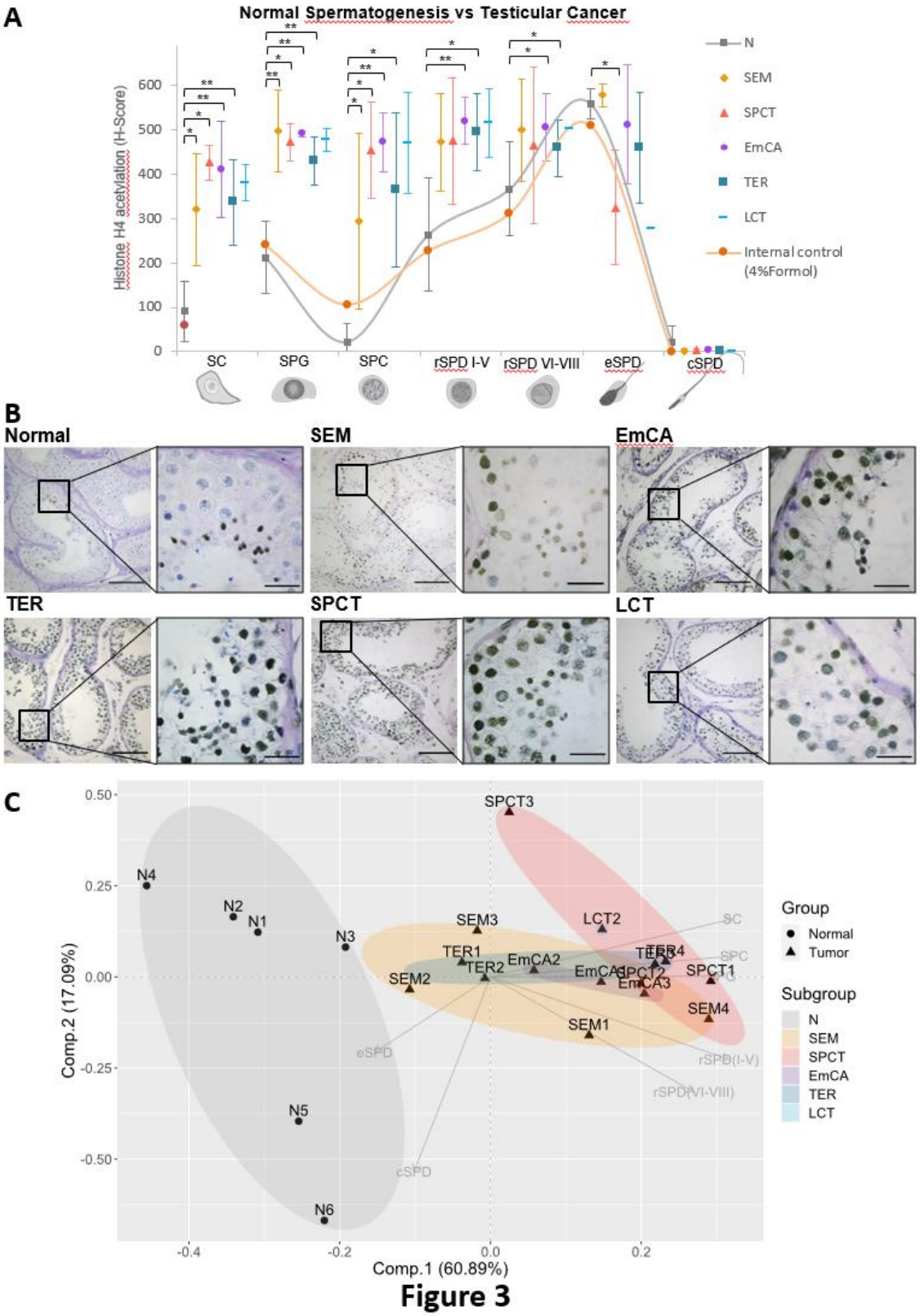
A
Normal Spermatogenesis vs Testicular Cancer
Histone H4 acetylation (H-Score)
0
100
200
300
400
500
600
SC
SPG
SPC
rSPD I-V
rSPD VI-VIII
eSPD
cSPD
N
SEM
SPCT
EmCA
TER
LCT
Internal control (4%Formol)
B
Normal
SEM
EmCA
TER
SPCT
LCT
C
Comp.2 (17.09%)
Comp.1 (60.89%)
0.50
0.25
0.00
-0.25
-0.50
-0.4
-0.2
0.0
0.2
N1
N2
N3
N4
N5
N6
SEM1
SEM2
SEM3
SEM4
TER1
TER2
EmCA2
EmCA3
LCT2
SPCT1
SPCT2
SPCT3
SC
SPC
eSPD
rSPD(I-V)
rSPD(VI-VIII)
cSPD
Group
Normal
Tumor
Subgroup
N
SEM
SPCT
EmCA
TER
LCT

Figure 3

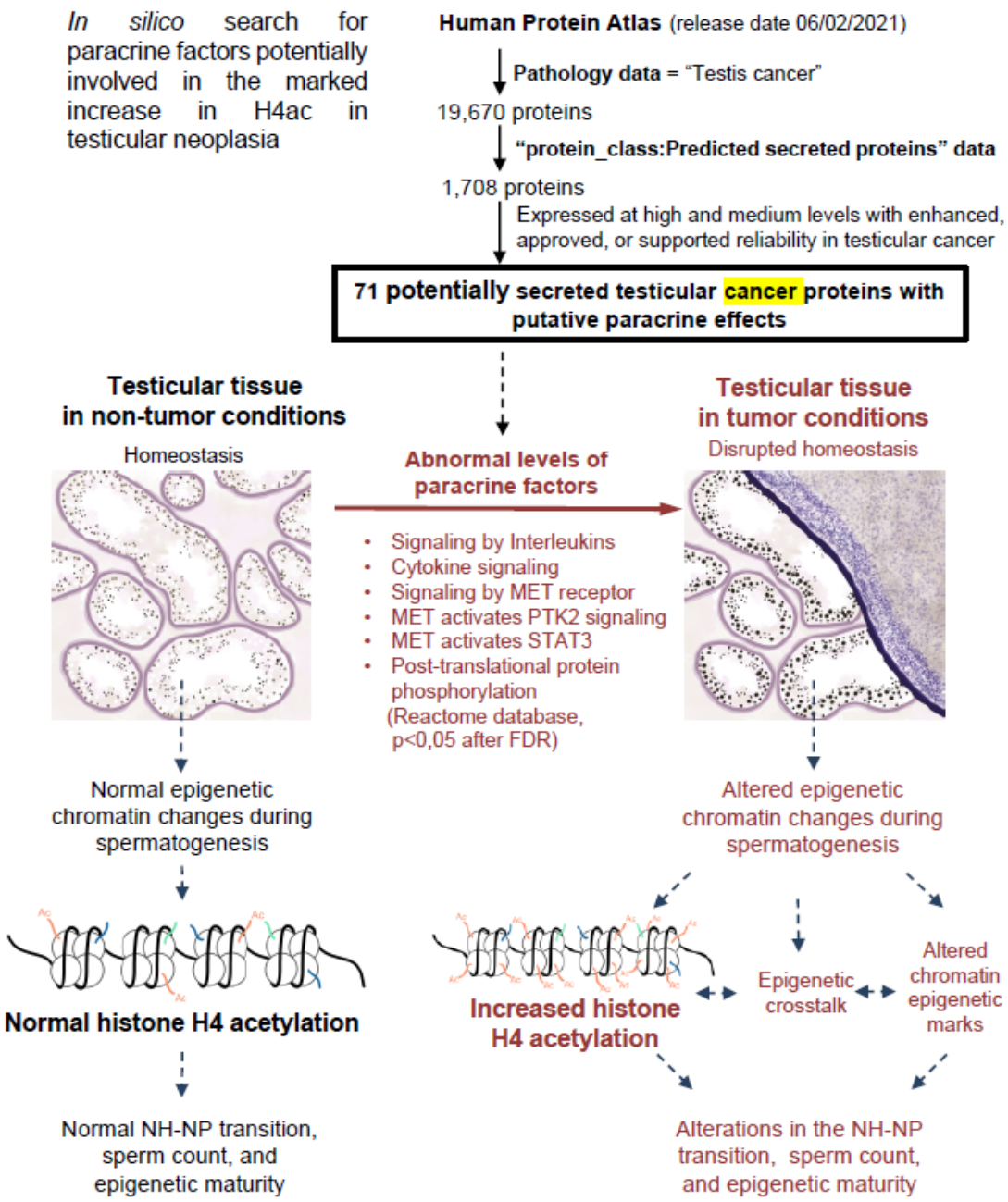
In silico search for paracrine factors potentially involved in the marked increase in H4ac in testicular neoplasia
Human Protein Atlas (release date 06/02/2021)
Pathology data = "Testis cancer"
19,670 proteins
"protein_class:Predicted secreted proteins" data
1,708 proteins
Expressed at high and medium levels with enhanced, approved, or supported reliability in testicular cancer
71 potentially secreted testicular cancer proteins with putative paracrine effects
Testicular tissue in non-tumor conditions
Homeostasis
Abnormal levels of paracrine factors
Signaling by Interleukins
Cytokine signaling
Signaling by MET receptor
MET activates PTK2 signaling
MET activates STAT3
Post-translational protein phosphorylation
(Reactome database, p<0,05 after FDR)
Testicular tissue in tumor conditions
Disrupted homeostasis
Normal epigenetic chromatin changes during spermatogenesis
Altered epigenetic chromatin changes during spermatogenesis
Normal histone H4 acetylation
Increased histone H4 acetylation
Epigenetic crosstalk
Altered chromatin epigenetic marks
Normal NH-NP transition, sperm count, and epigenetic maturity
Alterations in the NH-NP transition, sperm count, and epigenetic maturity

Figure 4