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Ki67 and LSD1 Expression on Testicular Germ Cell Tumors: correlation with pathological and clinical features

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Resumo

Introdução: Os tumores de células germinativas do testículo representam uma forma de cancro que acomete essencialmente homens jovens, sendo potencialmente tratável com intuito curativo. Contudo, uma vez que se associam a uma morbilidade significativamente alta, é impreterível definir a biologia destes tumores de forma a estabelecer a melhor abordagem terapêutica. Ki67, um marcador prognóstico envolvido em mecanismos de proliferação celular, tem demonstrado uma correlação heterogénea com os *outcomes* dos TCGT, impondo a necessidade de corroborar os resultados com *cohorts* maiores. LSD1, uma enzima epigenética envolvida no processo de malignidade, e recentemente relacionada com *checkpoints* imunológicos, representa um futuro alvo de fármacos epigenéticos, ferramentas inovadoras, as quais, combinadas com as terapêuticas já implementadas, poderão reduzir a morbilidade e melhorar o prognóstico dos TGCT.

Objetivos: Este estudo pretendeu avaliar a imunoexpressão do Ki67 e do LSD1 em todos os subtipos histológicos dos TGCT e correlacionar tal expressão com as características clinicopatológicas destes tumores. Houve ainda a intenção de corroborar os resultados com o conhecimento científico atual, recorrendo para tal a uma comparação com estudos *in silico*.

Material e métodos: Foram aplicadas técnicas de imunohistoquímica a 157 amostras de tumor para deteção da marcação nuclear do Ki67 e do LSD1, com processamento automático e manual, respetivamente. As lâminas imunomarcadas foram digitalizadas com o sistema VENTANA 200. A expressão dos dois marcadores foi avaliada através do sistema de análise digital de imagem (QuPath), que permitiu quantificação objetiva, cálculo do H-score e exclusão da positividade das células imunes infiltradoras e das células do estroma adjacente. A base de dados do The Cancer Genome Atlas (TCGA) foi usada com o objetivo de uma análise *in silico*.

Resultados: A percentagem média de núcleos positivos foi 15,2% para o Ki67 (IQR: 3.87-31.6) e 9,19% para o LSD1 (IQR: 2.98-16.0), com um H-score médio de 16,8 (IQR: 4.45-37.8) e 9,27 (IQR: 3.22-16.3), respetivamente. Verificou-se uma correlação significativa entre os valores de H-score do Ki67 e do LSD1 ($r_s = 0.182$, $p=0.037$). De acordo com as características clinicopatológicas, entre as quais o estadio da doença, invasão vascular, a classificação prognóstica IGCCCG e o tempo de recidiva/progressão livre de doença, a expressão de ambos os marcadores não foi significativamente diferente. Os resultados obtidos corroboraram o proposto pela análise *in silico.*

Discussão e conclusão: Apesar de estudos prévios terem sugerido um papel prognóstico significativo para o Ki67, através de uma quantificação não digital, estimada pelo olhar do patologista, o presente estudo não demonstrou tal efeito. Ainda que este estudo não tenha demonstrado um impacto significativo no prognóstico, os TGCT expressaram o LSD1 de forma

global, o que poderá demonstrar sensibilidade aos inibidores do LSD1, propondo a necessidade de estudos quanto à sua relação com o microambiente tumoral e infiltração imunológica dos TGCT.

Palavras-chave: Tumores de células germinativas; Cancro de Testículo; Biomarcadores; Histopatologia; Prognóstico; Ki67; LSD1

Abstract

Introduction: TGCTs represent a model of curable disease afflicting especially young men, with an overall good prognosis. However, given the associated significant morbidity, defining tumor biological characteristics is crucial to increase current knowledge and tailor the best clinical management. Ki67, involved in cell proliferation and a potential prognostic marker, still exhibits heterogenous correlations with TGCT outcomes, thus bringing the need of corroboration with larger cohorts in clinical practice. LSD1, an epigenetic enzyme involved in malignant behavior, and recently related to immune key checkpoints, represents a future target for epigenetic drugs, which are a possible novel tool that, combined with standard therapy, may lower morbidity, and improve TGCT prognosis.

Objectives: This study aimed to assess Ki67 and LSD1 immunoexpression among all TGCT histological subtypes and correlate such expression with clinicopathological features. Results were compared with an *in silico* analysis of available databases, to corroborate our findings.

Material and methods: immunohistochemistry for Ki67 and LSD1 was carried out in a representative cohort of 157 TGCT tumor samples, previously characterized and validated. All slides were scanned using VENTANA DP200 system. The expression of both markers was assessed using a digital image analysis system (QuPath), allowing for objective quantification, H-score calculation and elimination of positivity in infiltrating immune and stromal cells. The Cancer Genome Atlas (TCGA) database was used for carrying out the *in silico* analysis.

Results: Median percentage of positive nuclei was 15.2% for Ki67 (IQR: 3.87-31.6) and 9.19% for LSD1 (IQR: 2.98-16.0), with a median H-score of 16.8 (IQR: 4.45-37.8). and 9.27 (IQR: 3.22-16.3), respectively. There was a significant positive correlation between Ki67 and LSD1 H-scores (rs = 0.182, p=0.037). Ki67 positive percentage and H-score differed significantly between seminomas and nonseminomas (p=0.0316 and 0.0113, respectively), being higher in the latter. Expression was not significantly different according to clinicopathological features, including stage, IGCCCG prognosis-based system, or relapse/progression-free survival, which was corroborated by in silico analysis.

Discussion and conclusion: Despite few individual studies that may have suggested significant utility in evaluating Ki67 through subjective “eye-ball” estimation, our study, making use of digital imaging analysis and precise quantification, does not confirm such utility of these biomarkers in a daily practice cohort. Although not affecting patient outcome in our cohort, LSD1 is expressed overall in TGCTs, which could mean sensitivity to LSD1 inhibitors and should lead to study of its interaction with TGCTs microenvironment and immune infiltration.

Keywords: Germ Cell Tumors; Testicular cancer; Biomarkers; Histopathology; Prognosis; Ki67; LDS1

Lista de abreviaturas e siglas

AFP: alpha-fetoprotein
AJCC: American Joint Committee on Cancer
A-NED: alive with no evidence of disease
AWD: alive with disease
β- hCG: Human chorionic gonadotropin β
CH: choriocarcinoma
CT: chemotherapy
DFD: died from disease
D-NED: died with no evidence of disease
DSS: disease-specific survival
EC: embryonal carcinoma
GCNIS: germ cell neoplasia in situ
GCT: germ cell tumor
HR: hazard ratio
IGCCCG: International Germ Cell Cancer Collaborative Group
IHC: immunohistochemistry
IPO Porto: Instituto Português de Oncologia do Porto
IQR: interquartile range
LDH: lactate dehydrogenase
lncRNA: long noncoding RNA
LSD1: lysine-specific demethylase 1A
mRNA: messenger RNA
MAO: Monoamine Oxidase
NS: nonseminoma
PCR: polymerase chain reaction
RT: radiotherapy
RT-PCR: real time polymerase chain reaction
SE: seminoma
siRNA: small interfering RNA
TCGA: The Cancer Genome Atlas
TE: teratoma
TGCT: testicular germ cell tumor
US: ultrasonography
WHO: World Health Organization
YST: yolk sac tumor

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Introduction

Although rare, representing only 1% of male malignancies worldwide, testicular germ cell tumors (TGCTs) are the most common solid testicular neoplasms (95%) in men between the ages of 20 and 34 years, presenting a rising global incidence over the past decades [1, 2]. This is thought to be due to environmental changes related to Western lifestyle. Actually, the study of risk factors for TGCTs is under expansion; these cancers not only have a strong genetic component (with personal and family history of TGCTs substantially increasing the risk) but also have a strong environmental link, with risk factors including those related to some form of subfertility, such as cryptorchidism, Klinefelter's syndrome, hypospadias, testicular atrophy, perinatal factors, and others such as occupational exposure, trauma, HIV infection and lifestyle (including smoking, diet and low physical exercise) [2-4].

TGCTs exhibit a striking complex and heterogenous histology [5]. Actually, germ cell tumors overall are fascinating and very challenging cancers, that may arise not only in the gonads (ovary and testis) but also in extragonadal sites, being related to phenomena of embryonic and germ development (the reason why they are referred to as "developmental cancers") [1, 2, 6]. In practice, focusing on the so-called type II TGCTs - within the developmental-based classification of these neoplasms, the ones presenting in young-adults and by far the most frequent, with malignant behavior - they are divided into two main histologic categories, in the most recent WHO classification system, the seminomas (SE) and nonseminomas (NS) [1, 2, 7, 8]. The SE is the most common subtype, which makes up about half of all testicular tumors, with an incidence that peaks at around 35 years. SE are followed by Mixed Tumors (any tumor with at least two distinct subtypes present, which may include SE components), which compose the majority of the NS category. NSs overall tend to be diagnosed around 5-10 years earlier than SE [1, 3, 9]. NS comprise a complex array of subtypes, including embryonal carcinoma (EC), the most common NS occurring in the pure form, and choriocarcinoma (CH), yolk sac tumor (YST) and teratoma (TE), very rarely seen in their pure form. In fact, only less than 10% of TGCT are "pure", which means of a single tissue type. Importantly, germ cell neoplasia *in situ* (GCNIS) is the common precursor lesion of type II TGCTs, including both SE and NS, that can be detected in the adjacent parenchyma of most invasive tumors [1, 7-9].

At clinical presentation, most patients notice swelling, hardness or palpable nodules that may be painful or not [4]. However, as this presentation can also translate pathologies other than malignant tumors, such as infectious epididymitis or orchitis, further work-up is needed [4]. This includes scrotal

ultrasound and measurement of serum tumor markers – alpha-fetoprotein (AFP), human chorionic gonadotropin β (β-hCG) and lactate dehydrogenase (LDH) [4, 10]. These can support the diagnosis when testicular cancer is suspected, and should be serially measured during follow-up, contributing as well to staging and risk assessment and, later, to evaluation of response to therapy and early detection of relapse [9]. However, most importantly, these serum markers have important limitations; these are elevated in only 60% of patients at diagnosis and are dependent on histological subtypes present in the tumor [10]. Also, they may be elevated in many other medical conditions, hampering their sensitivity and specificity. For this reason, the definitive diagnosis of a TGCT can only be performed upon histological examination of the orchiectomy specimen [10].

Overall, TGCTs represent a model of curable disease, with the vast majority of patients showing good outcome. Not only most patients present with stage I disease (around 70%), but also around 75% of these are cured with orchiectomy alone, without need for subsequent adjuvant treatments, which is particularly true for SE [8, 11, 12]. The good prognosis is also partly explained by the extreme sensitivity of these cancers to cisplatin-based chemotherapy, as well as radiosensitivity, due to their unique molecular background, especially for those who require systemic therapy [7, 13] .

However, still a significant group of patients relapse, most frequently within the first 2 year after initial diagnosis, with approximately 5% of them occurring after 5 years or even after more than 10 years [12, 14]. Thus, inguinal orchiectomy followed by close surveillance may not be enough, implying the need for further treatments, which leads to higher morbidity [12]. Some patients even undergo through adjuvant chemotherapy *ad initio* to avoid disease relapse, which has been proven to be effective, with more than 90% of relapses being prevented [8, 15]. The main consequences of TGCT treatments include cardiovascular disease, second malignances, infertility, neuropathies, and emotional disorders [16, 17]. The negative impact on reproductive function is related to fertility impairment after orchidectomy, radiotherapy (RT), and chemotherapy (CT), mostly on short term, with the latter being dose-cumulative-dependent. Due to significant azoospermia, oligospermia and teratogenicity, semen preservation should be offered ideally before any treatment, or, at least, prior to CT and/or RT. Pre-treatment fertility assessment is also advised [15]. RT increases the risk of radiation-induced secondary non-germ cell malignancies, thus limiting its role for exceptional cases that are not suitable for CT, surveillance alone or elderly patients [15, 17, 18].

Hence, this creates a clinical dilemma, which is to determine which patients actually do benefit from cytotoxic chemotherapy in adjuvant setting to avoid disease relapse, discriminating them from those that can be safely put on surveillance, a strategy that is being increasingly preferred.

This is of extreme importance since these are very young patients, that most likely will have to endure the already designated short and long-term side effects from chemotherapy during their lifetime [11, 19, 20]. Furthermore, a significant subgroup of patients develops cisplatin-resistant disease, implicating challenging cases, and most of the TGCT related deaths [20-22]. Because of this, it is of paramount importance to identify reliable prognostic biomarkers that can predict patients that will most likely relapse, aiding in risk-stratification, particularly of stage I disease and allowing to better tailor subsequent clinical management [11, 20].

To date, the risk stratification of those patients has relied on clinicopathological parameters, such as tumor size and *rete testis* invasion for SEs and vascular invasion and EC proportion for NS [11, 12]. Vascular invasion is, so far, the most discriminative biomarker, and its value has been recently confirmed [11, 14, 23]. The prognostic significance of the others to guide treatment decisions is still under debate [14].

There have been attempts to come across further prognostic biomarkers. Importantly, such biomarker should be easily investigated with widely available techniques and its assessment should also be reproducible. For this, immunohistochemistry may be very relevant, since it is available in all Pathology Departments and can be performed at low cost with optimal positive and negative controls on histological slides already obtained for proper histological diagnosis [24]. Consequently, some biomarkers have been assessed, including markers related to cell proliferation (Ki67) and some related to immune microenvironment (CXCL12, CXCR4, beta-catenin). Such biomarkers showed a correlation with relapse-free survival rates, however its statistical significance changed when combined in multivariable analysis with histopathological features, such as vascular invasion [12].

Regarding Ki67, given its role as a proliferative status indicator of tumor cells, is a biomarker widely used to assess tumor proliferation rate, which is related to aggressiveness in some malignancies, as well as worth prognosis [25-30]. Some studies demonstrated that a poorer prognosis was associated with higher immunoexpression scores, with cutoffs between 40 and 70%, however others could not validate these cutoffs [12, 16, 23, 26, 31]. Despite this controversy, recent studies showed that, when focusing solely on patients without vascular invasion, Ki67 scoring of more than 50% could identify a group of patients with worse clinical outcomes. Still, such finding needs validation in much larger cohorts [12] .

Another concern which is also related to biomarkers is finding those that could predict response to more targeted (and possibly less toxic) therapies. The epigenetic landscape of TGCTs and its influence also on tumor aggressiveness and response/resistance to cisplatin treatment make epidrugs promising agents [8, 20-22, 32, 33]. In result of epigenetic targeted therapy studies in TGCT, other possible biomarkers have been identified, such has LDS1. LSD1 has been shown to be significantly elevated in pluripotent germ cell tumors, including TE, EC and SE [34]. Notably, Wang et al. demonstrated that such levels were considerably higher in TGCT cell lines than in those originating from somatic cells and were also higher in TGCT tissue than in normal testis tissue [35]. High levels of LSD1 are associated with overexpression of pluripotency genes, involved in cell growth and proliferation. When subjected to targeted therapy for LSD1, TGCT cell lines developed downregulation of those genes and growth arrest. Such finding can indicate a target for non-platinum-based therapies, with less side effects, which may translate a better prognosis, making LSD1 a possible prognostic epigenetic biomarker [35].

Interestingly, LSD1 and Ki67 have been positively correlated in a study on mantle cell lymphoma (MCL) and determined a poorer outcome, as the proliferative effect is enhanced. This correlation was only described by Zou et. al, which should be validated in further studies, especially in tissues with high expression of such molecules, in which it may become of additional prognostic information [36].

Other biomarkers have already been studied, such as p53 and MDM2. Cases with higher MDM2 immunoexpression were correlated with a statistically significant trend for platinum-based therapy resistance and recurrence [7]. Moreover, an association with the histologic type was found, where EC and CH showed higher levels of expression [7]. Although studies report distinct results regarding the association of p53 expression with stage at diagnosis, the fact that both p53 and MDM2 higher levels represent a more clinically aggressive histological subtype may be an indication that p53 is related to tumor progression, as proposed by Ehteshami et al in 1996. As the majority of TGCTs studies are based on subjective quantitative estimations of MDM2 expression, data regarding this prognostic biomarker remains controversial [7].

Due to the rich histological immune infiltration of TGCTs, immune checkpoints PD-L1 and CTLA-4 have also been assessed, focusing on a potential biomarker for targeted therapy and a possible prognostic biomarker [20, 37]. In fact, the majority of those tumors exhibited a positive expression of both immune checkpoint markers (85,5% and 96,3%) in infiltrating immune cells across all subtypes [5]. PD-L1 immunoexpression in immune cells was demonstrated to have an independent impact on

relapse-free survival rates [37] and patients with absent of PD-L1 positive infiltrating cells exhibited significantly worse prognosis [5]. To date, Lobo et. al demonstrated an association between PD-L1 and CTLA-4 immunoexpression and metastatic TGCTs (100% and 88,2% of the metastases, respectively), which can indicate the use of targeted agents in metastatic disease [5]. However, trials must be conducted given the failure of previous studies employing such single-agent therapies, indicating that combination (namely with epigenetic therapies) may be the way to go [5, 20]. Nonetheless, the combination of all these proposed prognostic factors has also been associated with a false prediction of higher stages and relapse of the disease, leading to over treatment with adjuvant therapy. Thus, the application of such prognostic markers still needs to be further optimized for the purpose of finding an optimal combination of the above to implement in the clinic [9].

In this study we aimed to determine the prognostic value of Ki67 (a marker of cell proliferation) and LSD1 (a histone lysine demethylase) in a well-defined cohort of TGCT patients, with the goal of finding a correlation with their outcomes. In addition, we attempted to verify a correlation between both markers related to tumor proliferation, and to assess the overall expression of LSD1 in TGCT subtypes, since this is a potential target for non-platinum targeted therapies. Furthermore, statistical analysis was conducted to compare with the results of a *in silico* analysis, with data from cBioPortal, in order to corroborate our findings. It is of cardinal importance to notice that the present study used QuPath as the image analysis software, a new tool emerging as a key component of modern scientific community.

Methods

Patients and samples

In this retrospective study, a cohort of 157 TGCT patients (previously validated in [3]), diagnosed and treated by the same multidisciplinary team at the Portuguese Oncology Institute of Porto – Portugal (IPO Porto) between the years of 2005 and 2016, was assessed. Clinical and histological data was reviewed according to the guidelines on the *American Joint Committee on Cancer* (AJCC) staging manual – 8th edition and the 2016 *World Health Organization* (WHO) *Classification of Tumour of the Urinary System and Male Genital Organs.* Patients with metastatic disease were further categorized according to the *International Germ Cell Cancer Collaborative Group* (IGCCCG)

prognostic system. Follow-up was last updated on January 2021. Formalin-fixed and paraffin-embedded TGCT samples were selected, of which representative blocks (containing at least 1cm^2 of tumor and interface with adjacent non-involved parenchyma) were thoroughly reviewed by a Pathologist experienced in TGCT evaluation. Four µm-thick slides were ordered for immunostaining. Consultation cases, type I or III TGCT cases, cases with no adequate representative blocks available, and cases with non-evaluable sample due to IHC problems (*i.e.,* of the manually performed LSD1 IHC) or to software error after scanning (*i.e.,* non-readable files on QuPath) were excluded from this study, downing the samples from 183 to 157 tumors. Each sample was assessed globally. In the presence of Mixed Tumors, each individual subtype was analyzed separately, resulting in a total of 221 individual TGCT histological samples.
This study is within the scope of a larger research project approved by the Ethics Committee of IPO Porto (Comissão da Ética para a Saúde, CES-IPO-1-018)

Immunohistochemistry

Ki67 immunohistochemistry was performed in automated fashion using Ventana platform as performed routinely for diagnostic purposes in the Department of Pathology, subjected to validation and quality control procedures. MIB-1 clone (DAKO) at 1:150 dilution was incubated for 1h and antigenic recovery was performed with cc1 for 36 minutes. Tissue of normal tonsil was used as positive control.

In case of LSD1, antigenic recovery was performed with EDTA buffer in a 30 minutes water-bath and endogenous peroxidase activity was blocked by hydrogen peroxide in 3% methanol. Nonspecific reactions were blocked with normal horse serum (dilution 1:50). Slides were incubated for one hour at room temperature with primary LSD1 monoclonal antibody (clone #2139, Cell Signaling; dilution 1:225). Both post-primary antibody and polymer were incubated for 30 minutes at room temperature (Novolink^{TM} Polymer Detection System – Novocastra, Product No. RE7150-K, Leica Biosystems, Newcastle, UK). Diaminobenzidine was used as chromogen and hematoxylin for nuclear counterstaining. Tissue of normal prostate was used as positive control.

Digital pathology analysis

The immunoexpression of Ki67 and LSD1 was assessed using a digital image analysis system (QuPath, Version 0.2.3, QuPath developers, The University of Edinburgh, Scotland) for nuclear immunostaining quantification, after proper scanning performed by VENTANA DP200 system. The

detailed QuPath assessment and study protocol is represented in Figure 1. Immunoexpression pattern was evaluated overall (per tumor) and also separately for each TGCT component (i.e., independently for each histological component within Mixed Tumors). Briefly, QuPath integrated tools were used for area selection within the tumor, excluding GCNIS, stromal, and inflammatory cells. The manual selection tools allowed for clear "cell-by-cell" separation of highly intricated tumor components such as EC and YST, and accurate elimination of non-tumor cells. A total cell count of 50,000 tumor cells was attempted for every tumor sample. Within each selected area, positive cell analysis was conducted, in which, after setting nuclear, cell and analysis parameters, such as positive intensity threshold, QuPath automatically attributed expression score annotations to every cell (blue for no expression, yellow for low expression (+1), orange for moderate (+2), and red for high expression (+3)). From the annotation analysis results, number of detected cells, number of positive detected cells (+1, +2, and +3), number of negative cells, positive cell percentage, and H-score (product of positive nuclei per each intensity score) were collected. Importantly, for mixed tumors, the analysis included two contexts: first, a case-basis analysis, where randomly selected areas of the whole tumor area with representation of all components present in the slide were read; and second a component-basis analysis, where labels were attached to each tumor component in the slide (SE, EC, YST, CH, or TE), and analysis was carried out separately, creating multiple analysis metrics (as much as the number of components present in the slide). Illustrative examples of Ki67 and LSD1 immunoexpression and analysis are depicted in Figure 2.

***In silico* analysis**

To find the most relevant DNA modifying enzymes to be subsequently validated in our own cohort, we explored The Cancer Genome Atlas (TCGA) database using the publicly available cBioPortal tool 38. The PanCancer Atlas database was used and mRNA expression values, according to tumor histological subtype and stage, were imported for *MIB1* and *LSD1* in the cohort of 149 patients.

Statistical analysis

Data was tabulated using Microsoft Excel for Office 365, and statistical analysis was performed using GraphPad Prism for Mac (version 9.1.1, GraphPad Software, San Diego, CA, USA) and SPSS Statistics for Mac (version 27.0.1.0, SPSS Inc, Chicago, IL, USA). The distribution of continuous variables between groups was compared using nonparametric Mann-Whitney and Kruskal-Wallis

tests. The same tests were applied for the analysis of BioPortal data. H-score correlation between Ki67 and LSD1 was assessed using Spearman test. Relapse/ progression free survival analysis was tested considering the 50^{th} and 75^{th} percentiles ($P < 50$, $P \geq 50$ $P < 75$ and $P \geq 75$), where survival curves were computed using the Kaplan-Meier method and log-rank test. Associations between categorical variables were assessed through Chi-square test. Statistical significance was set as $p<0.05$, with 95% confidence intervals (CI). Statistical significance was annotated as such: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Cohort characterization

As mentioned, in total, 221 primary tumor components were studied, corresponding to a cohort of 157 patients. A summary of all demographic and clinicopathological data is presented in Table I.
Median age at diagnosis was 31 years (interquartile range - IQR: 25-36) and median follow-up time was 69.0 months (95% CI 57.3-80.7). 52.2% of TGCT patients disclosed pure SE, followed by 38.9% having Mixed Tumors, in accordance with literature. Pure EC and pure TE represented 7.0% and 1.9% of TGCT tumors, respectively. YST and CH only occurred as part of Mixed Tumors. Regarding individual tumor components, 43.5% were identified as SE, 25.3% as EC, 14.5% as TE, 12.2% as YST, and 4.5% as CH. Median largest dimension of each tumor was 4,7 cm, with a minimum and maximum of 0,7 cm and 18 cm, respectively and vascular invasion was verified in 49.7 % of all cases. 64.1% of all cases were stage I, 20.5% stage II, and 15.4% stage III.
In total, 56 patients presented with metastatic disease, 75% corresponding to the IGCCCG good prognosis group, 14.3% to the intermediate group, and 10.7% to the poor prognosis group.

Immunoexpression of Ki67 and LSD1

An average 83040 (range: 2733-470056) and 76861 (range: 2231-496563) cells per sample were evaluated for Ki67 and LSD1, respectively.

Median percentage of positive nuclei for Ki67 was 15.2% (IQR: 3.87-31.6), with a median H-score of 16.8 (IQR: 4.45-37.8). Median percentage of positive nuclei for LSD1 was 9.19 % (IQR: 2.98-16.0),

with a median H-score of 9.27 (IQR: 3.22-16.3). There was a significant positive (weak) correlation between Ki67 and LSD1 H-scores (rs = 0.182, p=0.037).

Expression of Ki67 was significantly higher in NS compared to SE (Supplementary Table I, Figure 3), both considering percentage of positive nuclei (p=0.0316) and H-score (0.0113). Although LSD1 expression was overall higher in NS, it did not achieve statistical significance (p=0.1973 and p=0.1630 for percentage of positive nuclei and for H-score, respectively) (Supplementary Table I, Figure 3). Similar results occurred for the expression between pure forms (SE, EC, and TE) and Mixed type Tumors, with no statistical significance (p=0.1404 and p=0.1677 for percentage of Ki67 positive nuclei and for H-score, and p=0.9218 and p=0.8658 for LSD1 percentage of positive nuclei and for H-score, respectively), (Supplementary Figure 1)).

When considering individual tumor subtypes, expression of Ki67 was overall higher in CH, followed by EC, however, differences towards other components did not achieve significance (p=0.0548 and p=0.1292 for percentage of positive nuclei and H-score, respectively) (Supplementary Table I, Figure 4). Similarly, for LSD1, expression was higher in TE, CH and EC, but differences did not achieve significance (p=0.3275 and p=0.2824 for percentage of positive nuclei and H-score, respectively) (Supplementary Table I, Figure 4).

Association with clinicopathological features:

The expression of both Ki67 and LSD1 was not significantly associated with clinicopathological features, including stage of disease, the IGCCCG prognosis-based system and vascular invasion. The same analyses were computed individually only for SE and only for NS subgroups of patients, again depicting no statistically significant differences (Table II).

Regarding disease relapse/progression, expression of both Ki67 and LSD1 did not significantly associate with the event of relapse and there were no significant differences regarding relapse/progression-free survival (Supplementary Figure 2).

Survival analysis

A total of 10 patients had disease relapse, 9 of which relapsed in the first 2 years (median of 7 months, range: 0-14 months). Based on available data from the last follow-up, three patients died, two of them from the disease (DFD) and one from gastric cancer, with no evidence of disease (D-NED), and three patients were alive with disease (AWD) (Table I).

Using the P50 cutoff, relapse/progression-free survival did not differ significantly according to Ki67 or LSD1 H-score (p=0.457 and p=0.395, respectively). The same was verified using the P75 cutoff, with p=0.642 for Ki67 expression and p=0.689 for LSD1 (Supplementary Figure 2).

***In silico* study – cBioPortal data**

Corroborating our immunohistochemistry findings, in silico analysis of TCGA database showed no significant difference in the expression of these markers between different histological subtypes or according to AJCC disease stage (Table III).

Discussion

The present study used QuPath, short for Quantitative Pathology, for immunohistochemistry analysis. This software is a new platform created for image analysis, with an increasing interest in oncology research, whereas the key points that support the need for digital diagnosis include: the need for accurate biomarker analysis in leading reference hospitals, the need for a cost-effective tool, with reproducible, consistent and accurate results, universally applicable, and the requirement for solutions allowing remote pathology diagnosis, for instance, in the context of pandemics [39]. QuPath has been already applied in the elucidation of immune-evasive tumor microenvironmental mechanisms, in prognostic assessment of PD-L1 positiveness in patients with head and neck squamous cell carcinoma and in the prediction of RNA expression in unclassifiable or heterogenous cases of colorectal cancer [39-41]. This is the first study conducted in TGCT using QuPath, and one of the very few performed with this software in Portugal so far.

Initial studies indicated that Ki67, originally identified in the 1980s, is detected in dividing cells (G1, S, G2 and M phase) and not in quiescent cells (G0 phase) [27, 30, 42]. Its levels increase early in mitosis, decreasing in later phases of this process and in G1 and S phases [29, 42]. Recently, Ki67 has been shown to participate in ribosomal biogenesis, heterochromatin organization, and mitotic chromosome separation as well [28]. Thus, it is reasonable to assume that Ki67 reflects the proliferative activity of a cell population [43]. As Ki67 antigen analysis required fresh tissue processing, Gerdes et al. developed a monoclonal antibody, MIB-1, able to detect a Ki67 equivalent antigen in formalin-fixed paraffin-embedded tissues [30, 42]. In our study, it was possible to perform Ki67 immunohistochemistry in an automated fashion using a MIB-1 clone.

Despite poor understanding of the function and dynamic of Ki67, this protein is widely used as a proliferation index, which is assessed trough immunohistochemistry in tumor sections [44]. Many authors propose that the proliferative activity appraisal in several malignant tumor correlates with aggressiveness, progression, and metastatic behavior [43]. Consequently, Ki67 positive nuclei scores may predict prognosis, as well as the potential response to treatment and its benefit [44, 45]. The reliability of this marker has been shown in several types of cancer, such as of the breast, lung, prostate, cervix, soft tissue, and central nervous system [29, 44]. It is actually part of routine assessment of breast cancer and neuroendocrine tumors in routine diagnostics [42, 44].

Thus, to evaluate tumor aggressiveness, eventual outcomes and select the adequate treatment, Ki67 pretherapeutic assessment is becoming highly important for some cancers. According to the Ki67 labelling index, tumors can be classified as low, intermediate, and highly proliferating, with corresponding percentages varying with the types of cancers and respective histology [29]. Recent studies suggested that labeling a tumor as Ki67 positive or negative is an oversimplification, once the expression level of this marker is influenced by the time spent is each cell-cycle phase, namely the quiescence [45]. Thus, the level of expression (score) holds much more value in proliferation assessment [45]. The main reason for its proposed prognostic value is that Ki67 has been proven to correlate with the metastasis, tumor grade and the clinical stage of tumors, constituting an independent risk factor for survival rate for each stage [29]. As tumor proliferative activity has been correlated with occult metastatic disease in patients with low stage NS, Ki67 staining has been used to assess metastatic disease [43, 46].

However, in spite of statistically significant differences between patients with or without metastatic NS, Ki67 staining still has not shown clinical application to predict patients at high risk for metastasis [30.] This had been explained by the lack of precise discrimination between risk groups with highly proliferating tumors, such as NS, since Ki67 recognizes all cells that have entered the cell cycle [30]. Our study corroborated such findings, showing no significant association between Ki67 positive nuclei percentage or H-score and disease stage (Table II). Our mean Ki67 positive nuclei percentage was 15,2%, 19.8% for NS, which, according to previous studies with 50% to 100% Ki67 positively stained cells are much lower values [30]. This may be due to the antibody clone used and to the more precise digital methodology employed, with elimination of staining from infiltrating immune cells and stromal cells, which contribute substantially to the overall staining observed in tumor sections. Besides, our data was validated by the *in silico* analysis of cBioPortal, also showing no differences in expression according to stage (Table III). Although we showed higher Ki67 expression in NS

compared to SE in our study in line with the higher proliferative rates of more aggressive NS tumors, this was not observed in the analysis of TCGA data, which may be due to assessment of mRNA data only (compared to protein immunoexpression in our study) and to differences in cohort composition. Interestingly, higher expression levels were overall seen in highly aggressive tumor components, CH, and EC, which are frequently of poor prognosis. This may reflect some inner aggressiveness of these tumors related to their biology, but our results of Ki67 staining distribution among the several subtypes show that TGCTs are indeed highly heterogeneous, and a wide proportion of proliferating cells may be found in these tumors (Supplementary Table I, Figure 4).

Studies have proposed that Ki67 may also be a predictive biomarker that identifies subpopulations of patients who might benefit and respond to a given therapy. In example, Nishimura et al. found that patients with breast cancer with high levels of Ki67 revealed higher pathological complete response rates, which means that tumors with high cell proliferation (Ki67 > 25%) may be better candidates for neoadjuvant chemotherapy [47]. Regarding TGCT, Ki67 has not been useful in predicting a high risk group, but, interestingly, the assessment of tumor cell proliferation has been able to classify subgroups of patients at very low risk of metastatic disease, with an accuracy of 90% [30, 31].

Nevertheless, among several studies, Ki67 scoring in some cancers, including TGCT, is still controversial, partly because of the considerably heterogenous cutoffs, study, laboratorial and analytical methods [42, 44, 48]. Immunohistochemistry is a low-cost demonstration of a particular antigen expression in a tissue section, applying an antibody that targets that antigen, usually a protein [49]. The entire process implicates several steps, that include the selection of the specimen format and fixation - dependent on factors such as penetration, concentration, duration, and temperature -, the sectioning through microtomy, and antigen/epitope retrieval – with different protocols at disposition [49, 50]. The assessment of a marker expression also depends on the optimization of the chosen antibody, that varies according to the primary antibody concentrations, epitope retrieval solutions and its testing on several expected positive tissues [49]. Hence, it is logical that such assessments will differ among distinct laboratories, providing a full range of results. Given the many detection systems and interpretation methods, counting with the defined cutoffs and eyeball versus digital evaluation, the results among numerous articles on the same fields may differ in a way that precludes proper and definitive conclusions. For instance, considering the methodology of studies in TGCTs, the main differences start with the IHC, conducted in some by standard manual technics [23, 30, 31, 43, 51], while others applied automated methods, where the protocol was mechanically controlled [12, 16]. The type of sample used for assessment is also a

significant factor that may change the results. While some articles used whole tissue slides obtained with few µm sections of a representative tissue block [12, 23, 31, 43, 51], others used tissue microarrays (TMA) created with the obtained tissue, and further reembedded in new paraffin blocks in group with other cases [26]. Although more practical for screening purposes, TMAs may underestimate the proper assessment of a tumor, not representing adequately the tumors especially when focusing on such heterogenous cancers like TGCT [52, 53]. Specific subtypes of Mixed type Tumor may possibly be under-represented, implicating a variety of observations, with consequent different interpretations of clinicopathological features, namely the prognosis [53]. It must be taken in count that the cutoffs used for Ki67 expression in TGCT articles are also a contributing factor for the diverseness of results, particularly regarding prognostic evaluation. The most studied cutoff is 70% and the respective correlation with disease stage, metastatic disease, relapse, and the overall prognosis [12, 16, 23, 31]. Other cutoffs were also considered, such as 40% and 80% [12, 16, 43]. However, as further studies could not prove such correlation with the given cutoffs, others were proposed, such as 50% [12]. Nonetheless, the association with prognosis was not always verified, particularly when adjusting to other clinicopathological features like vascular invasion [12, 16]. Such heterogeneity of results is represented in Supplementary Table II, gathering some studies with each description of the samples, scoring and assessment methods, cutoffs, and significant findings according to the object of each study. Despite the efforts to standardize technical scoring, analytical interpretation, and data analysis steps, variations in protocols remain large, thus contributing to assay variability [42, 44, 48]. This is the exact problem with markers like Ki67, making the definition of IHC uniform protocols of utmost importance. While Ki67 seemed to be, initially, an interesting prognostic biomarker when subjectively assessed through rough “eye-ball” quantification, rapidly a myriad of different results was obtained, and its significance was lost particularly when adjusted for other variables. Our digital pathology and more objective analysis further confirms that Ki67 is not a promising prognostic biomarker to be introduced in the clinical setting for guiding treatment decisions for TGCT patients.

Epigenetic chromatin modifications are widely known to play an important role in tumorigenesis [54, 55]. Lysine-specific demethylase 1 (LSD1), also known as KDM1A, is one of the many epigenetic markers currently in study and is composed of several domains shared by many chromatin regulatory complexes, thus participating in a dynamic process that interferes with cell regulation [54, 55]. LSD1 is the first identified histone lysine demethylase responsible for histone demethylation, specifically of monomethyl and dimethyl histone 3 at lysine 4 (H3K4) and 9 (H3K9), where H3K4 marks an active chromatin transcription state and H3K9 a repressive one [55]. The consequence of histone demethylation, depending on the location and degree of residue methylation, is the

regulation and control of transcription, differentiation, stemness, cell motility, epithelial-to-mesenchymal transition, metabolism, autophagy, and senescence [55-57]. Indeed, high levels of LSD1 are associated with the expression of pluripotent stem cell markers, such as OCT4 and SOX2 [58]. As the expression of SOX2 confers stem cell properties to cancer cells, targeting LSD1 may interfere with stemness through these cell markers as well [34]. Hayami et. al, using real-time PCR, IHC and microarray data, described that LSD1 levels were significantly high even in early tumors, indicating that it can be a factor involved in the initiation process of carcinogenesis [54]. Higher levels were identified in bladder and lung cancer cell lines and, using small interfering RNA (siRNAs) targeting LSD1 in similar cell lines, significant growth suppression was found. Moreover, after treatment with those siRNAs, transfection of LSD1 on the same lines was performed, resulting in recovery of expression and cell growth [54]. Suppression of TE and EC cells, that expressed SOX2 and OCT4, was reported with the use of a new LSD1 inhibitor [34].

LSD1 has been reported to be overexpressed in a variety of cancers, such as lung, bladder, prostate, brain, colorectal and breast cancers, and certain hematologic malignancies (namely, acute myeloid leukemia and multiple myeloma), determining poor prognosis [55, 56]. In studies with pluripotent germ cell tumors, LSD1 exhibited significant expression levels, namely in teratocarcinoma, EC and SE cells, in contrast with normal testicular tissue [34, 59]. Our study analyzed comprehensively LSD1 immunoexpression in a large and well characterized TGCT tissue cohort, which was not done before. In our study, however, we found no significant differences in expression of LSD1 among the various histological subtypes, and this was confirmed in TCGA cohort analysis. Higher levels were overall seen in very distinct subtypes, such as differentiated TE and more undifferentiated subtypes like EC (Supplementary Table I, Figure 4). There was also no significant correlation between LSD1 positive nuclei percentage or H-score and the stage of disease, nor with relapse/progression-free survival, limiting its use as a prognostic marker. However, finding LSD1 expression overall in all histological subtypes of TGCTs in such a large cohort still may lead to exploring LSD1 inhibitors as therapeutic targets for all TGCT patients regardless of histology. Indeed, using LSD1 inhibitors with the purpose of exploring the biological function of this enzyme, Wang et al. found that such inhibition selectively blocked the growth of TE, EC, SE, and embryonic stem cells, also seen in recent studies with new LSD1 inhibitors [58].

Due to the potential of LSD1 as a target for cancer therapy, there are several LSD1 inhibitors being explored, which can be grouped in 5 subcategories: monoamine oxidase (MAO) inactivators, natural products, peptide inhibitors, polyamine-based inhibitors, and metal-based inhibitors. The overall observed effects consisted of the inhibition of cell growth, proliferation, and mobility, and

also regulation of transcription, including up- and downregulation of particular genes [57]. Although promising as a target therapy, with significant results in clinical trials for small cell lung cancer, glioma, prostate and breast cancer, issues related to safety and pharmacodynamics still need to be overcome [57].

This epigenetic enzyme presents an additional role in non-histone proteins, such as p53, and in long noncoding RNA (lncRNAs) complexes as well, which mechanisms are involved in sustaining stem cells, and upregulation of some receptors related with oncogenic pathways and of other oncogenic factors [55, 56]. p53 demethylation has effects in repression of transcriptional upregulation and apoptosis-promoting action of p53 itself [54]. Intriguingly, demethylase-independent mechanisms of LSD1 have recently been reported, namely in acute myeloid leukemia and prostate cancer, which promoted positive oncogenic effects such as stem cell and treatment-resistant cell survival [60, 61]. Further investigation should be conducted in order to verify if such demethylase-independent mechanisms occur in other malignances [55].

Recently, studies have reported a novel function of LSD1 in regulation of tumor immunogenicity, seen in breast cancer and melanoma, where LSD1 ablation resulted in the upregulation of key immune checkpoint regulators such as CCL5, CXCL9, and CXCL10, and in higher expression of PD-L1 in tumor infiltrating lymphocytes [5, 55]. Consequently, given the PD-L1 expression correlation with the outcomes of TGCT, and focusing on the previous documented link between LSD1 and PD-L1, LSD1 inhibition may be assumed as a pivotal way to convert some malignancies, including TGCT, in responsive tumors to PD-L1 targeted therapy, with improved prognosis [55]. Future studies should expand the knowledge in TILs and deeply characterize immune microenvironment of TGCT, since it remains scarcely studied, despite recent promising findings in this field [5].

Notably, we found a significant correlation between the expression of Ki67 and LSD1 H-score. Although this correlation was week, it seems to corroborate that tumors with higher proliferation are those with higher LSD1 expression in relation to stemness properties.

As previously discussed, given the fact that the findings from the present study resulted from digital pathology analysis application, in contrast to former studies on the focused biomarkers, our results may depend on the used methods and must be prospectively confirmed. Digital pathology is a promising tool that may potentially provide greater accuracy, reproducibility, and standardization of pathology-based analysis, thus enhancing the possibility of the uniformization of results [62]. The concept of digital pathology is recently revealing special utility in the current COVID-19 pandemic

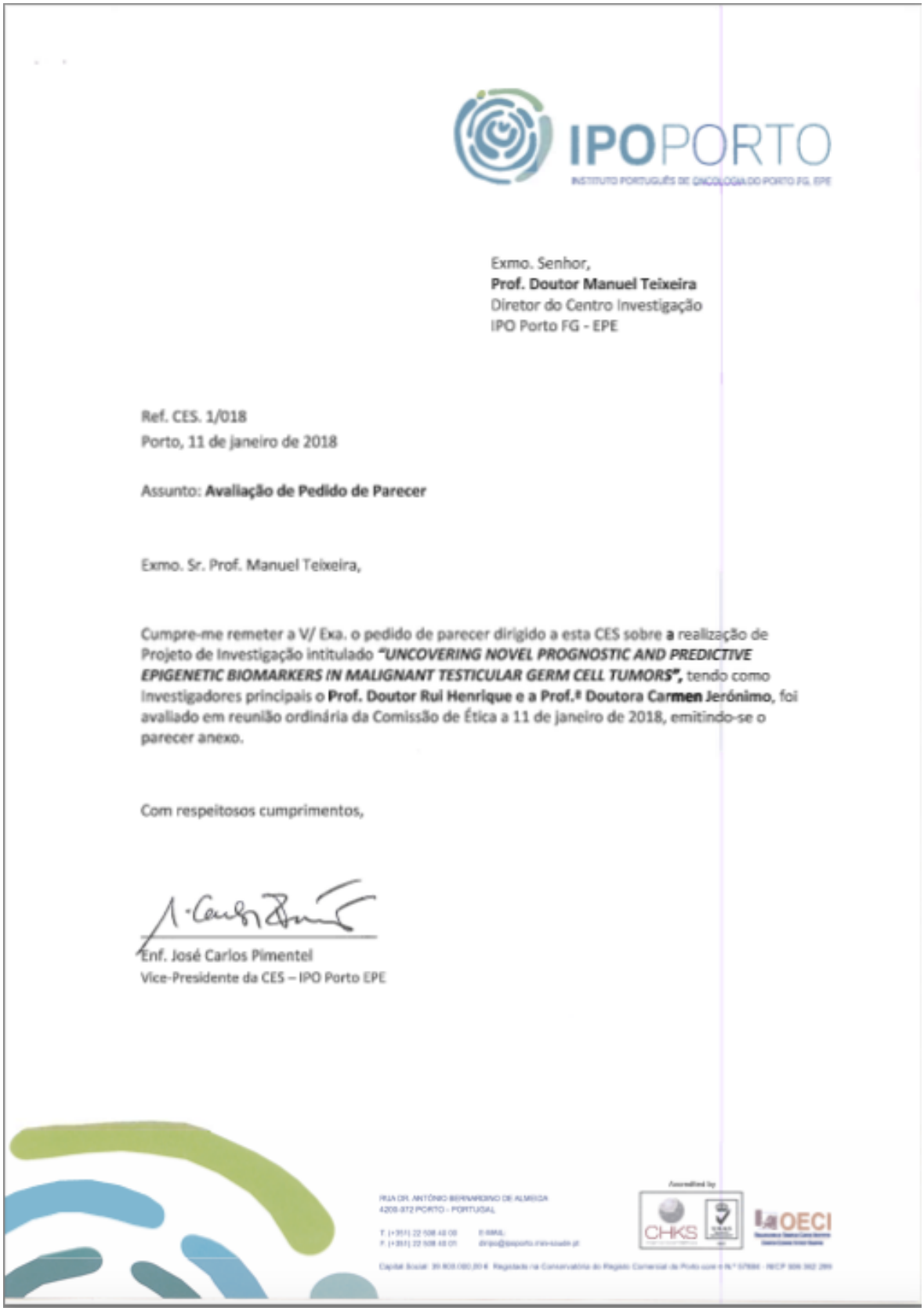
situation [63]. Firstly promoted with the intention of security and prevention of infection, such digital methods bring the possibility of several advantages: increasing quality of biomarker studies and clinical trials in tertiary healthcare, larger studies with higher accuracy, the cost-effectiveness, procedure standardization with crescent quality, the integration of artificial intelligence into routine pathology, and time and storage saving capacities for many pathology and/or investigation departments [63].

Conclusions

Malignant behavior is defined by stepwise changes in tumor cell biology, therefore the understanding of such characteristics and their correlation with clinical outcomes is of paramount importance. Regarding TGCTs, a disease afflicting especially young men that, despite its high cure rates, brings significant morbidity, defining morphologic factors and biological mechanisms is crucial to improve current knowledge with a great clinical impact.

Despite individual studies that may have suggested significant utility in evaluating Ki67 through the pathologist eye by a non-digital quantification, our study shows that such utility in a random cohort, from daily practice, cannot be demonstrated. *In silico* analyses corroborates our results regarding both markers and the lack of association with outcome and other clinical features. LSD1, while limited as a prognostic biomarker, may be an eventual therapeutic target expressed overall in various histological subtypes. It requires more well-defined studies, given the promising role of such treatments, including the discussed modulation of the immune microenvironment.

A – Parecer da Comissão de Ética para a Saúde do IPO Porto



IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Exmo. Senhor,
Prof. Doutor Manuel Teixeira
Diretor do Centro Investigação
IPO Porto FG - EPE

Ref. CES. 1/018
Porto, 11 de janeiro de 2018

Assunto: **Avaliação de Pedido de Parecer**

Exmo. Sr. Prof. Manuel Teixeira,

Cumpre-me remeter a V/ Exa. o pedido de parecer dirigido a esta CES sobre **a** realização de Projeto de Investigação intitulado ***"UNCOVERING NOVEL PROGNOSTIC AND PREDICTIVE EPIGENETIC BIOMARKERS IN MALIGNANT TESTICULAR GERM CELL TUMORS",*** tendo como Investigadores principais o **Prof. Doutor Rui Henrique e a Prof.ª Doutora Carmen Jerónimo**, foi avaliado em reunião ordinária da Comissão de Ética a 11 de janeiro de 2018, emitindo-se o parecer anexo.

Com respeitosos cumprimentos,

Enf. José Carlos Pimentel
Vice-Presidente da CES – IPO Porto EPE

RUA DR. ANTÓNIO BERNARDINO DE ALMEIDA
4200-072 PORTO - PORTUGAL

IPOPORTO
INSTITUTO PORTUGUÊS DE ONCOLOGIA DO PORTO FG, EPE

Parecer CES IPO: 1/2018
Assunto: Avaliação de pedido de realização de Projeto de Investigação intitulado ***"UNCOVERING NOVEL PROGNOSTIC AND PREDICTIVE EPIGENETIC BIOMARKERS IN MALIGNANT TESTICULAR GERM CELL TUMORS"***
Investigadores principais: **Prof. Doutor Rui Henrique e a Prof.ª Doutora Carmen Jerónimo**
Data: 11 de janeiro de 2018

PARECER

É parecer desta CES, não existir impedimento de natureza ética ao desenvolvimento do referido estudo de Investigação.

Enf. José Carlos Pimentel
Vice-Presidente da CES – IPO Porto EPE

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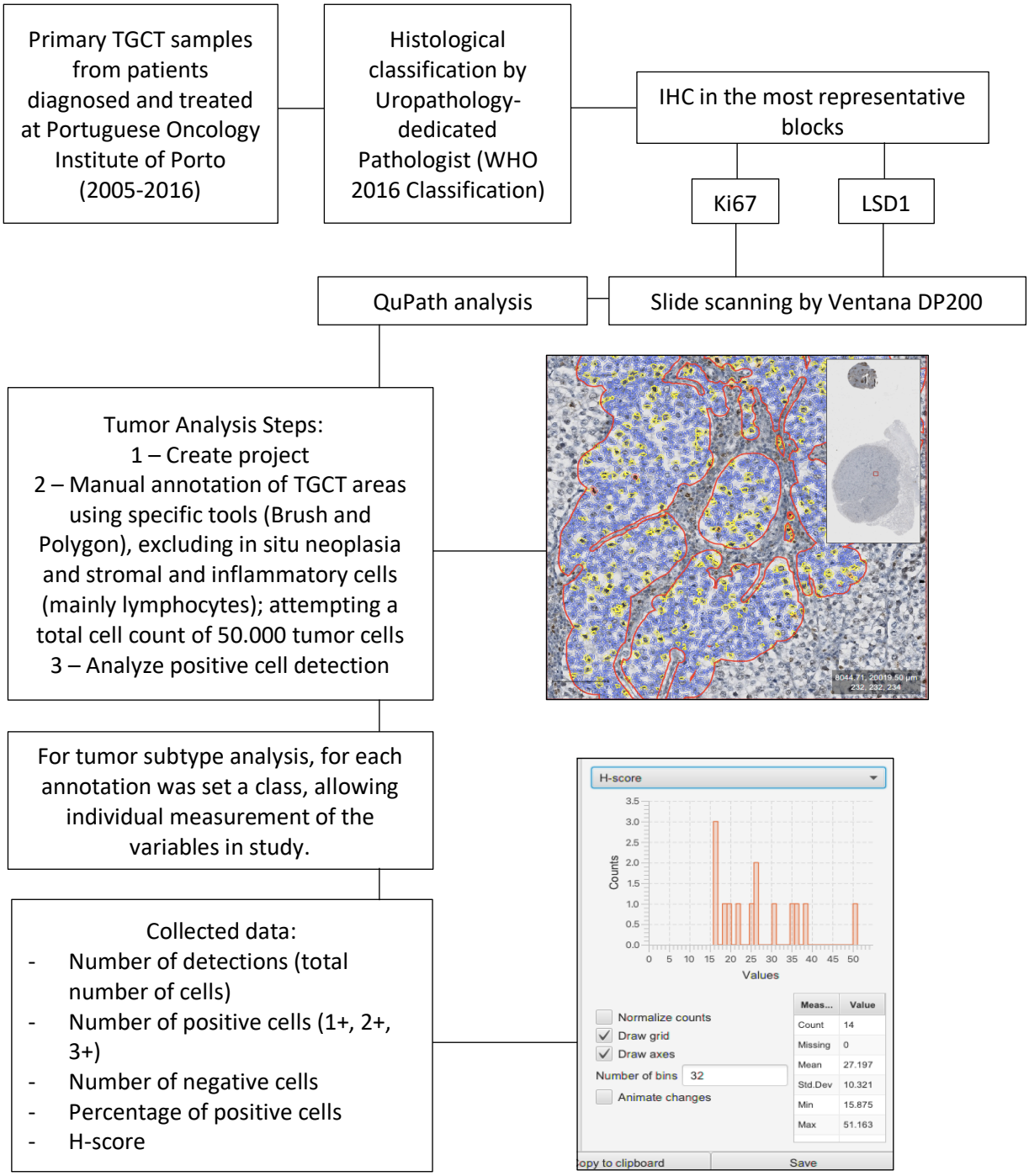


Figure 1. Study protocol. IHC, immunohistochemistry, TGCT, Testicular Germ Cell Tumor, WHO, World Health Organization

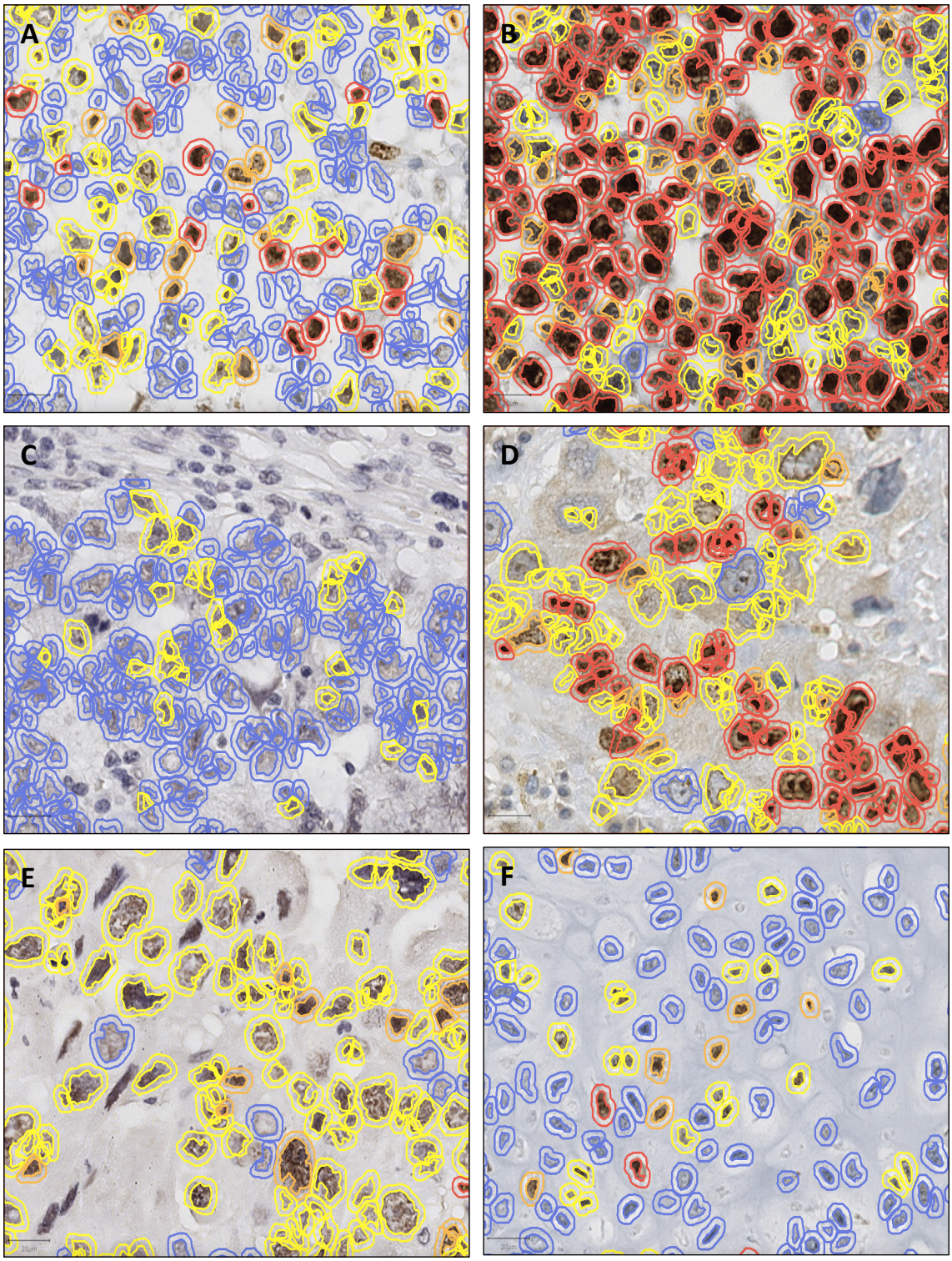


Figure 2. Illustrative examples of Ki67 and LSD1 immunoexpression across different testicular germ cell tumor subtypes, obtained through QuPath software with 40x magnification (scale of 20 µm): A. Case of pure SE, with Ki67 immunostaining; B-C. Two cases of pure EC, with Ki67 and LSD1 immunostaining, respectively. Notice the several immune cells and stromal cells that were eliminated from the quantification; D-E. CH components from the same Mixed Tumor case, with Ki67 and LSD1 immunostaining, respectively. Some cells are left uncounted for better appreciation of the nuclear DAB staining. F. TE component of a Mixed Tumor case, with Ki67. The colors represent the intensity of expression (blue – no expression, yellow – low, orange – moderate, and red – high expression).

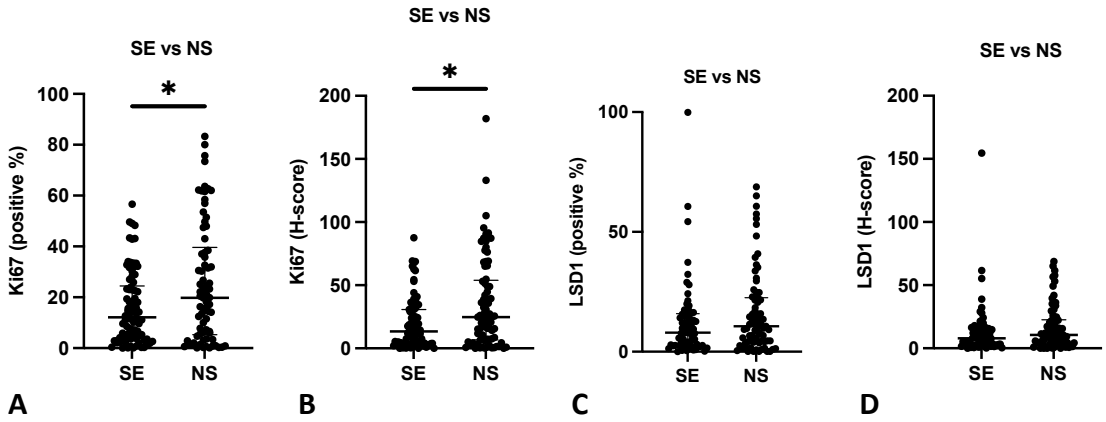


Figure 3. Immunoexpression in Seminomas and Nonseminomas. A-B. SE versus NS; A. Ki67 positive %; B. Ki67 H-score. C-D. SE versus NS; C. LSD1 positive %; D. LSD1 H-score. Median and interquartile range are presented. *p<0.05. NS Nonseminoma, SE Seminoma. *significant values

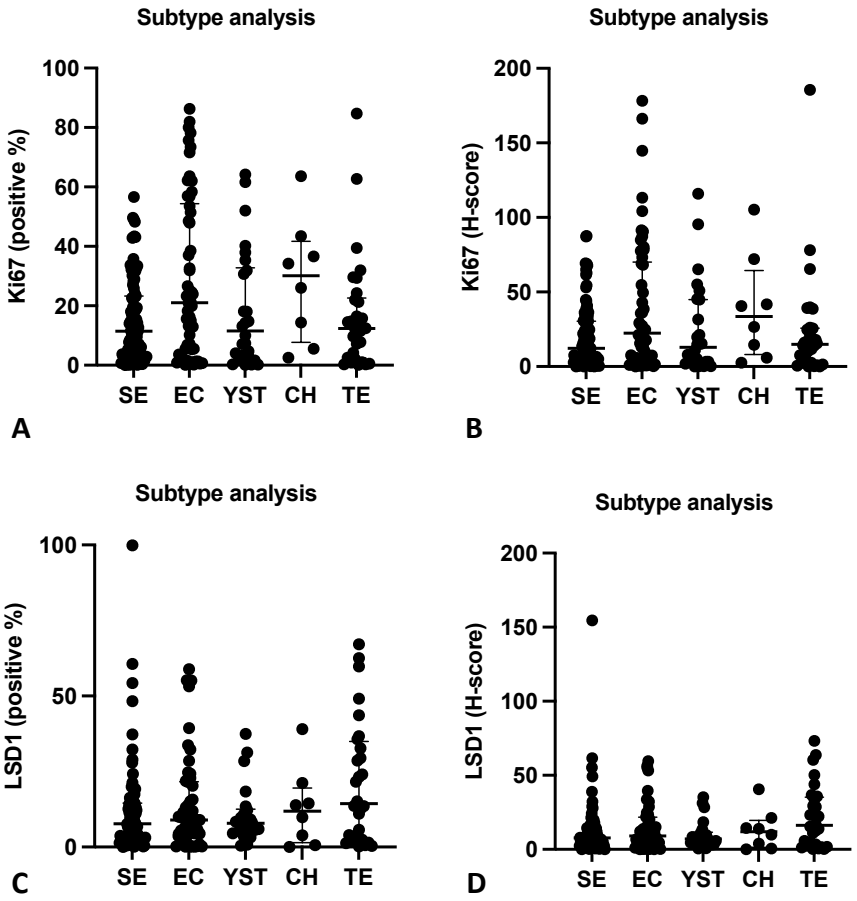
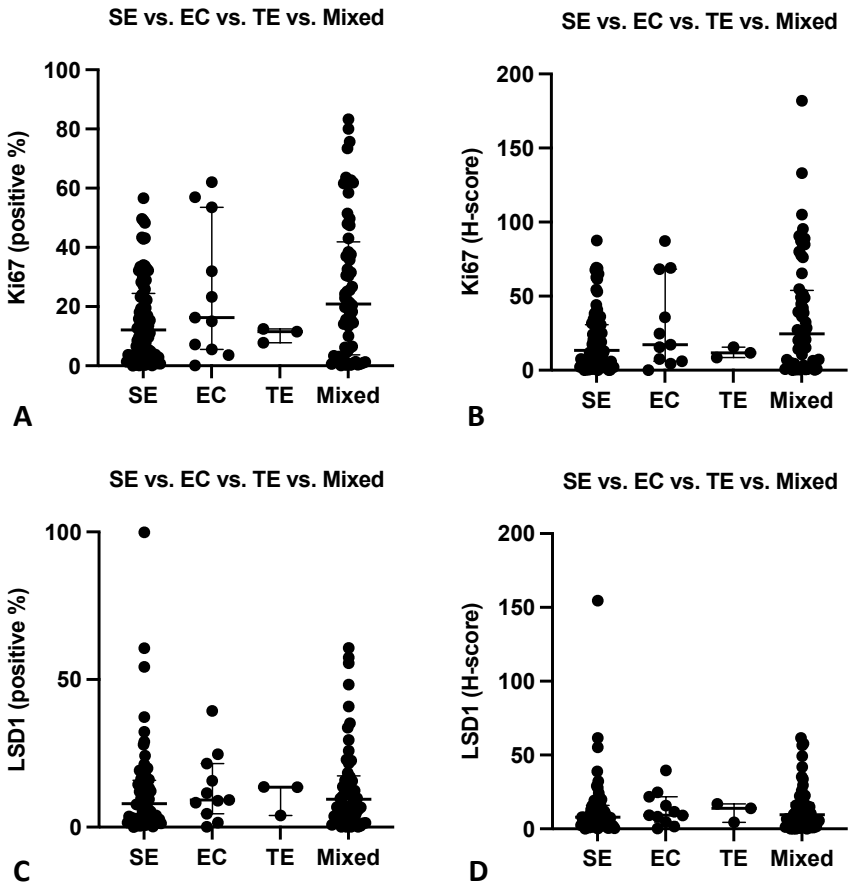
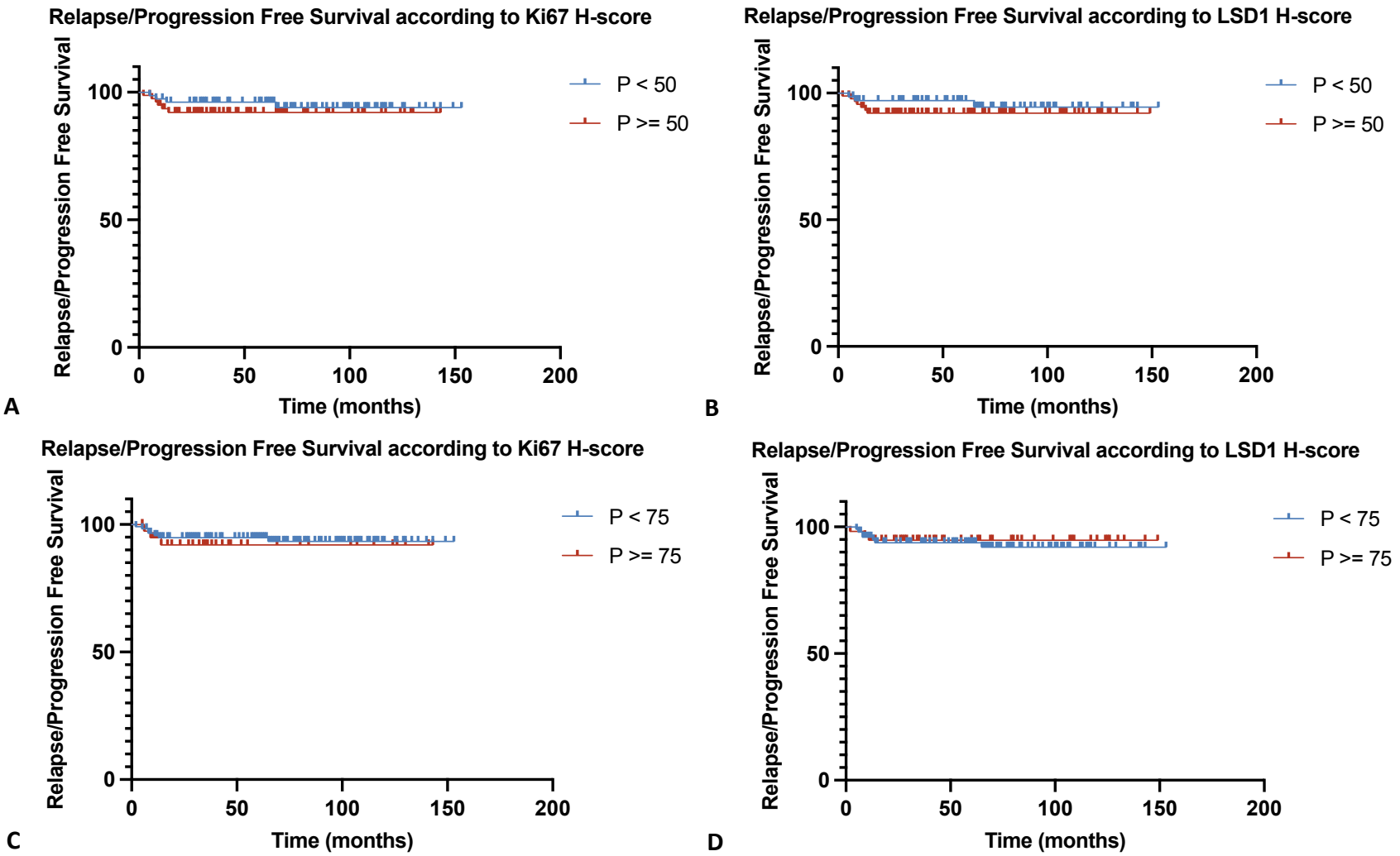


Figure 4. Immunoexpression among TGCT histological subtypes (SE, EC, YST, CH, and TE). A. Ki67 positive %; B. Ki67 H-score; C. LSD1 positive %. D. LSD1 H-score. Median and interquartile range are presented. CH Choriocarcinoma, EC Embryonal Carcinoma, NS Nonseminoma, SE Seminoma, TE Teratoma, YST Yolk-sac tumor.



Supplementary Figure 1. Immunoexpression in pure TGCT subtypes (SE, EC, and TE) versus Mixed type. A. Ki67 positive %; B. Ki67 H-score; C. LSD1 positive %. D. LSD1 H-score. Median and interquartile range are presented. EC Embryonal Carcinoma, NS Nonseminoma, SE Seminoma, TE Teratoma.



Supplementary Figure 2. Relapse/Progression free-survival according to biomarkers 50th (P50) and 75th (P75) percentiles. A-B Relapse/progression free survival according to P50. A. Ki67 H-score B. LSD1 H-score. C-D Relapse/progression free survival according to P75. C. Ki67 H-score D. LSD1 H-score.

Table I. Demographic and clinicopathological characteristics of the study cohort.

Variables	Patient cohort (n=157) Tumor samples (n=221)
Age (years) (median, IQR)	31 (25-36)
Laterality (n, %)	
Right	83/153 (54.2)
Left	68/153 (44.5)
Bilateral synchronous	2/153 (1.3)
Pre-operative AFP (n, %)	
Within normal range	97/151 (64.2)
Elevated	54/151 (35.8)
Pre-operative β-HCG (n, %)	
Within normal range	73/151 (48.3)
Elevated	78/151 (51.7)
Pre-operative LDH (n, %)	
Within normal range	71/124 (57.3)
Elevated	53/124 (42.7)
Histologic subtypes (n, %)	
Pure SE	82/157 (52.2)
Pure EC	11/157 (7.0)
Pure TE	3/157 (1.9)
Mixed Tumor	61/157 (38.9)
Tumor components (n, %)	
SE	96/221 (43.5)
EC	56/221 (25.3)
CH	10/221 (4.5)
YST	27/221 (12.2)
TE	32/221 (14.5)
Stage AJCC8 (n, %)	
I	100/156 (64.1)
II	32/156 (20.5)
III	24/156 (15.4)
Vascular Invasion (n, %)	
Yes	78/157 (49.7)
No	79/157 (50.3)
Tumor largest dimension (cm) (median, range)	4.7 (0,7-18)
Metastatic disease at diagnosis (n, %)	
Yes	56/156 (35.9)
No	100/156 (64.1)
IGCCCG prognosis group (with metastatic disease) (n, %)	
Good	42/56 (75)
Intermediate	8/56 (14.3)
Poor	6/56 (10.7)
Relapse/Progression (n, %)	
Yes	10/157 (6.4)
No	147/157 (93.6)
Additional treatments (n)	
RT	46
CT	99
Vital status (n, %)	

Alive with no disease	151/157 (96.2)
AWD	3/154 (1.9)
Dead	3/157 (1.9)
DFD	2/157 (1.3)
D-NED	1/157 (0.6)

AJCC American Joint Committee on Cancer, *AFP* Alpha-fetoprotein, *AWD* Alive with disease, *CH* Choriocarcinoma, *CT* Chemotherapy, *DFD* Died from disease, *D-NED* Died with no evidence of disease, *EC* Embryonal Carcinoma, *IGCCCG* International Germ Cell Cancer Collaborative Group, *IQR* Interquartile Range, *LDH* Lactate dehydrogenase, *RT* Radiotherapy, *SE* Seminoma, *TE* Teratoma, *YST* Yolk-sac Tumor.

Table II. Immunoexpression of Ki67 and LSD1 according to clinicopathological features

Stage	**I**	**II**	**III**	***p*-value**
Ki67				
Positivity (%)				
(median, [IQR])	15.4 (4.7-32.2)	10.8 (3.4-24.0)	21.7 (4.3-30.1)	0.593
Only SE	13.9 (3.87-25.4)	6.6 (3.19-17.6)	11.1 (5.17-31.2)	0.791
Only NS	22.8 (5.9-58.5)	14.1 (3.4-32.0)	22.1 (3.6-30.5)	0.278
H-score (median, [IQR])	17.6 (5.0-42.1)	11.2 (3.9-26.6)	24.6 (5.1-35.9)	0.490
Only SE	14.5 (4.2-31.5)	6.8 (3.5-19.9)	12.3 (6.4-31.9)	0.715
Only NS	25.5 (6.3-76.2)	14.2 (4.4-35.8)	24.8 (4.4-38.6)	0.274
LSD1				
Positivity (%)				
(median, [IQR])	8.2 (2.8-17.2)	9.4 (2.3-13.7)	10.6 (3.2-15.6)	0.894
Only SE	7.8 (2.6-15.8)	9.8 (2.5-15.7)	13.4 (3.1-16.2)	0.953
Only NS	9.8 (3.7-21.6)	9.0 (1.6-13.7)	9.2 (3.2-15.1)	0.692
H-score (median, [IQR])	8.2 (3.1-17.3)	9.4 (2.1-13.9)	10.6 (3.2-15.6)	0.879
Only SE	7.9 (2.6-15.8)	9.8 (2.5-15.7)	13.4 (3.1-16.2)	0.960
Only NS	9.8 (4.3-22.2)	9.1 (1.6-14.0)	9.3 (3.2-15.3)	0.644
IGCCCG	**Good**	**Intermediate**	**Poor**	***p*-value**
Ki67				
Positivity (%)				
(median, [IQR])	10.4 (3.2-26.1)	20.7 (4.9-41.5)	23.9 (17.6-28.4)	0.207
H-score (median, [IQR])	11.9 (3.7-27.7)	23.3 (5.0-75.1)	29.3 (20.1-35.8)	0.179
LSD1				
Positivity (%)				
(median, [IQR])	10.6 (1.6-15.6)	5.8 (4.2-40.9)	6.7 (3.3-13.5)	0.977
H-score (median, [IQR])	10.7 (1.6-15.6)	5.8 (4.2-41.9)	6.7 (3.4-13.7)	0.979
	Stage I		**Metastatic Disease**	***p*-value**
Ki67				
Positivity (%)				
(median, [IQR])	15.4 (4.7-32.2)		15.0 (3.5-28.4)	0.477
H-score (median, [IQR])	17.6 (5.0-42.1)		15.7 (4.1-34.1)	0.411
LSD1				

Positivity (%)			
(median, [IQR])	8.2 (2.8-17.2)	9.8 (2.9-15.1)	0.676
H-score (median, [IQR])	8.2 (3.1-17.3)	9.8 (2.9-15.3)	0.646
	Vascular Invasion	**No Vascular Invasion**	***p*-value**
Ki67			
Positivity (%)			
(median, [IQR])	17.6 (3.6-33.4)	13.9 (4.6-29.3)	0.364
H-score (median, [IQR])	18.6 (4.3-41.7)	15.4 (4.9-36.3)	0.470
LSD1			
Positivity (%)			
(median, [IQR])	9.0 (2.4-16.1)	9.4 (3.3-15.1)	0.738
H-score (median, [IQR])	9.1 (2.4-16.2)	9.4 (3.6-16.5)	0.684

IGCCCG International Germ Cell Cancer Collaborative Group, *IQR* Interquartile Range, *NS* Nonseminoma, *SE* Seminoma.

Table III. In silico analysis of TCGA data

SE vs. NS

	SE	**NS**	**p-value**
Ki67 mRNA expression (median, [IQR])	888 (635-1088)	758 (505-1122)	0.262
LSD1 mRNA expression (median, [IQR])	4012 (3220-4426)	3903 (2735-5650)	0.311

Stage

	I	**II**	**III**	**p-value**
Ki67 mRNA expression (median, [IQR])	806 (635-1115)	592 (424-1054)	856 (424-1133)	0.216
LSD1 mRNA expression (median, [IQR])	3956 (3214-5018)	4216 (2636-5340)	4762 (3208-5841)	0.437

AJCC American Joint Committee on Cancer, *IQR* Interquartile Range, *mRNA* messenger RNA*, NS* Nonseminoma, *SE* Seminoma.

Supplementary Table I. Expression of Ki67 and LSD1 according to histology

	SE	NS	*p*-value
Ki67			
Positivity (%)			
(median [IQR])	12.2 (3.7-24.5)	19.8 (5.3-39.6)	0.0316*
H-score			
(median [IQR])	13.4 (4.0-87.5)	24.7 (6.7-53.9)	0.0113*
LSD1			
Positivity (%)			
(median [IQR])	8.0 (2.6-15.9)	10.6 (4.2-22.6)	0.1973
H-score			
(median [IQR])	8.0 (2.6-15.9)	10.6 (4.3-22.6)	0.1630

	SE	EC	YST	CH	TE	*p*-value
Ki67						
Positivity (%)	11.5	21.0	11.6	30.1	12.4	
(median, [IQR)	(3.6-23.3)	(4.9-54.3)	(2.8-32.8)	(7.7-41.7)	(2.43-22.6)	0.0548
H-score	12.3	22.5	12.9	33.6	14.9	
(median, [IQR])	(4.0-30.4)	(5.4-70.2)	(2.9-44.9)	(8.1-64.4)	(2.5-25.7)	0.1292
LSD1						
Positivity (%)	7.8	9.0	7.9	11.9	14.4	
(median, [IQR])	(2.5-14.6)	(4.1-21.6)	(4.7-12.5)	(1.5-19.5)	(2.7-34.9)	0.3275
H-score	7.8	9.0	7.1	11.9	16.2	
(median, [IQR])	(2.5-14.6)	(4.1-21.8)	(4.3-11.6)	(1.5-19.5)	(2.7-35.2)	0.2824

CH Choriocarcinoma, *EC* Embryonal Carcinoma**,** *IQR* Interquartile Range, *NS* Nonseminoma, *SE* Seminoma, *TE* Teratoma, *YST* Yolk-sac tumor, *significant values

Supplementary Table II. Review of literature regarding TGCT and the expression of Ki67

Reference	Cohort size, Sample type and Stage of Disease	Number of SE and NS (SE/NS)	Date of Diagnosis (interval)	IHC	Methodology	Outcome
Lobo et al. (2020)12	70 TGCT with Stage I	28/42	1993-2018 (median follow-up of 42 months)	Automated	Performed by a TGCT-dedicated investigator, grading from 0 to 3, using 40 and 70% cutoffs for Ki67/MIB-1	Although relapse free survival was better for patients with ≤70%, no significant association was found between Ki67/MIB-1 staining percentage and the event of relapse (p=0.127). Adjusting for the effect of vascular invasion, MIB-1 staining loses its impact on relapse-free survival. Considering patients without vascular invasion, those with Ki67 > 50% experienced worse outcomes (p=0.042).
Gilbert et al. (2015)16	190 NS with stage I, with additional TMAs (59 and 80, managed with surveillance and minimum follow-up of 2 years, from different hospitals)	0/190	From TE08 trial: 1998-2003 (median follow-up of 40 months)	Automated	Performed by two independent pathologists, recording intensity of staining (from 0 to 3), and percentage of positive staining, using 40 and 70% cutoffs for Ki67/MIB-1	MIB-1 expression was significantly associated with decreasing likelihood of the tumor containing SE, and increasing likelihood of vascular invasion. There was no additional prognostic value for MIB-1 staining, for either one of the used cutoffs. Patients with weak staining had better prognosis, but such finding was associated with vascular invasion (independent value was not corroborated in multivariable analysis).

Gallegos et al. (2011) 26	62 Pure Seminomas: 43 in stage I 17 in stage II 2 in stage III	68/0	1996-2005	Automated	Reviewed by 2 pathologists, using tissue microarrays (TMA)	There was no significant association between Ki67 and metastatic disease at diagnosis. There was a significant inverse association between *rete testis* invasion and Ki67 expression higher than 50%.
P. Albers et al. (2003) 23	152 patients with NS, assigned for retroperitoneal lymph node resection (RPLNR)	0/152	1996-2002 (median follow-up of 34,5 months)	Performed on paraffin sections (5 µm) stained with MIB-1 antibody by standard technique.	Performed by one pathologist, who counted and calculated the percentage of MIB-1 positively stained nuclei of the total number of tumor cells in more than 500 tumor cells, at 400x magnification (MIB score: MIB-1 positive tumor cells/total number of tumor cells). A cutoff of 70% was used to test the accuracy of its predictive value. When possible, MIB-score was assessed	Mean MIB-1 score was 57.9 and 71.9 for Stage I and II, respectively. It was significantly higher for stage II (p=0.003). Using a cutoff of 70%, a negative predictive value of 78% and a positive predictive value of 47.3% were obtained. The combination of MIB-1 score with vascular invasion was able to predict a low-risk group at the 86.5% level.

					only in EC components.	
Albers et al. (1996)31	78 NS with clinical stage I (50 and 28 with pathologic Stage I and II, respectively)	0/78	1983-1994 (median follow-up 58.2 months)	Performed on paraffin sections stained with MIB-1 antibody by standard technique	Performed by one pathologist, who manually counted MIB-1 positively stained tumor cells at 1400 and calculating the percentage of positively stained nuclei of the total number of tumor cells. A cutoff of 70% was used. When possible, MIB-score was assessed only in EC components.	The MIB-1 mean value was significantly higher in stage II disease (p=0.02). A cutoff value of 70% MIB-1 positive nuclei was best to predict the pathologic stages. The prediction of a low-risk group was possible with an accuracy of approximately 90%. However, MIB-1 was not useful in predicting a high-risk group, because half of the patients with MIB-1 values > 70% had no metastasis (positive predictive value of 55%).
Albers et. al (1995)43	62 Nonseminomatous TGCT (with 45 in Stage A and 17 with metastatic disease)	0/62	1992 (median follow-up of 19.9 months)	Performed on paraffin sections stained with MIB-1 antibody by standard technique, using a modification of the method of Cattoretti et al.	Performed by 2 pathologists experienced in this practice, who manually assessed MIB-1 scoring of the positively stained nuclei, with a cutoff of 80%, performed in the most intensely stained well-fixed areas of the tumors with the exclusion of seminomatous,	The mean values of MIB-1 positive expression were 66.3% and 80.2% for stage A and metastatic-group patients, with a statistically significant difference (p=0.0032).

					teratomatous and/or stromal components.	
Mazumdar et al. (2003) 51	95 NSGCT (all containing EC alone or in association with other NS components). Only EC components were used in this study.	0/95	1975-1996 (median follow-up of 9.3 years)	Performed on paraffin sections (4 μm), from paraffin-embedded archival tissue blocks) by standard technique using streptavidin-biotin antibodies against Ki67 (diluted to 1:100)	Not described	Hierarchical agglomerative cluster analysis was used with the intention of discovering a subgroup of patients, assessing Ki67, p53, apoptosis, AFP and β-HCG. Cluster A patients (37) show higher Ki67 values, of whom 70% and 60% had good and poor prognosis, respectively. The 5-year survival rate was 94%. Data suggested that Ki67, p53 and apoptosis signaling pathways would be relevant to clinical decision making. EC not in cluster A, with poorer outcomes, exhibited lower Ki67 expression.

AFP Alpha-fetoprotein, *β- hCG* Human chorionic gonadotropin β, *CH* Choriocarcinoma, *CT* Chemotherapy, *EC* Embryonal Carcinoma, *IHC* immunohistochemistry, *IQR* Interquartile Range, *LDH* Lactate dehydrogenase, *RT* Radiotherapy, *SE* Seminoma, *TE* Teratoma, *YST* Yolk-sac Tumor

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