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REVIEW

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Biomarkers of prostate bladder and testicular cancers: current use in anatomic pathology and future directions

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Abstract

Urologic pathology is rapidly evolving to adopt growing knowledge of molecular pathways involved in genitourinary neoplasm. Many prognostic and predictive biomarkers are under active research and some of them have been incorporated in clinical practice. In this review, we will discuss recent developments of Molecular Pathology of prostate, bladder and testicular tumors with special emphasis on prognostic and predictive biomarkers.

Keywords Biomarker, Urinary bladder neoplasms, Prostate neoplasms, Testicular neoplasms, Pathology, Molecular, Classification

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Introduction

In recent years, the practice of anatomic pathology has experienced significant changes, in part due to the widespread adoption of diagnostic and prognostic biomarkers, both circulating and tissue-based. Specifically, implementation of molecular assays and novel immunohistochemical markers (such as mutation-specific and fusion protein-specific antibodies) has led to the identification of new entities with distinct biologic and clinical features, resulting in changes in tumor classification across different subspecialties of oncologic pathology. Routine assessment of biomarkers has also had substantial impact on clinical management, since many of them are accurate predictors of disease-specific or disease-related outcomes (prognostic biomarkers) and/or response to specific treatment modalities.

The booming field of biomarker research has identified some with proven clinical value, which have already been incorporated into routine practice, as well as others with promising diagnostic or prognostic performance that are likely to enter the clinical arena in the near future. However, much of the research data produced over the last few years pertains to biomarkers that are purely

experimental and of unknown clinical value. The amount of information on biomarkers can be overwhelming, being difficult to parse out what is truly relevant. In this review article we include a curated selection of diagnostic and prognostic biomarkers, highlighting those that are clinically relevant. Of note, some of them are well-established, whereas others are emerging and, therefore, not entirely validated. The objective is to present the reader with information useful for daily clinical practice and provide future perspectives in the field of oncologic uropathology.

Prostate cancer

In recent years, active surveillance has become the management of choice for patients with very low risk and low risk prostate cancer, being increeasingly considered a viable option for selected patients with favorable-intermediate risk disease. Biomakers with predictive value may further refine risk assessment within these categories, helping clinicians to adopt treatment strategies tailored to individual patients. Given their impact on clinical practice, some predictive biomarkers such as PTEN, Ki67 and mRNA-based gene expression signatures will be discussed below.

In the scenario of advanced disease, biomarkers of defects on DNA repair pathways, androgen receptor signaling, and neuroendocrine differentiation are the most important in current practice. The first identifies patients who may benefit with Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) and the latter may prompt the clinician to switch anti-androgen therapies toward chemotherapy or enrollment in clinical trials.

Biomarkers in the scenario of active surveillance and localized prostate carcinoma

Most patients diagnosed with prostate cancer have a low-grade, Gleason 6 (3+3), grade group (GG1), organ-confined acinar adenocarcinoma. Most of these patients will benefit from the adoption of active surveillance, an option that avoids or postpones definite treatment (and their complications) for years before disease progresses to a higher-risk condition requiring intervention. Active surveillance has gained ground in clinical practice and is usually selected as the best option depending on patient´s personal preference, clinical data, PSA serum levels / density and absence of higher-grade component (no Gleason pattern 4 or 5) in biopsy specimens. Active surveillance may also be included as an option in selected cases, or in some centers for adenocarcinomas Gleason 3+4 (GG2) with low volume of high-grade component and absence of cribriform morphology or intraductal carcinoma (Klotz et al. 2015).

The current scenario, however, may still be biased in favor of overtreatment. As for 2018, the US task force estimated that for each 1000 men undergoing PSA screening, 240 will have serum elevated levels, 100 a positive biopsy and 80 will eventually be treated with a definite treatment. This picture results in avoiding death in 1 man and metastatic disease in 3, while 5 men would die of disease despite treatment, and 50 and 15 men would live with erectile dysfunction and urinary incontinence after treatment, respectively (Grossman et al. 2018).

In a scenario in which both active surveillance and definite treatment are considered, additional tools for risk stratification are needed to safely discuss with patients their best option. Many tissue-based prognostic factors have been studied in this scenario including immunohistochemical markers, mRNA-based genomic signatures and proteomics.

PTEN immunohistochemistry

PTEN (Phosphatase and tensin homolog) is one of the most frequently inactivated tumor suppressor genes in human cancers. PTEN protein acts as a lipid phosphatase opposing the PI3K/AKT signaling pathway. In prostate cancer, PTEN loss is most caused by genomic deletion. PTEN loss increases with tumor grade and there is a high concordance between fluorescent in situ hybridization with (more readily available) immunohistochemical assays for PTEN (Lotan et al. 2016; Picanco-Albuquerque et al. 2016; Lotan et al. 2017; Jamaspishvili et al. 2018). In prostatectomy specimens, about 20% of acinar adenocarcinomas show PTEN loss but this finding rises to 40% in metastatic tumors. In prostate adenocarcinoma, there may be genetic heterogeneity regardless PTEN status and about 40% of tumors with PTEN loss is detected as a subclonal finding coexisting with areas of intact PTEN expression (Krohn et al. 2014, Jamaspishvili et al. 2018) (Fig. 1).

There is an abundance of studies on the prognostic value of PTEN as a cheap, widely available, non-centralized immunohistochemical test. PTEN loss at biopsy samples (defined as markedly decreased or entirely negative across > 10% of tumor cells compared with surrounding benign glands and/or stroma) with GG1 prostate cancer showed to be more associate with upgrading at radical prostatectomy specimen (Lotan et al. 2015). Among patients with GG2 tumors at biopsy, PTEN loss in these samples predicted non-confined disease in prostatectomy specimen and biochemical recurrence after surgery (Guedes et al. 2017). In a retrospective study in patients treated with radical prostatectomy, PTEN loss at biopsy could predict development of metastasis and

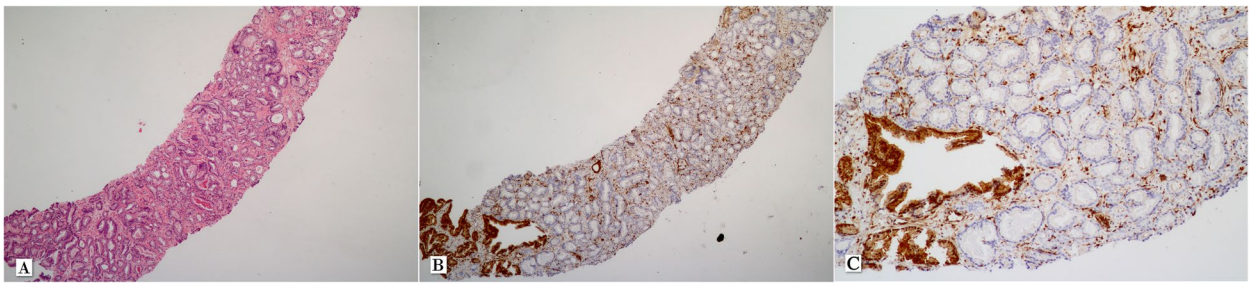

Fig. 1 PTEN expression can be lost in low-grade GG1 acinar adenocarcinomas of the prostate. This finding may identify patients under active surveillance who will experience grade reclassification and switch conduct for definitive treatment. It may be used as one factor—among other clinical, laboratorial and pathologic findings at biopsy—for decision making. HE (**A**) and PTEN immunohistochemistry (**B** and **C** 40x and 100x, magnification)

prostate cancer-specific mortality after radical prostatectomy (Mithal et al. 2014).

Few studies have compared PTEN immunohistochemistry with RNA-based genomic signatures. In surgical cohorts, PTEN loss lost its prognostic value (to predict biochemical recurrence) in models including Oncotype Dx and Prolaris. Importantly, these studies did not include a cost–benefit analysis. In one study, the single immunochemical reaction for PTEN outperformed Prolaris as an independent prognostic factor for metastasis and death (Lokman et al. 2018). Other study showed the Prolaris score, but not PTEN or Ki7 status by immunohistochemistry, predictors of recurrence of prostatectomy. The model of this study, however, was limited by the inclusion of many specimens from before 2005 ISUP consensus Gleason grading – probably using and outdated grading approach (Léon et al. 2018).

In the specific setting of active surveillance of patients with GG1 disease, a case–control (132 patients) study from John Hopkins, US, showed that PTEN loss was more common in patients who underwent grade reclassification during follow up. PTEN loss seems to be rare in patients with GG1 eligible for active surveillance (5% in this study) and is still uncommon in patients who underwent reclassification although much more common than in those patients who were maintained under active surveillance (9% versus 2%) (Tosoian et al. 2019). In a Finish cohort (231 patients with GG1 adenocarcinoma) undergoing active surveillance, PTEN loss at biopsy predicted rebiopsy GG upgrade, treatment change, and adverse histopathology in prostatectomy specimens (Lotan et al. 2020).

PTEN loss seems to be more common in prostate adenocarcinomas that are visible in Magnetic resonance imaging (Eineluoto et al. 2020). As a consequence, PTEN expression should be evaluated in target biopsy cores in addition to specimens selected by pathologic findings (highest grade and tumor extent). A growing field of study in prostate cancer is radiogenomics – the integration of Imaging and Molecular Characterization (Ferro et al. 2021; Banerjee et al. 2022).

Ki67 index

Ki67 is a protein encoded by the *MK167* gene that is highly expressed in cycling cells and not in resting G0-phase cells. Its biological function is not well understood but it is used in Surgical Pathology as a prototype immunohistochemical marker of cell proliferation. The proportion of Ki67 positive cells within a tumor is named Ki67 proliferative (or labeling) index. It is commonly used in Pathology and is used in many scenarios including risk stratification in breast invasive carcinoma (commonly used to indicate neoadjuvant chemotherapy) and key criterion for grading well differentiated neuroendocrine tumors.

Ki67 index has been proved of prognostic value in prostate cancer in different scenarios. In a large study enrolling 1,004 patients who underwent prostatectomy, the cutoff of > 5% Ki67 index in the prostatectomy samples was associated with recurrence and reduced cancer-specific and overall survival (Tretiakova et al. 2016). In preoperative biopsies (279 patients) Ki67 index with a cutoff of 10% was predictive of biochemical recurrence after prostatectomy and outperformed serum PSA or tumor extent at biopsy in both the scenarios of low-volume or low-grade tumor (GG1) (Zellweger et al. 2009).

In a large cohort (756 patients) of patients treated conservatively (any tumor grade), Ki67 index as a continuous variable (or with a 5% cutoff) was an independent risk factor for prostate cancer death – along with tumor grade, tumor extent at biopsy and PSA levels (Kammerer-Jacquet et al. 2019).

In the specific setting of patients under active surveillance for low or intermediate risk tumors (GG1/2, serum PSA < 15 ng/dL and tumor extent at biopsy ≤ 50%), a 60-patients cohort showed that PSA density, Ki-67 index

and grade were independent predictors of progression to radical treatment (Jhavar et al. 2009).

There is no established threshold, but the most appropriate value appears to be between 5 and 10%. In the 2019 ISUP survey, 45% of pathologists believe Ki67 to be useful for decision making in the setting of active surveillance versus definite treatment.

Use of immunohistochemical markers in routine practice

Guidelines for oncologists and urologist currently do not recommend the use of immunohistochemical prognostic markers in the setting of localized low-grade or intermediate-grade prostate adenocarcinoma. The recommendations of the International Society of Urological Pathology (ISUP) from the 2019 Consultation Conference included the following statements:

- Ki67 proliferative (or labeling) index and PTEN loss are potentially useful prognostic biomarkers in the GG1 (and some GG2) localized prostate adenocarcinoma specially in core biopsies in which clinicians and patients are evaluating the eligibility for active surveillance.
- Both Ki67 proliferative (or labeling) index and PTEN loss would be one factor among many others considered for the decision on active surveillance versus definitive treatment.
- Testing could be performed by immunohistochemistry for Ki67 and either immunohistochemistry or fluorescent in situ hybridization for PTEN (loss of expression or deletion, respectively) in core biopsies with highest grade and/or volume of tumor with optional additional evaluation in other samples.

Special attention should be undertaken in core biopsy from targeted lesions with suspicious findings in magnetic resonance imaging.

Immunohistochemical markers such as PTEN and Ki67 are inexpensive, globally established in Pathology laboratories worldwide, and need to be compared in more studies with centrally tested and expensive commercial RNA-based genomic assays.

mRNA-based genomic signatures

mRNA-based signatures have been developed and validated as prognostic factors in localized prostate carcinoma. In recent years, they have gained ground as tools for decision making in the setting of active surveillance. Currently, the NCCN guidelines acknowledge that three centrally tested commercial assays may be considered for patients for risk stratification. These tests are Oncotype Dx (Genomic Health), Prolaris (Myriad Genetics) and Decipher (GenomicDx Biosciences) (NCCN 2024a, b). They are considered prognostic with level of evidence IB, IIIC and IIIC; respectively. They are not cited in current EAU / ESMO guidelines (Motter et al. 2023).

Decipher is a 22 gene-expression assay that can be applied for formalin-fixed paraffin-embedded for biopsy and prostatectomy specimens. The test can predict adverse pathology in patients with low- and intermediate-risk prostate cancer as stratified by clinical evaluation (NCCN risk groups) (Herlemann et al. 2020). In prospective studies, patients with a biopsy-based diagnosis of prostate cancer, high-risk scores on the Decipher Biopsy test predicted shorter time to treatment in patients undergoing active surveillance and shorter time to treatment failure in patients receiving local treatment (Press et al. 2022; Vince et al. 2022). In a recent United state database analysis enrolling 572,545 patients (8,927 patients tested for Decipher), the use of molecular test were associated with higher likehood of option for conservative treatment. High Decipher scores were associated with option for radiation therapy. For those opting for prostatectomy, high Decipher scores were associated with adverse pathology (Zaorsky et al. 2023).

Apart from the localized cancer scenario, it has also been tested in advanced disease. A meta-analysis covering 855 patients showed that Decipher can predict 10-year metastasis risk in the post-prostatectomy scenario (Spratt et al. 2017). Also in radical prostatectomy specimens (NRG/RTOG 9601 trial), which randomized patients with prostate carcinoma with biochemical recurrence and pT3N0 or pT2N0 disease with positive margins, to receive salvage radiotherapy alone versus salvage radiotherapy with antiandrogen therapy. Decipher was independently prognostic for distant metastasis, cancer specific mortality, and overall survival (Feng et al. 2021). Importantly, the same study suggested that patients with lower Decipher scores had little or no benefit from the addition of antiandrogen therapy to salvage radiotherapy, whereas those patients with higher scores had much more benefit from the antiandrogen therapy. Currently, NCCN guidelines now recommend consideration of Decipher testing to aid decision making in the postoperative setting (NCCN et al. 2024).

An additional test is the Decipher PORTOS score. It evaluates 24 genes and was validated in a retrospective study which demonstrated that high PORTOS scores were significantly associated with decreased 10-year metastasis risk in patients who received postoperative radiotherapy. As a consequence, Decipher PORTOS is the only genomic classifier with predictive value regarding response to adjuvant or salvage radiotherapy but is not recommended for this decision making yet in the absence of prospective studies (Eggener et al. 2020).

Oncotype DX GPS is a panel consisting of 12 prostate cancer-related and 5 housekeeping genes (score 0 to 100), suitable for formalin-fixed biopsy specimens. This assay has been assessed in a cohort ($n = 431$) low- to intermediate-risk prostate cancer biopsies, showing correlation with adverse pathologic features (GG3 or extraprostatic extension), biochemical recurrence, and metastasis (Cullen et al. 2015). However, a more recent study in a prospective cohort ($n = 432$) treated with active surveillance failed to validate the GPS test and suggested that adding GPS to a model containing Prostate specific antigen (PSA) kinetics and diagnostic Gleason grading did not significantly improve stratification of risk for adverse pathology over the clinical factors (Lin et al. 2020). In a recent retrospective cohort, pathological classification was concordant to Oncotype DX GPS risk stratification in 84% of all cases. The study showed that risk stratification by accurate pathologic reporting of biopsy Gleason grade and PSA levels, is equivalent to Oncotype DX testing in low-risk patients. Additionally, the clinicopathologic stratification is superior to Oncotype DX in predicting the outcome of intermediate-favorable risk patients (Renavikar et al. 2023).

Prolaris is a gene-expression panel including 31 cell cycle-related and 5 housekeeping genes, which can be performed on formalin-fixed biopsy specimens and has shown prognostic value when applied to biopsies and prostatectomy samples. It can predict 10-year metastatic risk after prostatectomy and cancer-specific mortality after conservative treatment (Lin et al. 2018; Akhoundova et al. 2022).

The NCCN guidelines propose the use of Decipher or Prolaris to support risk assessment in patients with unfavorable intermediate- to high-risk localized prostate cancer and a life expectancy of at least 10 years and allow the use of any of the 3 tests (Decipher, Prolaris, or Oncotype DX Prostate) for patients with low to favorable intermediate risk.

The recommendations of the International Society of Urological Pathology (ISUP) from the 2019 Consultation Conference included the following statements:

- Genomic signatures are of potential benefit for additional information in the scenario of active surveillance and post radical prostatectomy settings
- they should be compared with robust / detailed pathologic assessment and use of immunohistochemical biomarkers which requires further validation
- the issue of tumor heterogeneity should be addressed including the evaluation of best sampling strategies (e.g., targeted lesion samples).

The second item above is interesting to see in detail. The pathology report usually gives the information on the diagnosis of adenocarcinoma and grade. Grade groups are used in prognostic nomograms and are crucial to select treatment. It must be emphasized, however, that additional information is given in Pathology reports that are usually not used in risk assessment. Both GUPS and ISUP recommend giving the percentage of Gleason pattern 4 and informing whether cribriform morphology is present in Pathology reports of GG2/GG3 tumors (Epstein et al. 2021; van Leenders et al. 2020a). A body of literature highlights the prognostic importance of these findings (Sharma and Miyamoto 2018, van Leenders et al. 2020b, Delahunt et al. 2022, Seyrek et al. 2022). The literature is almost entirely omissive on which extent genomic signatures would give additional prognostic information if this detailed pathologic evaluation were taking into account. In a retrospective study, 37% of the Oncotype DX score could be predicted by morphologic features at biopsy including cribriform morphology (Greenland et al. 2019).

Similarly, few studies have compared commercial mRNA-based tests with single immunohistochemical markers. In a cohort enrolling 424 patients treated by radical prostatectomy, PTEN loss outperformed Prolaris as a prognostic risk factor or metastasis or cancer related death (hazard ratio of 5,3 versus 2,2) (Leapman et al. 2018). More studies with similar design are urgently needed.

Two additional interesting points are the variability between tests. There is plenty of studies focusing on interobserver variability among pathologists to assign a Gleason grade for prostate adenocarcinoma. Risk assessment variation among genomic classifiers are much less scrutinized. In a small series of 22 patients who were tested for two or three commercial tests, the agreement in risk assignment were lower than expected: Decipher and Prolaris showed 67% agreement (kappa = 0.31), Prolaris e Oncotype Dx 75% (kappa = 0.39) and Decipher and Oncotype Dx 50% (kappa not assessable) (Alam et al. 2019). A study from Michigan, USA, showed the real-life picture of mRNA based gene tests. It started with 3966 patients with an initial diagnosis of prostate adenocarcinoma: 747 (19%) underwent some commercially available testing: 59% (439) Prolaris, 30% (227) Decipher and 11% (81) OncotypeDx. Active surveillance was the option for 58% of patients not tested at all, 76% tested with low-risk results and 46% of those tested with high-risk results. The molecular test with a low-risk result made one patient out of nine who would initially opt for definitive treatment to switch option for active surveillance. On the other hand, the molecular test with a high-risk result made one patient out of 26 who would initially opt

for active surveillance to switch the choice for definitive treatment. Risk assessment was variable between different tests with patients with GG1 adenocarcinoma being defined as high-risk in 14% of those tested by Prolaris and 58% of those tested by Decipher (Hu et al. 2018). Studies with similar designs are important to evaluate the accurate importance of such tests in clinical routine practice.

As also commented above, a growing field of study in prostate cancer is radiogenomics and it should be emphasized that molecular alterations of prognostic/predictive importance may be better represented in targeted lesions from areas that are visible at magnetic resonance imaging (Ferro et al. 2021; Banerjee et al. 2022).

Biomarkers in the scenario of advanced prostate carcinoma

Metastatic prostate cancer is frequently treated with anti-androgen therapy (alone or in combination of other treatments) and most patients will show a remarkable response at first. Disease progression in patients with castrate levels of serum testosterone is termed castration-resistant prostate cancer (CRPC). Although initially believed to be independent of androgen receptor signaling, CRPC usually remains dependent on androgen receptor activation pathways. Additional therapies against androgen receptor signaling are used in the CRPC setting such as blockers of androgen synthesis (abiraterone) and direct antagonists of the androgen receptor (enzalutamide, darolutamide and apalutamide). In prostate cancer, DNA repair defect are targetable pathways. In addition, some androgen receptor-related biomarkers may have prognostic effect. Advanced adenocarcinomas in a selective pressure of long-term anti-androgen therapy may undergo transformation into neuroendocrine carcinoma with dramatic prognostic and treatment implications.

Homologous recombination defects

Almost 20% of metastatic prostate cancer show genomic alterations in homologous recombination repair pathway including BRCA1, BRCA2 and ATM genes (Robinson et al. 2015). About half of these case show germline mutations which comprises 10% of all men with CRPC (Robinson et al. 2015; Pritchard et al. 2016). This pathway defect is twice more frequent in advanced tumors compared to localized disease (Cancer Genome Atlas Research Network 2015). Germline and somatic mutations of genes involved in the homologous recombination repair pathway are common in aggressive histologic findings such as invasive ductal adenocarcinoma, intraductal carcinoma and acinar adenocarcinoma with Gleason pattern 5 (Lotan et al. 2020). Germline mutations in *BRCA2* and *ATM* genes are more common in lethal prostate cancer when compared to localized disease (Na et al. 2017) and are associated with tumor reclassification and treatment switch in patients under a active surveillance protocol (Carter er al. 2019).

Overall, germline DNA repair mutations have been reported with the lowest frequencies seen in patients with lower-risk localized prostate cancer (1.6%–3.8%), higher frequencies in those with higher-risk localized disease (6%–8.9%), and the highest frequencies in those with metastatic disease (7.3%–16.2%)0.36,38–44 One study found that 11.8% of patients with metastatic prostate cancer have germline mutations in 1 of 16 DNA repair genes: *BRCA2* (5.3%), *ATM* (1.6%), *CHEK2* (1.9%), *BRCA1* (0.9%), *RAD51D* (0.4%), *PALB2* (0.4%), *ATR* (0.3%), and *NBN*, *PMS2*, *GEN1*, *MSH2*, *MSH6*, *RAD51C*, *MRE11A*, *BRIP1*, or *FAM175A* (Pritchard et al. 2016).

In retrospective studies, CRPC patients with germline homologous recombination defects have improved responses to chemotherapy mirroring the scenario seen in ovarian cancer. More recently, poly (ADP-ribose) polymerase (PARP) inhibitors have been approved as an option for treatment with metastatic prostate cancer who progress during anti-androgen therapy (Mota el al. 2020; Al-Akhras et al. 2024).

Since the journey of the patient with metastatic prostate cancer is usually long (> 10 years) it is not unusual that tissue specimens may be stored for many years before being used for molecular evaluation. Also, the failure rate using an NGS assay is higher in metastatic bone samples, and decalcification contributes to increasing failure. Several studies have investigated the role of circulating tumor DNA (ctDNA) in metastatic disease. A recent genomic analysis of ctDNA in 3.334 advanced prostate cancer patients has been reported showing that 94% of patients had detectable ctDNA. In this analysis, 837 patients had both liquid and tissue (archival or metastatic) available for NGS. Moreover, the median tumor fraction in those samples was 7.5%; however, the threshold for detection of gene amplification in this analysis was ≥ 20%, meaning that information about amplification/deletion was possible in only 38% of the overall samples (Giunta et al. 2021).

As a consequence, there is a considerable rate of inconclusive results due to poor preservation. Early testing in high-risk patients is advised. The ISUP currently recommends:

- a germline panel for defects on homologous recombination pathway for patients with localized prostate cancer with high grade (≥ GG4), any grade with PSA levels ≥ 20 ng/mL or known metastatic disease.

– a somatic panel for defects on homologous recombination pathway for patients with known distant metastases with at least testing for BRCA1 and BRCA2.

An additional comment is that NCCN (2024a, b) further indicates germline mutation testing for patients with intraductal carcinoma at biopsy (Isaacsson et al. 2018; Risbridger et al. 2015; Taylor et al. 2017). This observation emphasizes the importance of this diagnosis at biopsy specimens regardless of incorporating it or not in final grading score as it is recommended and not recommended by the two major international societies of Urological Pathology – ISUP and GUPS, respectively (Epstein et al. 2021; van Leenders et al. 2020a, b). Germline testing should also be considered for patients of intermediate risk with invasive cribriform and/or invasive ductal morphology although evidence of association between these morphologies and germline mutations in homologous recombination DNA repair pathways is less compelling.

Tests are preferentially performed in metastatic tissue but, if unavailable, in primary tumor samples. NCCN (2024a, b) recommends Multigene tumor testing for alterations in homologous recombination pathway, including but not limited to *BRCA1*, *BRCA2*, *ATM*, *PALB2*, *FANCA*, *RAD51D*, *CHEK2*, and *CDK12*, is recommended in patients with metastatic prostate cancer. This testing can be considered in patients with regional prostate cancer. The PROFOUND trial included patients with *BRCA1*, *BRCA2*, *ATM*, *BRIP1*, *BARD1*, *CDK12*, *CHEK1*, *CHEK2*, *FANCL*, *PALB2*, *PPP2R2A*, *RAD51B*, *RAD51C*, *RAD51D*, and *RAD54L* gene alterations. This study showed the effect of PARP inhibitor Olaparib on progression-free survival in pateints with metastatic castration-resistant prostate cancer (de Bono et al. 2020).

PARP inhibitors act by trapping PARP on DNA, which is key as it presents a physical obstacle to the replication machinery. To resolve the PARP-DNA interaction, HRR is necessary. Therefore, in HRR-deficient cancer cells, trapped PARP results in replication fork collapse and finally cell death. Non neoplastic cells have both pathways preserved and, so, PARP inhibition has no lethal effect due to alternative homologous recombination activation.

Olaparib is a poly (ADP-ribose) polymerase) inhibitor (iPARP) that rose in recent years as an option for patients with metastatic CRPC with mutated genes in homologous recombination pathways. Patients treated with Olaparib had longer progression-free survival intervals when compared to patients treated with abiraterone or enzalutamide (NCCN 2024a, b). Patients with BRCA2 mutations show improved responses when compared to other genetic alterations in this pathway. Olaparib with abiraterone may be used in some patients with metastatic CRPC (PROS16) and a pathogenic BRCA1 or BRCA2 mutation (germline and/or somatic) who have not yet received a novel hormone therapy (Saad et al. 2023a).

Rucaparib is an option for patients with metastatic CRPC and a pathogenic BRCA1 or BRCA2 mutation (germline and/or somatic) who have been treated with androgen receptor-directed therapy and a taxane-based chemotherapy. Progression-free survival was significantly longer in the group that received rucaparib than in those who received a control medication (abiraterone, enzalutamide, or docetaxel). In the pre-docetaxel setting, rucaparib is a preferred option for patients with BRCA1 or BRCA2 mutations. If the patient is not fit for chemotherapy, rucaparib can be considered even if taxane-based therapy has not been given (Fizazi et al. 2023).

Talazoparib plus enzalutamide is a treatment option for patients with metastatic CRPC and a pathogenic mutation (germline and/or somatic) in an HRR gene (*BRCA1, BRCA2, ATM, ATR, CDK12, CHEK2, FANCA, MLH1, MRE11A, NBN, PALB2*, or *RAD51C*) who have not yet had treatment in the setting of CRPC, depending on prior treatment in other disease settings (Saad et al. 2023b). Median progression-free survival was improved in the talazoparib group compared with controls. There may be heterogeneity of response based on the specific gene mutation.

Niraparib plus abiraterone is a treatment option for patients with metastatic CRPC and a pathogenic BRCA1 or BRCA2 mutation (germline and/or somatic) who have not yet had treatment in the setting of metastatic CRPC, depending on prior treatment in other disease settings. Progression-free survival was improved for those receiving niraparib in the HRR mutation group overall and in the BRCA mutation subgroup.

DNA mismatch repair

Defects in mismatch repair pathway (MMR) are also more common in metastatic prostate cancer than in localized disease. Pathogenic mutations in MMR genes are detected in 10% of CRPC compared to < 3% of primary tumors of all grades. These alterations are more common in aggressive histologies such as ductal adenocarcinomas and primary adenocarcinomas with Gleason pattern 5. Only about 20% of MMR defects derive from germline mutations. The risk of prostate carcinoma is raised in patients with Lynch syndrome.

Pembrolizumab (monoclonal antibody against programmed death receptor-1, PD-1, a immune checkpoints inhibitor) is approved for all progressing tumors with MMR defects or microsatellite instability.

Immunotherapy responses are lower in prostate cancer than in other primary sites.

The ISUP currently recommends:

- a somatic test for defects in mismatch repair pathway for patients with known distant metastases which can be done by immunohistochemistry (MLH1, PMS2, MSH2, MSH6) with or without microsatellite instability testing and gene sequencing.

CDK12 deficiency

CDK12 encodes cyclin-dependent kinase 12, a tumor suppressor protein with diverse functions related to genomic stability. At first, CDK12 role in DNA repair was attributed to regulation of homologous recombination DNA repair genes (*BRCA1*, *FANCD2*, and *ATR*), with a suggestion that genetic inactivation of *CDK12* was associated with PARP inhibitor sensitivity in preclinical models. More recently, however, it was proposed that in prostate cancer, CDK12 may function primarily in DNA replication-associated repair, with biallelic inactivation of *CDK12* resulting in a genomic signature with widespread focal tandem duplications which generates fusion–induced neoantigens and, as a consequence, sensitivity to immune checkpoint inhibitors (Wu et al. 2018).

Cyclin-dependent kinase 2 is encoded by *CDK12* gene which is altered in 2% and 5% of localized and metastatic prostate carcinomas, respectively (Chung et al. 2019) and is associated with aggressive disease (Nguyen et al. 2020). These *CDK12*-altered tumors showed poor responses to androgen-receptor signaling inhibitors and taxane-based chemotherapy, did not respond to PARPi and showed variable responses to checkpoint inhibitors (Antonarakis et al. 2020; Schweizer et al. 2020).

Androgen receptor-related markers

About half of CRPC harbor androgen receptor gene mutations or amplifications. From studies in tumor cell lines, it was identified that a splice variant AR*v7* is possibly associated with resistance to anti-androgen therapy. The expression rate of AR*v7* varies as function of methods employed (RT-PCR, sequencing, immunohistochemistry) or the specimen tested (tissue samples or circulating tumor cells) (Lotan et al. 2020). Currently, there is no role of AR*v7* testing in tumor samples because it is a very common finding in tumors exposed to anti-androgen therapy. Most studies showing potential to predict resistance to anti-androgen therapy is derived from studies that detected androgen receptor amplification or splice variant AR*v7* testing in cell free DNA.

Diagnosis of neuroendocrine prostate cancer

Small cell or large cell neuroendocrine carcinoma is a rare diagnosis in the localized disease setting. In advanced CRPC, neuroendocrine carcinoma is seen in up to 10% of the cases in which a biopsy was performed. It is well recognized that transformation of acinar adenocarcinoma in neuroendocrine carcinoma may develop as a form of lineage plasticity for acquisition of resistance to therapies targeting androgen receptor pathway. Indeed, the WHO classification creates as a different entity the Treatment-related neuroendocrine prostatic carcinoma (t-NEPC). In more than half of the patients in whom it develops, t-NEPC develops within 24 months of androgen-deprivation therapy and the median survival time after transformation into t-NEPC is only 7 months (Rubin et al. 2022).

Neuroendocrine carcinoma responds poorly to anti-androgen receptors and this diagnosis prompt the switch of treatment for limited option of platinum chemotherapy or enrolling patients in clinical trials.

Neuroendocrine markers are commonly used in Pathology laboratories (chromogranin, synaptophysin, CD56) but are not specific for neuroendocrine carcinomas. Variable expression of these markers is seen in low and intermediate grade localized acinar adenocarcinomas of the prostate, and this finding has no clinical implications. As a consequence, routine immunohistochemical testing for neuroendocrine differentiation is not advised in this scenario.

Similar to what is seen in lung neoplasms, neuroendocrine prostate adenocarcinoma usually shows *TP53* and *RB1* inactivation. These changes, however, can be observed in high-grade acinar adenocarcinoma, especially in the CRPC setting.

The ISUP Conference consultation of 2019 recommended:

- to not test expression of neuroendocrine markers in localized prostate cancer unless it shows suggestive neuroendocrine morphology
- the term neuroendocrine differentiation is best reserved for high-grade prostate cancers (with clinical implication being evaluated) and not for well differentiated neuroendocrine tumor or low-grade acinar adenocarcinoma
- advanced metastatic CRPC may manifest a range of morphologic features and in then future biomarker-driven clinical trial may define the better treatment options for tumors in the spectrum of acinar adenocarcinoma and neuroendocrine carcinoma

There is not enough data to infer clinical implications of expression of neuroendocrine markers in otherwise ordinary high-grade prostate adenocarcinomas.

A recent retrospective series enrolled 17 patients with prostate carcinomas GG ≥ 2 sharing expression of prostate (androgen receptor, PSA, NHK3.1) and neuroendocrine marker synaptohysin (the largest to date). This phenotype seems to have no prognostic value in de novo setting, while neuroendocrine differentiation arising in the setting of patients with prior diagnosis of prostate cancer during treatment (so called treatment-emergent amphicrine prostate cancer) had a poor prognosis (5.3-month survival). The treatment-emergent neuroendocrine transdifferentiation was detected in a mean interval of 41.1 months after initiation of anti-androgen therapy (Graham et al. 2023).

From a morphologic point of view, the diagnosis of Gleason pattern 5 in the absence of Gleason patterns 3 or 4 should be made after cautiously excluding benign mimickers, urothelial carcinoma and neuroendocrine carcinoma. A low threshold should be considered to use immunostains in this context. Neuroendocrine carcinoma, notably the small cell type, should be considered in tumors with prominent mitotic activity, numerous apoptotic bodies, high nuclear-to-cytoplasmic ratio, nuclear molding, extensive (geographical necrosis) and absence of central prominent nucleoli. Expression of neuroendocrine markers in the absence of adequate morphology should not lead to overdiagnosis of small cell carcinoma. Low-grade and high-grade prostate adenocarcinoma show variable expression of neuroendocrine markers (discussed above). Indeed, in a tumor morphologically indicative of neuroendocrine carcinoma, the absence of prostatic acinar differentiation markers (NKX3.1, prostein/P501S, PSMA and PSA) is much more compelling evidence than expression of neuroendocrine markers such as synaptophysin, chromogranin and INSM1 (Baraban and Epstein 2022). Additional valuable stains are TTF1 (expressed in 50% of small cell carcinomas of prostate origin) and a Ki67 index higher than 70% (high-grade acinar adenocarcinoma usually show a proliferative index below 50%) (Epstein et al. 2014a). The use of PSA as a single marker of prostatic differentiation is limited by the observation that it is expressed in 85–90% of Gleason 10 (5 + 5) acinar adenocarcinoma (Epstein et al. 2014b). The sensitivity for PSA, PSAP, PSMA and NKX3.1 was 64–94%, 98.6%, 100% and 98.6–100%, respectively. Currently, NKX3.1 is the best single marker for prostatic acinar differentiation with high sensitivity and very high specificity (99,7%) (Gurel et al. 2010; Huang et al. 2018) (Fig. 2).

From a clinical perspective, accurate diagnosis of neuroendocrine carcinoma is of crucial importance. Since the diagnosis come with the prediction of resistance to anti-androgen, it will exclude a large options new drugs developed targeting the androgen receptor pathway – that may show remarkable responses in patients with metastatic high-grade adenocarcinoma who experienced progression after initial anti-androgen therapy.

Since neuroendocrine transformation is a well-known phenomenon in prostate cancer progression leading to resistance to anti-androgen therapies, it is not surprising that some advanced tumors commonly show a spectrum of chances that does not fit in the prototypical poles of high-grade acinar adenocarcinoma and clearcut neuroendocrine carcinomas.

The current WHO (2022) criteria for the diagnosis of neuroendocrine carcinomas of the prostate are:

- for small cell carcinoma: characteristic high-grade histology including nuclear and architectural features (essential) and positive immunostaining for synaptophysin, chromogranin A, and/or additional neuroendocrine markers (desirable).
- for large cell carcinoma: characteristic high-grade histology combined with positive immunostaining for synaptophysin and chromogranin A (essential).

Treatment-related neuroendocrine prostatic carcinoma*:* prostatic carcinoma with neuroendocrine differentiation, pure or admixed with generally high-grade adenocarcinoma; confirmatory immunohistochemical stains such as synaptophysin and chromogranin) history of antiandrogen therapy (essential).

Use of artificial intelligence in prostate cancer

A rapidly growing in field is the application of artificial intelligence in Surgical Pathology, and its utility of prostate cancer using this tool has gained much attention in recent years. Deep leaning models were developed and applied for diagnosis, grading, outcome prediction and prediction of genomic signatures, and have been introduced in the workflow of pathologists worldwide. A review of the current state of the art of this topic is beyond the scope of the review and can has been reviewed in detail elsewhere (Rabilloud et al. 2023).

Biomarkers in bladder cancer

Bladder cancer is the ninth most common cancer in the world (614,298 cases/year), occurring mainly in North Africa, Southern Europe and North America; it has a mortality rate of 220 thousand cases/year (International Agency for Research on Cancer 2021), with a higher prevalence in males and a 5-year survival rate of 77% (WHO 2022).

Urothelial carcinoma is the most common histological type of bladder cancer (comprising 90% of cases) and they may occur anywhere in the urothelial tract.

Understanding the histology of urothelial carcinoma is necessary for the diagnosis and treatment strategies, since they can have various histological patterns, some of which are associated with poorer prognosis and aggressive behavior (like micropapillary, sarcomatous, nested patterns) (WHO 2022).

They can present themselves as flat lesions (carcinoma in situ), or papillary lesions, and it is already well known that the paths of development of lesion patterns are different: non-muscle-invasive lesions are related to changes in molecular pathways involving *FGFR3, H-RAS* and *PIK3CA;* Muscle-invasive lesions are associated with mutations in tumor suppressor genes, such as *TP53, P16 and RB* (Netto 2012, Robertson et al. 2017, Inamura 2018).

Molecular subtypes of urothelial carcinoma

Bladder cancer is a heterogeneous disease with various molecular subtypes that influence its behavior, prognosis, and response to treatment. Understanding these subtypes is crucial for developing targeted therapies and personalized treatment plans for patients (Inamura 2018).

Luminal-papillary subtype (35% of cases)

Luminal-like bladder cancer is characterized by the expression of genes associated with luminal epithelial cells. These tumors often show a more differentiated phenotype and are typically non-invasive or low-grade. They may have mutations in genes such as *FGFR3* (fibroblast growth factor receptor 3) and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), which are associated with cell signaling pathways and cell proliferation. For these cases, the suggestive treatment is *FGFR3* inhibitors and early cystectomy without neoadjuvant chemotherapy (Inamura 2018).

Luminal Infiltrated subtype (around 19% of cases)

Infiltrated bladder cancer is characterized by the presence of immune cell infiltration within the tumor microenvironment, and they also express smooth muscle and myofibroblasts gene signatures. These tumors may exhibit high levels of immune checkpoint molecules such as PD-L1 (programmed death-ligand 1) and may respond well to immunotherapy agents targeting the PD-1/PD-L1 axis (Inamura 2018).

Luminal infiltrated subtype also has alterations in the *TP53* gene and dysregulation of p53 pathway signaling. These tumors often have a high mutational burden and may be associated with a more aggressive phenotype and poorer prognosis. Neoadjuvant chemotherapy or molecular target therapy or immune molecular checkpoints treatments can be used in these cases (Inamura 2018).

Basal-squamous subtype (35% of cases)

Basal-squamous bladder cancer is more aggressive and tends to be invasive and high-grade. These tumors resemble basal cells of the bladder epithelium and can exhibit features of squamous cell carcinoma. Basal-squamous tumors often show mutations in tumor suppressor genes such as *TP53* (tumor protein p53) and *RB1* (retinoblastoma 1), which are involved in cell cycle regulation and DNA repair. The suggestion of treatment for these patients is with neoadjuvant chemotherapy and immune checkpoint molecules such as PD-L1 (programmed death-ligand 1) and may respond well to immunotherapy agents targeting the PD-1/PD-L1 axis (Inamura 2018).

Neuronal subtype

This subtype is characterized by tumors that express genes associated with neuronal differentiation. Neuronal subtype bladder cancers may have a distinct clinical course and response to therapy compared to other subtypes. They may exhibit neural markers such as synaptophysin and chromogranin A and are associated with a neuroendocrine phenotype. The treatment of choice for these cases are etoposide plus cisplatin-based therapy (Inamura 2018).

Immune checkpoint molecules (PDL1)

Programmed cell death ligand 1 (PD-L1) inhibitors have emerged as a promising therapeutic option for the treatment of bladder cancer, especially in the setting of local advanced or metastatic disease. Bladder cancer is known to be immunogenic, with tumor cells expressing PD-L1 to evade immune surveillance. PD-L1 inhibitors work by blocking the interaction between PD-L1 on tumor cells and PD-1 on immune cells, thereby restoring the immune system's ability to recognize and attack cancer cells (Alsaab et al. 2017).

PD-L1 expression has been identified as a potential predictive biomarker for response to PD-L1 inhibitors in bladder cancer, but its use is currently controversial and tends to be more restrictedly used. Tumors with high levels of PD-L1 expression was associated with poor overall survival, higher tumor stage and distant metastasis (Zhu et al. 2019).

As of this date, with the approval of first-line treatment with enfortumab vedotin in combination with pembrolizumab for patients with locally advanced or metastatic disease, regardless of PD-L1 status, routine immunohistochemical evaluation in this setting is no longer

performed, limiting the role of PD-L1 expression (Powles et al. 2024).

Evaluation of PD-L1 by pathologists:

The present guidelines for requesting PD-L1 testing in UC suggest informing the desired immune checkpoint blockade (ICB) drug indicated. This allows pathology laboratories to offer ideal testing conditions. If no specification is provided, the diagnosing pathologist should search for the information with the oncologist. This exchange between clinicians and pathologists is essential for providing precise and prompt test results. Standardized structured reporting is also advised for enhanced quality of individual pathology reports (Eckstein et al. 2019).

CPS (combined positive score) is a scoring algorithm used to assess first-line treatment eligibility with Pembrolizumab for patients with metastasized or locally advanced urothelial carcinomas of the bladder and upper urinary tract. It focuses on the total amount of PD-L1 positive immune cells and tumor cells in proportion to the total number of tumor cells. The cut-off for CPS is 10, and it is capped at 100 (Eckstein et al. 2019).

FGFR3 inhibitors

The *FGFR3* (fibroblast growth factor receptor 3) molecular pathway plays a significant role in various cellular processes, including cell proliferation, differentiation, survival, and migration. Aberrations in the *FGFR3* pathway have been implicated in the development and progression of several types of cancer, including bladder cancer. The activation of *FGFR3* triggers several signaling pathways, including the RAS-MAPK (mitogen-activated protein kinase), PI3K-AKT (phosphatidylinositol 3-kinase-protein kinase B) pathways, that regulate various cellular processes, including cell proliferation, survival, differentiation, angiogenesis, and metabolism.

The dysregulation of the *FGFR3* pathway can happen through mutations, amplifications, or overexpression (Ornitz and Itoh 2015).

Several *FGFR* inhibitors, including small-molecule tyrosine kinase inhibitors (TKIs) and monoclonal antibodies, have been developed and evaluated in clinical trials for the treatment of *FGFR*-altered cancers.

In bladder cancer, activating mutations in *FGFR3* are found predominantly in low-grade non-muscle-invasive tumors, particularly in papillary urothelial carcinomas *FGFR* inhibitors such as erdafitinib (Loriot et al. 2023) have shown improved outcomes in patients with invasive tumors with *FGFR* alterations, including *FGFR3* mutations, who have progressed on or are ineligible for standard chemotherapy. That said, it is important to emphasize that research into FGFR3 mutations must be carried out in the invasive component of neoplasms, to avoid false positive results.

Wang et al. examined the impact of a mutated gene found in a subset of urothelial cancers on response to treatment with immunotherapy and found that patients with tumors harboring mutations in the gene FGFR3 respond to immunotherapy similarly to patients without such mutations (Wang et al. 2019).

HER-2 target therapy

HER-2, also known as human epidermal growth factor receptor 2, is a member of the *HER* family of receptor tyrosine kinases. Abnormalities in the *HER-2* gene, including overexpression, amplification, and mutation, play a critical role in the pathogenesis of various cancers (just like breast, lung, colorectal and gastric cancers).

In 2024, the first tumor-agnostic approval of a HER2-directed therapy and antibody drug conjugate (trastuzumab—deruxtecan) by the Food and Drug Administration Food (United States) in patients with HER2-positive tumors with immunochesmtry 3 + score.

Studies have shown that in urothelial carcinomas, especially those of micropapillary subtype, may show HER2 overexpression. Within tumors with this morphology, HER2 overexpression occurs in 68% of cases and there is a high correlation between positive immunohistochemistry 2 + or 3 +, with gene amplification. Therefore, subtype recognition in urothelial carcinoma may be in near future to better select patients for target therapies (Ching et al. 2011; Behzatoğlu et al. 2018; Zinnall et al. 2018; Sanguedolce et al. 2019).

her-2 overexpression

Refers to an increase in the production of her-2 protein without an accompanying increase in gene copy number. This can occur due to transcriptional upregulation or post-translational stabilization of the HER2 protein. These overexpression guide to an excessive activation of downstream signaling pathways involved in cell proliferation, survival, and differentiation, contributing to tumor growth and progression. Targeting HER2 with specific inhibitors (trastuzumab, pertuzumab) has become a cornerstone of treatment for HER2-positive cancers, offering improved outcomes and quality of life for affected patients (Sanguedolce et al. 2023).

HER-2 amplification

HER2 gene amplification involves an increase in the number of copies of the *HER2* gene, resulting in higher levels of her2 protein expression on the cell surface, resulting in an uncontrolled cell proliferation and tumor growth.

HER-2 mutation

Genetic alterations in various regions of the *HER-2* gene that result in the production of a mutant her2 protein with altered structure and function, that can increase receptor dimerization and phosphorylation, promoting oncogenic signaling pathways or generate a ligand-independent activation of *HER-2* pathway.

Assessment of HER-2 status by the pathologist

To date, there is no consensus on the best way to evaluate *HER-2* status in bladder cancer, as there are several studies with different evaluation methodologies: some evaluate overexpression using immunohistochemistry (Scherrer et al. 2022), others evaluate *HER-2* amplification using situ hybridization (ISH) (Kamoun et al. 2020) and others search for the presence of *HER-2* mutations with genetic sequencing. In addition to the different ways of accessing HER-2 status, we must take into account the variables of tumor heterogeneity and variations in intra- and inter-observer interpretations.

A tumor that presents overexpression of *HER-2* does not necessarily have amplification of the gene detected, or even the presence of its mutation, which makes it difficult to establish an algorithm for evaluating the status of HER-2 in these neoplasms, making new multicenter studies necessary.

Most studies that evaluate *HER-2* overexpression by immunohistochemistry (IHC) evaluate using criteria already established in breast (Wolff et al. 2018) and gastric cancer, that is, they evaluate the percentage of cells with membrane labeling, classifying tumors as *HER-2* negative (0+), *HER-2* low (1+) or *HER-2* positive (2+ or 3+). Generally, the tumors subjected to this research are locally advanced or metastatic bladder cancer. The guidelines recommend trastuzumab, for gastric cancer, with chemotherapy only for patients with IHC 3+ and IHC 2+ with evidence of *HER-2* amplification by ISH (HER2 /CEP17 ratio ≥ 2). Trastuzumab is not recommended if the IHC score is 0 or 1+ (Ajani et al. 2013).

Biomarkers in germ cell tumors of the testis

The vast majority (> 95%) of testicular cancers correspond to germ cell tumors (GCTs), which are the most common solid neoplasms in young-adult Caucasian men aged 20–40 years (Trabert et al. 2015). These are grouped by the World Health Organization (WHO) as germ cell neoplasia in situ (GCNIS)-derived tumors (i.e. postpubertal-type tumors, the most common, typical of the young-adult male and showing malignant behavior) and GCNIS-unrelated tumors (including both prepubertal-type tumors and spermatocytic tumors) (Berney et al. 2022). Testicular germ cell tumors (TGCTs) are called developmental cancers, since they reflect the various steps of embryonic and germ cell development, retaining features of their cell of origin, including their epigenetic traits (Lobo et al. 2019a, b, c). This has led to the proposal of a classification of GCTs which includes all genders and age groups, and focusing on distinct pathobiology, cytogenetic and epigenetic background. This classification scheme comprises seven types of GCTs, of which only types I, II and III occur in the testis (corresponding to prepubertal-type tumors, postpubertal-type tumors and spermatocytic tumors, respectively) (Oosterhuis and Looijenga 2019). The study and better understanding of developmental biology has not only led to new classification proposals, but also contributed to the discovery of most biomarkers that are clinically useful for TGCT patients (Tavares et al. 2023). These include, overall, the classical serum tumor markers (alpha fetoprotein [AFP] and human chorionic gonadotropin [HCG]), which are secreted and are critical during embryogenesis; the pluripotency factors (for instance, OCT3/4, among others) which are used by Pathologists in their daily routine for diagnosing and characterizing the different histological types of TGCTs; and the embryonic microRNAs of the miR-371 ~ 373 cluster, which have emerged in recent years as the most promising non-invasive biomarker of TGCTs (Almstrup et al. 2020).

In the next sections we will review the current use of TGCT diagnostic and prognostic biomarkers, both in tissue and liquid biopsies.

Classical serum tumor markers

The so-called "classical serum tumor markers" include AFP, HCG and lactate dehydrogenase (LDH). These are used every day in the clinic and play important roles in the management of TGCT patients, being measured at diagnosis, post-orchiectomy and in follow-up visits (Murray et al. 2016b). These markers are part of the TNM-S staging for TGCTs, with post-orchiectomy markers integrating the "S" parameter of such staging (Oldenburg et al. 2022). Pre-orchiectomy serum tumor marker elevations are important for Pathologists, and should guide the grossing of testicular masses, looking for components that explain such elevations above the reference levels (Verrill et al. 2017). Also, serum tumor markers are part of the International Germ Cell Cancer Collaborative Group (IGCCCG) risk classification for metastatic patients, determining prognosis and, therefore, treatment (International Germ Cell Consensus Classification 1997).

HCG, which is produced by syncytiotrophoblast cells, is of importance during embryonic development, and its measurement is routinely used for diagnosis of pregnancy or for diagnosis and monitoring of molar disease

(Keay et al. 2004). It has a half-life of 12 to 36 h. HCG is elevated (usually several hundreds to thousands over the upper reference limit) in patients with choriocarcinoma, and such high elevations may exceptionally be used as indications to give pre-operative chemotherapy (Salem and Gilligan 2011). However, minor elevations may be seen in any TGCT subtype harboring foci of isolated syncytiotrophoblast cells (which do not merit the designation of choriocarcinoma). About 15–30% of seminomas can, therefore, show minor elevations (Dieckmann et al. 2018; Dieckmann et al. 2019). HCG elevations lack specificity, as they may also be seen in somatic malignancies (for instance in some forms of bladder cancer), and also secondarily in patients with hypogonadism (Stenman et al. 2004; Germa et al. 1987).

AFP is a glycoprotein detected in high concentrations in embryonal/fetal serum, being synthesized in the yolk sac, the site of embryonal hematopoiesis (Gitlin and Boesman 1967). Therefore, it is not surprising that it is a biomarker of yolk sac tumor histology. During the fetal stage hematopoiesis is transferred to the liver (and can occur for a brief period in the gastrointestinal tract as well), also explaining why around 20–25% of teratomas may secrete AFP (especially the ones showing hepatoid or intestinal features). AFP has a half-life of 5–7 days (7). However, AFP is physiologically elevated in the first year of life despite the absence of any TGCT (Blohm et al. 1998), and a proportion of the population shows constitutional minor elevations of this serum marker (Houwert et al. 2010), which can be a confounding factor. Like for HCG, AFP lacks specificity, being a tumor marker of hepatocellular carcinoma and of other carcinomas, namely those with enteroblastic differentiation (Murakami et al. 2016). Moreover, patients undergoing chemotherapy may show elevations of AFP due to liver injury, which could erroneously be interpreted as tumor recurrence (Germà et al. 1993) (a dilemma shown in one study to be resolved by microRNA testing, see below).

LDH is by far the least specific of the classical serum tumor markers. LDH is increased in many conditions with elevated cell turnover (including many cancers, but also stroke, myocardial infarctions, infections, etc.) (Jialal and Sokoll 2015). Also, its half-life is variable among institutions, depending on the type of assay and isoform detected. These reasons make it the least specific of TGCT serum markers (Ackers and Rustin 2006).

All in all, and despite their usefulness, classical serum tumor markers have several limitations. They are only elevated in around 60% of TGCTs at diagnosis, and elevations of specific markers are dependent on the histological composition of the tumor. There is an urgent need of additional non-invasive biomarkers which can overcome these limitations and complement these markers (Lobo et al. 2023a). This is particularly important in the testis, since approach to a testicular mass does not usually involve testicular biopsy (for the associated risks, including tumor seeding), and diagnosis of a malignant TGCT is only confirmed after orchiectomy is performed. This raises the need for liquid biopsy biomarkers with excellent sensitivity and specificity for TGCT diagnosis, with microRNAs emerging in the latest years as the most promising candidates (see section below for discussion on microRNAs).

Immunohistochemistry markers

Pluripotency-related transcription factors are among the most robust biomarkers of TGCTs (Gillis et al. 2011). They are detected by immunohistochemistry easily in every Pathology Department, and help define and confirm specific histological subtypes, being quite reliable in interpretation (with some caveats) (Siegmund et al. 2023, Ulbright et al. 2014) (Fig. 3).

One of the most used markers is SALL4, which is a pan-GCT marker, which can be quite useful in confirming a GCT origin in the event of cancers of unknown origin (Miettinen et al. 2014). It is upstream of OCT3/4 (also known as POU5F1), another very useful marker, which is an essential factor in the maintenance of embryonic stem cell and primordial germ cell pluripotency (Cheng et al. 2007), being used in the clinic as a marker of embryonal carcinoma and seminoma. Additionally, it is often employed in the work-up of testicular biopsies for infertility on during follow-up of contralateral testis, to rule out GCNIS (Oosterhuis et al. 2011). Negativity in other histological subtypes with extra-embryonic differentiation (yolk sac tumor, choriocarcinoma and teratoma) is consistent and very useful in practice. For instance, OCT3/4 negativity is especially useful for pinpointing small foci of yolk sac tumor, which may be difficult to discern from embryonal carcinoma. Like OCT3/4, NANOG is also part of the pluripotency network and signals both seminoma and embryonal carcinoma. LIN28, which is involved in microRNA maturation, was also found positive in GCNIS and seminoma (and also in additional subtypes) (Cao et al. 2011). However, these are less available in most laboratories compared to OCT3/4.

Following a better understanding of reprogramming phenomena (i.e. transitions between cells with different potency states, like the seminoma – embryonal carcinoma transition), members of the SOX family have also entered routine evaluation of GCTs by immunohistochemistry. SOX17 and SOX2 are, therefore, specific markers of seminoma and embryonal carcinoma, respectively (de Jong et al. 2008). Likewise, CD117 (KIT), a major factor regulating germ cell development, is used in routine as a marker of seminoma and GCNIS. This

tyrosine kinase receptor binds to stem cell factor and constitutes the most commonly mutated gene in TGCTs (Kemmer et al. 2004). KIT is a reliable marker of seminoma independent of mutational status. However, focal staining in yolk sac tumor has been reported. In addition, placental-like alkaline phosphatase (PLAP) is also used primarily as a seminoma marker, but it can also show some positivity in other TGCT subtypes and even in non-GCT cancers (Wick et al. 1987). Of interest, strong expression of D2-40 is quite characteristic of seminoma, being negative in other GCT subtypes. On the other hand, CD30 is used frequently for supporting the diagnosis of embryonal carcinoma, especially if strong and diffuse (Ranjitha et al. 2022). Very focal positivity may be admitted in seminoma (which may also show focal staining with cytokeratins, particularly in a dot-like fashion, an important pitfall). It may be argued that these seminomas already show evidence of initial reprogramming, and such intermediate phenotype of seminomas is supported also by molecular studies (Lobo et al. 2018). Recapitulating uses in serum, HCG and AFP are also detected by immunohistochemistry for pinpointing choriocarcinoma/syncytiotrophoblast cells or yolk sac tumor, respectively. AFP is specific, but it lacks sensitivity, while glypican is more sensitive for detecting yolk sac tumor foci, but instead lacks specificity (also staining syncytiotrophoblast cells, for instance) (Zynger et al. 2006). GATA3 is also useful for supporting trophoblastic differentiation, but also may stain yolk sac tumor.

There is a need of a specific teratoma marker, namely one that is discriminative from yolk sac tumor. Both tumors are challenging given their heterogeneity of morphologies. To date, no perfect immunohistochemistry biomarker exists, and distinction is many times morphological. HNF1ß (Gallo et al. 2020) and FOXA2 (Ricci et al. 2023) have been recently proposed as specific markers of yolk sac tumor, but are not currently widely disseminated. CDX2 is a sensitive marker of yolk sac tumor and may be useful for its wide availability in all laboratories, although it also stains glandular components of teratomas (Osman et al. 2016). SSX has also been advocated as a biomarker of spermatocytic tumors (Anderson et al. 2021), which is important to discriminate from seminoma, which may have a somewhat similar morphology.

Cytogenetic biomarkers

The hallmark of type II GCTs is the presence of gains of chromosome 12p, often in the form of isochromosome 12p, so this has become a robust biomarker of these tumors. Gains in 12p (a region including important TGCT markers, such as *NANOG*, *GDF-4* or *STELLA*) mark the transition from GCNIS to seminoma and have several applications in practice for Pathologists (Atkin & Baker 1982). First, they can support a GCT origin in the event of a somatic malignant transformation, especially in the metastatic setting (which almost invariably shows poor prognosis) (Fichtner et al. 2021). The histology of such tumors is indistinguishable from somatic cancers morphologically and by immunohistochemistry, and therefore showing presence of isochromosome 12p may truly support a GCT origin (Lobo et al. 2022). Likewise, absence of 12p gains supports a diagnosis of (most) spermatocytic tumors (which also display gains in chromosome 9, leading to *DMRT1* amplification), although a subset of spermatocytic tumors with aggressive behavior have been shown to harbor gains in 12p (Gupta et al. 2024). Also, it can also aid in resolving if a pure teratoma is a postpubertal-type or a prepubertal-type tumor, which is of clinical relevance since the former are malignant, while the latter show a benign behavior and there is often no need to continue close surveillance. Importantly, prepubertal-type teratomas may often be detected later in life, in adults (Wagner et al. 2020). Total embedding of adjacent parenchyma is recommended, looking for GCNIS or another histological component and, if not found, absence of gains in 12p may be the final proof that this is a type I (prepubertal-type) teratoma. Gains in 12p may be searched for using fluorescence in situ hybridization (FISH), polymerase chain reaction (PCR), next generation sequencing (NGS), comparative genomic hybridization or single nucleotide polymorphism array (Freitag et al. 2021).

MicroRNAs

MicroRNAs have been the hot topic at the moment regarding biomarkers of TGCTs. Since the identification of members of the 371 ~ 373 microRNA cluster as specific biomarkers of this family of tumors, innumerous studies have accumulated, generating the evidence required for this biomarker to enter clinical trials (NCT04914026 and NCT04435756, which are ongoing) (Tavares et al. 2024) and, more recently, for the approval of an IVD test for miR-371a-3p testing in the clinic, the M371 test. It is fair to say that this biomarker is approaching clinical implementation, and therefore it is important for Pathologists to be acquainted with this molecular test. In this section we will give an overview of the panorama of microRNA testing in TGCTs.

MicroRNAs are part of the family of non-coding RNAs. Once considered "junk RNA", it is now known that the non-coding fraction of our genome plays fundamental roles in physiological and disease states, being part of the epigenetic mechanisms of modification of gene expression. MicroRNAs have important roles in tumorigenesis, functioning either as oncogenic microRNAs (oncomiRs) or as tumor suppressors (tumor suppressor miRs) (Peng

and Croce 2016). The reason why microRNAs are so attractive as biomarkers has to do with a combination of features. They are small molecules, meaning that they are very stable in circulation and easily detected by low-cost and rapid techniques such as PCR, making them obvious candidates for non-invasive monitoring of disease. Also, they have a very short half-life and reflect disease burden. Besides, available pipelines for their detection (PCR-based) are overall simple and widely available in hospitals (Constâncio et al. 2023).

In 2006, members of the miR-371 ~ 373 cluster (specifically, miR-372-3p and miR-373-3p) were pinpointed as oncogenic in TGCTs (Voorhoeve et al. 2006), and paved the way for dozens of studies focused on these microRNAs. The reason for the success of this microRNA cluster has to do with its specificity for GCTs, which is the reason these are called "embryonic microRNAs". Testing in several other male tissues or cancers, non-GCT tumors or healthy volunteers with different features resulted in negative or negligible levels in circulation and in tissue (Boellaard et al. 2019; Belge et al. 2021), very contrasting with elevated levels in most TGCT subtypes, with the remarkable exception of teratoma. Despite the multiple studies on clinical application, it is curious to realize that knowledge about the biology of these microRNAs is still scarce, apart from the establishment of a role in neutralizing p53 activity by interacting with *LATS2* gene (Voorhoeve et al. 2006).

The first studies were conducted on tissues, also supported by in vitro studies, and confirmed presence of high levels of miR-371a-3p, miR-372-3p and miR-373-3p across histological subtypes of TGCTs (including GCNIS), in contrast to negative levels in normal parenchyma and non-GCT masses (Belge et al. 2021; Vilela-Salgueiro et al. 2018; Palmer et al. 2010). These studies also concurred on the decline of miR-371a-3p in differentiated teratoma tissues, almost always being negative. This has propelled researchers to seek additional markers specific for teratoma (Yodkhunnatham et al. 2024), which could be clinically useful in the metastatic setting after chemotherapy, and despite some candidates, like miR-375 (Nappi et al. 2021a; Lafin et al. 2021) or hypermethylated *RASSF1A* (Lobo et al. 2021b), such a marker is still not available. Studies on tissue are also useful for shedding light on rare tumor entities which are poorly understood, for instance cystic trophoblastic tumor, which was shown to harbor levels of miR-371a-3p closer to teratoma, denoting a maturation process and approximating these two entities which have indolent behavior, very different from choriocarcinoma (Lobo et al. 2023b).

Given the practical advantages of microRNAs as liquid biopsy biomarkers, studies in bodily fluids were rapidly undertaken with high success. Quantification of members of the miR-371 ~ 373 cluster was accomplished in serum or plasma with > 90% sensitivity and specificity for diagnosis of TGCT at diagnosis, surpassing the combined sensitivity of all three classical serum tumor markers available in the clinic (for review of these studies, see (Leão et al. 2021). Such performance was maintained in studies from several groups and in prospective, multi-institutional investigations, using slightly different pipelines (Nappi et al. 2021b; Dieckmann et al. 2019b). MicroRNA elevations are seen across histologies, being less dependent on histological composition of the tumor, and were not detected in other conditions (for instance, patients with hepatocellular carcinoma with high AFP or individuals with constitutive elevated AFP were negative for miR-371a-3p (Sequeira et al. 2022). Also, these microRNAs could also be used to diagnose GCTs in cerebrospinal fluid and pleural effusions and hydrocele fluid, while results for seminal plasma have been less impressive (Murray et al. 2016a; Radke et al. 2019; Dieckmann et al. 2016; Spiekermann et al. 2015).

The half-life of miR-371a-3p was shown to be less than 4 h (Lobo et al. 2019a), and levels were shown to reflect disease burden, signaling relapses with better performance than classical serum tumor markers, which display poor sensitivity in this setting. This was particularly validated in active surveillance cohorts (with 94% of relapsed patients showing elevated miR-371a-3p, but only 38% showing elevation of any classical serum tumor marker), and in one study also signaled relapses earlier than standard imaging modalities (Lobo et al. 2021a; Fankhauser et al. 2022a; Belge et al. 2024). A cost analysis (Charytonowicz et al. 2019) indicated that introducing microRNA testing in clinical practice had the ability to reduce costs with patient follow-up by reducing the frequency of imaging scans, also reducing exposure to ionizing radiation.

MicroRNA testing was also employed in the metastatic setting, where it correlated with response to chemotherapy and predicted histology at retroperitoneal lymph-node dissection (RPLND), discriminating non-teratomatous viable GCT elements with high accuracy (Leão et al. 2018). This is important since such patients merit additional chemotherapy, while masses exclusively with teratoma only benefit from surgery.

From many of these studies, miR-371a-3p was consistently the member of the cluster with best performance, and is currently the marker most explored in current times, in some investigations showing the same performance as the combination of the three members (Piao et al. 2021).

Current challenges in the field of microRNA testing in TGCTs include bringing together all the studies and defining a universal standard operating procedure (SOP) and pipeline for sample storing and selection, RNA extraction, PCR reaction, normalization and quantification, all of which have had variations by the different studies (Nappi et al. 2019a, b, Lafin et al. 2023). The impact of hemolysis and pre-analytics has to be addressed if one wants to move the test definitely to the clinic. This is an area where Pathologists can be of much help as part of a multidisciplinary team, with their expertise in sample features, diagnostics and molecular tests (Fonseca et al. 2022). Additional discussions are ongoing regarding obtaining optimal sensitivity for detecting minimal residual disease immediately after orchiectomy and predicting relapse, which has not been achieved to date, or even approaching small testicular masses or GCNIS (Fankhauser et al. 2022b).

Additional biomarkers

The search for clinically relevant TGCT biomarkers has been tackling multiple fronts, but these are not ready for prime-time yet. For instance, additional epigenetic mechanisms besides microRNAs have been explored. Despite the rich epigenetic landscape of TGCTs and the overwhelming differences in DNA methylation between subtypes (Shen et al. 2018), namely between seminomas and non-seminomas (the former being largely hypomethylated), methylation-based markers are still not in use. There is also evidence of differential hypermethylation and changes in histone marks in the event of cisplatin-resistance, which could be therapeutically targeted with demethylating agents (Lobo et al. 2021c; Fazal et al. 2021). The study of TGCTs microenvironment has also led to interesting results, namely PD-L1 expression in tumor cells and in immune cells correlating with poorer and better survival, respectively) (Lobo et al. 2019b; Cierna et al. 2016), but results of trials using immunotherapy have been less than satisfactory at the moment (Tsimberidou et al. 2021). Likewise, studies have explored homologous recombination (and other DNA repair pathway markers) as biomarkers of sensitivity to PARP inhibitors (Lobo et al. 2021c), but trials with these drugs have also showed little success (De Giorgi et al. 2020). Other biomarkers commonly used in routine assessment of other neoplasms by Pathologists include MDM2 amplification, which correlated with aggressive disease (Lobo et al. 2020; Bagrodia et al. 2016), mismatch repair (MMR) deficiency (Honecker et al. 2009), which were linked to treatment failure, and proliferation index (Ki67), which was not of prognostic value in a study using digital image analysis (Lourenço et al. 2022).

Biomarkers in tumors of the testicular stroma and sex cords

Testicular sex cord stromal tumors (TSCSTs) are less frequent that germ cell tumors, representing approximately 5% of testicular neoplasms overall (Dilworth et al. 1991). Proper identification of TSCSTs is clinically relevant because, unlike GCNIS-derived germ cell tumors, most of them are indolent and potentially amenable to conservative surgical management (i.e., testis-sparing surgery) in prepubertal children and patients of reproductive age (Nicolai et al. 2015). Additionally, some histologic subtypes of TSCTs have been associated with disorders of sex development and inherited cancer predisposition syndromes; therefore, their correct identification may have significant impact on patients and their families (Ulbright et al. 2007; Al-Obaidy et al. 2022; Siegmund et al. 2023; Yu et al. 2023). It is crucial to recognize primary TSCSTs with features that portend a high risk of metastases, since timely surgical intervention (including upfront retroperitoneal lymph node dissection as well as early resection of suspected metastatic lesions) is currently the best available therapeutic option for patients with these notoriously chemotherapy-resistant neoplasms (Mosharafa et al. 2003; Featherstone et al. 2009; Calaway et al. 2019; Nicolai et al. 2015).

TSCSTs encompass a diverse group of tumors with distinct histological features and clinical behavior; in recent years, several tissue-based biomarkers have emerged as valuable tools for improving our understanding of their biologic characteristics and guiding clinical management. In this section, we will focus on adjunctive biomarkers useful for diagnosis and prognostication of the most common histologic subtypes of TSCSTs. Some exceptionally rare entities will not be discussed, since they are beyond the scope of this succinct review. Also, for the sake of brevity, histopathologic and clinical characteristics of these tumors (including criteria for malignancy) will not be extensively reviewed herein. The reader is referred to other texts for detailed and comprehensive clinicopathologic descriptions of these tumors (Cheng et al. 2020; Ulbright et al. 2022; Cheville 1999; Acosta et al. 2024a; Dashora et al. 2022; Al-Obaidy et al. 2021; Colecchia et al. 2022).

Leydig cell tumor

Leydig cell tumor (LCT) is the most common TSCST subtype, affecting both pediatric and adult patients (~20% and ~80% of cases, respectively) (Dilworth et al. 1991; Cheville 1999; Conkey et al. 2005). Pediatric tumors are invariably indolent; in contrast, up to 10% of adult LCTs behave aggressively (Fankhauser et al. 2020). Although there are no specific diagnostic biomarkers for LCTs, molecular alterations with potential value for

predicting clinical behavior have been recently identified (Colecchia et al. 2021; Necchi et al. 2019; Rizzo et al. 2021). Predictive biomarkers are particularly relevant because there are no clinically validated criteria for malignancy, and the behavior of some LCTs may be unpredictable (Fankhauser et al. 2020; Colecchia et al. 2021; Necchi et al. 2019; Rizzo et al. 2021).

LCTs are typically positive for one or more of the immunomarkers used to support a sex cord stromal "lineage". Of note, none of these markers are specific for sex cord stromal tumors in general or for LCTs in particular. Among them, the most sensitive are alpha inhibin and SF1 (positive in > 95% of LCTs each), followed by calretinin (positive in approximately 80%) (23,24). FOXL2, WT1 and SOX9, which typically mark sex cord derivatives, are positive a minor subset of LCTs (up to 20%). Expression of markers such as AR, Melan A, and synaptophysin has been described in the past, but their clinical utility is limited (Lau et al. 2021; Iczkowski et al. 1998). Perhaps one exception is the use of AR to differentiate between LCTs (typically positive) and the testicular tumors of the androgenital syndrome (expected to be negative) (Wang et al. 2011). Focal keratin expression can be seen in some LCTs, representing a potential pitfall, especially when these tumors are found in extratesticular (i.e., metastatic) sites.

Nuclear expression of beta-catenin, which has been initially posited to be specific for Sertoli cell tumor, not otherwise specified (SCT-NOS), is also frequently seen in LCT (~ 40–50%) (Lau et al. 2021; Iczkowski et al. 1998). However, the expression pattern is different in these tumor types, being focal or multifocal in LCT and characteristically diffuse in SCT-NOS (Rizzo et al. 2021; Lau et al. 2021; Kitagawa et al. 2024) (Fig. 4). This pattern of nuclear beta catenin expression correlates well with findings of genomic studies (see below) (Rizzo et al. 2021; Kitagawa et al. 2024). Among predictive immunomarkers, FH and MDM2 have been proposed as potentially useful adjunctive tests to identify primary (i.e., testicular) tumors with metastatic potential, and their assessment is suggested in testicular LCTs with worrisome histologic findings (Colecchia et al. 2021; Necchi et al. 2019; Rizzo et al. 2021). More specifically, loss of FH expression and overexpression of MDM2 have been identified as recurrent findings in subsets of aggressive LCTs (see below) (Colecchia et al. 2021; Necchi et al. 2019; Rizzo et al. 2021) (Fig. 5).

Molecular analyses have suggested that LCTs of pediatric and adult patients may harbor different genomic alterations. A recurrent somatic gain-of function mutation of the receptor for luteinizing hormone and human chorionadotropin (*LHCGR* p.R578H) has been described in pediatric LCTs (Liu et al. 1999). This variant leads to the activation of downstream Gs signaling, inducing proliferation of Leydig cells (Acosta et al. 2024a, b). In line with these findings, occasional adult LCTs with hotspot codon 201 *GNAS* variants have been described (Libé et al. 2012). However, most LCTs in adult patients lack *LHCGR* and *GNAS* variants (Carvajal-Carmona et al. 2006). Instead, a significant proportion of adult LCTs harbor gain-of-function *CTNNB1* variants (typically affecting exon 3) (Rizzo et al. 2021; Gao et al. 2017). Comparison of tumor cellularity and variant allele frequencies (VAF) suggests that *CTNNB1* are present mostly as subclonal events in LCTs, explaining the focal or multifocal (rather than diffuse) expression of nuclear beta-catenin seen with immunohistochemistry (Rizzo et al. 2021; Kitagawa et al. 2024). *FH* variants were initially described in 2 LCTs from adult patients, one of whom had evidence of hereditary leiomyomatosis and renal cell carcinoma (Carvajal-Carmona et al. 2006). More recent studies have suggested that *FH* variants correlate with the presence of aggressive histopathologic features and malignant clinical behavior in this tumor type (Rizzo et al. 2021; Carvajal-Carmona et al. 2006; Acosta et al. 2023b). Another recently posited predictive biomarker is *MDM2*, with recurrent amplifications being identified by genomic DNA sequencing in 30–50% of clinically malignant LCTs in two separate studies (Colecchia et al. 2021; Rizzo et al. 2021). Of note, these amplification events can be also detected by fluorescence in-situ hybridization or suggested by immunohistochemistry (likely requiring confirmation with other techniques) (Rizzo et al. 2021). Recurrent gene fusions involving exon 2 of the *TERT* gene have also been described in malignant LCTs (Kruslin et al. 2021). In line with this finding, Rizzo et al. described *TERT* amplifications in examples of aggressive LCTs, suggesting that activation of this gene may play a role in biologic progression (Rizzo et al. 2021).

Sertoli cell tumor, not otherwise specified (SCT-NOS)

SCT-NOS is the second most common type of sex cord stromal tumor in men, representing ~ 1% of all testicular neoplasms (Dilworth et al. 1991; Cheng 2020). Sex cord stromal tumors with signet ring cell morphology, regarded as a separate entity in the latest WHO classification of tumors of genitourinary and male reproductive organs (2022), may represent a variant of SCT-NOS (see below) (Michalova et al. 2017; WHO 2022). Like LCT, SCT-NOS may affect adult and pediatric patients, and ~ 10% may exhibit malignant clinical behavior (Grogg et al. 2020a). Of note, malignant SCT-NOS with morphologic features resembling those of seminoma have been well-recognized (Acosta et al. 2023a; Carrillo-Ng et al. 2024). A highly recurrent genomic alteration has been identified in these cases, suggesting that they likely

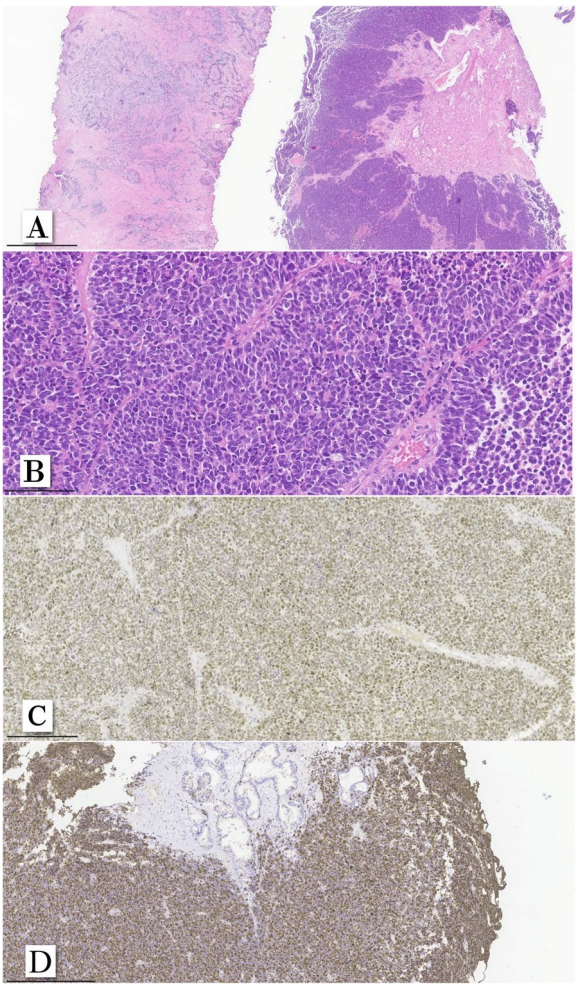

Fig. 2 Small cell neuroendocrine carcinoma can be distinguished from high-grade acinar adenocarcinoma of the prostate by morphology (**A** HE stain showing acinar adenocarcinoma at right and small cell neuroendocrine carcinoma at left). In addition to extensive / geographical necrosis and high cellularity, small cell carcinoma is characterized by nuclear moulding, speckled chromatin (salt-and-pepper pattern), with small or absent nucleoli, and numerous figures of mitosis and apoptosis (**B** HE). These tumors usually express neuroendocrine markers and no or little expression of prostatic differentiation. Other useful findings in small cell neuroendocrine carcinoma of the prostate is the expression of TT1 (**C**) and a Ki67 proliferative index higher than 50% (**D**)

represent a distinct entity (Acosta et al. 2023a; Carrillo-Ng et al. 2024).

SCT-NOS express one or more of the typical markers used to determine sex cord stromal lineage; among them, the most sensitive ones are SF1 (~80%) and SOX9 (~60%) (Lau et al. 2021; Mesa et al. 2017; Zhao et al. 2018). WT1, calretinin, and FOXL2 are also useful, but have somewhat limited analytic sensitivity (~40% to 50% each), whereas inhibin has been described as typically negative in some studies (Lau et al. 2021; Mesa et al. 2017; Zhao et al. 2018). Beta-catenin is a relatively sensitive marker, with diffuse nuclear expression seen in ~70% of SCT-NOS overall (Lau et al. 2021; Perrone et al. 2014; Kao and Ulbright 2020; Rizzo et al. 2023; Zhang and Ulbright 2015). Studies suggest that nuclear beta-catenin expression is seen in the vast majority (>90%) of typical SCT-NOS with non-aggressive clinicopathologic features. This suggests that the diagnosis of SCT-NOS should be questioned if there is no nuclear beta catenin expression in a seemingly indolent TSCST without obvious morphologic features of SCT-NOS (e.g., absence of noticeable tubular or corded architecture) overall (Lau et al. 2021; Perrone et al. 2014; Kao and Ulbright 2020; Rizzo et al. 2023; Zhang and Ulbright 2015). Conversely, histologically indolent sex cord stromal tumors with unusual architectural patterns (e.g., reticular or microcystic) and diffuse nuclear beta catenin expression show genomic methylation profiles indistinguishable from those of typical SCT-NOS, suggesting that they may represent morphologic outliers of this entity (Siegmund et al. 2022a). Malignant sex cord stromal tumors resembling seminoma are positive for SF1 and/or other sex cord-stromal lineage markers, and they frequently express CD30 (Acosta et al. 2023a; Carrillo-Ng et al. 2024).

From a molecular perspective, nuclear expression of beta catenin is associated with gain-of-function exon 3 *CTNNB1* variants in most SCT-NOS (Gao et al. 2017; Rizzo et al. 2023; Siegmund et al. 2022a). More specifically, *CTNNB1* mutations have been identified in ~70% of all SCT-NOS (Perrone et al. 2014). Of note, these analyses pre-date the identification of a recurrent gene fusion in malignant TSCTs that resemble seminoma, which likely represent a different entity; hence, the overall frequency of beta-catenin alterations in true SCT-NOS may be slightly higher. (Perrone et al. 2014; Rizzo et al. 2023). Of note, a minor subset of SCT-NOS harbor loss-of-function *APC* variants which, like *CTNNB1* variants, are expected to result in upregulation of Wnt signaling (Rizzo et al. 2023). Importantly, some *APC* variants in SCT-NOS are of germline origin and associated with familial adenomatous polyposis (Rizzo et al. 2023; Siegmund et al. 2022a; Siegmund et al. 2023). Some syndromic SCT-NOS may be bilateral or multifocal; otherwise, their histologic features are indistinguishable from those of sporadic SCT-NOS (Rizzo et al. 2023; Siegmund et al. 2022a; Siegmund et al. 2023). Genetic counseling or germline assessment should be considered in bilateral or multifocal SCT-NOS in patients with unknown germline/syndromic status. Excluding the gene fusion mentioned below, highly recurrent genomic alterations useful for prognostication have not been identified in malignant SCT-NOS. Clinically aggressive cases with *CTNNB1*

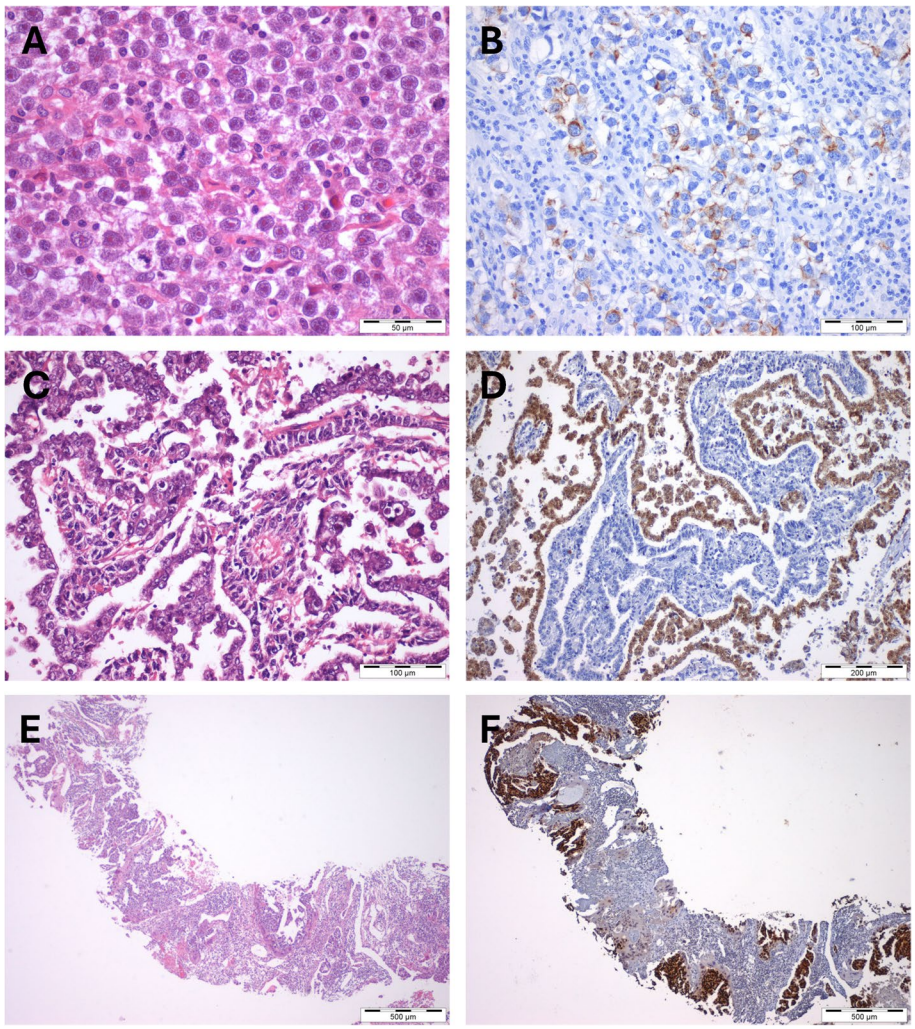

Fig. 3 Seminoma with immunoexpression of cytokeratins (CK8/18). Expression of cytokeratins may be seen in seminomas (sometimes with a dot-like pattern), and constitutes a diagnostic pitfall, especially in the case of loss of the typical clear cytoplasm and in the event of increased pleomorphism and foci of necrosis, where it can be mistaken for embryonal carcinoma, as in the present case (**A** and **B**). Utility of OCT3/4 in the diagnosis of mixed tumors. This mixed tumor is composed of embryonal carcinoma and yolk sac tumor. The two components are intermingled and may be difficult to distinguish on H&E sections. OCT3/4 is useful in this differential, highlighting the embryonal carcinoma cells, and being negative in the yolk sac tumor areas (**C** and **D**). Metastatic germ cell tumor to the lung. The patient presented with a lung mass and had a history of a previous testicular tumor. OCT3/4 was positive (as was CD30 and cytokeratins, not shown), confirming the diagnosis of an embryonal carcinoma component. There were also foci of syncytiotrophoblast cells represented in the biopsy, which were negative for OCT3/4 (and positive for HCG, not shown), and explained the high serum levels of HCG (**E** and **F**)

mutations (unlike benign counterparts) exhibit multiple chromosomal imbalances (i.e., aneuploidy), which likely underlie biological progression (Necchi et al.2019; Zhao et al. 2018; Rizzo et al. 2023). As mentioned above, a subset of malignant TSCSTs with morphologic characteristics mimicking those of seminoma have been well described in the literature (Acosta et al. 2023a; Carrillo-Ng et al. 2024). These tumors were initially interpreted as examples of malignant SCT-NOS, likely because they show some evidence of sex cord differentiation (Acosta et al. 2023a; Carrillo-Ng et al. 2024). Recent analysis of these neoplasms using DNA sequencing and fluorescence in-situ hybridization demonstrated highly recurrent *EWSR1::ATF1* gene fusions that encompass exons 1–6/7 of *EWSR1* and exons 4–7 of *ATF1* (Acosta et al. 2023a). These fusions are expected to produce protein products that contains the transactivation domain EWSR1 and the DNA binding domain of ATF1 (Acosta et al. 2023a; Carrillo-Ng et al. 2024). Hence, this event is hypothesized to result in a change in gene expression

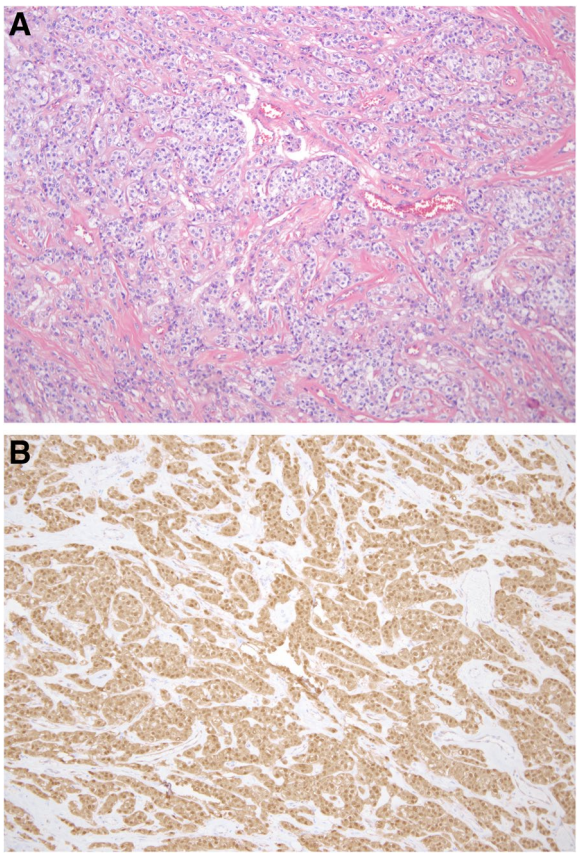

Fig. 4 Sertoli cell tumor, NOS (**A** 100x magnification) with nuclear beta-catenin positivity (**B** 100x)

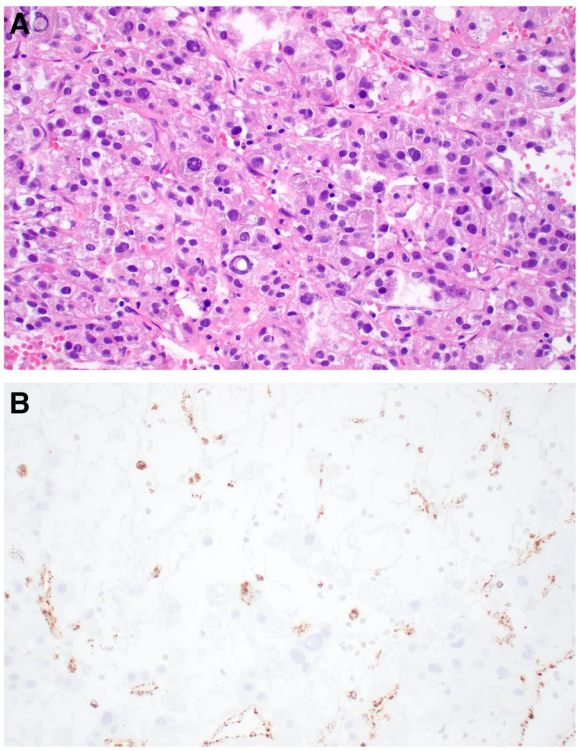

Fig. 5 Leydig cell tumor (**A** HE 200x magnification) with FH loss (**B** 200x)

patterns by a mechanism of "promoter-hijacking". Given the unique morphologic, clinical, and molecular features of these neoplasms, they have been proposed to represent a distinct entity.

Large cell calcifying Sertoli cell tumor (LCCSCT)

Large cell calcifying Sertoli Cell (LCCSCT) is a specific subtype of Sertoli cell tumor characterized by large polygonal neoplastic cells with abundant eosinophilic cytoplasm, myxoid stroma containing neutrophilic infiltrates and variably abundant laminated "mulberry-like" calcifications. LCCTs may occur sporadically or in the context of inherited cancer predisposition syndromes, including Carney complex, Peutz-Jeghers syndrome and neurofibromatosis type 1 (4,5,45,46). The frequency of its association with the Carney complex has ranged from ~ 10%-40% in different publications (Yu et al. 2023; Al-Obaidy et al. 2022). Approximately 10% of LCCSCTs are malignant, and aggressive cases seem to be largely sporadic (Abdulfatah et al. 2024).

LCCSCT express one or more of the non-specific markers used to determine sex cord stromal lineage (Anderson et al. 2022; Petersson et al. 2010). Sporadic counterparts of other tumors associated with the Carney complex, such as malignant melanocytic nerve sheath tumor, typically exhibit functional loss of the regulatory subunits of the protein kinase A tetramer (*PRKAR1A*), leading to constitutive activation of the catalytic subunits of the complex (Al-Obaidy et al. 2022; Anderson et al. 2022; Petersson et al. 2010). This seems to occur also in LCCSCT, which demonstrate loss of PRKAR1A expression demonstrated by immunohistochemistry in > 90% of cases (Yu et al. 2023; Al-Obaidy et al. 2022; Anderson et al. 2022; Petersson et al. 2010). This immunostain seems is specific for LCCSCT, since morphologic mimics consistently demonstrate retained expression of the marker (Anderson et al. 2022; Sato et al. 2005). From a genomic perspective, all LCCSCTs analyzed to date (both sporadic and syndromic) have shown pathogenic *PRKAR1A* variants (Yu et al. 2023; Abdulfatah et al. 2024; Anderson et al. 2022; Petersson et al. 2010). Hence, the presence of *PRKAR1A* alterations, detected by molecular studies or immunohistochemistry, is a desirable diagnostic feature for this entity, especially when candidate tumors that lack prototypical morphologic features (Fig. 6). Malignant progression seems to be associated with the acquisition of chromosomal imbalances/aneuploidy, biallelic inactivation of important tumor

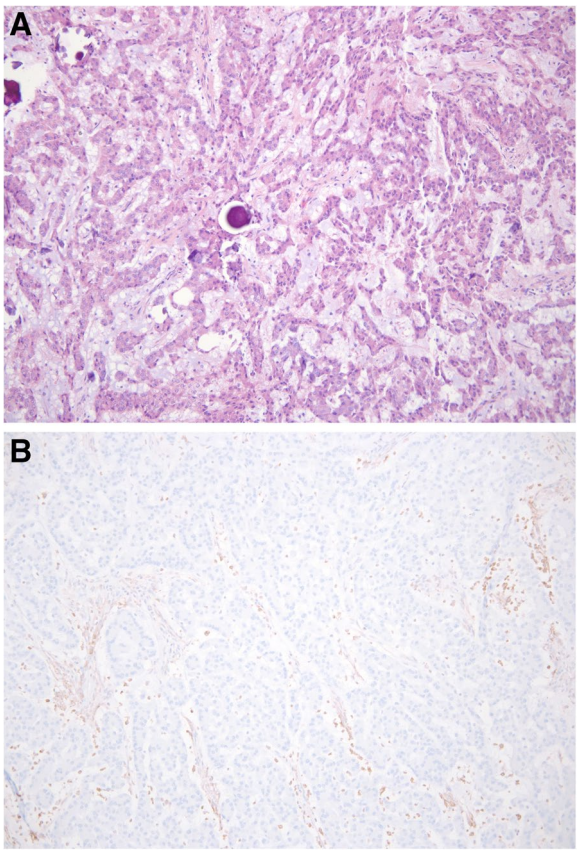

Fig. 6 Large cell calcifying Sertoli cell tumor (**A** HE 100x magnification) with PRKAR1A loss (**B** 100x)

suppressors (*CDKN2A*), or additional mutations (Yu et al. 2023; Abdulfatah et al. 2024; Anderson et al. 2022).

Granulosa cell tumors

Granulosa cell tumors of the testis are defined by their morphologic resemblance to ovarian counterparts, being divided into adult-type (AGCT) and juvenile-type (JGCT) (Cheng 2020; WHO 2022). Testicular AGCT comprise a very wide morphologic spectrum, including tumors with prominent cystic change and spindle cell components. Unlike ovarian AGCT, they typically do not produce sex hormone, presenting instead as a painless testicular mass. They affect post-pubertal adult men and approximately 10–20% metastasize, with poor clinical outcomes (Grogg et al. 2020b; Cornejo and Young 2014). Adult granulosa cell tumors stain for sex cord markers (such as WT1, calretinin, FOXL2) as well as for inhibin and SF1 (not mutation-specific) (Grogg et al. 2020b; Cornejo and Young 2014), but there are currently no specific immunomarkers (Lau et al. 2021). Of note, a significant subset expresses keratins (up to ~ 60%) and S100 (up to ~ 60%) (Costa et al. 1994). EMA has been proposed as a useful marker to distinguish between AGCT and JGCT, with the former being positive and the latter being negative (Costa et al. 1994; McCluggage 2005; Riopel et al. 1998). In AGCT, histochemical studies highlight reticulin fibers surrounding groups of cells rather than individual tumor cells (as seen in fibromas) (Stall and Young 2019). From a molecular perspective, ovarian AGCT consistently harbor a gain-of-function *FOXL2* variant (p.C134W), which has been in more than 90% cases across different studies (Lima et al. 2012; Shah et al. 2009; Pilsworth et al. 2021). In contrast, testicular AGCT are genomically heterogeneous, with only a minority harboring *FOXL2* p.C134W (Grogg et al. 2020b; Cornejo and Young 2014; Siegmund et al. 2022b). The single recurrent finding in testicular AGCTs is heterozygous copy number losses involving the long arm of chromosome 22 (~ 70%); this alteration is frequent across different cancer types and most likely represents a random recurrent finding (Siegmund et al. 2022b). Given the morphologic and molecular heterogeneity of testicular AGCTs, it is possible that this diagnostic category comprises a miscellaneous group of TSCST that cannot be classified into other defined histologic subtypes (e.g., LCT or SCT-NOS).

Testicular JGCTs represent largely an infantile entity, with 90% of cases occurring in patients of up to 6 months of age (Grogg et al. 2020b; Kao et al. 2015). They only rarely occur in children older than 1 year and are always benign (Grogg et al. 2020b; Kao et al. 2015). Unlike ovarian counterparts, they are not hormonally active, and subsets are associated with undescended testes or gonadal dysgenesis (Kao et al. 2015). Immunohistochemistry is rarely needed for diagnostic purposes, but testicular JGCTs express general sex cord stromal tumor markers (SF1, WT1, calretinin) (Grogg et al. 2020b; Kao et al. 2015; Collins et al. 2023a). Ovarian JGCTs typically harbor internal tandem duplications in the ankyrin homology domain of *AKT1*, gain-of-function codon 201 *GNAS* variants, and/or mutations in genes that regulate chromatin structure (*KMT2D*, *ARID1A*) (Collins et al. 2023a; Kalfa et al. 2006; Auguste et al. 2015). In contrast, testicular JGCTs are mutationally silent and exhibit monosomy 10 in ~ 60% of cases (Collins et al. 2023a).

Tumors with pure or prominent gonadal stromal components

This category includes tumors with pure or predominant spindle cell components that are thought to derive from the gonadal stroma, including fibroma/thecoma, myoid gonadal stromal tumor, and mixed tumors with sex cord and stromal components (sometimes referred to as "Sertoli-stromal cell tumors" in the literature) (Ulbright et al. 2022; WHO 2022; Zhang et al. 2013;

Jones et al. 1997; Kao and Ulbright 2014). These tumors express at least one of the markers used to establish sex cord stromal lineage, with the caveat that myoid gonadal stromal tumors may be consistently negative for SOX9 (although the number of cases analyzed to date is very small) (Zhang et al. 2013; Jones et al. 1997; Kao and Ulbright 2014). Myoid gonadal stromal tumor has been recently introduced as a distinct entity in the WHO, being defined as a gonadal stromal tumor with pure spindle cell histology and co-expression of SMA and S100 (WHO 2022; Kao and Ulbright 2014). Fibroma/thecoma lack a specific immunoprofile and are defined largely based on their resemblance to ovarian counterparts (Zhang et al. 2013; Jones et al. 1997). A small subset of mixed sex cord stromal tumors (including "Sertoli-stromal cell tumors") exhibits nuclear beta catenin expression limited to the sex cord components. Except for the small number mixed sex cord stromal tumors with nuclear beta-catenin expression, which harbor *CTNNB1* variants, recurrent mutations or gene fusions have not been identified in these TSCSTs (Siegmund et al. 2022a; Acosta et al. 2024b; Collins et al. 2023b). Genomic analyses of tumors with pure or predominant spindle cell components that were originally classified as myoid gonadal stromal tumor, Sertoli-stromal cell tumor, and unclassified sex cord stromal tumor have demonstrated a recurrent pattern of chromosomal gains suggestive of a global shift in ploidy. Hence, it is possible that these different tumor types may represent part of a biologic and histopathologic spectrum (Siegmund et al. 2022a; Acosta et al. 2024b). Importantly, tumors with pure spindle cell components classified as fibroma/thecoma and myoid gonadal stromal tumor are invariably indolent (Kruslin et al. 2021; Zhang et al. 2013; Jones et al. 1997; Kao and Ulbright 2014).

Conclusion

Molecular pathology is developing fast in the field of many types of Urologic Cancers and can be essential in adopting precise therapy. Pathologists should be familiar with recent updates on prognostic and predictive biomarkers that will be increasingly more relevant and requested in daily practice.

Abbreviations

AJCC	American Joint Committee on Cancer
AR*v7*	Androgen receptor splice variant-7
ATM	ATM serine/threonine kinase or Ataxia-telangiectasia mutated
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
CAP	College of American Pathologists
CDK12	Cyclin-dependent kinase 12
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4)
CRPC	Castrate-resistance prostate cancer
EAU	European Association of Urology
FDA	(United States) Food and Drug Administration
ICCR	International Collaboration on Cancer Reporting
iPARP	Poly (ADP-ribose) polymerase) inhibitor
ISUP	International Society of Urological Pathology
GCNIS	Germ cell neoplasia in situ
GG	Grade Group
GUPS	Genitourinary Pathology Society
HRD	Homologous recombination defects
ISUP	International Society of Urological Pathology
LCCSCT	Large cell calcifying Sertoli cell tumor
LCT	Leydig cell tumor
NCCN	National Comprehensive Cancer Network
MMR	DNA mismatch repair
PARP	Poly(ADP-ribose) polymerase (PARP)
PD-1	Programmed Cell Death (receptor)
PD-L1	Programmed Cell Death Ligand 1
PTEN	Phosphatase and tensin homolog
SCT-NOS	Sertoli cell tumor, not otherwise specified
TGCT	Testicular Germ Cell Tumor
TSCST	Testicular sex cord stromal tumor
TURBT	Transurethral resection of a bladder tumor
WHO	World Health Organization

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MAM, DAA, JL and MBMF drafted the manuscript. LFG, JMM, SMB and AA revised the manscript and gave significant contribution of intellectual content. All authors approved the manuscript.

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