TESTICULAR CANCER: NEW INSIGHTS ON THE ORIGIN, GENETICS, TREATMENT, FERTILITY, GENERAL HEALTH, QUALITY OF LIFE AND SEXUAL FUNCTION

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EDITORIAL
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Editorial: Testicular Cancer: New Insights on the Origin, Genetics, Treatment, Fertility, General Health, Quality of Life and Sexual Function

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Keywords: testis cancer, genetics, male infertility, sexual function, cancer treatment

Editorial on the Research Topic

Testicular Cancer: New Insights on the Origin, Genetics, Treatment, Fertility, General Health, Quality of Life and Sexual Function

About 95% of all testicular cancers are represented by testicular germ cell tumors (TGCTs), which include seminoma and non-seminoma histological types. TGCT is the most common solid tumor among males 15–34 years of age, with an estimated 8,850 new cases and 410 deaths during 2017 in the United States. The highest incidence rates of testicular cancer are in Norway (11.8 per 100,000) and the lowest are in India (0.5 per 100,000) and Thailand (0.4 per 100,000). The annual incidence of Testicular cancer (TC) has doubled over the past 40 years with an increasing trend over time, particularly in Caucasian males (1).

The pathogenesis of TC is poorly known. The identification of pathogenic mechanisms and risk factors involved in testicular carcinogenesis still represent topics of extremely high clinical interest. The origin of TGCT, probably starting at early stages of embryogenesis, seems to be a part of the Testicular Dysgenesis Syndrome (TDS) where some early PGC/gonocytes are blocked in their differentiation, are tightly regulated by epigenetic modification in terms of microRNA expression and DNA methylation, retaining their early marker profile (Baroni et al.).

It is now clear that genetic, environmental and hormonal risk factors concur and mutually influence both the development of the disease and its prognosis. Indeed, the probability of developing TC is the result of a combination of a number of factors that can be distinguished into genetic, environmental and hormonal factors. The most common risk factor for testicular cancer is undescended testis (cryptorchidism); others risk factors are personal or family history of testicular cancer, age, ethnicity, and infertility.

Moreover, a possible causal relationship between viral infections and TGCTs was firstly evoked almost 40 years ago and is still a subject of debate. Recent efforts in oncological and virological research have brought to light the oncogenic potential of different virus species. Evidences from a systematic review and meta-analysis support an oncogenic effect of HIV and EBV on the human testis, but the evidence was insufficient to establish causality (Garolla et al.). Therefore, the exposition to different environmental agents, such as pesticides and non-steroidal estrogens can increase the risk of developing this neoplasm. The increased exposure to environmental factors, particularly chemical pollutants with endocrine disrupting activity, can alters the major

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hormonal axis that drives testis development and function from gestational age. The susceptibility to these alterations further depends on genetic factors that justify the strong familiarity of TC (De Toni et al.). The presence of testicular microlithiasis in patients with such risk factors increases more the risk of cancer. Testicular microlithiasis (TM) represents itself a risk factor for TGCT, because in infertile men the presence of TM is associated to an ∼18-fold higher prevalence of testicular cancer as reported in a full meta-analysis of eight carefully selected studies (Barbonetti et al.). Longitudinal studies are warranted to elucidate whether this cross-sectional association actually reflects a higher susceptibility of infertile men with TM to develop testicular cancer over time.

This special issue, entitled "Testicular Cancer: New Insights on the Origin, Genetics, Treatment, Fertility, General Health, Quality of Life and Sexual Function," provides an overview of clinical diagnosis and disease management and an approach to explain the molecular development of TC. The limited number of studies and the resulting lack of exact knowledge about development, differentiation, and treatment of TC leaves several clinical problems regarding treatment and follow-up unsolved. The aim of this special issue intends to give an update on the most controversial issues in research areas of TGCT, as well as new results and express the opinions of a selection of specialists who have expanded the field with their recent discoveries. Both clinical and basic researches are reported and many questions are addressed. One of these deals regard the diagnostic strategies which still remain problem to be solved. Diagnosis for TGCTs is greatly based on detecting serum markers such as alpha-fetoprotein, beta-human chorionic gonadotropin, and lactate dehydrogenase but only 60% of all patients show elevations of these markers. For this reason tumor markers alone are not able to detect many recurrences, indeed in about 40% of men with disease recurrence the levels of these markers are usually "normal." Therefore, the discovery of novel clinical biomarkers of TC would clearly help the early detection and the monitor of the disease. The use of proteomic platforms permit to discover putative prognostic and diagnostic markers of testicular cancer. A panel of proteins for early detection, identified by proteomics technique, might be used for prognostic evaluation and for follow-up of TC. Moreover, the molecular mechanisms revealed by these proteomic studies might represents molecular targets for anticancer treatments (Milardi et al.).

With effective treatment, the overall five-year survival rate of TGCT is 97%. Men diagnosed with GCT have excellent survival rates due to advances in the multimodal treatment paradigm of chemotherapy, radiation therapy, and surgery. Despite the good response of these tumors to platinum-based chemotherapy, some patients are refractory to treatment and present poor clinical outcomes. During carcinogenesis and tumor development, cancer cells reprogram energy metabolism toward a hyper-glycolytic phenotype, with over-expression of metabolism related proteins, like glucose and monocarboxylate transporters, pH regulators, and intracellular glycolytic enzymes (Warburg effect). The alterations in the expression of proteins related with the Warburg effect and hyper-glycolytic and acid-resistant phenotype are associated with aggressive clinicopathological parameters (Bonatelli et al.). Other molecular findings are also associated with local cancer invasiveness as OCT4, KLF4, and PTTG1 expression in seminoma (Grande et al.).

Retroperitoneal Lymph Node Dissection (RPLND) is generally considered as a treatment option for non-seminomas, when lymph nodes are compromised. There are three different RPLND techniques: open, laparoscopic, and robotic. The open approach is as effective as the other two in its oncological efficiency. Recent studies have been pointing out a slight increase of advantages on the robotic approach. Also, it is noteworthy that new technologies are on the rise, improving the laparoscopic approach, requiring further studies after their uses are consolidated (Vaz et al.).

Testicular function usually gets worse after treatment for testicular cancer, so the patients must be carefully followed for signs of hypogonadism and associated comorbidities. Hypogonadism has been often reported in testicular tumor survivors because of the radio- and/or chemo-induced Leydig cell damage. Longitudinal studies have revealed a higher negative impact of chemotherapy on Leydig cell function than radiotherapy or orchiectomy alone, leading to a higher risk for hypogonadism and its related complications, including cardiovascular, metabolic and bone mineralization impairment, and sexual dysfunction in testicular tumor survivors. Compared to orchiectomy alone, combined or high-dose chemotherapy and radiotherapy increase the risk for metabolic syndrome, DM, and cardiovascular events (La Vignera et al.).

These cancer survivors will therefore have to live with the long-term physical and psychological consequences of both their treatments (surgery, chemotherapy, radiotherapy) and the diagnosis itself. Invasive and destructive surgery such as retroperitoneal lymph node dissection increases the frequency of such dysfunctions (2). However, reports of thorough longitudinal follow-up from diagnosis to long-term survivorship are rare and confirm that TC and its treatment have a significant effect on sexuality. The absence of a clear correlation with biochemical hypogonadism suggests that this may to a large extent be due to the surgical procedure itself, or to the psychological impact of a cancer diagnosis (Pallotti et al.).

Individual health, sexual relationships affect several important aspects of survival and significantly influence the QoL of long-term survivors. Physical, psychological, work-related problems and changing perspectives about work and life in general influenced life and career decisions among testicular cancer survivors (Schepisi et al.).

Finally cancer treatment is not an individual experience, but induces deep effects on patients' families, who often have to assume a caregiving role for the duration of and following treatment for cancer. The role of a more integrated system of the patient and his social support, with the purpose of improving QoL not only during active treatment, but also in the follow-up period, and to encourage a less traumatic return to the everyday life (De Padova et al.).

We would like to express our sincere gratitude to all authors and referees for their contribution to this issue. Multidisciplinary

and collaborative efforts in recent years have clearly improved our understanding of the pathogenesis of testicular cancer but this disease remains an enigma in many aspects especially the environmental etiology.

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REVIEW
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Psychosocial Issues in Long-Term Survivors of Testicular Cancer

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Testicular cancer is the most frequent tumor in young males aged 15–39 years. As cure rates are currently around 90%, the prevalence of survivors is increasing. However, a disease-free condition does not necessarily correspond to a life free of physical and psychosocial health problems. The aim of this review was to explore psychosocial morbidity among testicular cancer survivors. A literature search was conducted in three electronic databases (PubMed, Medline, and Embase). The results of the search on cancer survivors were then combined with those of the search on psychosocial concerns and work performance. Eighty-four publications met the inclusion criteria. Physical, psychological, work-related problems and changing perspectives about work and life in general influenced life and career decisions among testicular cancer survivors. Individual health, sexual relationships and work problems, affect several important aspects of survival and significantly influence the QoL of long-term survivors.

Keywords: testicular cancer, survivors, psychological concerns, sexual problems, reentry

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INTRODUCTION

Testicular cancer (TC), which represents 1% of male tumors, is the most frequent tumor in young males aged 15–39 years, and its incidence is increasing worldwide (1).

The most frequent histology is germ cell tumor (which represents 90–95% of cases): there are several subgroups of germ cell tumors: Seminoma (Pure, Spermatocytic), Embryonal Carcinoma, Choriocarcinoma, Yolk Sac Tumor, Teratoma (mature, immature, with malignant component). Some epidemiological risk factors has been detected: cryptorchidism, hypospadias, decreased spermatogenesis, familial history, or personal history of contralateral TC. Overall, this is often a treatable tumor, but prognosis and consequently mortality depend on the risk categories defined by the 1997 International Germ Cell Consensus Classification (**Table 1**) (2). TC can be cured by surgery, in case of localized disease, or by chemotherapy, in case of metastatic disease. Platinum-based chemotherapy regimens are the standard treatment because they allow to obtain complete responses, even in metastatic patients (3). As cure rates for TC are currently around 90%, the prevalence of TC survivors (TCSs) is increasing and their life expectancy is considered comparable to that of the age-matched male general population (4, 5). However, a disease-free condition does not necessarily correspond to a life free of physical and psychosocial health problems.

TABLE 1 | Prognostic groups based on the IGCCCG Consensus Classification (2).

Prognostic group	Seminoma	Non-seminoma	5 year survival
Good	• Any primary site • Normal AFP • Any hCG and LDH • No non-pulmonary visceral metastases	• Testis/retroperitoneal primary • No non-pulmonary visceral metastases • AFP < 1,000 ng/mL • hCG < 5,000 IU/L (1,000 ng/mL) • LDH < 1.5 × ULN	∼90%
Intermediate	• Any primary site • Non-pulmonary visceral metastases • Normal AFP • Any hCG and LDH	• Testis/retroperitoneal primary • No non-pulmonary visceral metastases • Tumor Markers S2 ○ hCG 5,000–50,000 mIU/ml ○ AFP 1,000–10,000 ng/ml ○ LDH 1,5–10 × ULN	∼75%
Poor	No patients	• Mediastinal primary + • Tumor Markers S3 ○ hCG >50,000 mIU/ml ○ AFP >10,000 ng/ml ○ LDH >10 × ULN • Non-pulmonary visceral metastases	∼45%

Although the majority of TCSs experience good levels of functioning and enjoy a health-related quality of life (HRQoL) comparable to that of the general population, a minority of survivors are faced with the long-term psychosocial effects and somatic sequelae of their disease history and previous treatments (platinum-based chemotherapy, radiotherapy and/or retroperitoneal lymphoadenectomy) (6–8).

It has been estimated that the overall incidence rate of late effects among TCSs is 66.3 per 1,000 persons/year, and that a higher risk is observed for hypercholesterolemia, infertility, and orchitis (9). Fertility issues, fatigue, chronic peripheral neuropathy, hearing loss, Raynaud-like phenomenon, tinnitus, cardiovascular toxicity, decreased pulmonary function, hypertension, and hyperthyroidism have also been reported (6, 10–19).

Furthermore, TCSs have a slightly higher risk than normal of developing germ cell tumors and/or treatment-induced non-germ cell tumors (15). There is also evidence that TC diagnosis and treatment can cause psychosocial problems in survivors (20) such as anxiety, fertility distress, fear of recurrence (21–23), all of which reduce overall life satisfaction and negatively affect social contacts and family relationships (20).

The aim of this review was to explore psychosocial morbidity among TCSs, focusing on 3 levels of concern: physical, psychological, and reentry, as conceptualized by Holland et al. (24).

METHODS

A literature search was conducted in three electronic databases (PubMed, Medline, and Embase) and original studies published up to October 2017 were identified. The term "survivor(s)" was combined with "testis cancer," "testicular cancer," and "germ-cell tumors" to facilitate the retrieval of abstracts about TCSs. The terms "physical," "psychological," and "quality of life" were used to search for material on physical and psychosocial issues. For work, the terms "work," "job," "worker(s)," "absenteeism," and "reentry problems" were used.

In this narrative review, the results of the search on cancer survivors were then combined with those of the search on psychosocial concerns and work performance.

RESULTS

Physical Issues

Physical concerns include continued preoccupation with illness and hypervigilance regarding minor symptoms, aches and pains, fears of disease recurrence or relapse, increased feelings of physical damage and infertility, and concerns about sexuality and attractiveness (24). Main physical concerns are summarized in **Table 2**. Over 70% of TCSs assessed their general health as good (8) and perceived their overall quality of life as equal to or slightly better than that of healthy men (6, 8, 29, 30). However, it has been seen that the quality of life of TCSs can be compromised by some disease-related health issues and physical limitations (30). According to some studies the greatest changes are physical (31, 32) and, among these, the main long-term sequelae are impairment of sexual life and fertility (29).

The uneasiness related to physical issues can take different forms. The experience of TC may compromise the person's sense of "invulnerability" and safety and replace it with fear of recurrence, concerns about other tumors, and death (33). Anxiety about follow-up diagnostic tests and cancer recurrence are known to be very common in cancer survivors and to persist a long time after the end of treatments (34, 35), despite the good prognosis for TC and the rarity of late recurrence (1–4% of cases) (36, 37). Fosså et al. (38) found that 17% of TCSs reported a worsening of anxiety related to relapse 2 years after the baseline measurement, whereas 36% of patients displayed improved global quality of life as compared with baseline Skaali et al. reported that almost one out of 3 TCSs reported fear of recurrence an average of 11 years after diagnosis (22). Moreover,

TABLE 2 | Physical issues in long-term TCSs.

	Physical issues	Percentage of patients	References
Sexual problems	Problems with ejaculation	29–44	(25) (26)
		25.7	(27)
	Reduction of sexual activity	27.3	(27)
		9–24	(25) (26)
	Loss of desire	17.3	(27)
		7–20	(25) (26)
	Feeling less attractive	15	(28)

higher levels of fear of recurrence (FoR) were significantly associated with higher levels of psychological distress, but not with cancer histotypes or treatment modalities. However, treatment-induced neurotoxicity, fatigue and severe somatic symptoms were significantly associated with level of FoR. The level of FoR was negatively correlated with quality of life (QoL) scores (22).

TC and related treatment strategies can cause both physiological changes and emotional reactions that affect or interfere with sexual functioning.

Impairment of sexual life and fertility represent the main long-term physical sequelae with respect to healthy controls (29), with sexual problems after TC therapy present in around 20% of patients (25, 39).

TC usually occurs during a man's most sexually active years when the impact of disease and treatments on sexual functioning, fertility, identity, and body image can be devastating (26). There is evidence that perceived attractiveness, retaining fertility, having children, and living with a partner are among the most important predictors of good health-related quality of life for men 3–13 years post-treatment (8). Dahl et al. reported that, although TCSs experience more problems with sexual drive, erection and ejaculation than healthy men, sexual satisfaction is not decreased and is even better than control for younger survivors (20–39 years). Moreover, whilst increasing age, lack of a partner, and high levels of anxiety are associated with compromised overall sexual function, this is also known to be true of males in general (26).

A varying percentage of TCSs report having physical sexual problems: orgasmic problems (10–20%); ejaculatory failure (29–44%), which is related to surgery in the retroperitoneal area; and erectile dysfunction (around 10%), which is linked to radiotherapy. In addition to physical sexual issues, some survivors also report psychosexual dysfunction after treatment such as decreased libido (7–20%), decreased sexual activity (9–24%) and dissatisfaction (5–20%) (40, 41). Some studies have reported different data on erectile dysfunction, the prevalence of which after TC is similar to that found in the general population (20, 26).

A review and meta-analysis of 36 studies sexual functioning after treatment for TC, covering a mean follow-up period of 2.0–6.9 years, revealed no deterioration over the course of time apart from a decrease in sexual desire and an increase in sexual satisfaction (40). Nazareth et al. (41) stated that, in general, sexual dysfunction linked to treatment of TC persists for about 2 years post-treatment, after which functioning seems to recover. In a recent Danish study, conducted in a cohort of 2,260 TCSs with a median follow-up of 17 years, a relationship among chemo—(bleomycin, etoposide, and cisplatin) radiotherapy and increased risk of erectile dysfunction was found (42).

Sexual dysfunction may be due to biological or psychological causes or a combination of both. According to Jonker-Pool et al. (40), a distinction can be made: impairment in physiologic domains such as erection and ejaculation are associated with extent of disease and treatment modalities (i.e., surgery, radiotherapy, or chemotherapy), while psychological domains such as sex drive and satisfaction are treatment-independent. However, treatment strategies for TC can result in physiologic changes and at the same time trigger emotional reactions. Thus, decreased sexual functioning (e.g., reduction in or inhibition of libido) may be due to treatment-related somatic factors such as fatigue, general malaise, hair loss, and excessive weight changes attributable to emotional factors including about sexual performance, fear of loss of control, and uncertainty about the future (12, 43).

Psychological factors arising from having a life-threatening, genitourinary disease play a strongly mediating (if not determining) role in sexual functioning; the traumatic experience of having cancer may affect the sexuality of TCSs, influencing more subjective aspects such as sexual desire, sexual activity, and sexual satisfaction (20, 40, 44).

Because of the symbolic nature of the testes, the loss of this organ may affect masculinity, sexual identity, and body image. Castration or (hemi)castration is linked to fantasies, beliefs, myths, and cultural values about the testes that can have a severely traumatic effect and psychological consequences on the person/patient. Thus, concerns related to sexual and reproductive functioning may generate feelings of inadequacy, hopelessness, and emotional distress (45).

After removal of a testicle by orchiectomy, TCSs may have long-lasting feelings of loss or shame. Skoogh et al. (46) found that such feelings were more common among younger and single men than among older and non-single men. There was no correlation between feelings of loss or uneasiness and shame and having or not having a prosthesis, although offering a testicular prosthesis may help to reduce the trauma induced by this experience.

TC involves a male organ that is highly associated with perceptions of masculinity, attractiveness and body image. Patients undergoing removal of a testicle may see it as a disembodying procedure, especially during the period in their life when there is a heightened fixation on the "perfect body" and a striving for physical fitness (45).

Body image is a seldom explored topic in TC survivorship. In a work by Rossen et al., negative changes in body image, i.e., perceived reduced masculinity, following TC and its treatment, were reported in 17% of long-term TCSs and were associated with various aspects of sexual dysfunction, i.e., reduced sexual

interest, reduced sexual activity, reduced sexual enjoyment, erectile dysfunction, ejaculation dysfunction, and increased sexual discomfort (28). Similar results have been reported in other studies. Tuinman et al. (47) found that 16% of survivors expressed concern about their body image and reported feeling anxious at the thought that other people notice the missing testicle. Rudberg et al. (48) reported that 15% of Swedish TCS felt less attractive. Thus, although the surgical removal of a testicle generally has a negative impact on body image, this tends to less over time (21) and most TCSs do not report feeling less attractive (8) or less masculine than before their experience of TC (38).

Another aspect that affects the emotional experience of TC patients is the meaning they give to the disease. TC attacks an organ intrinsically associated with sexuality and reproduction at a time of life when sexual desire and performance, sense of masculinity, body image, and fertility are central issues (49). A Norwegian study conducted on a cancer survivor population (including TCSs) reported a significant reduction in paternity in the TCSs compared to non-cancer males (27).

Recently, a Greek study of 53 TC patients submitted to full bilateral, non-nerve-sparing post-chemotherapy retroperitoneal lymph node dissection (RPLND) observed that orgasmic function, intercourse and overall sexual satisfaction were significantly impaired post-operatively (50). However, as a subjective perception, a substantial number of patients reported higher levels of sexual desire and no difference in erectile function poorer orgasmic function and satisfaction post-operatively (50).

In a Serbian cross-sectional study involving 202 TCSs, 27.3% experienced decreased sexual function compared to the period before chemotherapy, 20.8% reported no erectile function impairment and 25.7% had problems with ejaculation. Loss of desire was reported by 17.3% of TCSs (51).

An Italian study evaluating the effects of several kinds of treatment on erectile function found that only adjuvant radiotherapy was as an independent predictor of non-recovery of normal function. Adjuvant chemotherapy alone, chemotherapy plus RPLND or RPLND alone did not significantly impair the recovery of normal erections (52).

Psychological Concerns

Although there is evidence to suggest that the majority of cancer survivors adjust well in terms of QoL and psychological well-being, emotional problems have been reported in a substantial minority of long-term TCSs (53). Main psychological concerns are summarized in **Table 3**. The experience of cancer and long-term physical sequelae of treatment can affect the psychological well-being and may lead to increased levels of psychological distress in those living with a history of cancer (57, 58). Psychosocial morbidity among cancer survivors include an increased sense of vulnerability and uncertainty about the future, feelings of personal inadequacy, fear of social rejection and stigmatization, anxiety, depression, and symptoms of post-traumatic stress disorder (24). Increased levels of anxiety and depression may also be present years after diagnosis (59, 60). A recent study by Inhestern et al. (61) confirmed the conclusions of a meta-analysis in long-term cancer survivors conducted by Mitchell et al. in which anxiety, rather than depression, was a more widely perceived problem in long-term cancer survivors than in healthy controls (62).

TABLE 3 | Psychological issues in long-term TCSs.

Psychological issues	Percentage of patients	References
Fear of recurrence	33	(20)
	17	(36)
Increase in anxiety	25	(54)
	19	(55)
	6.1	(56)
Depression after treatment	20	(55)
	9–11	(54)
	7.9	(56)

Several studies on the lives of TC survivors have found that the psychosocial aspects of the disease, such as anxiety about the future, coping and work reentry, are more important determinants of distress, morbidity and QoL than the type of treatment undergone and the time since its completion. It is thus possible that subjective evaluations are more important determinants of functioning and contributors to distress than a patient's actual medical history (7, 55, 63, 64).

There is evidence that cancer may precipitate post-traumatic stress (PTS) conditions, including PTS symptoms or PTS disorders (PTSD). Literature also show that cancer may also facilitate post-traumatic growth such as positive perceptions of oneself, emotional growth, improving relationships with others, and greater appreciation of life (65–67), although little is known about this area is TC.

Several studies have shown that TC patients treated with chemotherapy are at risk of long-term lower cognitive performance (22, 68–70). Chemotherapy, especially platinum-based treatment, is associated with paraesthesia, hypogonadism, hypercholesterolemia, and hypertension (71), and also with memory problems and lower cognitive performance in TCSs (68). This condition, known as "chemo-brain" or "chemo-fog" has been described in other tumor types (72). In some cases, the prevalence of cognitive difficulties in TCSs is unexpectedly high, especially in terms of neuropsychological outcomes (73). Amidi et al. (74) found impairments related to verbal learning and memory (29–33% of TCSs), visual learning and memory (14–28%), processing speed (8–24%), executive functioning (17%), and attention and working memory (4–15%). However, it is worthy of note that no correlation between cognitive impairments and type of treatment has been identified (22, 68–70, 74, 75). Fung et al. consider that cognitive impairment could be related to anxiety and depression that are prevalent in this kind of patients (76).

Some studies evaluated the influence of different treatment modalities on quality of life: men who received the most aggressive treatment perceived the lowest HRQoL (8, 77). Each individual's experience with cancer is different. Cancer type and stage, type and severity of treatment, and subsequent physical effects may all contribute to how survivors "live" the experience of cancer, as well as to their levels of distress, personal growth,

and QoL (78). TC, albeit curable in a high percentage of cases, has some aspects that make it an invasive emotional event and a particularly distressing experience. It mainly affects young men aged between 15 and 45 years during a central phase of their life cycle in which they are still constructing their own personal identity. The threat to existential continuity represented by cancer and the profound effect it has on body image and on personal values make this evolutionary transition a difficult one.

This in an important period of life, characterized by major life changes and specific developmental tasks. Indeed, these men are at or are near their prime of life, when interpersonal relationships, long-term work goals, and the desire to start a family may be major concerns (21, 54, 79, 80). Moreover, in this period of life health is generally taken for granted and life-threatening illnesses and dying are rarely considered possibilities (33).

The experience of TC may continue to influence the well-being of survivors and to interfere with the normal course of daily life months or even years after they are cured (54).

Physical and psychological consequences of treatment may interfere with life plans made before cancer, obliging survivors to review their short- and long-term goals. This may lead to impaired psychosocial functioning and more cancer-related distress (33).

A review of the literature on psychological and social domains showed that the majority of TCSs experience good levels of functioning and good post-therapeutic QoL, although a small percentage reported psychosocial problems such as anxiety, depression, fertility distress, sexual problems, and work-related problems (21, 40).

Qualitatively strong studies reviewed by Fleer et al. (21) indicated that the levels of psychological distress reported by TCSs varied between 9 and 27% (8, 23, 81). The study by Dahl et al. found that around 25% of TCSs became more anxious after diagnosis and treatment and that the distress experienced by TCSs is significantly higher than that of controls (81). However, other studies had different outcomes, e.g., a survey on QoL reported a slightly poorer mental health in TCSs than in a control group (7).

In the past, numerous studies have reported that the most frequent symptoms of emotional distress are tension, anxiety, restlessness, nervousness, and health worries (79). It has been seen that long-term TCSs continue to have significantly higher levels of anxiety (but not depression) many years after treatment compared to age-adjusted healthy males (1, 12, 23, 56). Substantially increased levels of anxiety among TCSs with respect to controls are associated with peripheral neuropathy, fear of recurrence, economic concerns, alcohol abuse, sexual difficulties, younger age at diagnosis, and a history of treatment for mental problems (12).

Among the possible causes of the increased anxiety experienced by a considerable proportion of TCS are a feeling of unsafety (54), a paradoxical perceived loss of protection by medical providers, a decrease in medical surveillance, and the perception of being completely on one's own. Symptoms of anxiety often occur before follow-up visits. Although the prevalence of depression among long-term TCSs corresponds to that observed in the general population (6, 56), some studies report a different frequency of self-reported depressive symptoms (79, 82). For example, Dahl et al. reported that depression was prevalent in 9–11% of TCSs up to 5 years after the end of treatment (56).

Thus, the overall picture regarding depression in TCSs is somewhat unclear (82). In general, differences in study results can be attributed to different aspects of distress evaluated, sampling differences in survivors, and the use of different validated questionnaires. A recent study assessed the prevalence of anxiety/depression in long-term TCSs, reporting anxiety in 6.1% of survivors and depression in 7.9%. Younger age at diagnosis and a shorter time since diagnosis were significantly associated with higher anxiety (83). A Polish study evaluated levels of anxiety/depression in 111 TC patients (57 undergoing chemotherapy and 54 patients at least 6 months after treatment). The prevalence of anxiety disorder was 40% during chemotherapy and 18.5% after treatment. Depression was present in 14.6% of patients during chemotherapy and in 9.3% after treatment. The prevalence of aggressiveness was 5.6% in patients during chemotherapy and 18.9% in the post-treatment group (84).

An Australian study conducted on 486 eligible TC survivors found small but significant increases in mean levels of anxiety and depression, a greater prevalence of extremely severe anxiety (19%) and depression (20%), and significant deficits in mainly mental aspects of generic HRQoL**.** The majority of TCSs reported one or more unmet needs regarding existential issues, more frequent than in breast and gynecological cancer survivors and probably correlated to the young age of the TCSs (64).

A diagnosis of cancer and associated treatments may represent a potentially ongoing threat and trigger recurring challenges. Indeed, this life-threatening illness is conceptualized as a potentially multidimensional traumatic event that risks compromising body integrity, leading to disability, disfigurement, pain and loss of social and occupational functioning, and creating dependence on others (24). Although most cancer survivors do not meet the criteria to be diagnosed with post-traumatic stress disorder, they may nevertheless report painful re-experiencing of the cancer diagnosis and treatment-related events. Traumatic stress symptomatology in terms of intrusive thoughts about the disease, avoidance of reminders of cancer, and hyper-vigilance are commonly reported by survivors after completion of treatment (85).

Little is known about cancer-related stress symptoms in TCSs. Some studies (31, 55) have focused on the cancer-related stress symptoms of intrusion and avoidance, the core symptoms of PTSD (86). Fleer et al. (55) reported that a minority (13%) of TCSs experience clinically elevated levels of cancer-related stress symptoms. In particular, TCSs with a lower level of education and unemployed survivors reported higher levels of cancer-related stress symptoms than their counterparts. The authors also reported that the impact of the illness felt by TCSs on their current lives and their anxiety about the future contributed more significantly to distress than objective illness variables. Mykletun et al. (31) observed that TCSs who experienced more TC-related stress were more likely to report reduced QoL, but concluded that the stress was not attributable to treatment strategies. A

recent study reported that in a sample of TCSs who were 11 years post-diagnosis, just over 10% had either subclinical or full PTSD. Probable PTSD was not related to time since TC diagnosis, but was significantly associated with cisplatin-related side effects, probable anxiety disorder, and poor self-rated health at 11 years post-diagnosis (87).

A recent study by Norwegian researchers evaluated the prevalence of chronic fatigue among 812 TC survivors. The risk of this disorder increased 3- to 4-fold for high levels of neuropathy vs. no neuropathy, and 2- to 3-fold for high levels of Raynaud-like phenomena and when testosterone levels were in the lowest quartile. Conversely, moderate to high physical activity had a protective effect against the syndrome (88).

Neuroticism in TCSs undergoing long-term follow-up is significantly associated with somatic and mental morbidities, self-esteem, concerns about not being able to father children, sexual problems, use of alcohol, sedatives and hypnotics, frequent visits to their G.P., and seeing a psychologist/psychiatrist (63).

Partnered Relationships

Cancer has considerable psychosocial implications related to the impact of the disease and its treatment on the individual from a psychological and spiritual point of view, and also from the perspective of interpersonal and social relationships (89, 90). The disease, far from being individual experience, also exerts a profound effect on patients' families. In particular, the partners of cancer patients are subject to a wide range of both emotional and practical repercussions throughout the course of the disease. For couples who face the survivorship phase, the main tasks include resuming a sexual relationship, discussing changes in life plans, deciding on health behavior changes, dealing with disease and treatment-related late effects that may influence patient functioning, managing worry about disease recurrence, and reflecting on the impact the cancer has had on themselves and the relationship (91).

Affective-relational life is an important theme in TC survivorship. It has been found that romantic relationships are associated with both positive (e.g., improved physical and emotional function) and negative aspects (e.g., new conflicts) (91). The majority of long-term TCSs and their wives report that their experience with cancer draws them closer as a couple, strengthening their mutual ties, trust, understanding, commitment to each other, and intimacy (92, 93). This aspect was also highlighted in a review on sexual functioning of TCSs and their partners (94). Although sexuality may be restricted or impaired by the experience of cancer, the decline in sexual satisfaction is usually very limited (44). As Jankowska explained, it is possible that patients who are facing a life-threatening disease may reorient their life's priorities and values with regard to sexuality and the relationship with their partner, reaping positive benefits such as greater intimacy and closeness (94). Thus, the psychological and affective aspects of the dyadic dimension may play a protective role in sexual function.

Relationship status (partnered vs. unpartnered) can play an important role in adjustment outcomes (91). Men who were involved in a relationship at the time of TC describe a better physical and emotional adaptation to the cancer experience (95, 96). Tuinman et al. also described positive outcomes and a higher level of functioning for survivors with a continuing relationship after diagnosis. In particular, they reported greater levels of social support, self-esteem and overall mental health compared with single TCSs and survivors who met their partner after the completion of treatment (97).

There is ample evidence in the literature about the relational difficulties experienced by individuals with a history of TC. For young adults involved or thinking about becoming involved in intimate relationships, the effect of TC treatment on sexual function, fertility, and overall future health may represent significant barriers to successful romantic and sexual relationships. Carpentier et al. describe 4 recurring themes related to testicular cancer diagnosis that can interfere and influence satisfaction in the romantic relationships of survivors: feeling different, viewing their differences as "damaged goods," struggles with cancer-related disclosures, and feelings of embarrassment (98).

Survivors unpartnered during treatment express worry about their history of cancer affecting future interpersonal relationships (95, 99).

New relationship difficulties reported by TCSs and spouses concern communication problems centered on a fear of talking about the cancer, problems in understanding and expressing feelings with their respective partners, the possibility of recurrence, and implications for the future (93).

In some cases, TC exacerbates pre-existing relationship conflicts or creates new conflicts which ultimately lead to relationship dissolution. A cancer experience leads to a greater appreciation of life in which conflicts no longer have a place, thus leading survivors to end conflict-plagued relationships (91). The majority of TCSs (70–90%) are in partnered relationships when TC is diagnosed and the majority of follow-up studies show that the rate of divorce and broken relationships for TCSs is 5 to 10% (100). Conversely, Joly et al. reported that friendships were more likely to remain intact for TCSs than for controls (29). Similar results were obtained by Syse in terms of marriage percentages (101).

Reentry Problems

Social and functional life (work and study) issues faced by cancer survivors may include difficulties in transitioning from patient to healthy status, being regarded by others as "special," feeling that one's job is not secure, experiencing discrimination and/or negative peer and employer attitudes (24). Several studies have been conducted to investigate reentry problems in cancer survivors and employment rate data vary considerably, ranging from 35 (102) to 67% for long-term survivors (103, 104). The differences in percentages may be attributable to different cancer types examined. However, a Finnish study reported only a 9% lower employment rate than that of the cancer-free population (105). This discrepancy may be correlated with the fact that people with a higher level of education have a greater chance of being employed after their cancer diagnosis than less educated patients job type is also a factor, e.g., manual labor is negatively associated with a return to work due to its physically strenuous nature (106, 107). With regard to TCSs, coping behavior is

not only needed for human relationships, but also for work situations. Rutskij et al. reported that TCSs with poorer avoidance coping skills fared worse in terms of paired relationships and paid work than TCSs with a better approach to coping (108). Another study showed that TCSs diagnosed <5 years earlier reported more absenteeism than controls, whereas there was no difference between controls and survivors diagnosed >5 years earlier (109). An interesting study conducted among breast, testicular and prostate cancer survivors in Northern Europe (NOCWO trial) did not reveal any differences in work engagement between cancer survivors and other employees, despite all of the problems reported by survivors, i.e., poorer health status, physical QoL, and work ability, more anxiety, and significantly higher neuroticism (110).

Within this context, recent studies have highlighted the importance of post-treatment psychosocial and behavioral interventions (111). In particular, knowledge that one's job is secure should be acknowledged as a prerequisite for normal living conditions (112). Improving communication at the workplace and developing supportive leadership practices are needed to avoid isolating behavior in cancer survivors (113).

CONCLUSIONS

TC is perhaps the paradigm of cancers with problems related to a long-term survival. There are 2 fundamental reasons for this, i.e., the curability of a high number of patients, leading to better long-term survival, but also the onset at a young age, leading to problems that differ from those arising from tumors diagnosed later in life. Such problems, including individual health, sexual relationships and work problems, affect several important aspects of survival and significantly influence the QoL of long-term survivors. Recently, a web-based computer-tailored intervention, the Kanker Nazorg Wijzer (Cancer Aftercare Guide), was developed in the Netherlands with the aim of providing psychosocial and lifestyle support for cancer survivors. It not only provides the most appropriate advice regarding physical activity, diet etc., but also measures psychosocial well-being by assessing QoL, psychological distress, mental adjustment to cancer, fatigue, work limitations, and social support. This tool is not yet suitable for use for TCS-related problems (114, 115).

As far as we know, QoL evaluations in long-term TCSs have only been conducted in single country trials. Recently, however, some studies have begun assessing the feasibility of collecting QoL data among TCSs recruited from different countries (116, 117). To better understand the impact of TC on QoL it is important to know how sociocultural differences in sexuality, masculinity and fertility influence the survivors. This problem exist and is still very wide. Educational events, patients associations and the development of Cancer Aftercare Guides, as like as the abovementioned Dutch project, could provide some solutions to a better awareness about the importance of QoL in TCSs.

AUTHOR CONTRIBUTIONS

GS and SD have collaborated in the conception, in the data retrieval, and in the drafting of the text. DD and CC have collaborated in the revision of the text and in the completion of the bibliographic research. EM, FR, GRo, CL, GRa, VC, AF, LG, and UD revised the manuscript.

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SYSTEMATIC REVIEW
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Testicular Cancer in Infertile Men With and Without Testicular Microlithiasis: A Systematic Review and Meta-Analysis of Case-Control Studies

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Background: An association between testicular microlithiasis (TM) and both carcinoma *in situ* (CIS) of the testis and testicular germ cell tumors (TGCTs) has been reported. Furthermore, TM seems to be significantly more prevalent in men with male-factor infertility, representing itself a risk factor for TGCT. Nevertheless, the evidence of the association of TM with a higher prevalence of testicular cancer in infertile men remains inconclusive. The aim of this study was to systematically evaluate whether, and to what extent, TM is associated to a significantly higher prevalence of testicular cancer in infertile males.

Methods: A thorough search of MEDLINE, SCOPUS, CINAHL, WEB OF SCIENCE, and Cochrane Library databases was carried out to identify case-control studies comparing the prevalence of testicular cancer in infertile men with and without TM. Methodological quality of the studies was assessed using the Newcastle-Ottawa Scale. In the absence of heterogeneity, odds ratios (ORs) with 95% confidence intervals (CIs) for testicular cancer were combined using a fixed effect model. Funnel plots and trim-and-fill analysis were used to assess publication bias.

Results: Eight studies met the inclusion criteria and provided information on 180 infertile men with TM and 5,088 infertile men without TM. The pooled OR indicated that the presence of TM is associated with a ∼18-fold higher odd for testicular cancer (pooled OR:18.11, 95%CI: 8.09, 40.55; $P < 0.0001$). No heterogeneity among the studies was observed ($P_{\text{for heterogeneity}} = 0.99$, $I^2 = 0\%$). At the sensitivity analysis, similar pooled ORs and 95%CIs were generated with the exclusion of each study, indicating the high degree of stability of the results. The funnel plot revealed a possible publication bias and the trim-and-fill test detected two putative missing studies. Nevertheless, even when the pooled estimate was adjusted for publication bias, there was a still significantly higher odd for testicular cancer in the TM group (adjusted pooled OR: 16.42, 95%CI: 7.62, 35.37; $P < 0.0001$).

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Conclusions: In infertile men the presence of TM is associated to an ∼18-fold higher prevalence of testicular cancer. Longitudinal studies are warranted to elucidate whether this cross-sectional association actually reflects a higher susceptibility of infertile men with TM to develop testicular cancer over time.

Keywords: testicular microlithiasis, testicular cancer, germ cell tumor, male infertility, ultrasonography

INTRODUCTION

Testicular microlithiasis (TM) usually represents an incidental finding during a scrotal ultrasonography (US) examination which shows a typical speckled pattern of the testicular parenchyma with multiple, tiny, bright non-shadowing echogenic foci, involving one or both testes, due to intratubular microcalcifications (1).

Testicular microlithiasis in itself does not represent a malignant condition and, in different series of patients referred for urologic evaluation, albeit infrequent, it has been found in association with a number of non-neoplastic disorders such as cryptorchidism (2–4), epididymitis (5, 6) and testicular torsion (5, 7). Nevertheless, as suggested by some authors, TM should be regarded as a visible sign of a premalignant condition, since an association between TM and both carcinoma *in situ* (CIS) of the testis (3, 8–10) and testicular germ cell tumors (TGCTs), i.e., seminomas and non-seminomas (5, 6, 11–17), has been reported.

The relationship between TM and testicular cancer would be of special concern in infertile male population, as male-factor infertility in itself has been associated with an increased risk of TGCT (18, 19). In particular, in a large retrospective cohort study by Hanson et al. (19), men with oligozoospermia had a >10-fold increase in the risk of testicular cancer when compared to fertile men. Indeed, TM and male-factor infertility due to poor spermatogenesis, together with other closely related clinical conditions, such as cryptorchidism and urogenital malformations, could share common pathogenetic mechanisms related to testicular dysgenesis syndrome (20). This could explain the reported higher prevalence of TM in infertile men populations when compared to men referred for scrotal complaints or young asymptomatic males (21, 22).

However, whether and to what extent the presence of TM in infertile men actually confers a significantly higher risk of testicular cancer remains unclear, as, in this population, an association of TM with a higher prevalence of testicular cancer has been reported by some studies (23–26) but not by others (2, 7, 27, 28).

Hence, we carried out a systematic review with meta-analysis of the available case-control studies, aiming to answer the following question: "Does, and to what extent, TM is associated to a significantly higher prevalence of testicular cancer in infertile males?"

MATERIALS AND METHODS

The study was conducted according to the Cochrane Collaboration and the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (29). It also complies with the guidelines of Meta-Analyses and Systematic Reviews of Observational Studies (MOOSE) (30). PRISMA and MOOSE Checklists have been presented as **Supplementary Tables 1, 2**.

The study is registered in the International Prospective Register of Systematic Reviews (PROSPERO) with the registration number CRD42019121488.

Systematic Search Strategy

We conducted a systematic search in MEDLINE, SCOPUS, CINAHL, WEB OF SCIENCE, and Cochrane Library databases to identify all relevant studies in the English language with the terms: ("testicular microlithiasis") AND ("testicular cancer" OR "testicular tumor*" OR "testicular neoplasm*" OR "germ cell cancer" OR "germ cell tumor*" OR TGCT OR "germ cell neoplasm*" OR seminoma* OR nonseminoma*). If it was not clear from the title and abstract whether the paper contained relevant data, the full paper was retrieved. We scrutinized the reference lists of the identified articles to find possible additional pertinent studies.

Inclusion and Exclusion Criteria

The outcome of interest was a difference in the prevalence of testicular cancer between infertile men with and without TM. The eligibility criteria used for the inclusion were: (1) observational case-control studies involving adult men undergoing scrotal US as a part of diagnostic work-up for infertile marriage with (cases) and without (controls) TM; (2) availability of data for the calculation of odds ratios (ORs) with a 95% confidence interval (CI) for testicular cancer in both the groups. As variable ultrasonographic definitions of TM have been reported in literature, no restrictions in diagnostic criteria for TM were used when assessing the eligibility of the studies.

Two independent reviewers (AM and EM) assessed the eligibility of each selected article and any disagreement was resolved via discussion involving a third reviewer (AB).

Data Extraction

Data from the selected articles were extracted by including the first author, publication year, country, the total number of cases (infertile men with TM) and controls (infertile men without TM), and the number of events (number of patients with testicular cancer) in each group. Additional information, when available, included: mean age or age range of the participants, testicular volumes, semen characteristics, and the percentage of cases with bilateral TM and/or cryptorchidism history. Wherever

quantitative data were missing or inconsistent, the authors were contacted to obtain the necessary information.

Quality Assessment

The quality of studies included in the quantitative analysis was assessed using the "star system" of the Newcastle-Ottawa Quality Assessment Scale (NOS) (31). The minimum score was 0 stars and the maximum that could be awarded was 9 stars. Studies getting scores ≥6 stars were regarded as good quality studies. The quality assessment was performed by two reviewers (AB and SDA) and any disagreement was resolved by a third reviewer (SF) who re-evaluated the original study.

Statistical Analysis

The relationship between TM and testicular cancer was assessed using ORs and a 95% CI as well as by Mantel-Haenszel estimates. In the absence of heterogeneity between the studies, data were combined using a fixed effect model. The Cochrane Chi-square (Cochrane Q) test and the I^2 test were carried out to analyze the heterogeneity between the results of different studies. An $I^2 > 50\%$ and/or $P < 0.05$ indicated substantial heterogeneity (32).

Sensitivity analysis was performed by sequential omission of individual studies to determine the contribution of each study to the pooled estimate and evaluate the stability of the results.

Publication bias was graphically identified using a funnel plot, wherein a symmetric inverted funnel shape arises from a "well-behaved" data set, in which publication bias is unlikely (33). The funnel plot was also subjected to the Duval and Tweedie's "trim-and-fill" analysis, which, in the presence of asymmetric shape, detects putative missing studies to rebalance the distribution. This analysis also provides an adjusted pooled estimate taking the additional studies into account, thus correcting the analysis for publication bias (34).

The extracted data were analyzed using the package "metafor" of the statistical software R (version 3.0.3; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study Selection

The electronic search yielded a total of 1,062 studies. After removal of duplicate, 763 studies were left, of which 662 were excluded based on titles and abstracts. Hence, as shown in **Figure 1**, a total of 101 studies were identified, of which 8 met the inclusion criteria (2, 7, 23–28).

Main details of the articles included in the quantitative synthesis are reported in **Table 1**.

Quality of the Included Studies

The NOS score-based quality ratings of the studies are presented in **Table 2**. Quality scores ranged from 3 to 7. Seven articles were considered to be of good quality (2, 7, 24–28) scoring ≥6 and one article (23) was assessed to be of poor quality. In particular, in all studies except that by Pierik et al. (23), diagnostic criteria for TM used for the definition of cases were clearly reported: according to Backus et al. (8), TM was defined as ≥5 randomly distributed non-shadowing hyperechogenic foci with diameters <3 mm per transducer field. In most studies a full comparability could not be ensured by adjusting either on age or other variables (**Table 2**).

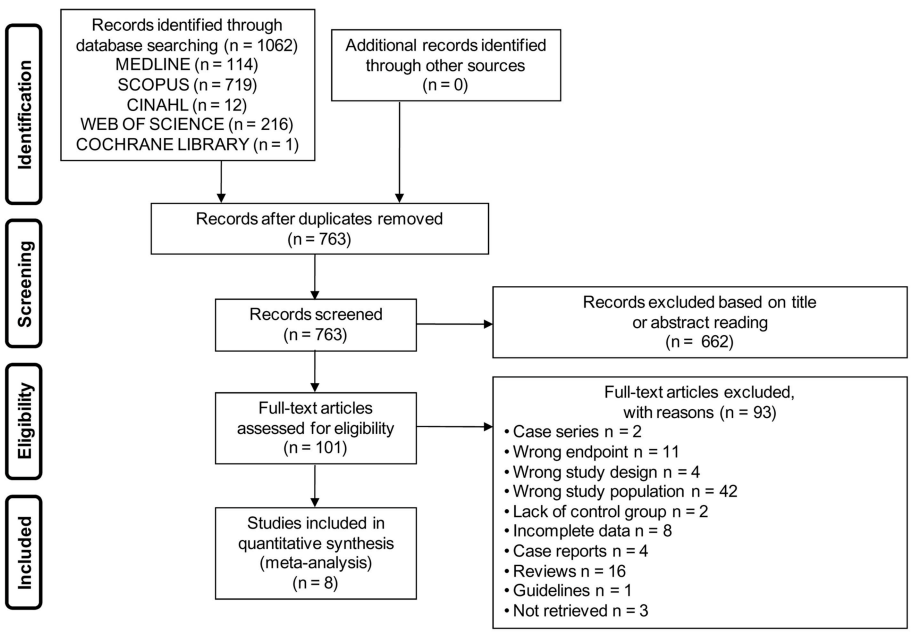

FIGURE 1 | Flow diagram showing an overview of the study selection process.

TABLE 1 | Main characteristics of the eight studies included.

Study	Region	Mean age or age range of participants (years)	TM group *n* (%)	Confirmation of cancer diagnosis after US	Bilateral testicular volume (mean ± SD)	Cryptorchidism history (%) in TM group	Bilateral TM (%)	Semen abnormalities
Aizenstein et al. (2)	USA	37	5 (2.8)	NA	NR	40	50	All participants had oligo and/ or astheno
La Vignera et al. (26)	Italy	43.3	60 (18.8)	Histology	NR	NR	NR	Oligo/astheno and/or terato in 66.6% of the TM group
Mazzilli et al. (27)	Italy	NA	13 (4.6)	NA	Whole population: 18.0 ± 4.5 ml	NR	NR	In the whole study population: oligo: 23.8% azo: 5.6% astheno: 70.4% terato: 20.3%
Negri et al. (25)	Italy	37	31 (1.4)	Histology	NR	13	NR	NS
Pierik et al. (23)	NL	20–58	12 (0.9)	Histology	NR	33.3	NR	NS
Qublan et al. (28)	Jordan	31	23 (9.8)	NA	Whole population: 14.0 ± 3.6 ml	NR	NR	All participants had oligo or azo
Sakamoto et al. (24)	Japan	35.8	31 (5.6)	Histology	TM group: 9.0 ± 5.2 ml Controls: 9.8 ± 5.1 ml	3.2	90.3	Oligo or azo in 64.5% of whole study population
Thomas et al. (7)	UK	29–51	5 (3.1)	NA	NR	0	0	NS

Astheno, asthenozoospermia; Azo, azoospermia; Oligo, oligozoospermia; Terato, teratozoospermia; TM, testicular microlithiasis; NA, not applicable (no testicular masses were detected at US); NL, Netherlands; NR, not reported; NS, not specified; SD, standard deviation; US, ultrasonography.

TABLE 2 | Newcastle-Ottawa assessment scale for case-control studies.

Study	Selection				Comparability		Exposure			Total
	Definition of cases	Representativeness of cases	Selection of controls	Definition of controls	On age	On other risk factors	Assessment of exposure	Same methods of ascertainment for cases and controls	Non response rate	
Aizenstein et al. (2)	1	1	1	1	0	0	1	1	0	6
La Vignera et al. (26)	1	1	1	1	0	0	1	1	0	6
Mazzilli et al. (27)	1	1	1	1	0	1	1	1	0	7
Negri et al. (5)	1	1	1	1	0	0	1	1	0	6
Pierik et al. (23)	0	1	1	0	0	0	1	0	0	3
Qublan et al. (28)	1	1	1	1	0	0	1	1	0	6
Sakamoto et al. (24)	1	1	1	1	1	1	1	0	0	7
Thomas et al. (7)	1	1	1	1	0	0	1	1	0	6

Synthesis of Results

The eight studies included in the meta-analysis collectively provided information on 180 infertile men with TM and 5,088 infertile men without TM. As shown in **Figure 2**, pooled estimate indicated that the presence of TM is associated with a ∼18-fold higher odd for testicular cancer (OR: 18.11, 95% CI: 8.09, 40.55; $P < 0.0001$). No heterogeneity among the studies was observed ($P_{\text{for heterogeneity}} = 0.99$, $I^2 = 0\%$).

Sensitivity analysis was performed to assess the contribution of individual studies to the overall odd for testicular cancer.

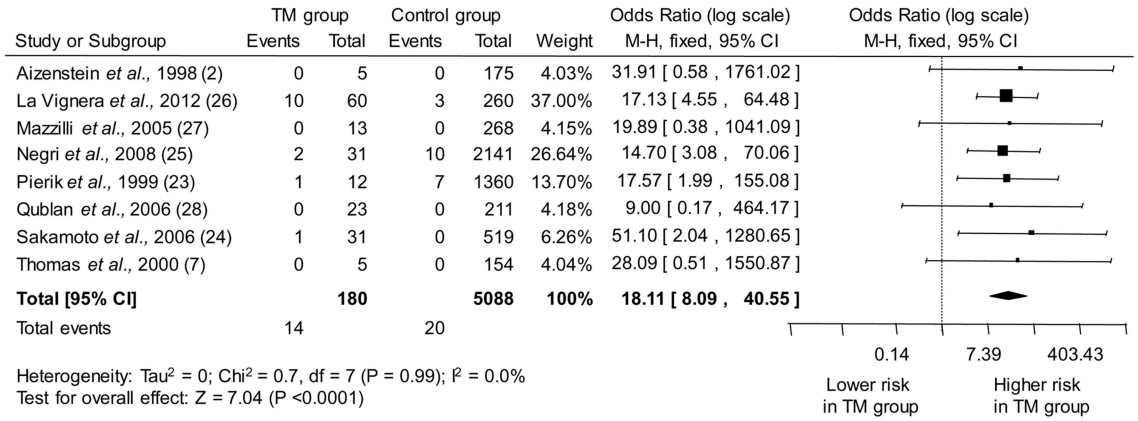

FIGURE 2 | Forest plots depicting the odds ratio for testicular cancer between infertile men with and without testicular microlithiasis (TM). Diamond indicates the overall summary estimate (width of the diamond represents the 95% CI); boxes indicate the weight of individual studies in the pooled analysis. CI, confidence interval; df, degrees of freedom; M-H, Mantel-Haenszel.

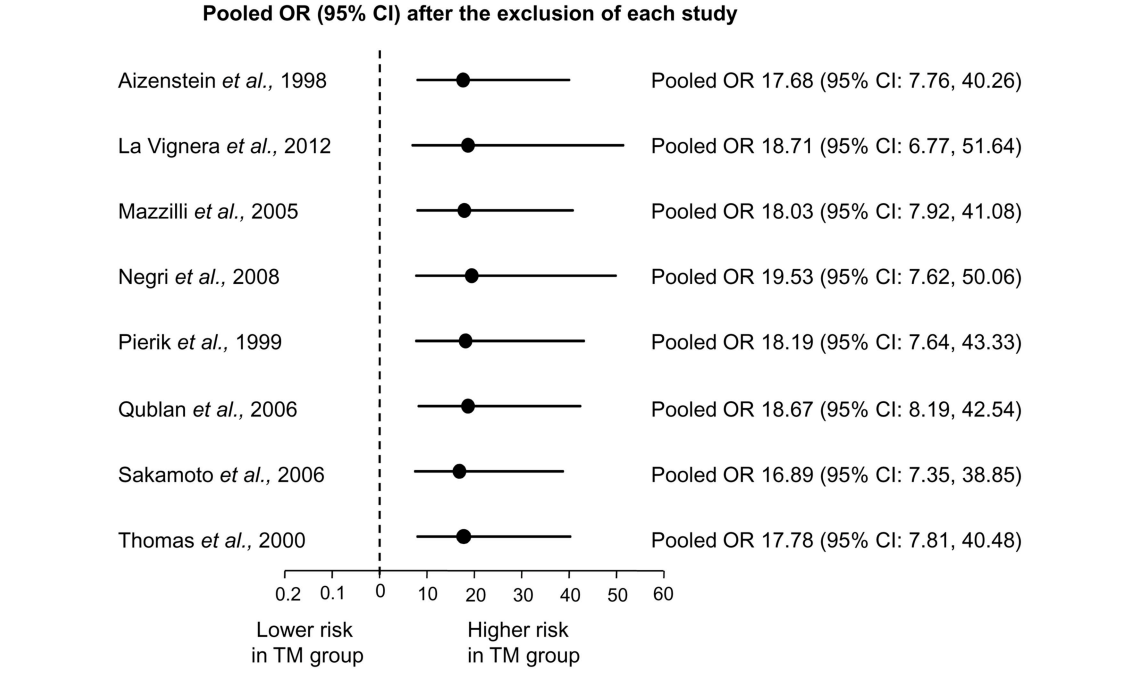

FIGURE 3 | Sensitivity analysis showing the influence of each individual study on the pooled odds ratio (OR) with 95% Confidence Interval (CI) for testicular cancer.

As shown in **Figure 3**, similar pooled ORs and 95% CIs were generated with the exclusion of each study, thus indicating the high degree of stability of the results.

Publication Bias

The asymmetry of the funnel plot suggested a possible publication bias (**Figure 4**). Accordingly, the trim-and-fill analysis identified two putative missing studies on the left side of the distribution. Nevertheless, when the funnel distribution was rebalanced by including these additional studies, the adjusted pooled estimate indicated a persistent significantly higher odd for testicular cancer in the TM group (adjusted OR: 16.42, 95% CI: 7.62, 35.37; $P < 0.0001$) with no heterogeneity ($P_{\text{for heterogeneity}} = 0.99$, $I^2 = 0\%$).

DISCUSSION

To date, inconclusive results have been reported by studies evaluating the association of TM with a higher prevalence of testicular cancer in infertile males. Many studies retrospectively explored the relationship between TM and testicular cancer in

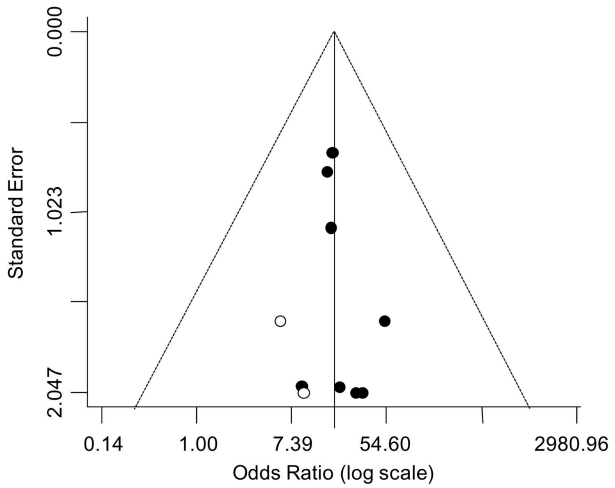

FIGURE 4 | Funnel plot for the analysis of the relationship between TM and testicular cancer in infertile men. The trim-and-fill analysis identified two putative missing studies (white circle) on the left side of the distribution.

heterogeneous series of patients referred for scrotal US due to different urological/andrological indications, also including (but not restricted to) infertility (6, 13, 15, 35–40); in most cases, data from individual sub-group of patients were not provided separately, thus making impossible to draw conclusions regarding infertile males. Some of these studies were meta-analyzed by Wang et al. (17), reporting a strong overall association of TM with an almost 13-fold increased risk of testicular cancer. In that analysis, a very large between-studies heterogeneity was found ($I^2 = 82.1\%$) and, interestingly, the studies with the most significant forest plot results (14, 16, 26, 41) included infertile patients in their samples. Actually, even the few studies providing information about the association between TM and testicular cancer in male infertility specifically, did not produce unequivocal results.

In the largest retrospective studies, by Pierik et al. (23) and by Negri et al. (25), that enrolled only infertile men, patients with TM, who represented approximately 1% of the study populations, exhibited significantly higher odds for testicular cancer when compared to TM-free infertile patients. A significantly higher association between TM and testicular cancer was also found by Sakamoto et al. (24) and by La Vignera et al. (26) in their infertile sub-group of patients, who showed TM in 5.6 and 18.8% of cases, respectively. However, in four studies, enrolling smaller-sized infertile men samples, where the prevalence of TM ranged from 2.8 to 9.8%, no cases of testicular cancer were detected in either patients with or without TM (2, 7, 27, 28).

In the present meta-analysis of these eight carefully selected studies, the overall prevalence of testicular cancer in infertile men with TM was 7.8%, corresponding to an odd of detecting a testicular cancer approximately 18-fold higher (pooled OR : 18.11, 95% CI: 8.09, 40.55) than in infertile men without TM. Given the retrospective design of the included studies, whether TM has to be regarded as a precursor of malignant disease or whether it develops as a result of malignant disease remains uncertain.

It is generally accepted that virtually all TGCTs arise from a CIS (42), the common precursor which eventually progresses to invasive cancer if not treated. Interestingly, in a study by von Eckardstein et al. (3), a CIS was diagnosed in 2 out 11 men with TM, whereas no CIS was found in biopsies from 65 individuals without TM. The cross-sectional association between TM and CIS was subsequently confirmed by De Gouveia et al. (10) on a larger retrospective series of 263 subfertile men. Although longitudinal studies are lacking, these data suggest that, as TM has a significant predictive value for the presence of CIS (which is a precursor of TGCT), TM might also predict the development of overt testicular cancer.

It has been hypothesized that the complex relationship linking together CIS/testicular cancer, poor semen quality and closely related conditions, such as cryptorchidism and hypospadias, could reflect the testicular dysgenesis syndrome, a common underlying entity with an origin in fetal life (20). Events involved in the development of a testicular cancer in adult life are likely to occur during embryogenesis and CIS cells, which closely resemble fetal gonocytes both morphologically and immunochemically (43–45), are presumed to derive from primitive primordial germ cells or gonocytes that escaped normal differentiation *in utero*, and instead entered a neoplastic transformation (46). Similarly, any disturbance in early fetal life of the development/differentiation of Leydig and Sertoli cells may lead to an impairment of both production of testosterone and insulin-like factor 3 (INSL3) and germ cell development, resulting in genital malformations (such as hypospadias and cryptorchidism) and, later in life, impaired spermatogenesis (47). The model of the testicular dysgenesis syndrome not only could explain why infertility represents a risk factor for testicular cancer but also why the presence of TM is associated with an even higher risk: TM might be the expression of an already existent CIS.

LIMITATIONS

Some limitations of this meta-analysis, other than the aforementioned retrospective design of the included studies, have to be recognized. Firstly, overall, meta-analyzed studies included very few patients with TM (only 180 individuals) and very few events (only 14 testicular cancers in cases and 20 in controls) resulting in quite imprecise ORs, as indicated by their wide confidence intervals. However, it should be recalled that both TM and testicular cancer represent relatively uncommon conditions. For instance, in the aforementioned study by Hanson et al. (19), who reported a more than 10-fold increase in the risk for testicular cancer in oligozoospermic men, only 30 cases of testicular cancer were found in a large sample of 20,433 subfertile men. In any case, in spite of the low number of events registered in our quantitative synthesis, at the sensitivity analysis, similar pooled ORs and 95% CIs were generated when the studies with the highest weight in contributing in the pooled estimate (23, 25, 26) were excluded, thus indicating the very high degree of stability of the results.

As another limitation of this meta-analysis, the largely incomplete information about the occurrence of other recognized risk factors, such as testicular hypotrophy and cryptorchidism, in cases and controls (**Table 1**), did not allow sub-group analyses for the assessment of a further increase in the risk of cancer.

Finally, the funnel plot revealed a possible publication bias, suggesting that published studies could be a not fully representative sample of the available evidence. Nevertheless, the value of the corrected pooled OR, taking into account two putative missing studies identified by the trim-and-fill analysis, demonstrated that the publication bias did not substantially affect the overall estimate.

In conclusion, the results from the present meta-analysis indicate that, in infertile men, TM is associated to an ∼18-fold higher odd of detecting testicular cancer. Longitudinal studies are warranted to elucidate whether this cross-sectional association actually reflects a higher susceptibility of infertile men with TM to develop testicular cancer over time.

AUTHOR CONTRIBUTIONS

AB conceived the study, participated in the assessment of the eligibility of each selected study, assessed the quality of the studies, performed the statistical analysis, and wrote the manuscript. AM and EM participated in the systematic search and in the assessment of the eligibility of the studies. SD assessed the quality of the studies. DB and CC were involved in data extraction. FF helped draft the manuscript and critically revised the manuscript. SF contributed to conception, participated in the assessment of the quality of the studies, and critically revised the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00164/full#supplementary-material

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ORIGINAL RESEARCH
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Long-Term Follow Up of the Erectile Function of Testicular Cancer Survivors

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The diagnosis of testicular cancer (TC) can have a considerable and persistent impact on a patient's sexuality, especially given its location. The high prevalence of TC in young adults, and the good prognosis, explain the great interest in sexual dysfunction and its influence on post-treatment quality of life. The aim of this study was to evaluate the impact of the diagnosis and treatments (inguinal orchiectomy and chemotherapy) on sex life. For this purpose, we recruited 241 TC patients attending the Laboratory of Seminology–Sperm Bank "Loredana Gandini" for sperm cryopreservation (mean age 31.3 ± 6.9 years) and 223 cancer-free healthy men who were undergoing andrological screening (mean age 32.0 ± 7.7 years). The IIEF-15 questionnaire was administered at the baseline (post-orchiectomy, pre-chemotherapy—T0) and at 6 (T1), 12 (T2), 18 (T3), 24 (T4), 48 months (T5) and >5 years (T6, median 96 months) after chemotherapy to all patients, to evaluate the following domains: erectile function (EF), orgasmic function (OF), sexual desire (SD), intercourse satisfaction (IS) and overall satisfaction (OS). A subgroup of patients also underwent blood sex hormone analysis for further correlations with IIEF scores. At the baseline, 37.7% of patients had erectile dysfunction (EF score <26) and all IIEF domains except OF showed significantly lower scores than in controls ($p < 0.001$). Long-term follow-up revealed persistently lower scores in TC survivors than in controls for EF, SD, IS, and OS. Furthermore, most IIEF domains did not improve significantly in TC patients during the duration of the follow-up, with the exception of EF, which showed a significant improvement from T2. Finally, no significant correlation was found between hormone levels (gonadotropin and testosterone) and IIEF-15 scores. In conclusion, TC and its treatment have a significant effect on sexuality. The absence of a clear correlation with biochemical hypogonadism suggests that this may to a large extent be due to the surgical procedure itself, or to the psychological impact of a cancer diagnosis.

Keywords: testicular cancer, cancer survivors, orchiectomy, sexual function, erectile dysfunction, IIEF

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INTRODUCTION

Alongside cardiovascular disease, cancer is currently the main cause of mortality worldwide. Italian cancer registers show that nearly 5% of the population has received a diagnosis of cancer (1). However, modern treatments mean that the life expectancy of about 60% of juvenile and young adult cancer survivors is comparable to that of the general population.

Men in reproductive age are mainly affected by testicular cancer (TC) and lymphomas, but despite the high incidence, their 5-year survival rates are above 80–90% (2, 3). These cancer survivors will therefore have to live with the long-term physical and psychological consequences of both their treatments (surgery, chemotherapy, radiotherapy) and the diagnosis itself (4, 5). This has important health, social and economic repercussions, as these long-term consequences affect men in their working and reproductive years, affecting their physical capabilities as well as their reproductive and sexual health. According to the WHO, reproductive health is defined as a "state of complete physical, mental and social well-being and not merely the absence of disease or infirmity, in all matters relating to the reproductive system, and to its functions and processes" (6). Sexual health should in fact be considered as a complex interaction of multiple factors including social and cultural aspects, individual experiences, and self-image. Cancer and its treatments should certainly be considered as capable of disrupting sex life, but many patients find it difficult to discuss these problems and there is a lack of consensus on valid outcome measures for assessing sexual function in cancer patients on the basis of a broader definition of sexual health (7)—issues yet to be faced in common practice or research.

Most knowledge of male sexual dysfunction after cancer pertains to prostate cancer after invasive surgical procedures and hormonal treatments: however, this is not representative of other situations (8). The sexological features of testicular cancer have been investigated by several authors, revealing associations with perceived loss of masculinity and sexual function, which paves the way for psycho-organic sexual dysfunctions (4). Orchiectomy itself can alter the perception of body image, potentially manifesting as reduced libido and sexual gratification linked to the psychological stress of not "being normal" (9). The cancer diagnosis itself is a moment of intense psychological distress and, although literature reports vary widely, about one-third of patients with testicular cancer report erectile dysfunction and/or ejaculation disorders (10). Invasive and destructive surgery such as retroperitoneal lymph node dissection increases the frequency of such dysfunctions (10–12). However, most data focus on either the short- or the long-term consequences of therapy and reports of thorough longitudinal follow-up from diagnosis to long-term survivorship are rare. The aim of this study is to evaluate the effect of TC after orchiectomy and provide a complete follow-up in order to highlight possible short- and long-term sexological changes after treatment.

MATERIALS AND METHODS

Patients

The study was approved by our University Hospital's institutional review board (Ethical Committee Policlinico Umberto I—University of Rome "Sapienza") and all patients gave informed written consent. We recruited 241 sexually active consecutive patients (mean age 31.3 ± 6.9 years, range 18–52) with a recent diagnosis of testicular cancer who attended the Laboratory of Seminology—Sperm Bank "Loredana Gandini" between 2006 and 2018 for sperm cryopreservation before any cancer treatment. All patients had undergone orchiectomy within the previous 30 days.

As the control group, we recruited 223 healthy subjects (mean age 32.0 ± 7.7 years, range 18–55) who attended the Endocrinology and Andrology outpatient clinic of the Department of Experimental Medicine in the same period for idiopathic primary infertility. Subjects with hypogonadism and other endocrine disorders, diabetes, hypertension, cryptorchidism, history of cancer and/or previous chemo/radiotherapy, history of urogenital surgery, Klinefelter Syndrome and other chromosomal abnormalities or any genetic diseases were excluded. Both patients and controls underwent a thorough medical history and a general and andrological physical examination, and were administered the International Index of Erectile Function 15 questionnaire (IIEF-15) to evaluate sexual function. The IIEF-15 was administered to TC patients at the post-orchiectomy baseline before chemotherapy (T0) and at 6 (T1), 12 (T2), 18 (T3), 24 (T4), and 48 months (T5) after chemotherapy, with a final follow-up between 5 and 12 years post-chemotherapy (T6, median 96 months). Each patient underwent the baseline evaluation and at least one follow-up. A subgroup of TC patients also underwent blood hormone tests (FSH, LH, total Testosterone) for later comparison with healthy controls to investigate any correlations with IIEF scores. This subgroup patients underwent blood hormone analysis at T0, T1, and T2.

Hormone Analysis

Blood samples were collected at 8.00 a.m. after at least 8 h of overnight fasting for measurement of FSH, luteinizing hormone (LH) and total testosterone. Serum FSH, LH, and testosterone were measured by chemiluminescent microparticle immunoassay (CMIA, Architect System; Abbott Laboratories, Abbott Park, IL, USA), with detection limits of 0.05 mIU/ml, 0.07 mIU/ml, and 0.28 nmol/l, respectively. Intra- and inter-assay coefficients of variation were 3.1 and 7.0% at 3.2 mIU/ml (FSH), 3.6 and 5.1% at 3.3 mIU/ml (LH), and 2.1 and 3.6% at 10.08 nmol (total testosterone). Normal ranges for adults were 1.38–9.58 mIU/ml (FSH), 1.80–8.16 mIU/ml (LH), and 9.4–33.5 nmol/l (total testosterone).

IIEF-15

Sexual function can be evaluated in a reassuring and comfortable setting with self-administered questionnaires. One of the most widely used in both clinical practice and research is IIEF-15. This multidimensional tool enables the rapid, reliable and reproducible measurement of several domains of sexual function (13). It was developed to enable the evaluation of patients' sexuality in clinical trials for erectile dysfunction with high sensitivity and specificity. The advantage of self-administration is that it is perceived by patients as less invasive and burdensome than a direct interview. The classic form has 15 items grouped into five domains: erectile function (EF), questions 1–5 and 15; orgasmic function (OF), questions 9–10; sexual desire (SD), questions 11–12; intercourse satisfaction (IS), questions 6–8; and general satisfaction (GS), questions 13–14. Generally, a

score below 26 in the EF domain is considered diagnostic for erectile dysfunction.

Statistical Analysis

Continuous variables are presented as mean, median and standard deviation. Differences between groups were evaluated by ANOVA or Kruskal-Wallis test, based on data distribution as evaluated by Kolmogorov-Smirnov test. *Post-hoc* results were corrected using the Bonferroni method for multiple comparisons. Categorical variables are presented as counts and percentages and were compared by χ^2 test. Statistically significant correlations among the variables examined were evaluated using Spearman's rank correlation test. The probability values are 2-sided and a p-value <0.05 was considered statistically significant. All computations were carried out with Statistical Package for the Social Sciences (SPSS) 25.0 (SPSS Inc., Chicago, USA).

RESULTS

Pre-therapy

Table 1 describes the demographics of the recruited TC patients and control subjects. The TC and control groups were comparable in age, BMI and percentage of smokers. The baseline prevalence of erectile dysfunction as self-reported through the IIEF (EF domain score <26) was 37.8% (91/241) in TC patients against 9.9% (22/223) in the control group (χ^2 $p < 0.001$). Erectile dysfunction was severe in 23.2% (56/241), moderate in 4.1% (10/241) and mild in 10.4% (25/241) of TC patients, while all cases in the control group were mild. The baseline comparison of TC and CTR groups is presented in **Table 2**: all IIEF-15 domain scores were significantly worse in patients than in controls (all $p < 0.001$), with the exception of orgasmic function ($p = 0.334$). No significant correlations were found between IIEF scores and age, BMI, smoking status, cigarettes smoked/day and years of smoking in either group.

TABLE 1 | Testicular cancer and Control group demographics: continuous data are presented as mean ± SD, median (in brackets) and 25–75th percentile of data distribution; categorical data as percentage and count.

	Testicular cancer (241 pts)	Controls (223 pts)
Age at diagnosis (years)	31.3 ± 6.9 (31.0) 26.0–36.0	32.0 ± 7.7 (32.0) 26.0–37.0
BMI (kg/m^2)	24.9 ± 3.0 (24.5) 23.0–26.7	24.6 ± 2.7 (24.1) 22.7–25.9
Smokers	19.5% 47 pts	23.3% 52 pts
Cigarettes/day[a]	11.4 ± 8.5 (10.0) 5.0–15.0	11.3 ± 8.5 (10.0) 5.0–15.0
Years of smoking[a]	12.3 ± 6.6 (10.0) 7.0–16.0	10.6 ± 6.2 (10.0) 6.0–15.0
Occupation	Office worker (23.6%) Factory/heavy worker (15.3%) Freelance professional (13.9%) Student/university (9.7%) Police/military (2.8%) Driver (5.6%) Healthcare professional (4.2%) Unemployed (5.6%) Other (19.3%)	Office worker (18.1%) Factory/heavy worker (16.9%) Freelance professional (20.5%) Students/university (14.5%) Police/military (3.6%) Driver (3.6%) Healthcare professional (9.6%) Unemployed (2.4%) Other (10.8%)
Histological diagnosis	58.5% Seminoma pT1-pT2 30.7% Mixed germ cell tumor pT1-pT2 8.0% Embryonal carcinoma pT1-pT2 2.8% Yolk sac tumor	/
Chemotherapy regimen	BEP 1-3 cycles cysplatin 1 cycle	/

[a] *smokers only.*

Post-therapy

All patients underwent a chemotherapy regimen only, as indicated in **Table 1**. IIEF scores from longitudinal follow up are compared against healthy controls are shown in **Table 3**. Kruskal Wallis test with *post-hoc* corrections for multiple comparisons (Bonferroni) revealed that:

- ED domain scores had improved significantly 1 year post-chemotherapy (T0 vs. T2: $p = 0.001$), with further improvements at T3 and T4 (T0 vs. T3: $p = 0.014$; T0 vs. T4: $p = 0.002$) (**Figure 1**, **Table 3**). However, there was an increase in the prevalence of erectile dysfunction at T5, with a significant reduction in ED domain scores; this seemed to persist at T6. Compared to controls, ED scores remained significantly worse at T1 then returned to a level comparable with healthy controls (**Table 3**).
- OF domain scores showed a trend of improvement from the baseline ($p = 0.070$), but pairwise comparisons against both baseline and controls did not reach statistical significance (**Table 3**).
- SD, IS, and GS domain scores, although showing a trend of improvement, did not differ significantly from the baseline at any time points; however TC patients scored significantly worse than the controls for the entire duration of the study (**Table 3**).

Sex Hormone Analysis

The prevalence of biochemical hypogonadism (total testosterone <8.0 nmol/l) was 4.1% in the TC group; there were no hypogonadal patients in the control group. The Kruskal Wallis test with *post-hoc* corrections for multiple comparisons (Bonferroni) revealed that gonadotropin (both FSH and LH) levels in the TC patients were higher both pre-chemotherapy and at T1 and T2 than in the controls (all $p < 0.001$). Total testosterone at T0 was significantly lower than in the controls ($p < 0.001$), but there was no difference from the control groups at T1 or T2 (**Table 4**). Finally, no significant correlation was found between total testosterone levels and the score of any IIEF-15 domain.

DISCUSSION

The trend of reduced mortality for various cancers, including testicular cancer, has increased clinicians' awareness of the importance of long-term quality of life after surgery and chemo- and radiotherapy. Recent literature has focused on cancers involving the testes and genitalia (8, 12, 14, 15), but sexual function in male survivors of other frequent cancers has also been investigated (16, 17). In general, while TC survivors maintain or recover a good quality of life, investigation of their sex life reveals marked changes (18–20). Carpentier et al. highlighted that the diagnosis and therapy stages are associated with peak levels of anxiety and concern, which then drop in the post-treatment period. Similarly, stress-related central inhibition of sexual function results in a rise in libido, erection, and ejaculation disorders during cancer treatments (4). In fact, sexual dysfunctions in TC patients may arise from a combination of treatment-related physical side effects (genital mutilation, reduced testosterone levels, chronic pain, and other residual side effects) and psychological vulnerability (anxiety, fear, mood disorders, etc.) (**Figure 2**) (21). A possible underlying cause may be the induction of iatrogenic hypogonadism: in fact, orchiectomy, chemotherapy, and radiotherapy may all induce gonadal dysfunction. Our group recently found that a cohort of orchiectomized TC patients prior to cancer treatment had increased levels of gonadotropins and reduced

TABLE 2 | Baseline testicular cancer IIEF scores vs. control group: continuous data are presented as mean ± SD, median (in brackets) and 25–75th percentile of data distribution.

	ED domain	OF domain	SD domain	IS domain	GS domain
TC	22.7 ± 9.1	8.2 ± 1.9	7.5 ± 1.9	8.3 ± 4.7	7.4 ± 2.6
241 pts	(27.0)	(10.0)	(8.0)	(10.0)	(8.0)
	20.0–29.0	8.0–10.0	6.0–9.0	7.0–12.0	6.0–10.0
CTR	27.9 ± 2.6	8.9 ± 1.2	8.9 ± 1.2	12.6 ± 1.9	9.0 ± 1.3
223 pts	(28.5)	(10.0)	(9.0)	(13.0)	(9.0)
	27.0–30.0	8.0–10.0	8.0–10.0	11.5–14.0	8.0–10.0
P-value	<0.001	0.334	<0.001	<0.001	<0.001

(Mann Whitney U test). EF, erectile function; OF, orgasmic function; SD, sexual desire; IS, intercourse satisfaction; GS, general satisfaction.

TABLE 3 | IIEF scores of testicular cancer and control group: continuous data are presented as mean ± SD, median (in brackets) and 25–75th percentile of data distribution, while categorical data as percentage and counts.

	ED domain	OF domain	SD domain	IS domain	GS domain	Erectile dysfunction (%)
T0	22.7 ± 9.1[a]	8.2 ± 1.9	7.5 ± 1.9[a]	8.3 ± 4.7[a]	7.4 ± 2.6[a]	37.8%[d]
241 pts	(27.0)	(10.0)	(8.0)	(10.0)	(8.0)	(91/241)
	20.0–29.0	8.0–10.0	6.0–9.0	7.0–12.0	6.0–10.0	
T1	24.1 ± 8.6[c]	8.8 ± 2.4	8.0 ± 1.7[b]	8.7 ± 4.2[b]	7.9 ± 2.3[a]	28.4%[d]
74 pts	(28.0)	(10.0)	(8.0)	(10.0)	(8.0)	(21/74)
	24.0–30.0	9.0–10.0	7.0–9.0	8.0–11.0	8.0–10.0	
T2	25.8 ± 7.1	8.8 ± 2.3	7.6 ± 1.7[b]	9.4 ± 3.6[b]	7.9 ± 2.2[a]	23.6%[d]
110 pts	(29.0)	(10.0)	(8.0)	(10.0)	(8.0)	(26/110)
	26.0–30.0	9.0–10.0	7.0–9.0	8.0–12.0	7.0–10.0	
T3	26.5 ± 6.3	8.7 ± 2.3	7.6 ± 1.6[b]	9.7 ± 3.7[b]	8.0 ± 2.2[b]	18.3%[d]
60 pts	(29.0)	(10.0)	(8.0)	(10.0)	(8.0)	(11/60)
	27.0–30.0	8.0–10.0	7.0–9.0	9.0–12.0	7.0–10.0	
T4	26.9 ± 5.7	9.2 ± 1.7	8.0 ± 1.5[b]	10.0 ± 3.3[b]	8.5 ± 1.7[b]	16.0%[d]
75 pts	(29.0)	(10.0)	(8.0)	(11.0)	(9.0)	(12/75)
	27.0–30.0	9.0–10.0	7.0–9.0	9.0–12.0	8.0–10.0	
T5	24.9 ± 8.0	8.0 ± 2.9	7.5 ± 1.8[b]	8.9 ± 4.0[b]	7.8 ± 2.3[a]	25.4%[d]
67 pts	(28.0)	(10.0)	(8.0)	(9.0)	(8.0)	(17/67)
	25.0–30.0	6.0–10.0	6.0–9.0	8.0–12.0	7.0–10.0	
T6	25.2 ± 7.3	9.0 ± 2.0	7.7 ± 1.6[b]	9.8 ± 3.6[b]	8.2 ± 2.0[c]	30.5%[d]
36 pts	(28.0)	(10.0)	(8.0)	(10.0)	(8.0)	(11/36)
	24.0–30.0	8.0–10.0	7.0–9.0	9.0–12.0	8.0–10.0	
CTR	27.9 ± 2.6	8.9 ± 1.2	8.9 ± 1.2	12.6 ± 1.9	9.0 ± 1.3	9.9%
223 pts	(28.5)	(10.0)	(9.0)	(13.0)	(9.0)	(22/223)
	27.0–30.0	8.0–10.0	8.0–10.0	11.5–14.0	8.0–10.0	
P-value	<0.001	0.068	<0.001	<0.001	<0.001	//

(Kruskal Wallis test with Bonferroni correction for multiple comparisons). EF, erectile function; OF, orgasmic function; SD, sexual desire; IS, intercourse satisfaction; GS, general satisfaction.
[a] $p < 0.001$ vs. Controls
[b] $p < 0.01$ vs. Controls
[c] $p < 0.05$ vs. Controls
[d] χ^2 $p < 0.001$ vs. Controls.

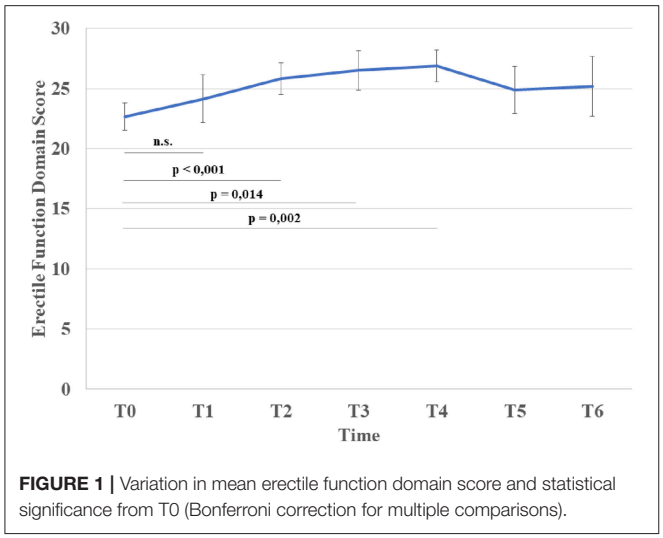

FIGURE 1 | Variation in mean erectile function domain score and statistical significance from T0 (Bonferroni correction for multiple comparisons).

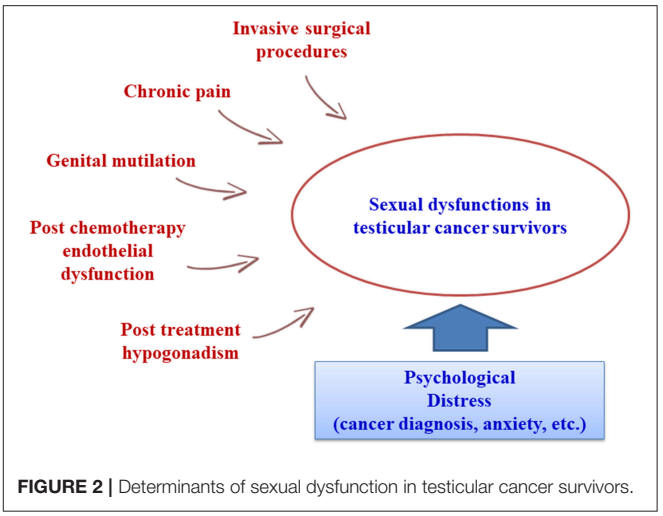

FIGURE 2 | Determinants of sexual dysfunction in testicular cancer survivors.

TABLE 4 | FSH, LH and total testosterone of testicular cancer and Control group: continuous data are presented as mean ± SD, median (in brackets) and 25–75th percentile of data distribution, while categorical data as percentage and counts.

	FSH (mUI/ml)	LH (mUI/ml)	Total testosterone (nmol/l)	Biochemical hypogonadism (%)
T0	7.6 ± 6.3[a]	4.6 ± 4.1[a]	17.9 ± 6.1[a]	4.1%
194 pts	(6.1)	(4.0)	(17.0)	(8/194)
	4.0–9.8	2.7–5.6	13.3–20.9	
T1	14.3 ± 8.9[a]	6.6 ± 3.5[a]	19.2 ± 6.7	4.4%
68 pts	(12.3)	(5.6)	(19.0)	(3/68)
	7.3–19.7	3.7–8.2	15.0–21.6	
T2	13.0 ± 8.5[a]	6.8 ± 6.1[a]	17.9 ± 5.7	2.8%
71 pts	(10.0)	(5.3)	(17.9)	(2/71)
	7.1–18.5	3.8–7.4	13.5–20.5	
CTR	4.7 ± 4.5	3.7 ± 1.8	20.2 ± 7.1	0.0%
223 pts	(3.5)	(3.3)	(19.4)	(0/223)
	2.3–5.4	2.4–4.6	15.1–24.4	
P-value	<0.001	<0.001	0.002	//

(Kruskal Wallis test with Bonferroni correction for multiple comparisons).
[a]p < 0.001 vs. CTR.

testosterone in comparison with healthy controls, albeit still within the normal range (22). Some authors have shown that gonadotropin alterations persist after chemotherapy, while about 10% of patients may suffer from low total testosterone levels after treatment or have a higher risk of late onset hypogonadism (20, 23–26). However, other authors found that chemotherapy had only mild effects on hormone levels (27, 28). In contrast, radiotherapy may affect testosterone levels for up to 5 years in TC patients who received testicular irradiation for contralateral carcinoma *in situ* (29), but direct testicular irradiation is not a standard treatment for TC patients and current radiotherapy protocols probably have only a minor effect on testicular function (20). In any case, whether or not altered gonadotropin and testosterone levels are the only determinant of sexual dysfunctions in TC patients is still under debate. The already cited study by Huddart et al. found that about 10% of post-therapy TC patients had biochemical hypogonadism, with worse sexual function than observed in non-hypogonadal TC survivors (20). However, a later study by Lackner et al. (30) found a higher percentage of post-treatment hypogonadism (26%). These authors could not identify an unambiguous threshold level for testosterone associated with the onset of sexological symptoms, and hypothesized that each patient might have an individual threshold (30). In 2009, Eberard et al. published a caseload of 129 TC survivors 3–5 years post-therapy compared to an age-matched group of men without cancer, observing that TC survivors had a higher likelihood of low sexual desire (OR 6.7) and erectile dysfunction (OR 3.8) compared to controls, but that these conditions could not be predicted from the presence of hypogonadism (31). However, these results are limited by the lack of the pre-treatment status of the TC patients. Subsequent studies also failed to find a clear association between sexual dysfunctions and biochemical hypogonadism (28, 32–34). In conclusion, most of the literature evidence suggests that the high prevalence of sexual dysfunctions cannot be justified by the relatively low prevalence of biochemically detected hypogonadism. Another hypothesis could link sexual dysfunctions to specific treatment modalities. The side effects of several chemotherapy drugs include endothelial damage, angiopathy, and peripheral neuropathy, which may be linked to erectile and ejaculatory disorders (35, 36). Radiotherapy can cause sexual dysfunctions by inducing damage to the cavernous nerve and/or progressive fibrosis of the cavernous tissue and endothelial damage, which can become clinically evident through the onset of erectile dysfunction even several years post-treatment (37). However, the literature data are inconsistent, as several studies report no significant associations between sexual dysfunctions and specific treatment modalities (31), while others report the significant influence of either chemo- or radiotherapy (14, 15, 32, 38). The reason for this variability could be the use of different combinations of these therapies with different surgical procedures (tailored to the patient in relation to various clinical parameters such as stage, etc.), while individual variability might also induce different outcomes. Kim et al. reported

that surgery combined with chemotherapy produced a higher incidence of reduced libido and ejaculatory disorders, while surgery combined with radiotherapy was followed by a greater incidence of erectile dysfunction (32). More recently, Bandak et al. observed that each treatment modality carried an increased risk of erectile and orgasmic dysfunctions, with multimodal treatment associated with the highest risk (15). Invasive surgical procedures such as retroperitoneal lymph node dissection are known to have a strong impact on sexual function (especially ejaculatory and orgasm disorders and impaired satisfaction) (12). Several studies have confirmed a worse sexological outcome after retroperitoneal surgery (lymph node dissection and/or re-surgery for relapse after chemotherapy) as a consequence of ejaculatory nerve damage during the procedure (10, 12, 21, 39, 40). Another issue is the trend of sexual dysfunction over time in TC survivors, as most literature studies are cross-sectional and only a few longitudinal studies are available. We currently expect a higher incidence of SD soon after the orchiectomy and the end of cancer treatment. Tuinman et al. found low IIEF scores post-orchiectomy and 3 months post-treatment, with significant improvements after 1 year of follow-up (41). These results were comparable to those of other studies focusing on SD within the first year after treatment (5, 42, 43). Long-term pre- and post-therapy comparisons are rare. Aass et al. found that sexual problems persisted in about 30% of TC survivors 36 months post-treatment (39), while Böhlen et al. reported no significant pre- vs. post-therapy differences in sexual function after at least 32 months of follow-up (44). Despite the general agreement on the presence of sexual dysfunctions in TC survivors, the absence of longitudinal long-term follow-up and the lack of standardization in the measurement of sexual function limit the assessment of their true patient burden. Data comparison and generalization are difficult, as different papers use a variety of tools and methods to evaluate sexual function. Moreover, in common with most sexological questionnaires, IIEF is poor at discriminating to what extent sexual dysfunctions are secondary to the organic sequalae of cancer treatments (45). In accordance with most of the available literature data, our results clearly show that TC patients undergoing orchiectomy and chemotherapy suffered from a higher degree of sexual dysfunctions than a control population of cancer-free subjects. These mainly presented as erectile dysfunctions, but also as impaired sexual desire and satisfaction. The incidence of orgasmic dysfunction did not differ significantly from controls. It is worth noting that the presence of these sexual dysfunctions at the baseline suggests that they might be induced by orchiectomy. However, it is difficult to find a biological relationship. We found a lower incidence (about 4%) of biochemical hypogonadism at the baseline (total testosterone $<$8.0 nmol/l) than in other reports, but like them, we found no significant correlation with sexual function domains (20, 30, 31). This suggests that sexual dysfunctions are not explained by abnormal hormone levels consequent to orchiectomy and might instead be more closely associated with a psychological burden in these patients, which might coexist and be synergistic with treatment-induced hypogonadism. As the testicles are associated with masculinity, orchiectomy might well induce changes in body perception. In a caseload of 407 TC patients, Rossen et al. observed that about 17% had a reduced perception of masculinity induced by orchiectomy. This was associated with a 9-fold increased risk of erectile dysfunction and a 15-fold increased risk of sexual discomfort (10). Wortel et al. reported that after orchiectomy, up to 50% of patients might complain of a distorted perception of body image (42). All of our patients underwent the insertion of a testicular prosthesis. This may have positively influenced their body perception, as suggested in a study by Catanzariti et al. (43), but whether and to what extent this might have contributed to the improved IIEF-15 scores in our study is unknown, and should be investigated in further studies. We also detected a significant improvement in erectile function post-therapy. Although some degree of erectile dysfunction persisted at all time points, the incidence constantly dropped in the first 2 years after treatment (up to T4) and the IIEF-15 erectile dysfunction scores improved and were comparable to the controls 1 year after treatment (T2). There was also a trend of improvement in sexual desire and in both intercourse and general satisfaction, but follow-up showed that they remained significantly worse than in the controls. To our knowledge, the present study is the longest monocentric follow-up currently available for the sexological evaluation of TC patients. Unfortunately, the generalizability of data comparisons against healthy controls might be reduced for long term follow-up, as the increased percentage of patients with erectile dysfunction at T5 and T6 may simply be due to their increased age and the consequent possible onset of other factors (hypertension and other cardiovascular diseases, use of medications, etc.) increasing the incidence of sexual dysfunctions, independently of TC and its treatments.

In conclusion, our data, from a large caseload compared to a control group of similar age and strengthened by the use of a validated psychometric tool, indicate that TC patients need adequate sexological counseling following diagnosis/orchiectomy and prior to chemotherapy. Discussing these aspects with patients could help them to cope with the disease and to understand that their erectile function should improve within a year after the end of treatment. Future studies should identify subjects who are more likely to suffer from SDs, thus permitting the better follow-up of these patients and enabling them to be offered all the support they need to maintain a satisfactory sex life and, consequently, a good general quality of life.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available. If required, the data are available in our database upon request. Requests to access the datasets should be directed to donatella.paoli@uniroma1.it.

AUTHOR CONTRIBUTIONS

FL, FP and DP conceived the study; FP and FL wrote the article; FP, AP and FC acquired and analyzed the data; FL,

FP and DP contributed to data interpretation; AR and AL manuscript revision.

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REVIEW
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Severe Complications in Testicular Germ Cell Tumors: The Choriocarcinoma Syndrome

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Testicular germ cell tumors (TGCTs) represent the most common solid tumor in young men and is a model of curable cancer. The effectiveness of cisplatin-based chemotherapy secures more than 95% of patients' 5-years survival rate. However, some high-risk patients with a very advanced disease develop choriocarcinoma syndrome (CS) connected with acute respiratory failure with poor prognosis and high mortality rate shortly after beginning systemic chemotherapy. CS was first described as a syndrome with hemorrhage from metastatic sites in patients with TGCTs with significantly high choriogonadotropin level. Acute hemorrhage to lung metastases is typical, but hemorrhage can occur from any metastatic site. Patognomic of choriocarcinoma cells is an invasion of small blood vessels within CS. The incidence of CS in patients with TGCTs are not well-defined and can vary across the world. To date, there are a few case reports and small retrospective series reporting a connection between systemic chemotherapy and the development of CS in metastatic TGCTs. CS is known to be triggered by massive tumor cell lysis as a result of chemotherapy and cytokine release, aggravated with alveolar hemorrhage. This can lead to a consecutive superinfection, furthered with neutropenia after chemotherapy, acute respiratory distress syndrome, rising to systemic inflammatory response, resulting in multiorgan failure and death. A reasonably effective approach in patients with extensive disease could be a shortened course of chemotherapy as well as a reduction of dosage in induction chemotherapy before full-dose chemotherapeutical regimen; however, current data regarding optimal treatment approach are limited. Patients' referral to tertiary centers and the administration of induction chemotherapy in an intensive care unit setting could further improve the treatment outcome.

Keywords: choriocarcinoma syndrome, testicular germ cell tumor, choriogonadotropin, acute respiratory failure, lung metastases

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INTRODUCTION

Despite the rarity of testicular germ cell tumors, with a count of just 1% from all male malignancies, these tumors represent the most common type of solid tumor in reproductive men between the ages of 20 and 40 years, with an incidence of up to 10 in 100,000 men (1). Due to a unique chemosensitivity to chemotherapy, they represent one of the most curative malignancies overall.

More than 95% of patients achieve a 5-years survival rate because of the effectiveness of combined cisplatin-based chemotherapy (2, 3). Germ cell tumors can be classified as either seminomatous or non-seminomatous histologic types, whereby choriocarcinoma is the most aggressive subtype of non-seminomas due to their biologic characteristics (4, 5). International Germ Cell Cancer Collaborative Group (IGCCCG) developed a clinically based prognostic classification for germ cell tumors, which has been used since 1997 (6). The IGCCCG classification stratifies patients into good-, intermediate-, and poor-prognosis subgroups on the basis of three criteria: the primary tumor site, the levels of serum tumor markers, and whether extra-pulmonary visceral metastases are present. The majority of the patients with metastatic disease are allocated in the good-risk group, representing 56% of patients with a non-seminomatous germ cell tumor (NSGCT) with a high curability rate >90%, while patients in the intermediate risk (representing 28% of patients with NSCGTs) and poor prognosis groups (representing 16% of patients with NSCGTs) have 5-years survival rates of only 80 and 48%, respectively. The poor-risk NSGCT is defined by the presence of a mediastinal primary tumor, non-pulmonary visceral metastases, or any of the following serum tumor marker elevations: alpha-fetoprotein (AFP) >10,000 ng/mL, human choriogonadotropin (hCG) >50,000 IU/L and/or lactate dehydrogenase (LDH) >10 times the upper limit of normal rely on IGCCCG classification.

The current understanding is that there exists a subgroup of patients with poor-risk NSGCTs, the so-called super-high-risk patients. They are characterized by widespread lung metastases, pure choriocarcinoma, and a high choriogonadotropin level as defined by the European Germ Cell Cancer Collaborative Group EGCCCG (**Figure 1**) (7, 8). These patients are at a high risk to develop the so-called choriocarcinoma syndrome, which was described for the first time in 1984 by Logothetis et al., as a syndrome with hemorrhage from metastatic sites in patients with advanced germ cell tumors with high-volume of choriocarcinoma elements, especially those with a choriogonadotropin level over 50,000 IU/l (9). Acute hemorrhage to lung metastases is typical, but hemorrhage from any metastatic sites can occur. The patognomic characteristic of choriocarcinoma cells is their invasion to small blood vessels. Typically, choriocarcinoma syndrome occurs shortly after the administration of chemotherapy and is connected with a high risk of fatal bleeding from metastatic lesions and frequently with acute respiratory failure with a high mortality rate at an early phase of the treatment induction (10). Mostly, the hemorrhage appears shortly after the introduction of the chemotherapy, but there are also cases with pretreatment onsets reported in literature (11). The choriocarcinoma syndrome was not only described in pure choriocarcinomas; cases with embryonal tumors or seminomas have also been recorded (12, 13).

The incidence of acute respiratory failure in patients with testicular germ cell tumors (TGCTs) with massive lung involvement and choriocarcinoma syndrome is not well-defined (14). The available literature is missing data on the total percentage of the super-high-risk patient population from the poor-risk group of GCTs, e.g., Moran- Ribon et al. counted 20% from the total of the poor-risk group, but the authors also pointed out the careful interpretation due to selection bias, which could be introduced by the presentation of these patients to a referral center (10).

Until now, there are still controversies on the definition of super-high-risk patients or so-called choriocarcinoma syndrome, as well as the optimal therapeutical approach for this group of patients. Available retrospective data are based on different patients' characteristics, different high-risk features of this patient's group, as well as different primary goals or the study end-points, including different conclusions. Therefore, it is also difficult to determine the incidence of choriocarcinoma syndrome, or ARDS connected with this so-called choriocarcinoma syndrome or mortality rate.

The aim of this article is to describe severe complications in TGCTs related to the choriocarcinoma syndrome, a patognomic unit which deserves a deeper understanding of its etiopathogenesis, clinical manifestation, and the selection of high-risk patients corresponding to better (optimal) therapeutical approach for them, in order to decrease mortality in the early phases of induction chemotherapy without compromising their survival.

PATHOPHYSIOLOGY

The exact pathogenesis of choriocarcinoma syndrome is currently unknown. Choriocarcinoma is the rarest subtype of germ cell malignancy with pure examples representing under 1% of the total number of NSGCT, while 7–8% of testicular tumors contain a choriocarcinoma component (15). Choriocarcinoma is composed of cytotrophoblast, intermediate trophoblast and syncytiotrophoblast cells. Cytotrophoblasts represent a trophoblastic stem cell, whereas the syncytiotrophoblast represents a more terminally differentiated cell. The typical pattern is a plexiform arrangement of syncytiotrophoblast cells with mononucleated, mostly cytotrophoblast cells around foci of hemorrhage, although some examples may have a relatively inconspicuous syncytiotrophoblast component. In developmental embryology, these cell types secrete hCG to promote the maintenance of corpus luteum and they are essential for the implantation and subsequent placentar development (16). Pure or choriocarcinoma-predominant tumors are characterized by high levels of hCG, which may be associated with a different clinical signs and symptoms (15). Choriocarcinoma is characterized by rapid proliferation, invasiveness, vascularity, and a tendency to outgrow its blood supply with subsequent necrosis of tumor (14). Metastases to regional lymph nodes, as well as hematogenous spread to lung, liver and brain, occur at an early stage (5). A propensity for hemorrhage is well-recognized in patients with gestational trophoblastic disease, the female counterpart of testicular choriocarcinoma (17). Although choriocarcinoma in the male is a less common entity, a similar tendency for hemorrhage exists. Hemorrhage has been implicated as the cause of death in 44% of patients with testicular choriocarcinoma at autopsy (4). These processes reflect the biological behavior of choriocarcinoma cells, which directly

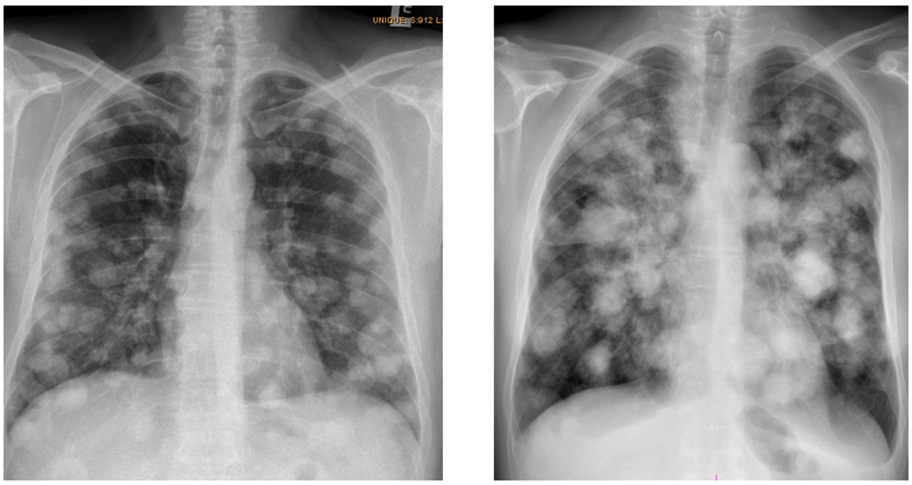

FIGURE 1 | Typical chest X-rays of patients with choriocarcinoma syndrome (patient with multiple lung metastases, histologically proved choriocarcinoma, hCG level 1,600,0000 IU/L, who developed shortly after the administration of the 2-days of EP chemotherapy-choriocarcinoma syndrome), archive of National Cancer Institute, 2017.

invade, erode and destroy blood vessels. It is also believed that some products of tumor cells elaborate on the damage of blood vessels without their direct invasion (12). The basic mechanism of choriocarcinoma syndrome is probably massive tumor lysis, resulting from chemotherapy. Subsequent cytokine release, aggravated with alveolar hemorrhage, can lead to acute respiratory failure (ARDS) and death (18). The pathogenesis of ARDS in patients with TGCTs is probably multifactorial and involves massive lung metastases, massive intra-alveolar tumor-lysis, early necrosis of tumor cells, and consecutive superinfection, which can be furthered with neutropenia after chemotherapy. The pathogenesis of intra-alveolar tumor-lysis is probably the same as in the case of tumor-lysis syndrome (TLS), which is characterized by the release of potassium, phosphorus, nucleic acids, and their metabolites, such as ureic acid and cytokines (19). While the occurrence of TLS with hematological malignancies and some aggressive solid tumors is consistently described, such events have not exactly been reported in poor-risk germ cell tumors. Case reports in TGCTs described as an early death due to TLS, could be related to pulmonary distress with multifactorial pathogenesis in super-high-risk patients without metabolic signs of TLS in this group of patients (20). In case of choriocarcinoma syndrome; releasing of the cytokines is probably raising to systemic inflammatory response directing to multiorgan failure (21).

Another factor that could play the additive role in the pathogenesis of choriocarcinoma syndrome is paraneoplastic hyperthyroidism induced by a very high level of choriogonadotropin. In patients with hCG over 50,000 U/l, the incidence of hyperthyroidism could be present in more than 50% of patients (22). hCG is dimeric molecule composed of two subunits, an alpha subunit which is identical with an alpha subunit of the thyroid-stimulating hormone (TSH) and hormone-specific beta subunit. Due to the structural similarity of the alpha subunits TSH and hCG, the stimulation of the thyroid-stimulating hormone- receptor (TSH-r) by hCG can lead to thyrotoxicosis and worsen the patient's condition, as well as the course of acute respiratory failure (23).

TREATMENT OUTCOME

Treatment Approaches Without a Reduced-Dose Intensity During Induction Chemotherapy

There is no effective preventive precaution against the choriocarcinoma syndrome. To date, there are just few case reports and small retrospective series recording the connection between systemic chemotherapy and the development of the choriocarcinoma syndrome and ARDS in metastatic TGCTs (10, 11, 13, 18, 21, 24).

The first retrospective study by Moran-Ribon et al., published in 1994, involved 11 super-high-risk patients with TGCTs treated between 1982 and 1989 at the Gustave Roussy Institute (10). All these patients developed poor-risk TGCTs after 35 days of chemotherapy administration, and experienced acute respiratory failure with the need of mechanical ventilation (MV). All of them died within the first 5 weeks of chemotherapy, despite their admission to the Intensive Care Unit (ICU) before the beginning of therapy. Eight of them had extensive pulmonary metastases, three patients had hilar lymphadenopathy, and three others had bulky mediastinal adenopathy with atelectasis and pleural effusion. Six patients had hCG over 100,000 IU/L, and in 9 patients there was a presence of hypoxemia PaO_2 <60 mm Hg (8 kPa) at the time of their admission to ICU. Different cisplatin-based chemotherapeutical protocols were used.

During the period of observation all patients developed fever, and septicemia was documented in 7 of them, with the empiric antibiotic coverage in all of them. In 5 patients, the antibiotics

were not active and in 2 patients just one demonstrated some activity, when compared the results of antibiograms of the isolated microorganism, retrospectively. None of the patients had a tumor lysis syndrome. In these patients, there was an increase of alveolar abnormalities and the objective response on the lungs was a minor response in four, a stable disease in four, and progression in three patients. At the time of the patients' death, the lungs became "uniformly white" on chest X-rays. Four (36%) patients had an autopsy, with the results indicating the presence of necrotic and fibrotic tissue but with the viable residual tumor in all of them. Authors hypothesized multifactorial etiology of ARDS in this super-high-risk group of patients and the clinical presentation was compatible to the choriocarcinoma syndrome reported by Logothetis (9).

In 2003, Kirch et al. published a retrospective study of 16 poor-risk patients with TGCTs, who were referred to the ICU for treatment due to respiratory distress and a high risk of complications after the administration of chemotherapy during the 10-years period of time (18). They were identified using a computerized database, based on the heavy tumor burden, disseminated lung metastases, and elevated tumor marker levels. All patients had pulmonary metastases, nine (56%) patients also had extrapulmonary involvement, with a median level of hCG 81,700 IU/l. Most patients were hypoxemic, and all of them had dyspnea at the time of the admission to the ICU. Treatment regimens consisted mainly of either etoposide-cisplatin, or adriamycin, cyclophosphamide and cisplatin, or adriamycin, cyclophosphamide and vincristine. Only 1 patient was treated with the regimen containing bleomycin. Nine patients developed ARDS, requiring mechanical ventilation within 3 days after the initiation of the chemotherapy, while seven patients improved immediately after the administration of induction chemotherapy. On admission to the ICU, the median PaO_2 under the room air was 46 mm Hg (range 44–63 mmHg) for the patients who required intubation, compared to a median PaO_2 of 80 mmHg (range 70–109 mmHg) for those who remained stable after the chemotherapy. Six patients also experienced tracheal/bronchial hemorrhage after the course of chemotherapy. Two patients were afflicted with the biological tumor lysis syndrome, as the study reported. Seven ventilated patients were febrile during the observation, while fever was microbiologically documented in five of them. Seven patients experienced chemotherapy-induced aplasia, with a median duration of neutropenia of 10 days. Three (19%) patients developed nosocomial pneumonia and in two of them it was the leading cause of death. Six (86%) patients who did not require MV were discharged alive. Only one patient out of nine requiring MV remained alive, while seven patients died under mechanical ventilation and the last one was weaned off MV but died due to the sepsis arising from a superinfected necrotic tumor on day 41 after chemotherapy administration. Five patients received an autopsy, which indicated that ARDS was the first cause of death in three of them, and pulmonary aspergillosis in one, whereas a massive tumor invasion with hemothorax due to metastases rupture was observed in another autopsied patient. In the study, the development of ARDS was independent of the level of hCG as well as with the applied chemotherapeutical regimen. The only one predictor of the intubation was the initial PaO_2/FiO_2 ratio at the time of the admission to the ICU. Refractory hypoxemia and ventilator-associated pneumonia were the leading cause of death. Bleomycin, which toxicity can be aggravated with high oxygen concentration, could not be responsible for the worsening of the respiratory functions because only one patient was actually treated with it.

Treatment Approaches Utilizing Alternative Regimen During Induction Chemotherapy

Based on this knowledge, new alternative approaches have been applied for this group of patients due to the risk of evolution of the choriocarcinoma syndrome or acute respiratory failure, with the aim to improve their prognosis. The effective approach appeared to be either reduced or shortened course of the induction chemotherapy before the administration of the full-dose chemotherapeutical regimen. Three retrospective studies tested the alternative regimens during induction chemotherapy to decrease the mortality rate due to acute complications connected with administered chemotherapy (24–26)

Massard et al. retrospectively evaluated all patients treated for poor-risk TGCTs and multiple lung metastases at Institut Gustave Roussy from April 1982 to November 2006. A total of 25 patients with extensive lung metastases, dyspnea, at the time of the admission to the specialized intensive care unit or hypoxia (defined as a partial pressure of oxygen PO_2 <80 mmHg), or both criteria, were selected. Until 1997, a cohort of 15 patients was treated with full-dose cisplatin-based chemotherapy. Since 1997, a second cohort of 10 patients were treated with the reduced regimen of induction chemotherapy. The regimen consisted of 3 days of EP without bleomycin, and the remaining 2 days of chemotherapy were postponed to approximately day 15. After induction chemotherapy, the full-dose regimen with a standard dose of BEP on day 21 was started. This approach lowered the incidence of ARDS after 1997 by 57% (87 vs. 30%), as well as the mortality rate of ARDS by 40% (60 vs. 20%). Long-term survival increased from 27 to 40% (24).

Gillessen et al. retrospectively evaluated 20 patients with metastatic TGCTs (18 patients with poor prognosis), treated in St Bartholomew's Hospital, between 1998 and 2009 (25). Patients were treated with the low-dose induction chemotherapy baby-BOP (bBOP) on day 1 only, with subsequent chemotherapy BEP introduced 7–10 days after the bBOP regimen (25). bBOP was administered to 9 patients because of poor performance status ($\geq$3), due to an extensive retroperitoneal disease in 6 patients, brought about by pulmonary embolism at diagnosis in two patients and hydronephrosis and impaired renal function in four patients. Two patients were presented with the vena cava superior obstruction, and two patients had imminent or present respiratory failure and serious gastrointestinal bleeding due to stomach metastasis. bBOP was well-tolerated, without any toxic deaths, or neutropenic sepsis. In one patient with a massive pulmonary disease, tumor-related death occurred during the first cycle, due to cystic transformation growth, resulting in pulmonary insufficiency and death, while the level of tumor markers decreased. Response to bBOP was not inferior to the

standard first-line BEP regimen in this group of poor-risk patients. The 2-year PFS and OS rates for 18 poor-risk patients with induction bBOP were 72 and 79%, respectively, which were not significantly different compared to poor-risk patients treated without induction bBOP in their institution, with 2-year PFS and OS rates at the level of 75 and 80%, respectively (25).

In the study published by Tryakin et al. in 2018, authors retrospectively assessed 63 (24%) out of 265 patients with poor risk metastatic GCTs, all with ultra-high tumor marker levels and/or an ECOG performance status of 3–4 (26). Before 2005, these patients were treated with full dose BEP; after 2005, the patients were treated with abbreviated EP as the induction chemotherapy, followed by subsequent full-dose chemotherapy. Of these 63 patients, 50 (79%) had hCG ≥200,000 IU/L, 50 (79%) had lung metastases, and 50 (79%) had liver metastases, respectively. Respiratory insufficiency was observed in 28 of the patients; 22 patients had multiple pulmonary metastases with a high level of hCG, two patients presented with pulmonary embolism, while four had a massive mediastinal tumor at the time of the diagnosis. Hemoptysis was present in 26 patients with multiple lung metastases and 20 complained of grade ≥3 pain. Forty-five (71%) out of 63 patients were treated will full doses of cisplatin- and etoposide-based chemotherapy during the first cycle, while 18 (29%) received a reduced cycle of EP as induction chemotherapy. Patients who received the first cycle of reduced chemotherapy EP developed fewer acute life-threatening toxicities, compared to patients treated with the full-dose first cycle of chemotherapy (44 vs. 76%, $P = 0.01$). The rates of severe hematological toxicities for the reduced- compared to full-dose chemotherapy group (grade 4, 56% vs. 96%, $p = 0.0004$) and infectious complications (grade 3–4, 28 vs. 53%, $p = 0.09$) were lower. The 5-years OS rates was 52%, equal in both groups (HR 0.99, 95% CI 0.44–2.26, $p = 0.99$). However, OS in the group of patients with ultra-high tumor marker levels ($n = 63$), compared with the group of other patients with poor-risk ($n = 202$), did not differ significantly (HR 0.89, 95% CI 0.58–1.36, $P = 0.59$ (25, 26).

To date, there is no exact feature associated with the higher risk of the complications after the administration of the chemotherapy within this group of patients. It seems to be reasonable that massive lung involvement is the main risk factor in the pathogenesis of ARDS. But it is also possible that chemotherapy can be associated with the worsening of the respiratory functions, as the studies by Moran Ribon et al. and Kirch et al. concluded (10, 18). On the other hand, the evolution of ARDS in the Kirch et al. study was independent of the known prognostic factors as a histologic type of the tumor, stage of the disease, tumor marker levels, or given chemotherapeutical regimen (18). The only predictor of the need of MV was the initial PaO_2/FiO_2 ratio before chemotherapy administration. At the same time, tumor-associated ARDS can be explained as a consequence of administered chemotherapy, because the peak of tumor markers was observed 2 days after chemotherapy administration (18). Based on this knowledge, a reasonably effective approach can be a reduced respectively, shortened course of induction chemotherapy before the administration of consecutive full-dose chemotherapy as the eventual prevention of ARDS development in these patients. All three retrospective studies with modified induction chemotherapy showed non-inferior treatment results compared to full-dose induction chemotherapy (24–26). These studies suggest that reduced induction chemotherapy can be helpful to decrease the incidence of ARDS in this group of patients. However, the significant improvement of the reduction on the mortality of ARDS could probably be multifactorial and can be connected to improved supportive care and the preventive administration of the granulocyte-colony stimulating factor (G-CSF). The acute tumor and /or alveolar hemorrhage was present in more than 30% of the patients in all mentioned studies, which could eventually aggravate respiratory failure. Secondary infection in neutropenic patients, as well as interstitial lung fibrosis, can be the leading cause of consecutive ARDS and may be an associated cause of death.

In the Moran Ribon et al. and Kirsch et al. studies, neutropenia occurred in almost half of the patients; septicemia was also observed and described (10, 18). At the same, we need to realize that ventilator-associated pneumonia is hard to diagnose. Lung parenchyma is characterized by local immunosuppression because of necrotic tumor masses, which allow bacterial colonization and consequent infection. An early diagnosis of ventilator-associated pneumonia and consequent adequate antibiotic therapy is crucial in this situation. Bronchoscopy with bronchio-alveolar washing and bronchial brushing may allow the isolation of adequate bacteriological samples, as well as the removal of blood clots. Immunosuppression after chemotherapy can be an independent risk factor associated with a higher mortality rate of these super-high-risk patients with relative risk at 2.3% (27).

We need to realize that all mentioned studies had their limitations due to retrospective character, and different time frames of treatment with full-dose and reduced-dose chemotherapy, which could account for selection bias and at the same time, could also participate in better treatment results with reduced-dose chemotherapy. The biggest deficiency of all discussed studies was a low number and high heterogeneity of the patients (**Table 1**).

In the review by Reilley, the author brought a comprehensive view over molecular pathology, clinical features of choriocarcinoma, as well as diagnostic and therapeutic challenges in this unique but aggressive germ cell malignancy due to complications arising from an underlying disease and its treatment. He pointed out the importance of treatment individualization based on the extent of the disease, e.g., for the patient with high-volume lung metastases, etoposide and cisplatin without bleomycin as induction regimen, should be administered to avoid fatal respiratory failure (28).

CONCLUSION AND FUTURE DIRECTION

Choriocarcinoma syndrome is a rare but life-threatening condition in "super high-risk" patients with testicular germ cell tumors that are characterized with widespread lung metastases, choriocarcinoma histology, and a high choriogonadotropin level. Such patients are often ineligible for prospective trials. Their

TABLE 1 | Summary of studies of super high-risk patients.

References	Number of patients	Level of hCG IU/L	Pulmonary mts	Other mts	Initial pO2 mmHg	Full dose/reduced dose chemotherapy	Chemotherapeutical regimen	Infection complications no pts.	ARDS/ death from ARDS	Hemorhage no pts.	Death due to ARDS/survival
Moran-Ribon et al. (10)	11	(9,114–1,080,000) median >100,000	8 (73%)	Mediastinal LAP, hilar LAP, kidney, liver, pancreas, adrenal gland	<60 mmHg 9(82%) >60 mmHg 2 (18%)	11 (100%)–full dose	Different cisplatin-based regimens	7 (64%) 11 (100%) febrile	11(100%)	NR	All died
Kirch et al. (18)	16	(33–413,000) median 81,700	9 (56%)	Liver, bones, brain	<70 mmHg 16(100%) <46 mmHg 9(56%)	16 (100%)–full dose	EP, adriamycin, cyclophosphamide, or adriamycin, cyclophosphamide, vincristine	5 (31%) 7 (44%) febrile	9(56%)	6(38%)	8/9 (89%) (on MV) died 1/7 (14%) (without MV) died
Massard et al. (24)	25	(11–8,920,000) median >200,000	25 (100%)	NR	<80 mmHg 2(8%)	15 (60%)–full dose 10 (40%)–reduced EP	Cisplatin -based regimens-full dose EP-reduced	NR	13 (87%_–full dose 3 (30%)–reduced dose	NR	9/15 (60%) died–full dose 2/10 (20%) died –reduced dose
Gillessen et al. (25)	20	(1–1,250,000) median 35,195	NS, 1 (5%)-massive	RP LAP, stomach, brain, bones	NR	20 (100%)–reduced-bBOP	bBOP, BEP	NR-no toxic death, no neutropenic sepsis after bBOP	None	2 (10%)	None died due to ARDS
Tryakin et al. (26)	63	(0–20,525,000) median >200,000	50 (79%)	Liver, mediastinal LAP, RP LAP, brain	NR	45 (71%)–full dose 18 (29%)–reduced dose	BEP, EP	Gr.3,4 24 (53%)-full dose vs. 5 (28%)	NR	NR	NR

NR, non-reported; RP LAP, retroperitoneal lymphadenopathy; BEP, bleomycin, etoposide, cisplatin; EP, etoposide, cisplatin; ARDS, acute respiratory distress syndrome; LAP, lymphadenopathy; bBOP, baby BOP-bleomycin, vincristine, cisplatin; MV, mechanical ventilation.

eventual inclusion in reports as early deaths is recommended when interpreting the results of clinical trials in patients with poor-risk TGCTs. The optimal therapeutic approach for this group of patients remains to be defined. Current evidence suggests that the best treatment results in these "superhigh-risk" patients are obtained in high-volume reference centers (1). Because of the rarity and complexity of this super-high-risk group of patients, there is imperative need for their early referral to specialized centers (if possible within 24 h) to optimize their chances of survival, as indicated by retrospective data and by the recommendations of TGCT guidelines (7, 8). According to EGCCCG, patients with widespread lung metastases, pure choriocarcinoma, and high hCG should be treated by 2–3 days of full-dose cisplatin and etoposide with the continuation of chemotherapy after the patient's recovery (7, 8). Bleomycin should be avoided during induction chemotherapy since it may induce pulmonary fibrosis. However, it should be re-introduced once the patient's condition allows that, because its complete omission was demonstrated to be detrimental in a prospective randomized trial (7, 8).

Prospective translational trials that will lead to a better understanding of the pathophysiology of the choriocarcinoma syndrome and development of new biomarkers for better stratification of super-high-risk patients are warranted. Due to its low prevalence, international collaboration and clinical trials utilizing new treatment strategies are needed to decrease the mortality rate caused by this rare but highly fatal condition.

AUTHOR CONTRIBUTIONS

KR, MC, UD, and MM participated in the conception and design of this study. MM and KR drafted the article and all authors reviewed it critically for its important intellectual content.

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SYSTEMATIC REVIEW
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Hypogonadism and Sexual Dysfunction in Testicular Tumor Survivors: A Systematic Review

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Testicular tumor is the most common malignancy in men of reproductive age. According to the tumor histology and staging, current treatment options include orchiectomy alone or associated with adjuvant chemo- and/or radiotherapy. Although these treatments have considerably raised the percentage of survivors compared to the past, they have been identified as risk factors for testosterone deficiency and sexual dysfunction in this subgroup of men. Male hypogonadism, in turn, predisposes to the development of metabolic and cardiovascular impairment that negatively affects general health. Accordingly, longitudinal studies report a long-term risk for cardiovascular diseases after radiotherapy and/or cisplatin-based chemotherapy in testicular tumor survivors. The aim of this review was to summarize the current evidence on hypogonadism and sexual dysfunction in long-term cancer survivors, including the epidemiology of cardiovascular and metabolic disorders, to increase the awareness that serum testosterone levels, sexual function, and general health should be evaluated during the endocrinological management of these patients.

Keywords: hypogonadism, testicular tumor, testosterone, sexual dysfunction, cardiovascular risk

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INTRODUCTION

The testicular tumor is the most common solid malignancy in young adult men (aged 14–44 years) in Western countries and represents ∼1.5% of all tumor diagnosis worldwide (1). Its incidence has risen over the last decades, especially in industrialized countries (2). Testicular tumor affects from <1 per 100,000 males in many African and Asian nations to >9 per 100,000 men in the highest-incidence areas of Northern and Western Europe. Despite the highest incidence in more developed countries and particularly in Europe, the incidence-to-mortality ratio is 26:1 in northern Europe compared with 2:1 in Southeast Asia, South–Central Asia, and Africa. This indicates the need to improve the treatment strategy in some non-European countries (3).

Over the years, a multitude of classifications have been proposed for testicular tumor, reflecting a progressive better understanding of its pathogenesis. Despite the testis being a relatively small organ, it consists of many different cell types; thus, it may give rise to a large variety of neoplasms (**Table 1**). Nonetheless, more than 95% of testicular tumors are testicular germ-cell tumors (TGCTs) derived from germ cells (4). Sex cord stromal tumors and other non-germ-cell tumors are exceedingly rare. The most recent WHO classification, which has been published in 2016, represents a transition from an exclusively morphological system into one that takes into account

the histological composition, the age of onset, and the pathogenic mechanisms of testicular tumor development (5). This new classification recognizes two major types of TGCTs: those derived from germ-cell neoplasia *in situ* (GCNIS) and those unrelated to GCNIS (5).

Management of testicular tumor is controversial. After orchiectomy, subsequent management options include active surveillance, adjuvant chemotherapy or radiotherapy, and primary retroperitoneal lymphadenectomy (RPLND) (6). Treatment-related toxicity is crucial considering that the long-term survival rate of TGCTs is ∼99%, regardless of treatment strategy (6). For this reason, the most recent guidelines focus on minimizing unnecessary treatments to avoid adverse effects that are associated with them and to customize treatment for each patient considering patient's individual risks and his individual wishes (7). Each patient should be informed about the potential advantages and disadvantages of surveillance and adjuvant therapy (7). While surveillance allows most patients to avoid additional treatment, adjuvant therapy significantly lowers the relapse rate (7). Over the years, enthusiasm for adjuvant radiotherapy has been markedly reduced by the risk of radiation-induced secondary cancers. An increasing evidence suggests that active surveillance post-orchiectomy is a suitable alternative to adjuvant regimens in both stage I seminomas and non-seminomas (6). In the treatment of advanced testicular tumor, the current standard of care includes the use of platinum-based chemotherapy [bleomycin, etoposide, and cisplatin (BEP)] (6). A clear dose relationship has been established for the following BEP sequelae: pulmonary toxicity, fertility (8), neurotoxicity, ototoxicity, nephrotoxicity, metabolic syndrome, and hypogonadism (9, 10).

Hypogonadism has been often reported in testicular tumor survivors. Indeed, testicular tumor may represent a feature of the so-called testicular dysgenesis syndrome (TDS) (11, 12). The possibility exists that TDS may somehow impair Leydig cell function. Accordingly, studies indicate that germ-cell malignancy itself may be associated with poorer gonadal function in the remaining testis prior to other treatments (13). Also, the occurrence of microlithiasis (a feature of TDS) in the remaining testis has been shown to predict the incoming of hypogonadism in testicular tumor survivors (14). In addition, because of the radio- and/or chemo-induced Leydig cell damage, adjuvant therapy rises the risk of hormonal deterioration that results in increasing serum luteinizing hormone (LH) levels and decreasing serum testosterone concentrations (15, 16).

Therefore, the aim of this review was to gather together the current evidence of hypogonadism and sexual dysfunction in long-term testicular tumor survivors, including the epidemiology of cardiovascular and metabolic disorders, to increase the awareness to evaluate serum testosterone, sexual function, and general health in testicular tumor survivors.

METHODS

We performed a comprehensive review of the literature aimed at evaluating the occurrence of hypogonadism and its related complications, including cardiovascular, metabolic and bone mineralization impairment, and sexual dysfunction in testicular tumor survivors. A systematic search was made through PubMed, MEDLINE, Cochrane, Google Scholar, and Scopus databases. Data were independently extracted by RC and FB. The search strategy was based on the following keywords: "testicular cancer," "testicular tumor," "testosterone," "hypogonadism," "cardiovascular," "diabetes," "bone," "osteoporosis," "erectile dysfunction," "premature ejaculation," and "sexual dysfunction." Additional manual searches were made using the reference lists of relevant studies.

No language restriction was used for any literature search. Information on the year of publication, country, continent, study design, and mean age of patients was collected. Studies that met the following inclusion criteria were included in the qualitative synthesis:

- Full-length articles (including longitudinal, retrospective, cross-sectional, case–control studies, review, and meta-analysis) published between 1990 and 2019;
- Studies carried out on patients with testicular tumor of any histological type and stage, whose treatment (surgery, radiotherapy, and/or chemotherapy) was clearly reported;
- Studies having at least one among gonadotropins, total testosterone, cardiovascular health, metabolic profile, or bone mineralization as main outcome, collected at baseline and or at the follow-up counseling.

Studies that did not met the above-mentioned inclusion criteria were excluded.

HYPOGONADISM

Several longitudinal studies have been carried out to assess the Leydig cell function in testicular tumor survivors. The evidence suggests the vulnerability of Leydig cells to platinum-based chemotherapy and radiotherapy. Animal studies have shown Leydig cell apoptosis (as well as in Sertoli and germ cells) induced by cisplatin both on single administration and on a cumulative manner (17–19). In addition, patients receiving more than 20-Gy dose of radiation at the testicular level need testosterone replacement therapy after 15 years of follow-up, as for a half of patients receiving 16-Gr dose of radiation (20). Interestingly, infra-diaphragmatic radiotherapy when administered at the dose of 30 Gy, corresponding to 0.09–0.32 Gy testicular irradiation (21), is associated also with a slightly greater risk for developing testosterone deficiency, according to a study of meta-analysis (22). These findings suggest that Leydig cells are susceptible to minimal irradiation doses (22, 23).

A number of studies compared chemotherapy-, radiotherapy-, and orchiectomy-alone-dependent toxicity. The results are influenced by the length of follow-up, since those having a longer time of surveillance allow drafting of conclusions on the Leydig cell functional reserve. A summary of the risk of developing hypogonadism in testicular tumor survivors is reported in **Table 2**.

A prospective multicenter study on 1,235 testicular tumor survivors (mean age 44 years) investigated the risk for hypogonadism after a 11-year-long follow-up. While no difference in serum testosterone levels was found among patients

TABLE 1 | Classification of testicular tumors.

Testicular Tumors	Germ-Cell Tumor	GCNIS-derived	Type II	Seminoma	
				Non-seminoma	• Yolk sac tumor • Embryonal carcinoma • Teratoma, post-pubertal type • Choriocarcinoma
		Non GCNIS-derived	Type I	• *Yolk sac tumor, pre-pubertal type* • *Teratoma, pre-pubertal type*	
			Type III	• *Spermatocytic tumor*	
	Sex Cord/Stromal Tumor	Leydig cell tumor		• *Malignant Leydig cell tumor*	
		Sertoli cell tumor		• *Malignant Sertoli cell tumor* • *Large cell calcifying Sertoli cell tumor* • *Intratubular large cell hyalinizing Sertoli cell neoplasia*	
		Granulosa cell tumor		• *Adult Type* • *Juvenile Type*	
		Techoma/fibroma			
		Others		• *Myoid gonadal stromal tumor* • *Mixed* • *Unclassified*	
	Germ-cell and sex cord/gonadal stromal tumors	Gonadoblastoma			
		Unclassified			
	Miscellaneous			Hemangioma Hematologic neoplasms Secondary tumors Ovarian epithelial tumors Tumors of the collecting ducts and rete testis	
	Paratesticular tumors			Adenomatoid tumor Mesothelioma Epididymal tumor	
		Soft tissue tumors		*Lipoma and Liposarcoma Leiomyoma and Leiomyosarcoma Fibroblastic and Myofibroblastic Tumors*	

GCNIS, germ-cell neoplasia in situ.

and controls ($n = 200$), age-adjusted LH levels were higher in the former. In greater detail, the age-adjusted OR of hypogonadism was 3.8 in testicular tumor survivors and showed to increase with treatment intensity being marginally high for surgery alone, 3.5 for radiotherapy, and 4.8 and 7.9 for low- and high-dose chemotherapy, respectively (13). These findings suggest the occurrence of an age-dependent deterioration in Leydig cell function of testicular tumor survivors, with a higher effect of chemotherapy compared to radiotherapy.

A longitudinal cohort study on 307 patients with testicular tumor reported lower testosterone levels at all surveillance time points, which were done after a mean of 9 years (range: 5–21 years; S1) and after a mean of 18 years (range: 13–28 years; S2) (10). At baseline, the risk of testosterone deficiency was higher in the orchiectomy-alone group ($n = 69$; OR = 4.7) than for radiotherapy ($n = 130$; OR = 2.6) and chemotherapy ($n = 108$; OR = 1.9), when compared to controls. At S2, the risk of low testosterone levels was significantly higher in patients receiving chemotherapy (OR = 5.2) than in those treated with radiotherapy (OR = 3.3) or surgery alone (OR = 2). Similar results were found for the risk of high LH serum levels. Therefore, in contrast to surgery alone, both groups receiving radio- and chemotherapy (with a higher effect in the latter) had a lower Leydig cell function with time. In addition, the cumulative platinum dose was significantly associated with the risk of increasing LH levels for each cycle. These results suggest a functional reserve decrement in testosterone production of the remaining testis, which makes testicular tumor survivors vulnerable to the aging-related decline of Leydig cell function (late-onset hypogonadism). Furthermore, residual long-term serum platinum levels and the consequent chronic exposure of the testicular tissue may contribute to hypogonadism as well and may explain the reason why the group treated with chemotherapy has worse Leydig cell function (10).

In agreement with these findings, in a more than 5-year-long follow-up prospective study on 680 patients, low testosterone levels were found in 11% of the group of patients

TABLE 2 | Summary of available data from studies on hypogonadism in testicular cancer survivors.

References	Study design	Total sample	Time of enrollment/Follow-up	Results
Nord et al. (13)	Cross-sectional	1,235 patients and 200 controls	11 years	• No difference in testosterone level was found • Higher age-adjusted LH levels vs. controls • Age-adjusted ratio for hypogonadism = 3.8
Huddart et al. (24)	Case–control	680 patients	>5 years post-treatment	• Hypogonadism was more common in patients treated with chemotherapy plus radiotherapy (37%) vs. those treated with orchiectomy alone (6%) ($p < 0.01$) • High LH levels were found in 11% of patients treated with radiotherapy and in 10% of those treated with chemotherapy ($p < 0.01$ vs. orchiectomy alone) • Compared to baseline, a fall in testosterone levels was observed in patients treated with chemotherapy
Eberhard et al. (14)	Case–control	143 patients and 916 age-matched controls	0, 6, 12, 24, 36, and 60 months after therapy	• Chemotherapy and radiotherapy were both associated with risk for hypogonadism at T0, T6, and T12 • Microlithiasis predicted hypogonadism at all time points • Hypogonadism at T0 predicted the risk for hypogonadism at T6, T12, T24, and T36
Sprauten et al.(10)	Prospective	307 patients	18 years	• A significantly higher risk for low testosterone and high LH was found
Bandak et al. (22)	Meta-analysis	1,187 patients treated with chemotherapy and 671 patients treated with orchiectomy from 11 studies; 301 patients treated with chemotherapy plus non-conventional therapy and 531 patients treated with orchiectomy from 7 studies; 761 patients treated with radiotherapy and 494 patients treated with orchiectomy from 6 studies	1–12 years	• Compared to orchiectomy alone, risk for hypogonadism was significantly higher in chemotherapy (OR 1.8), non-conventional therapy (OR 3.1), and infradiaphragmatic radiotherapy (OR 1.6)
Kerns et al. (25)	Cross-sectional	1,214 patients treated with chemotherapy or post-chemotherapy RPLND	4.2 years post-treatment (range: 1 to 30 years)	• Hypogonadism occurs in 10.2% of patients

LH, luteinizing hormone; OR, odds ratio; RPLND, retroperitoneal lymph node dissection; T, time.

undergoing orchiectomy ($n = 169$), while a significantly higher portion of patients with low testosterone levels was found in patients receiving both radiotherapy and chemotherapy (37%, $n = 81$). Irradiated patients ($n = 158$) and those who received chemotherapy ($n = 272$) showed abnormally high LH levels in the 11% and in the 10% of cases, respectively. The results of this study confirmed that gonadal dysfunction is common in testicular tumor survivors even when managed with orchiectomy alone. Chemotherapy seems to result in an additional risk of testicular failure (24).

A meta-analysis of cohort studies definitively confirmed the occurrence of a higher risk for testosterone deficiency in TGCT patients treated with standard chemotherapy (≤4 platinum-based for chemotherapy cycle; OR 1.8), non-conventional chemotherapy (platinum-based combination chemotherapy with double dose of cisplatin, >4 cycles of platinum-based combination chemotherapy, or both chemotherapy and radiotherapy; OR 3.1), and radiotherapy (OR 1.6) when compared to patients with orchiectomy alone (22). The follow-up time of the studies included in this meta-analysis (22) ranged from only 2 months to 12 years, and some of them reported Leydig cell recovery in the years following the treatment. Accordingly, when patients are monitored for <5 years, the occurrence of hypogonadism is less frequently reported. In fact, a study carried out in 143 TGCT patients found a higher risk for hypogonadism in patients treated with radiotherapy or with three to four chemotherapy cycles when compared to adjuvant chemotherapy (≤2 cycles) at the 6 and 12th post-therapy month. Adjuvant chemotherapy consisted of no more than two cycles of combined therapy with bleomycin plus cisplatin plus etoposide or vinblastin, or carboplatin single administration, and it was offered to patients with a clinical stage I testicular tumor. High-dose chemotherapy consisted of three to four cycles, and it was administered to patients with more advanced disease. By contrast, no difference was found in further surveillance time points (24, 36, and 60 months). High doses of chemotherapy or radiotherapy seem to be, therefore, more harmful than the adjuvant chemotherapy on Leydig cell function, at least during the first-year post-treatment (14). This study also investigated whether any predictor of testosterone deficiency development in testicular tumor survivors does exist. Interestingly, while testicular volume, consistency, age, androgen

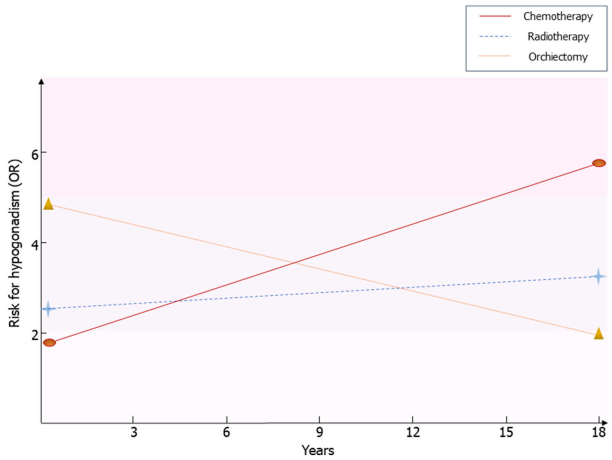

FIGURE 1 | Risk for hypogonadism in testicular tumor survivors. Results coming from the available follow-up studies are resumed. Risk of hypogonadism has been calculated in comparison with healthy men. OR, odds ratio.

receptor polymorphisms, and tumor stage have not been found to correlate with risk of hypogonadism, both mycrolithiasis in the remaining testis and the presence of low testosterone levels after orchiectomy but prior to any other treatment predicted the risk of developing hypogonadism (14). The reason why mycrolithiasis may somehow be associated with a higher risk of Leydig cell failure might be inherent to the possible existence of a tumor-dependent mechanism of Leydig cell damage. Testicular mycrolithiasis belongs to the TDS spectrum, the latter syndrome being considered to be involved in testicular tumor pathogenesis (26, 27).

Framing together these results, Leydig cell vulnerability to chemotherapy and radiotherapy results in an impaired function in the first post-treatment year (14), with an apparent restoration of the Leydig cell function after at least 5 years from the treatment (14). The subsequent later decline of the function, due to subtler damage of Leydig cell function, seems to initially arise with a first phase of subclinical hypogonadism, consisting of increased LH and normal testosterone levels (13, 24), until the full onset of testosterone deficiency (10). This more likely happens in older patients, due to greater susceptibility of Leydig cells to the aging-induced damage in testicular tumor survivors, as previously suggested (10, 13) (**Figure 1**).

Testicular Tumor Survivors: Long-Term Complications

Cardiovascular Diseases

The number of testicular tumor survivors has markedly increased through the decades. A multicenter study carried out on 1,214 testicular tumor patients treated with platinum-based chemotherapy has recently investigated the prevalence of adverse health outcomes (the so-called "Platinum Study"), in an attempt to assess long-term platinum-dependent toxicities. Mean age of patients was 37 years (range: 18–74 years), and the mean time from chemotherapy completion was 4.2 years (range: 1–30 years). Hypertension, peripheral artery disease, and a thromboembolic event were reported in the 9.4, 4.6, and 7.2% of cases, respectively. Coronary artery disease and cardiovascular events such as transient ischemic attack and stroke were negligible (1.6, 0.7, and 0.5%, respectively). Interestingly, the Reynaud phenomenon occurred in 33.4% of patients (25). In support of these findings, ongoing endothelial cell and vascular damage and hypertension could be related to the long-term serum platinum levels (28, 29).

The 10-year cardiovascular risk assessed by the Framingham Risk Score (3%) and the Systemic Coronary Risk Evaluation (1.7%) algorithms was comparable to controls and was independent of the treatment (30). By contrast, a greater risk of developing cardiovascular disease was found after 10.2 years of observation in 992 testicular tumor survivors (31). Other studies confirmed these findings (32). Similarly, a 20-year follow-up study carried out in 990 testicular tumor survivors and 990 age-matched controls more recently found a 5.7-fold higher risk for coronary artery disease in patients treated with chemotherapy (BEP) alone ($n = 364$) compared with surgery alone ($n = 206$) and a 3.1-fold higher risk for myocardial infarction in survivors treated with chemotherapy alone compared with controls. Both groups of patients receiving chemotherapy and radiotherapy ($n = 386$) showed an increased prevalence of administration with antihypertensive and antidiabetic drugs compared with controls. Atherosclerosis was observed only in 8% of patients, despite an increased risk for atherosclerotic disease observed in the chemotherapy and radiotherapy groups (both single and combined administration) compared with surgery alone. The risk was greater in the case of combined chemotherapy and radiotherapy (33). Summary of available data from studies on cardiovascular risk factors and diseases in testicular cancer survivors is described in **Table 3**.

In summary, these findings suggest the presence of a greater risk of developing cardiovascular diseases in testicular tumor survivors, especially after chemotherapy. Two hypotheses have been proposed to explain this association. The direct one suggests a chemotherapy-induced damage at the endothelial level. The indirect hypothesis ascribes the risk to the increased incidence of cardiovascular risk factors, such as hypertension, dyslipidemia, metabolic syndrome, and diabetes, which, in turn, raise the susceptibility to cardiovascular diseases (28).

Metabolic Diseases

According to the findings of the "Platinum Study," which investigated 1,214 testicular cancer survivors, the most frequent adverse outcome observed 4.2 years after chemotherapy completion was obesity, with a prevalence of 71.5%. Diabetes and hypertriglyceridemia rarely occurred (3 and 0.5%, respectively), and hypercholesterolemia was reported in 8% of cases (25).

A follow-up study (1998–2002) on 1,135 testicular tumor survivors younger than 60 years assessed the association between metabolic syndrome (the modified National Cholesterol Education Program definition was used) and type of testicular tumor treatment. The sample studied included patients treated with surgery alone ($n = 225$), radiotherapy ($n = 446$), and cumulative cisplatinum dose $\leq$850 mg ($n = 376$) and >850 mg ($n = 88$). A greater risk for metabolic syndrome was found

TABLE 3 | Summary of available data from studies on cardiovascular risk factors and cardiovascular diseases in testicular cancer survivors.

References	Study design	Total sample	Time of enrollment/Follow-up	Results
Meinardi et al. (32)	Cross-sectional	87 patients (long-term survivors of metastatic testicular cancer treated with cisplatin-based chemotherapy) and 40 controls (affected by stage I testicular tumor treated with orchiectomy alone)	>10 years post-therapy	• An increased observed-to-expected ratio for coronary artery disease was found in patients compared to general male Dutch population • The 33% of patients showed impaired diastolic left ventricular function • Patients has higher blood pressure, total cholesterol, and triglycerides and were more insulin resistant compared to controls
Haugnes et al. (34)	Prospective	1,135 patients (225 were treated with orchiectomy alone, 446 with radiotherapy, 376 with a cumulative cisplatin dose ≤850 mg, 88 with a cumulative cisplatin dose >850 mg)and 1,150 controls	9–12 years	• Increased odds for metabolic syndrome in patients treated with chemotherapy (cisplatin >850 mg) compared both to the surgery group (OR 2.8) and controls • The cisplatin ≤850 mg group had higher odds for metabolic syndrome compared only to the surgery group (OR 2.1) • Patients treated with radiotherapy did not show increased odds compared to the surgery group
Huddart et al. (31)	Prospective	992 patients	10.2 years	• Increased risk for cardiac events was registered after chemotherapy alone (RR 2.59), radiotherapy alone (RR 2.40), and chemotherapy plus radiotherapy (RR 2.78)
Haugnes et al. (33)	Prospective	990 patients (206 were treated with orchiectomy alone, 386 with radiotherapy alone, 364 with chemotherapy alone, 34 with combined radiotherapy, and chemotherapy) and 990 controls (healthy subjects from general population)	19 years	• Radiotherapy alone (OR 2.3) and radiotherapy plus chemotherapy (OR 3.9) groups showed and increased prevalence of diabetes mellitus compared to controls • Chemotherapy group has a 5.7-fold higher risk for coronary artery disease compared to surgery alone and a 3.1-fold higher risk for myocardial infarction compared to controls
Willemse et al. (30)	Cross-sectional	255 patients and 360 controls	7.8 years post-therapy	• Patients treated with combined chemotherapy had a higher risk for metabolic syndrome compared to controls
de Haas et al. (35)	Retrospective	370 patients treated with chemotherapy	≥3 years post-therapy	• Metabolic syndrome was detected in the 25% of patients
Kerns et al. (25)	Cross-sectional	1,214 patients treated with cisplatin-based chemotherapy	≥1 year post-therapy	• Obesity occurred in the 41.7% of patients • Patients had a high risk for hyperlipidemia, hypertension, and diabetes (OR 9.8)

OR, odds ratio; RR, risk rate.

in both groups of patients receiving chemotherapy compared with those who underwent to surgery alone. The group treated with the higher cisplatinum cumulative dose showed a greater risk compared to controls ($n = 1150$), even after adjusting for testosterone levels, thus suggesting that this risk is not dependent on hypogonadism but is due to cisplatinum-induced damage (34). However, other studies have shown that serum testosterone levels <15 nmol/L are associated with a greater risk for developing metabolic syndrome in testicular tumor survivors (35). Indeed, after a median follow-up of 5 years, testicular tumor survivors treated with chemotherapy showed a 2.2-fold higher risk of developing metabolic syndrome compared with controls, whereas the risk increased up to 4.1-fold in survivors whose testosterone levels were <15 nmol/L. Furthermore, among the entire cohort of patients, overweight, and hypercholesterolemia were both found in 24% of cases (35). Similar findings were also reported in a study showing a higher risk of metabolic syndrome in a cohort of 255 testicular tumor survivors 7.8 years after chemotherapy (36). The risk was 2.5-fold higher in survivors with hypogonadism (30).

In conclusion, several reports have found the presence of different dysmetabolic diseases [obesity, metabolic syndrome, and diabetes mellitus (DM)], hypogonadism, and other cardiovascular risk factors. Their early diagnosis and proper treatment are of paramount relevance to lower the long-term cardiovascular risk in testicular tumor survivors.

Bone Density

The occurrence of a decreased bone mineral density (BMD) has been suggested in testicular tumor survivors. A prospective study on 63 germ-cell testicular tumor patients (mean age: 33 years; range: 16–70 years) showed a significant bone loss (lumbar spine BMD: −1.52%; total hip BMD: −2.05%) after 1 year from combination chemotherapy in patients with metastatic testicular tumor ($n = 36$), with no sign of recovery up to 5 years of follow-up. The decrease in BMD was not related with

TABLE 4 | Summary of available data from studies on bone mineralization in testicular cancer survivors.

References	Study design	Total sample	Time of enrollment/Follow-up	Results
Murugaesu et al. (41)	Cross-sectional	39 patients	5–28 years	• Orchiectomy alone or orchiectomy plus chemotherapy predisposed to osteoporosis
Willemse et al. (39)	Cross-sectional	199 patients treated with chemotherapy and 45 newly diagnosed patients within 3 months after orchiectomy	7.4 years post-treatment	• The 25.8% of patients had Z-score between −1 and −2 SD, the 12% of patients has Z-score below −2 SD • Moderate and severe vertebral fractures were observed in 13.6% of cured-long term survivors and in 15.6% of newly diagnosed patients
Foresta et al. (38)	Case–control	125 normotestosteronemic patients treated with orchiectomy and 41 controls	NR	• Vitamin D serum levels was lower in patients than in controls • The 23.8% of patients had Z-score below −2 SD
Willemse et al. (37)	Prospective	63 patients (27 were treated with orchiectomy, 36 received chemotherapy)	5 years post-treatment	• Normal values of bone mineral density were detected in patients treated with orchiectomy only • Significant bone loss was observed in patients receiving chemotherapy
Isaksson et al. (40)	Case–control	91 patients and 91 controls	9.3 years	• Compared to eugonodal patients, patients with hypogonadism receiving or not testosterone replacement therapy had 6–8% lower hip bone mineral density
Ondrusova et al. (23)	Cross-sectional	1,249 patients (313 treated with orchiectomy, 665 with chemotherapy, 271 with radiotherapy)	35 years post-treatment	• Osteopenia or osteoporosis occurred in 136 patients treated with orchiectomy, 298 patients treated chemotherapy, and 139 patients treated with radiotherapy

NR, not reported; SD, standard deviation.

gonadal function, vitamin D levels, cisplatin cumulative dose, or corticosteroid administration. In contrast, stage I patients with no evidence of metastasis, treated with surgery alone or combined with a single dose of adjuvant chemotherapy, did not show any significant difference in BMD (37). In addition, lower BMD was observed in testicular germ-cell tumor patients treated with unilateral orchiectomy ($n = 125$) compared to age-matched controls ($n = 41$), despite the absence of hypogonadism (38). A cross-sectional study in 199 long-term testicular tumor survivors evaluated after a mean of 7.4 years from unilateral orchiectomy and in 45 newly diagnosed testicular tumor patients 3 months after orchiectomy showed an increased prevalence of mild and moderate vertebral fractures (40.2 and 31.1%, respectively) by the Genant's semi-quantitative method, independently of BMD, type of treatment, and gonadal function (39). Furthermore, osteopenia or osteoporosis was found in 43–51% of cases among a cohort of 1,249 long-term testicular tumor survivors. Hypogonadism more frequently occurred in patients with reduced BMD, but all survivors with osteopenia or osteoporosis showed lower testosterone levels. The patients treated with radiotherapy did not show a significantly worse BMD compared with those who received chemotherapy or surgery alone (23). Accordingly, the 9-year-long follow-up in 91 testicular tumor survivors (mean age: 31 years) revealed a significantly 6–8% lower hip BMD in both untreated and treated hypogonadal survivors compared to eugonodal ones and a significant 8% lower spinal BMD in untreated hypogonadal compared to eugonodal survivors (40), thus suggesting the increased risk of impaired bone health in hypogonadal testicular tumor survivors. By contrast, a single study on only 39 testicular tumor (TT) patients after a follow-up time ranging from 5 to 28 years did not find abnormal BMD in patients treated with surgery alone or with chemotherapy (41). Summary of available data from studies on bone mineralization in testicular cancer survivors is described in **Table 4**.

In conclusion, vertebral fractures and impaired BMD occur in testicular tumor survivors, but it is still unclear whether it is related to hypogonadism or to cancer therapy-induced bone damage. Osteological examination should be considered in the follow-up of these patients.

SEXUAL FUNCTION

Sexual dysfunction is often experienced by testicular tumor survivors. The available evidence on this topic is summarized in **Table 5**.

Erection

A number of reports have evaluated the erectile function among testicular tumor survivors (36, 46). A multicenter study encompassing more than 1,200 survivors reported a 4.2-fold higher risk of erectile dysfunction (ED) in testicular tumor survivors compared with controls (25). The prevalence of ED has been esteemed to range from 30 to 40% (45–47, 55) in testicular tumor survivors, mainly assessed by the International Index of Erectile Function (IIEF) questionnaire and largely due to the incapacity to maintain the erection (47).

A meta-analysis of controlled studies found a ∼2.5-fold greater risk of ED up to 2 years after treatment (42). Data from a longitudinal study showed a median time of erectile

TABLE 5 | Summary of available data from studies on sexual dysfunction in testicular tumor survivors.

References	Study design	Total sample	Time of enrollment/Follow-up	Methods	Results
Nazareth et al. (42)	Meta-analysis	709 patients from six controlled and 337 patients from seven uncontrolled studies	Up to 2 years post-treatment	Self-reported or structured questionnaire	• Significantly reduced or absent orgasm (OR 4.6) in patients vs. controls • ED (OR 2.5) in patients vs. controls • Ejaculatory dysfunction (OR 28.6) in patients vs. controls
Eberhard et al. (43)	Case–control	129 patients and 916 age-matched controls	3–5 post-treatment	NR	• Patients have a higher risk for ED (OR 3.3) and low sexual desire (OR 6.7) 3 to 5 years after TT treatment
Tuinman et al. (44)	Prospective	93 patients	1, 3, and 12 months after orchiectomy	IIEF	• Orgasm and EF decreased 3 months after orchiectomy and restored 1 year later • Singles reported more sexual problems compared to partnered patients and to controls
Tasdemir et al. (45)	Case–control	27 patients treated with chemotherapy and controls	>3 years post-treatment	IIEF-15	• The IIEF-15 score was significantly lower in patients vs. controls
Kim et al. (46)	Case–control	246 patients vs. 236 age-matched controls	>5 years post-treatment	BSFI	• Patients scored lower on sex drive, erection, ejaculation, and problem assessment vs. controls • Chemotherapy or radiotherapy increased the risk for sexual dysfunction vs. controls • Surgery-only treatment did not increase the risk for sexual dysfunction vs. controls
Pühse et al. (47)	Cross-sectional	539 patients	After completion of oncologic therapy	IIEF-15 BSFI	• ED occurred in the 31.5% of patients (due to inability to maintain erection on the 24.4% of cases) • Ejaculatory disorders (premature, delayed, retrograde, anejaculation) were reported in the 84.9% of cases • The 32.4% of cases experienced reduced intensity of orgasm • The 95.4% of cases referred reduced overall sexual satisfaction
Bumbasirevic et al. (48)	Cross-sectional	202 patients	47.3 ± 26.8 months	SF 36	• ED was reported by the 20.8% of patients • Loss of desire was reported by the 17.3% of patients • Ejaculatory dysfunction was reported by the 25.7% of patients
Alacacioglu et al. (49)	Case–control	41 patients vs. 38 controls	NR	GRISS	• Patients scored lower on satisfaction, erection, avoidance, and touch vs. controls
Wortel et al. (50)	Prospective	161 patients	Prior to radiotherapy and after 3 and 6 months	Dutch questionnaire	• ED was found in the 23% of patients
Capogrosso et al. (51)	Prospective cross-sectional	143 patients	86 months	IIEF-15	• ED occurs in the 25.5% of patients, being severe in the 11.2% of cases • Mean time of EF recovery is 60, 60, and 70 months after chemotherapy, radiotherapy, and RPLND, respectively • Adjuvant RT is an independent predictor of no recovery of normal EF
Catanzariti et al. (52)	Prospective	67 patients with prosthesis implantation	Before and 6 months after orchiectomy	IIEF-15 PEDT	• No change in questionnaire scores • The 22.4% of patients were dissatisfied about the prosthesis
Dimitropoulos et al. (53)	Prospective	53 patients treated with post-chemotherapy full bilateral non-nerve sparing RPLND	Before and 3 months after operation	IIEF-15	• No change in questionnaire scores • Orgasmic function and intercourse and overall sexual satisfaction were significantly impaired post-operatively
Bandak et al. (54)	Cross-sectional	2,260 patients (1,098) treated by orchiectomy alone, 788 with chemotherapy alone or post-chemotherapy RPLND, 300 with radiotherapy, 74 receiving more than one line of treatment	17 years	IIEF-15	• The risk for ED was higher in chemotherapy (OR 1.5), post-chemotherapy RPLND (OR 2.1), radiotherapy (1.7), and more than one line of treatment (OR 3.2) groups vs. orchiectomy alone group • Orgasmic dysfunction was associated with radiotherapy, post-chemotherapy RPLND, and more than one line of treatment
Kerns et al. (25)	Cross-sectional	1,214 patients treated with chemotherapy or post-chemotherapy RPLND	4.2 years post-treatment (range: 1–30 years)	Self-reported	• ED occurs in 28.4% of patients

BSFI, Brief Sexual Function Inventory; BSF, Brief Sexual Function Inventory; ED, erectile dysfunction; EF, erectile function; GRISS, Golombok–Rust Inventory of Sexual Satisfaction; NR, not reported; RPLND, retroperitoneal lymph node dissection; SF, short form; TT, testicular tumor.

TABLE 6 | Risk for cardiovascular and metabolic complications in testicular tumor survivors.

	Chemotherapy	Radiotherapy
Metabolic syndrome	• Increased for combined therapy or high-dose cisplatin-based chemotherapy • Non-increased for low dose cisplatin-based chemotherapy	Non-increased
Diabetes mellitus	• Increased for combined therapy	Increased
Cardiovascular events	• Increased for combined therapy	Increased

Combined therapy included PVB (cisplatin, vinblastine, and bleomycin) and BEP (bleomycin, etoposide, and cisplatin) schemes; low-dose cisplatin-based chemotherapy: ≤850 mg cumulative dosage; high-dose cisplatin-based chemotherapy: >850 mg cumulative dosage.

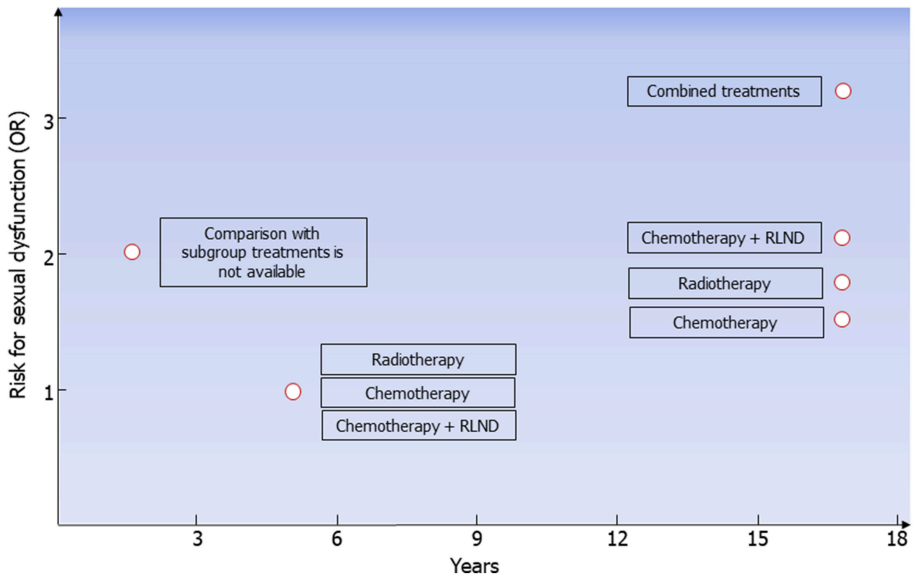

FIGURE 2 | Risk for erectile dysfunction in testicular tumor survivors. According to data coming from all the available follow-up studies, risk for erectile dysfunction (ED) is higher 2 years after treatment in testicular tumor survivors. At the fifth year following radiotherapy, chemotherapy, or chemotherapy plus retroperitoneal lymph node dissection (RLND), the erectile function is apparently restored. The risk for ED is higher in patients treated with chemotherapy (OR 1.5), radiotherapy (OR 1.7), chemotherapy plus RLND (OR 2.1), and combined treatments (OR 3.2) compared to those treated with surgery only.

function recovery of 60, 60, and 70 months in patients receiving radiotherapy, chemotherapy, and RPLND, respectively, after a ∼7.5-year-long follow-up in 143 Caucasian-European testicular tumor survivors. Only adjuvant radiotherapy emerged as an independent predictor of non-recovery (51). Accordingly, the Childhood Cancer Survivor Study indicated a negative impact of radiotherapy on erectile function, since a ≥10-Gr testicular irradiation dose was associated with a greater risk of ED (RR 3.55) among a cohort of 1,622 male cancer survivors (mean age: 37.2 years) (56). However, chemotherapy is also capable of negatively influencing sexual function. Data from a controlled study reported worse scores at the IIEF-15 and the Beck Anxiety questionnaire in patients receiving chemotherapy more than 3 years before evaluation compared to the age-matched controls who did not undergo to chemotherapy. The absence of any significant difference in serum gonadotropin and testosterone levels between the two groups suggests that the greater risk of ED is independent from hypogonadism. However, the small sample size ($n = 27$) limits the reliability of the study results (45). In addition, a longitudinal, cross-sectional study from 202 Serbian testicular tumor survivors followed-up for at least 1 year after platinum-based chemotherapy reported ED in 20.8% of cases (using the SF questionnaire). No patient of this cohort underwent testicular prosthesis implantation due to their socioeconomic background (48). Testicular prosthesis does not seem to affect sexual function *per se*; a part patients complain about is its consistence (52).

These results suggest that the type of testicular tumor has clear implications in the erectile function. Orchiectomy alone may be preferred to other treatment strategies, when possible. Moreover, following an initial post-therapy damage, the erectile function seems to re-establish itself 6 years after the treatment (51). However, a longer time of observation suggests different conclusions. Very recently, a comprehensive prospective study carried out in a cohort of 2,260 testicular tumor survivors reported an increased risk of ED after a 17-year-long follow-up. In greater detail, the study population included 1,098 patients who underwent orchiectomy alone, 788 treated with

chemotherapy (BEP) alone or post-retroperitoneal surgery, 300 patients treated with radiotherapy, and 74 receiving more than one treatment. ED was assessed by the IIEF-15 questionnaire. Compared to orchiectomy alone, the survey showed an increased risk of ED in patients who received chemotherapy (OR 1.5), chemotherapy plus post chemotherapy testicular surgery (OR 2.1), RT (OR 1.7), or more than one type of treatment (OR 3.2) (54), thus showing that additional treatments negatively impact the erectile function. Accordingly, data from other reports agree with the worse impact of RPLND following chemotherapy on erectile function (57, 58).

Orgasm and Ejaculation

About one third of testicular tumor survivors experience ejaculation dysfunction (45). In addition, a ∼2.3 higher risk of impaired ejaculation has been reported in these patients compared with controls, being even higher (OR 3.06) in non-seminoma patients (47). A meta-analysis of controlled studies found a decreased or absent orgasmic sensation associated with ejaculatory dysfunction in testicular tumor survivors up to 2 years after the treatment (59). After 17 years of follow-up, orgasmic dysfunction seems to persist and to associate with radiotherapy, chemotherapy plus post-chemotherapy RLND, and more than one line of treatment in 2,260 testicular tumor survivors (52).

Treatment options may also influence the ejaculatory function in testicular tumor survivors. Chemotherapy showed a greater risk of delayed ejaculation compared to radiotherapy and surgery (46). Full bilateral, non-nerve-sparing RLND may associate with ejaculatory disorders compared to other treatments, probably due to a damage on the sympathetic nerve fibers that control ante-grade ejaculation (53). Accordingly, despite no difference in erectile function following post-chemotherapy RLND observed, orgasmic function and satisfaction were significantly impaired post-operatively, compared to pre-operative function in a cohort of 53 patients (53).

CONCLUSION

Since the introduction of platinum-based chemotherapy and radiotherapy, the 10-year survival rate of patients with testicular tumor has exceeded 97%. The choice of treatment, especially in stage I, where treatment options include surveillance, adjuvant chemotherapy, or adjuvant radiotherapy (6), should take into consideration the risk for long-term complications. Longitudinal studies have revealed a higher negative impact of chemotherapy on Leydig cell function than radiotherapy or orchiectomy alone, leading to a higher risk for hypogonadism. Compared to orchiectomy alone, combined or high-dose chemotherapy and radiotherapy increase the risk for metabolic syndrome, DM, and cardiovascular events (**Table 6**). Furthermore, the long-term risk for ED is higher in patients treated with combined treatments, chemotherapy plus RPLND, radiotherapy, and chemotherapy compared to orchiectomy alone (**Figure 2**). On this account, orchiectomy and clinical surveillance should be preferred. Finally, management of testicular tumor survivors should include the evaluation of gonadal function, cardiovascular and metabolic profiles, BMD, and sexual function to timely detect any possible impairment.

AUTHOR CONTRIBUTIONS

RCa and RCo conceived the work and wrote the paper. RCa, FB, YD, GB, and MC identified the articles. AD and SL revised the paper critically and gave final approval. All authors read and approved the final manuscript.

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Corrigendum: Hypogonadism and Sexual Dysfunction in Testicular Tumor Survivors: A Systematic Review

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An author name was incorrectly spelled as Andrea Di (first name) Cataldo (last name). The correct spelling is Andrea (first name) Di Cataldo (last name). The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Testicular Cancer—Surgical Treatment

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Testicular Germ Cell Tumor (GCT) is the most common solid tumor in men between the ages of 20–44. Men diagnosed with GCT have excellent survival rates due to advances in the multimodal treatment paradigm of chemotherapy, radiation therapy, and surgery. When considering the adequate treatment, several variables should be investigated and known to select the proper procedure. Therefore, when considering Testicular Intra-Epithelial Neoplasia, organ-sparring treatment, such as radiotherapy or organ-sparring surgery should be considered, reaching a cure rate of 98%. However, when the case is of a seminoma or a non-seminoma, orchiectomy is usually the chosen procedure, reaching an oncological cure rate of 80–85%, when there is no metastasis. Retroperitoneal Lymph Node Dissection (RPLND) is generally considered as a treatment option for non-seminomas, when lymph nodes are compromised. There are three different RPLND techniques: open, laparoscopic, and robotic. The open approach is as effective as the other two in its oncological efficiency. Although, when considering both laparoscopic and robotic approach, hospital stays are significantly reduced, better cosmetic results, and less complications when compared to the open approach. Both laparoscopic and robotic approaches require extensive experience and have a steep learning curve, while also providing similar outcome, however, recent studies have been pointing out a slight increase of advantages on the robotic approach. Therefore, further studies are necessary to assert the robotic approach superiority. Also, it is noteworthy that new technologies are on the rise, improving the laparoscopic approach, requiring further studies after their uses are consolidated.

Keywords: testicular, cancer, surgical, treatment, lymph node, dissection, techniques

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Testicular germ cell tumor (GCT) is the most common solid tumor in men between the ages of 20 and 44. Men diagnosed with GCT have excellent survival rates due to advances in the multimodal treatment paradigm of chemotherapy, radiation therapy, and surgery (1).

Testicular cancer is divided into two large groups for treatment planning: seminoma and non-seminoma. Non-seminomatous testicular tumors include: embryonal carcinomas, yolk sac tumors, choriocarcinomas, teratomas, and mixed germ cell tumors. Teratomatous elements can be found within non-seminomas, increasing the odds of chemotherapy resistance; therefore, requiring surgical treatment for cure. However, pure seminomas do not contain such elements. Accordingly, surgical treatment plays a larger role in the treatment of non-seminomas than in the treatment of seminomas (2).

In patients suspected to have malignant cancer, radical orchiectomy is the chosen diagnostic and therapeutic procedure. History and physical, alpha-fetoprotein, beta-hCG, LDH, chemistry profile, and testicular ultrasound should be performed before the surgery. The access is made

through an inguinal incision, allowing the complete removal of the ipsilateral testicle, epididymis, and spermatic cord at the height of the internal inguinal ring. The results after a single radical orchiectomy are between 80 and 85% of oncological cure, when there is no metastasis (2).

When comparing post-orchiectomy oncological outcomes, it is worth noting that non-seminomas present a higher relapse risk when compared to seminomas, especially when lymphovascular invasion has occurred (3). Therefore, post-orchiectomy surveillance is a feasible option in both cases; however, it has a greater importance when it comes to non-seminomas (3).

The International Germ Cell Cancer Consensus Group (IGCCCG) classifies patients into three different groups, depending on the place of disease and level of marker elevation: good, intermediate, or poor prognosis (4). This classification has been incorporated into the Tumor, Node, and Metastases (TNM) system. Approximately 65% of patients with metastatic non-seminomas in modern series are ranked into the good prognosis group, which has survival rates of roughly 97% when retroperitoneal lymph node dissection is performed with several different techniques, discussed below, and chemotherapy (4). The majority of patients (>95%) with metastatic seminoma are classified into the good prognosis group, having survival rates of 95% or more (4). The intermediate prognosis group include 20% of the metastatic non-seminoma and only 3% of seminomas, having an overall survival rate of about 90% (4). The only participant of the poor prognosis group, which comprises ∼20% of patients with metastatic disease, is the non-seminoma, with survival rates of 65–70% (4).

Testicular Intra-epithelial Neoplasia (TIN) is considered the precursor of GCTs (5). TINs have four important and particular characteristics that directly affect its management. The first is that TIN is frequently distributed over wide areas of the affected testicle; therefore, testicular biopsies are able to provide the diagnosis. The second is that TIN is frequently present in the testicle a reasonable amount of time before the cancer progression. Third, immunohistological methods are able to safely detect TIN. And lastly, if TIN is clinically found, organ-sparring treatment is possible (5).

Considering the latter, for TIN treatment options, local radiotherapy is the safest one, with a 98% success rate (5). Another available option is chemotherapy, although it has remarkably lower efficacy, with a success rate of only 76% after three cycles (5). Another possibility is performing an organ-sparing surgery, allowing testicle preservation.

Testis-sparing surgery is predominantly considered in patients with benign lesions and TIN, with tumor mass size of 1–1.5 cm or less, and who have either only one or both testicles afflicted with the disease. In these cases, orchiectomy could be considered an overtreatment, assuming that the patient would become infertile after the procedure (6). Recent studies have shown that in these cases of small scrotal masses, testis-sparing surgery is a reliable and secure option, although some articles point out the concern of multifocal tumors; therefore, an excision contemplating a 1 cm safety rim of normal testicle tissue should be performed in addition to the tumoral mass, diminishing the risk of leaving malignant satellite lesions (7).

Retroperitoneal lymph node dissection (RPLND) has been utilized for treatment of GCTs since the 1900s, and a great amount of data is available, demonstrating its long-term efficacy and safety (4). As opposed to chemotherapy, surgery is not associated with cardiopulmonary disease, metabolic syndrome of secondary malignancy. The surgery alone reduces the probability of requiring subsequent chemotherapy by 50% and excludes the need for abdominal computed tomography (CT) scans during follow-up (4). Nonetheless, primary RPLND does not exclude the risk of recurrence outside the retroperitoneum (5–8% of all recurrences in stage I and 30% of patients with pathological stage II disease), being the lungs the most affected organ (4, 8).

It is important to note that RPLND is mostly recommended as a non-seminoma treatment option, assuming that surveillance and chemotherapy are currently the most suited options for seminomas (9). However, if the patient is not willing to undergo surveillance, chemotherapy is more effective than RPLND, in the case of non-seminomas (9).

Still considering non-seminomas, in the case of salvage treatment on patients with recurrence during surveillance, 3–4 cycles of BEP chemotherapy should be performed (9). Afterwards, the need of postchemotherapy RPLND should be evaluated individually and performed if necessary (9).

RPLND requires extensive experience, as discussed below; therefore, when performed outside centers with a high volume of surgeries, it is associated with higher morbidity and higher infield recurrence rate. Additionally, if positive lymph nodes are detected on primary RPLND, patients still need to undergo adjuvant chemotherapy of two cycles of bleomycin/etoposide/cisplatin (BEP) (4). Therefore, European and Canadian consensus guidelines no longer recommend primary RPLND for stage I Non-Seminoma Germ Cell Tumors (NSGCTs), while the National Comprehensive Cancer Network (NCCN) guidelines still list it as a valid option (2, 4, 9).

RPLND has greatly improved throughout the years, especially with the introduction of the laparoscopic approach in 1992 and recently, in 2006, with the robotic-assisted approach (10).

Laparoscopic Retroperitoneal Lymph Node Dissection (L-RPLND), on its earliest reports, provided a reduced recovery time, less blood loss and lower complications rates when compared to Open Retroperitoneal Lymph Node Dissection (O-RPLND). However, the operation had a lower lymph node yield, a very steep learning curve, and little long-term oncologic outcomes studies (10).

In 2005, Nassar Albqami and Günter Janetschek published a study comparing O-RPLND and L-RPLND in the management of Clinical Stages (CS) I and II testicular cancer, focusing on mean operation time, mean blood loss, length of stay in hospitals and relapses during follow-up as well as surgical and oncologic efficacy, complication rates, morbidity, cosmetic results, diagnostic accuracy, and recurrence rates (10).

The obtained results strongly suggested that L-RPLND, when compared to O-RPLND, provided equivalent surgical and oncologic efficiency, with similar survival and tumor-recurrence rates. However, the patient satisfaction was clearly higher with

L-RPLND, because it delivers better cosmetic results, quicker convalescence, less postoperative mortality, less complications, and shorter operation times. It is noteworthy that the procedure is indeed difficult, but once the steep learning curve has been overcome, the advantages make L-RPLND better than O-RPLND (10).

Within the last decade, robotic-assisted technology has emerged in the field of urology as an alternative to the traditional laparoscopic surgery. The robot grants a greater extent of freedom of movement and better three-dimensional visualization, while still providing the perks of a minimally invasive approach. The greatest debate about the use of robotics lies in the increased cost of the technology (11).

In 2015, a study from Harris, Gorin, Ball, Pierorazio, and Allaf, from the John Hopkins' Urology Institute, published the first retrospective article comparing the results of both laparoscopic and robot-assisted approaches performed in their center from 2006 to 2014. In this period of time, 16 Robotic-Assisted Retroperitoneal Lymph Node Dissection (R-RPLND) and 21 L-RPLND were performed by a single surgeon, being all of them stage I NSGCTs (12).

The results denote that R-RPLND is equivalent to L-RPLND when comparing perioperative results and safety. Specifically, the analyzed parameters, from which all had similar values, were: complication rates, operative times, estimated blood loss, and conversions. Additionally, these parameters were also similar among groups: ejaculatory status, LN yield and frequency of LN positivity (12).

Supporters of the robotic technology state that, when analyzing prostatectomies and nephrectomies, the superior perioperative results are not the only advantage, but is also worth mentioning the improvement of intracorporeal suturing and better control around nerve plexuses and vessels. These technical improvements that the robotic technology provides are of great importance to the development of RPLND, particularly when considering the number of LNs resected and the success of nerve-sparing technique as evidenced by the protection of great vessels, nerve plexuses, and antegrade ejaculation (12).

In 2016, an article analyzing 20 R-RPLND performed on NSGCTs with clinical stages (CS) I and II and postchemotherapy, presented the advantages of the robotic-assisted approach as it is easier to be reproduced, whereas the conventional laparoscopic approach requires an experient surgeon and demands a steep learning curve. In addition, it enables bilateral access in supine position, upholding the oncological principles (13).

In 2018, a systematic review from John Hopkins' analyzed 36 articles until July 2017 comparing the three different surgical approaches (open, laparoscopic, and robotic-assisted) and concluded that the robotic-assisted approach enables equivalent or even superior oncological results compared to the other approaches when performed by experient surgeons. Moreover, the R-RPLND offers greater dexterity, superior visualization, a shorter learning curve for the surgeon and less complications overall, while still providing better recovery advantages compared to the L-RPLND, such as shorter length of stays in hospitals and reduced complication rates. However, larger prospective studies are still required to better evaluate long-term oncologic outcomes and complication rates in both the primary and post-chemotherapy settings (1).

In conclusion, further studies should be performed with a larger number of case reports to assert the superiority of the R-RPLND. It is noteworthy that the ever-growing technology evolution has been providing innovations for the conventional L-RPLND; for example, three-dimensional visualization and multi-articular clamps, which could, eventually, close the gap between L-RPLND and R-RPLND, because R-RPLND still has its disadvantages, such as lack of tactile feedback to the surgeon, inability to move the surgical table once the arms of the robot are fixed, and expenses related to the robot and its semi-disposable instruments. Therefore, further studies are required in the future to determine the best available technique, as they are currently improving (14).

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

MB organized, supervised, corrected, and submitted the manuscript. RV and GB researched and read the bibliography used, and wrote approximately half of the manuscript.

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Caregiver Emotional Burden in Testicular Cancer Patients: From Patient to Caregiver Support

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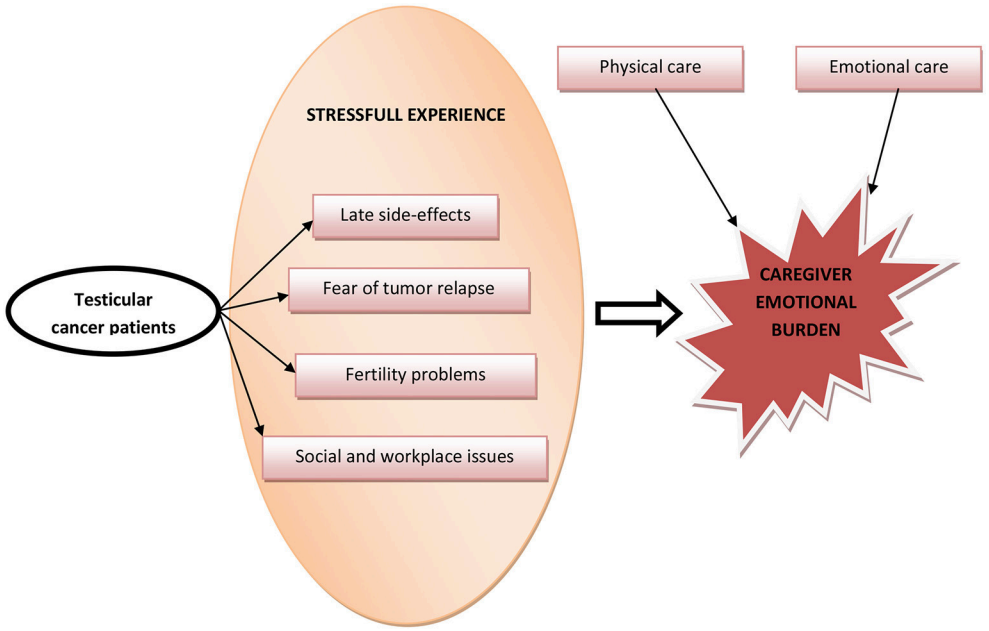
Testicular cancer is the most common tumor in young males aged 15–40 years. The overall cure rate for men with testicular cancer is >90%, so a huge number of these patients will become testicular cancer survivors. These people may feel some stress in the experience of diagnosis, treatment, and consequences that affects the quality of life, and during follow-up, especially when new issues and emotional distresses appear over time, such as late side-effects of treatments and emotional challenges including fear of tumor relapse, fertility and sexuality concerns, and social and workplace issues. The cancer experience has an impact not only on patients, but also on their relatives (e.g., spouses, parents, or siblings), who often have to assume a caregiving role for the duration of and following treatment for cancer. Moreover, the caregiver plays an important role in supporting a man with a testicular cancer, providing physical and emotional care. This review presents a summary of existing knowledge regarding the impact and the burden of testicular cancer on caregivers.

Keywords: caregiver, testicular, cancer, patients, long-term survivors

INTRODUCTION

Testicular cancer (TC) is the most frequent solid tumor in young adult men aged between 15 and 35 years, and is a highly curable cancer with survival rates close to 99% for stage I disease cases (localized tumor) and 80–90% for cases with metastatic disease treated with cisplatin-based chemotherapy and surgery on residual masses, when indicated (1). In addition, the 10–20% of metastatic patients who are not cured with first-line cisplatin-based chemotherapy, increase their chances of long-term remission in nearly 50% of cases treated with second-line treatments, such as high-dose chemotherapy (HDCT) or standard-dose chemotherapeutic regimens, and in nearly 15–30% of cases treated in the following lines with other salvage regimens (2–5). A young age at diagnosis and excellent prognosis, physical, psychological and social well-being represent a significant indicator for follow-up and survivorship of these people. In fact, despite the excellent prognosis, cured patients may experience long-term somatic sequelae and psychosocial distress



GRAPHICAL ABSTRACT | Factors influencing Caregiver emotional burden in TC patients.

according to the tumor and treatment burden (6, 7). However, type and duration of each treatment depends on initial stage of the disease (**Table 1**). As a consequence, different physical and psychological loads correlate with different treatment loads. Both tumor diagnosis and tumor treatment are usually stressful events affecting not only patients but the whole family system (**Graphical Abstract**) (8).

Role of Caregiver

Caregivers are individuals, usually family members or relatives (e.g., partner, parents, but also close friends), who have a significant relationship with the patient suffering from a life-threatening illness and provide assistance (9, 10). Along the whole process of the disease and its treatments, they are engaged in the practical help and psychological support for coping with the situation, including the emotions of uncertainty and fear (11). This role requires many abilities that may be physically, emotionally and financially demanding. The burden of caregiving has been defined a "multidimensional biopsychosocial reaction resulting from an imbalance of care demands relative to caregivers' personal time, social roles, physical and emotional states, financial resources, and formal care resources given the other multiple roles they fulfill" (12).

Cancer could determine major effects both on caregivers and patients, with literature clearly indicating that cancer affects the emotional, social, physical, and spiritual well-being of patients and their family members (13).

Most studies in family caregivers of cancer patients reported diverse problems as a consequence of their role, ranging from diminished physical health and psychological distress to an adverse impact on their work (14). The most prevalent physical problems included sleep disturbance, fatigue, pain, loss of physical strength, loss of appetite, and weight loss.

Cancer is a family experience that exerts a change in family's system, balance and identity, redefines the rules, changes the lives of all its members, brings an immense amount of stress, and presents many challenging situations. Cancer and the approaches used to treat it can introduce a complex array of lifestyle changes and emotional responses, which can be difficult for family members to handle. The diagnosis of cancer, its treatment and symptoms both of the illness and of the chemotherapy have an influence on how patients and their caregivers experience distress. A review on psychological impact of cancer on patients' partners and other relatives affirmed that an important minority of carers become highly distressed, clinically depressed and anxious: in particular, prevalence of clinically significant distress among caregivers was reported to be 20–30% in studies using self-report questionnaires, whereas in studies that used diagnostic interviews rates are approximately 10% (15). However, data concerning caregivers' distress are sparse. This review analyzes TC-related distress and burden of caregivers.

CAREGIVER BURDEN

The experience of illness perceived by the caregiver depends on some specific aspects of the disease such as the type of cancer and the stage of life in which it is diagnosed.

TC has a profound effect on body image and on the personal image of oneself and, and often occurs in adolescence and young adulthood, times characterized by significant life changes and psychosocial challenges. These men are in the prime of their lives, when health is often taken for granted, while interpersonal relationships and the desire to start a family may be major interests (16–19).

TABLE 1 | Therapeutic strategies, clinical complications, and correlation with QoL.

Histology	Stage	Primary treatment	Most common adverse events	Detrimental effect on QoL	Stage	Secondary treatment	Most common adverse events	Detrimental effect on QoL
Seminoma	I	Single agent Carboplatin (AUC7 for 1 cycle) or RT (20 or 25 Gy)	Myelotoxicity Fatigue	Low risk	Relapsed/Refractory TC	VeIP (4 cycles) or TIP (4 cycles) or HDCT ± RT	Myelotoxycity Fatigue Alopecia Vomiting Neurotoxicity Infertility Cardiovascular toxicity Pulmonary toxicity Solid secondary tumors Leukemia	High risk
	IIA	RT (30 Gy)	Fibrosis	Low risk				
	IIB	BEP (3 cycles) or EP (4 cycles) or RT in selected non-bulky cases	Myelotoxycity Fatigue Alopecia Vomiting Neurotoxicity Infertility Pulmonary toxicity Solid secondary tumors Leukemia	Intermediate risk				
	IIC, III	BEP (3 or 4 cycles)						
Non-seminoma	I	Surveillance or BEP (1 cycle) or RPLND	If BEP: Myelotoxicity Fatigue Vomiting Alopecia	Intermediate risk				
	II, IIIA	BEP (3 cycles) or EP (4 cycles) or Nerve-sparing RPLND	Myelotoxycity Fatigue Alopecia Vomiting Neurotoxicity Infertility Pulmonary toxicity Solid secondary tumors Leukemia	Intermediate risk				
	IIIB, IIIC	BEP (4 cycles) or VIP (4 cycles)						

RT, radiotherapy; BEP, Bleomycin, Etoposide, and Cisplatin; EP, Etoposide and Cisplatin; RPLND, retroperitoneal lymph-node dissection; VIP, Etoposide, Ifosfamide, and Cisplatin; VeIP, VeIP Vinblastine, Ifosfamide, Cisplatin; TIP, Paclitaxel, Ifosfamide, and Cisplatin; HDCT, high dose chemotherapy. Stage and primary treatment according to NCCN guidelines version 1.2019[1].

Diagnosis of TC causes a sort of emotional earthquake which involves relatives, partners and close friends. Cancer patients during treatment and also during the follow-up period experience many needs, and caregivers are often unprepared to respond to this important burden (20). Main psychological problems experienced by TC caregivers are summarized in **Table 2**. Of note is that the physical and mental health of patients and their caregivers are often related. In this contest, improving knowledge and social support to caregivers could help to ameliorate patients' global health. Confirming this, depression symptoms are less frequent in patients living in couples (21). Taking care of a patient with cancer is described as a full-time job (22) and caregivers themselves often are in great need of psychological support. Caregivers of TC patients usually are parents or partners, typically young females and it is recognized that the highest predisposition to symptoms of distress have been showed by female caregivers of young age and lower social status (19). Therefore, for female partners of cancer patients, there is a risk of developing psychological and psychiatric morbidity and lower quality-of-life (QoL) than women in healthy couples

[1]https://www.nccn.org/professionals/physician_gls/pdf/testicular.pdf

TABLE 2 | Main psychological issues in TC caregivers.

Main psychological problems in TC caregivers
Need of information
Anxiety
Depression
Inadequacy
Lack of practical and emotional support
Risk of infertility
Sexual difficulties

(15, 23). In this contest, TC survivors' caregivers are a high risk population. Several studies reported rates of anxiety of between 30 and 50% (24) among family caregivers in comparison to rates of anxiety of between 19 and 34% (25) in patient samples. Likewise, rates of depression are reported to be between 10 and 25% (25) in patients samples compared to between 12 and 59% (24) among family caregivers. This underlines that, in many cases, the psychological burden on caregivers is even greater than in patients (**Table 3**). Caregivers receive less practical and emotional support from friends and professionals than patients (26). However, their high self-efficacy can improve their own mental state and also the mental well-being of patients.

In their caregiver role, partners of TC patients have a social task: commonly they give information to family members and friends while her husband is in the hospital (27). Another considerable aspect of these women's burden is emotional experiences connected to the illness and to the period of life in which it occurs. Often the diagnosis of TC happens in an important period of life, characterized by major life changes and specific developmental tasks, when forming intimate and long-term emotional and sexual relationships, and starting families may be major concerns (28). Each member of the couple, both the patient and the partner, is faced with the possibility of treatment-related infertility and sexual difficulties in a period of life where partners are often focused on starting a family (27). However, only a minority of the couples experienced more serious and long lasting testicular cancer-induced disturbances in sexual and marital relationships. In general, couples felt their relationship became more tightly bonded and stronger following the confrontation with TC (29, 30).

Both psychosocial and QoL consequences occur years after the experience of the tumor and the end of treatments. Tuinman et al. (31) showed that spouses who experienced the diagnosis and treatment process had better physical QoL than the average woman. Their stress response levels were low and were related to the stress response level of TC survivors and to the duration of treatments received. However, these women, even years after the completion of treatment, were experiencing more stress response symptoms than the TC survivors.

Relevant components of the caregiver's burden consist of the support, assistance and information needs that, if not unmet, leads to reduced QoL and high levels of distress (32). Kim et al. (33) demonstrated that caregivers of cancer patients frequently have a variety of unmet needs and that unmet needs strongly predicted their QoL.

TABLE 3 | Differences in emotional burden between TC patients and their caregivers.

	Caregivers (%)	Patients (%)
Anxiety	30–50 (24)	19–34 (25)
Depression	12–59 (24)	10–25 (25)

Patients, caregivers and care providers had different expectations about TC survivorship: psychological distress was considered as highly relevant by 35% of patients and caregivers and 93% of care providers; the couple's relationship was quite or very difficult for 12% of patients and caregivers in comparison to 64% in the perception of care providers (34). A different perception of the illness experience could affect the recognition and ability to respond to the needs of patients and caregivers. In another study, close relatives of men suffering from TC highlight four themes: the disease and its course, normalization, the long-term consequences, and the social network (35). The results showed that relatives suffer from social isolation (35).

Another source of caregiver burden could be the fertility issue: the paternity rates among men who attempted to conceive a child after treatment were 71% at 15 years and 76% at 20 years after orchiectomy, but this rate ranged from 48% in the HDCT group to 92% in the surveillance group (36). Sandén and Söderhamn (37) reported a conversational interview to a young woman whose partner had TC using a semistructured guide with open-ended questions. Caring became primary for female partner, and she focused less on her own needs in order to support the patient; everyday life changed, as more time was spent at the hospital, the home, and the parent's home. The third keypoint was the shortness of time: from the discovery of the disease and the start of chemotherapy, time was reported as passing very quickly, and the felt like they spent a lot of time with their physicians.

In literature, little data exists about the role of mothers as caregiver of TC patients and the dramatic changes in their lives. Unlike their healthy peers, young TC patients often face greater challenges in life: they may experience delays in developmental milestones, difficulties in employment and interpersonal relationships, and medical and institutional problems (e.g., economy, education, transport). These challenges can hinder their transition to independence, which is not favored by mothers who continue to take care even when their sons progress to adulthood (38).

CAREGIVER THERAPY

Caregivers need a large volume of information, including: diagnosis-related information, prognosis-related information, treatment-related information, information on homecare, and information about impact on the family or on relationship with partner. Therefore, psychoeducational interventions have been conceived to increase their knowledge. Bultz et al. (39) reported that this sort of intervention had a positive impact on caregivers' ability to provide care and also improve marital satisfaction

of patients. Pelusi et al. (40) revealed that caregivers sharing their cancer experience with others in storytelling is essential to offer educational information and emotional support to those who hear it, but also care for self is an important component of managing the course of these events. A Chinese study explored the relationship between family resilience and the post-traumatic growth, and the quality of life of survivors of breast cancer, demonstrating that family resilience decreased caregivers' and patients' burden (41). One intervention used telephone interpersonal counseling, which was delivered to patients and their caregivers separately to improve cancer education and resulted in significant decreases in depression and anxiety levels in the caregivers group (42). Kozachik et al. (43) conducted a quasi-experimental study to describe the use of complementary therapy (such as reflexology, guided imagery, and reminiscence therapy) to cancer patients undergoing chemotherapy and their family caregivers. The authors were unable to draw conclusions regarding the impact of complementary therapy on caregiver burden, however they suggest that one complementary therapy may be incorporated into patients' and caregivers' courses of cancer treatment (43).

According to the different stage of the disease at diagnosis, several treatment strategies are recommended (**Table 1**). These modalities are associated with different complications and late toxicities and a negative impact on QoL. TC survivors have a high risk of leukemia; the relative risk, associated with the previous use of etoposide, ranges between 3.5 and 4.5 and appears often within 10 years following the end of treatment (44). Younger age at radiotherapy and/or chemotherapy increases risk for solid secondary tumors and remains elevated for at least 35 years (45). In long-term setting pulmonary toxicity, infections and cardiovascular events are higher compared with the general population (46, 47). For TC survivors and their caregivers, preserved fertility is a fundamental subject which has an important impact on their QoL (48). The prospect of paternity improves with the decreasing number of cycles of chemotherapy, therefore the correct management of TC requires a careful balance between the intensity of treatment and burden of disease, in order to limit short and long term adverse events (49). In sight of this, the correct management of late toxicities is essential in order to preserve the higher QoL of patients and their caregivers. A recent multidisciplinary consensus conference by the Italian Germ cell cancer Group (IGG) and the Associazione Italiana di Oncologia Medica (AIOM) has provided recommendations for surveillance and follow-up appointments of men with TC, suggesting a visit with caregiver at the beginning of follow-up and, eventually, a psychological consultation (50).

To date there are no evidence about the importance of the role of nurse in supporting caregivers of TC-patients. In our opinion, it is important to develop educational programs with the aim of creating a cancer clinical nurse specialist role, who could support patients and their families during the course of disease. This program could guarantee personalized nursing assistance and aid (both psychological and practical), leaving from the psychosocial contest of the patients. The role of nurse is expressed before, during and after the identification and the monitoring of signs and symptoms of the disease and the treatment, in order to promote the well-being of the patients and their caregivers.

TC patients and their families have to be included; they should have the opportunity to be involved in the planning of the assistance and in the decision making process through individualized services which could adapt to the changing psychophysical status of the patients (51).

CONCLUSIONS

Cancer treatment is not an individual experience, but induces deep effects on patients' families. Couples who achieve the survivorship phase often have to change life plans, deal with TC and treatment-related effects, and manage worries about future health (52). Patients partnered at diagnosis experience a better emotional and physical adaptation to disease (53, 54) and the majority of follow-up studies reported that the rate of divorce or broken relationship was 5% to 10% (55). The impact of disease on caregivers depends also on patients' story. TC patients who undergo, after orchiectomy, one cycle of chemotherapy probably have a lower burden of distress compared with patients who complete four standard cycles of chemotherapy, due to the reduced treatment load. Moreover, some patients are not cured with first-line chemotherapy and have to be treated with further intensified chemotherapeutic regimens, including standard dose chemotherapy supported by granulocyte-colony stimulating factor (G-CSF) (3) and/or HDCT with support of autologous peripheral-blood stem-cell (56, 57). HDCT is a stressful program both for patients and their family, due to long hospitalization periods and a high risk of treatment-related toxicity that requires the adoption of specific precautionary measures. These therapeutic options are able to lead to long-term remission of disease, but leave a stressful emotional burden on the patient and his caregivers. There is some data available about stressful burden of caregivers of elderly persons with physical dependence (58), but there are little evidence about young patients' caregivers, though often they are young people at risk of psychological distress which could have an impact on long-term effects (59, 60).

Unfortunately, there are little data available in literature concerning the role of caregiver in TC patients, probably because of the lower number of persons involved compared with breast cancer, for example. However, in our opinion, according to the young age of patients and the very good prognosis, it is important to consider a more integrated system of the patient and his social support, with the purpose of improving QoL not only during active treatment, but also in the follow-up period, and to encourage a less traumatic return to the everyday life.

AUTHOR CONTRIBUTIONS

SD, CC, and UD collaborated in the conception, in the data retrieval, and in the drafting of the text. CC, GS, and MC collaborated in the revision of the text and in the completion of the bibliographic research. AB, TB, AF, CM, SB, AA, SP, SM, MC, GB, LG, and MM revised the manuscript.

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On the Origin of Testicular Germ Cell Tumors: From Gonocytes to Testicular Cancer

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Human primordial germ cells (PGCs) have been described in the yolk sac wall around the beginning of the third week. From week 4 to 5, they migrate under control of SCF/c-KIT signaling pathway to the genital ridge, where they become gonocytes. PGCs and gonocytes express classic pluripotency markers, such as KIT, NANOG, and OCT3/4 that, during spermatogonia differentiation, are gradually suppressed, and substituted by the expression of some germ cell specific genes, such as VASA, SOX17, and TSPY. These genes, during normal development of germ cells, are tightly regulated by epigenetic modification, in terms of microRNA expression and DNA methylation. In adolescents and young adults, testicular germ cell tumors (TGCT) have a common precursor, the germ cell neoplasia *in situ* (GCNIS); the hypothesis of their origin from PGCs or gonocytes, whose maturation is altered, is widely accepted. The origin of TGCT, probably starting at early stages of embryogenesis, seems to be a part of the Testicular Dysgenesis Syndrome (TDS) where some early PGC/gonocytes, for still unclear reasons, are blocked in their differentiation, retaining their early marker profile. In this paper, current knowledge on the combination of epidemiological and genomic factors, involved in the development of testicular germ cell tumors, is reviewed.

Keywords: germ cell neoplasia *in situ* (GCNIS), primordial germ cell (PGC), testicular germ cell tumors (TGCTs), testicular dysgenesis syndrome (TDS), Sertoli cells, Leydig cells

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INTRODUCTION

The two main categories of testicular cancer fall into "germ cell," representing up to 95% of testis malignancies, vs. "non-germ cell." Tumors originated by germ cells are known as testicular germ cell tumors (TGCT) and can be divided into two main types: seminomas and non-seminomas according to their histological features. In about 10% of cases both seminoma and non-seminoma cells are present simultaneously in one testicle resulting in the so-called mixed germ tumors (1).

TGCT were extremely rare types of cancer until the second half of the twentieth century, when their prevalence arose dramatically and, for not yet elucidated reasons, have continued to steadily increase. In fact, the annual number of cases has more than doubled since the 1950s (2, 3).

TGCT, also known as Type II germ cell tumors (4), account for only 1% of all malignancies in males but in several Countries they are the most common solid tumors, occurring mainly in young men (18–35 years) (5) in which represent the leading cause of cancer-related death.

The incidence of this cancer shows geographic and ethnic differences: it is lowest (ranging from <0.5/100,000–5/100,000) in the majority of African and Asian Countries and highest (up to 12/100,000) in white populations of Northern European Countries. In particular, in the latter population, was observed, in 2012, significant differences in the incidence ranged from over 12/100,000 in Denmark and Norway to 5/100,000 in neighboring Finland or 5.4/100,000 in Italy and 3/100,000 in Spain (6).

Fortunately, the platinum-based chemotherapy has contributed to improve the mortality rate of TGCT worldwide from 1970 onwards and, today, the overall cure rate of TGCT is more than 90%; however about 10% of TGCT are unresponsive to chemotherapy, and 4–8% of relatively young patients, especially those with disseminated non-seminomas, die of the disease. These facts show the relevance to further improve our knowledge on the mechanisms underlying this disease.

Regarding TGCT etiopathogenesis, both inherited and environmental factors are thought to play a pivotal role, but at this time, there are insufficient evidences to make a risk assessment on any single individual factors. **Figure 1** (modified by Asian J. Andr.) summarizes the "genvironmental hypothesis" that could, probably, explain the development of TGCT with a combined action of epigenetic and environmental factors (8).

RISK FACTOR OF TGCT

TGCT are considered the result of an altered germ cell differentiation that can be linked to the Testicular Dysgenesis Syndrome (TDS), a complex syndrome resulting from an abnormal fetal development of male gonads due to genetic, environmental factors or both (9, 10).

Various aspects of TDS (gonadal malformations, testicular microlithiasis, cryptorchidism, previous TGCT in the contralateral testis, disorders of sex development), altered fertility (subfertility/infertility), or hypospadias, are associated with increased risk of TGCT (9). For example, patients with previous history of TGCT have a relative risk of developing a contralateral malignancy about 25-fold higher than the age-matched general population (11).

Inherited genetic aberrations leading to disorders of sex development (DSD) are considered to affect gonadal development increasing the risk for GCNIS and TGCT. For example, 15–30% of patients 45XO/46XY DSD and 46XY DSD (with different degrees of gonadal dysgenesis) show the highest risk for TGCT (12).

Beyond karyotype, environmental factors may influence the risk for TGCT such as an excessive exposure to estrogen or molecules with estrogenic activity or endocrine disruptors during pregnancy (13).

Previous studies showed that environmental estrogens altered the normal development of embryonic urogenital system, resulting in an increase in cryptorchidism in newborns, and a decrease of total sperm counts associated with an increase in testis cancer rates in young men (14).

As demonstrated by other studies, mothers of patients with TGCT had higher estrogen levels during pregnancy (15) or were exposed to organic pollutants (16).

In this regard, it's fundamental to analyze the role of somatic cells. Indeed, also somatic Sertoli and Leydig cells, besides germ cells, could be affected in dysgenetic gonads. Their functions are to provide the appropriate microenvironment and the correct endocrine and paracrine signals for a normal germ cell development. So, altered testosterone levels could affect the normal development of somatic Sertoli cells (17) leading them to an insufficient germ cell stimulation and to an abnormal differentiation. In particular, a recent study performed on normal and neoplastic adult human testes led to the hypothesis that, in GCNIS tumors, Sertoli cell phenotype is changed to a less mature state (18).

Moreover, Sertoli cells secrete stromal cell-derived factor 1 (SDF1/CXCL12), a chemokine implicated both in PGC migration and regulation and support of adult stem cell niches. SDF1/CXCL12 binds to CXCR4 receptor located on both normal and TGCT cells and the signaling system lead to survival and growth of transformed cells thus facilitating the metastatic colonization of other organs.

Other important signaling systems expressed by Sertoli cells are represented by activin and inhibin, two members of the transforming growth factor beta (TGFbeta) superfamily that play a well-known role in spermatogenesis and FSH secretion.

Recently, it was demonstrated that activin A target genes are differentially expressed in neoplastic adult human testes compared to normal testes, thus suggesting a modulatory role of activin in the tumor niche and in TGCT development (18).

Inhibin B production is stimulated by androgens thus constituting a link between the microenvironment in where germ cells reside and the cells themselves. Importantly, inhibin is involved in the regulation of gonadal tumor development and progression (19).

In addition, endocrine disruptors may disturb regulatory actions exerted by androgens on somatic and germ cells (5). The latter could continue to express embryonic genes related to the undifferentiated state and pluripotency. Consequently, fetal gonocytes undergo abnormal cell division and accumulate chromosome aberrations facilitating their malignant transformation.

Some observations support the hypothesis of an involvement of sex hormone signaling. For example, TGCT develops only after puberty when the activated hypothalamic-pituitary-gonadal axis induces the transformation of GCNIS; in fact, patients affected by hypogonadotropic hypogonadism have a low risk of TGCT in cryptorchid testis (5).

Nevertheless, there is no evidence that the development of seminoma or non- seminoma TGCT are directly induced by sex hormones after birth. It seems more likely that hormones have an indirect effect when, during spermatogenesis, they promote GCNIS cell divisions leading to amplification of transformed

cells bearing and accumulating various chromosome and genetic aberrations (20).

In summary, it is likely that imbalanced levels of maternal estrogens or environmental molecules with estrogenic activity during pregnancy could interact with specific genetic aberrations accumulated by GCNIS, playing a key role to promote tumorigenic pathology. In addition, affected Sertoli cells could create a defective microenvironment that allows arrested gonocytes to survive in the postnatal testes.

FROM GONOCYTES TO TESTICULAR CANCER

Previous studies about the origin of testicular cancers in adolescents and young adults (21, 22) demonstrated that TGCT have a common pathologic precursor, previously named carcinoma *in situ* (CIS) or Intratubular Germ Cell Neoplasia Unclassified (IGCNU) and, recently, according to an update of the 2016 World Health Organization classification (23), referred to as germ cell neoplasia *in situ* (GCNIS).

Further studies led to the currently most accepted hypothesis that GCNIS is an embryonic germ cell, that is a primordial germ cell (PGC) or a gonocyte, that failed to differentiate into a spermatogonium during development (24).

Normal Germ Cell Development: From PGCs to Spermatogonia

Human PGCs have been described in the yolk sac wall during the 3–4 weeks post conception. From week 4 to 5, they migrate under control of SCF/c-KIT signaling system (25) in the hind gut epithelium and then they colonize the genital ridges, the precursors of both ovary and testis, where they are surrounded by supportive cells deriving from the coelomic epithelium. During and early after their migration PGC express specific markers, and some of these markers, such as OCT3/4, c-KIT, placenta like alkaline phosphatase (PLAP) and NANOG could be used as diagnostic markers for TGCT and GCNIS (26).

At 6th week, the expression of SRY gene in the male embryo lead to differentiation of genital ridges into testes (27) inducing the expression of SOX9, a transcription factor that initiates the differentiation of supportive cells into Sertoli cells (28). Sertoli cells organize the microenvironmental niche regulating germ cell differentiation into spermatogonia until the first month after birth, when the mitotic arrest takes place (29).

At 7th week, primitive seminiferous cords, a particular structure in which germ cells and Sertoli cells are not yet organized, are formed. Subsequently, germ cells migrate toward the basal lamina of the seminiferous cords (if not migrate they undergo to apoptosis and cleared from the seminiferous epithelium) and, during the 13th week, germ cells start to lose the expression of some markers (c-KIT, OCT3/4, and PLAP). In particular, c-KIT can still be detected at a relatively low level, while OCT3/4 and PLAP disappear completely. On the contrary, VASA and SOX17 continue to be expressed remaining positive throughout life (**Figure 2**). In addition, at same time, gonocytes express TSPY, which regulates the normal proliferation of spermatogonia and remain positive up to meiotic division.

PGCs differentiation passes through three stages which three different types of germ cells: gonocytes, intermediate cells, and spermatogonia, concurrently present in the fetal testis and distinguishable by morphologic and immunohistochemical features (30).

In particular, gonocytes are large cells with spherical euchromatic nuclei with one or two nucleoli (31). At the 10th week of gestation, they are the more abundant type of germ cells located centrally within the seminiferous cords and separated from the basal lamina by Sertoli cells. Then, gonocytes become intermediate cells, with similar morphology but located peripherally within the seminiferous cords and in contact with the basal lamina. At gestational week 15, many intermediate cells are present together with gonocytes. It has been hypothesized that when these cells reach the basal lamina, they lose their pluripotency and start to differentiate into spermatogonia. From the 18th week onward, spermatogonia constitute the most common germ cell population. They are located peripherally to the basal lamina and enter mitotic arrest.

With regards to molecular features the three different types of germ cells populations (gonocytes, intermediate cells and spermagonia) express different markers of pluripotency (**Table 1**) and show different epigenetic modifications.

In particular, gonocytes express markers of pluripotency (OCT3/4, NANOG, and c-Kit), and are positive for placental alkaline phosphatase (PLAP). Normally, gonocytes are negative for melanoma-associated antigen 4 (MAGEA4); in fact this marker is expressed in the fetal germ cells from 17 weeks of gestation onward (30).

Intermediate cells are negative for both c-KIT and MAGEA4, and show low or negative staining for OCT3/4 and positive staining for proliferating cell nuclear antigen (PCNA), marker of proliferative activity. Beginning from late gestation (week 17–18) until about 1 year of post-natal life, spermatogonia loss fetal markers (the cells are negative for c-KIT and PCNA) and start the expression of germ cell specific markers such as MAGE4A, VASA, and testis-specific protein Y-encoded (TSPY) gene (32–34).

Regarding the epigenetic modifications, genes involved in germ cell development are strictly regulated by epigenetic changes, such as DNA methylation, and microRNA (miRNA) activity (see below) (35, 36).

In particular, gonocytes show loss of genomic methylation. *De novo* DNA methylation will start in spermatogonia to re-establish the parental imprinting pattern (35).

The maturation processes that lead from gonocyte to spermatogonia are not synchronized and therefore in seminiferous tubules are present germ cell populations expressing embryo/fetal markers, differentiation markers, methylated or un-methylated genes.

From Normal Germ Cells to GCNIS

The GCNIS cells, located above the basal lamina, show abundant cytoplasm and large spherical or irregular nucleus with tetraploid DNA content with 1 or 2 nucleoli (37). These cells are present in 0.4–0.8% of men among where spermatogenesis is

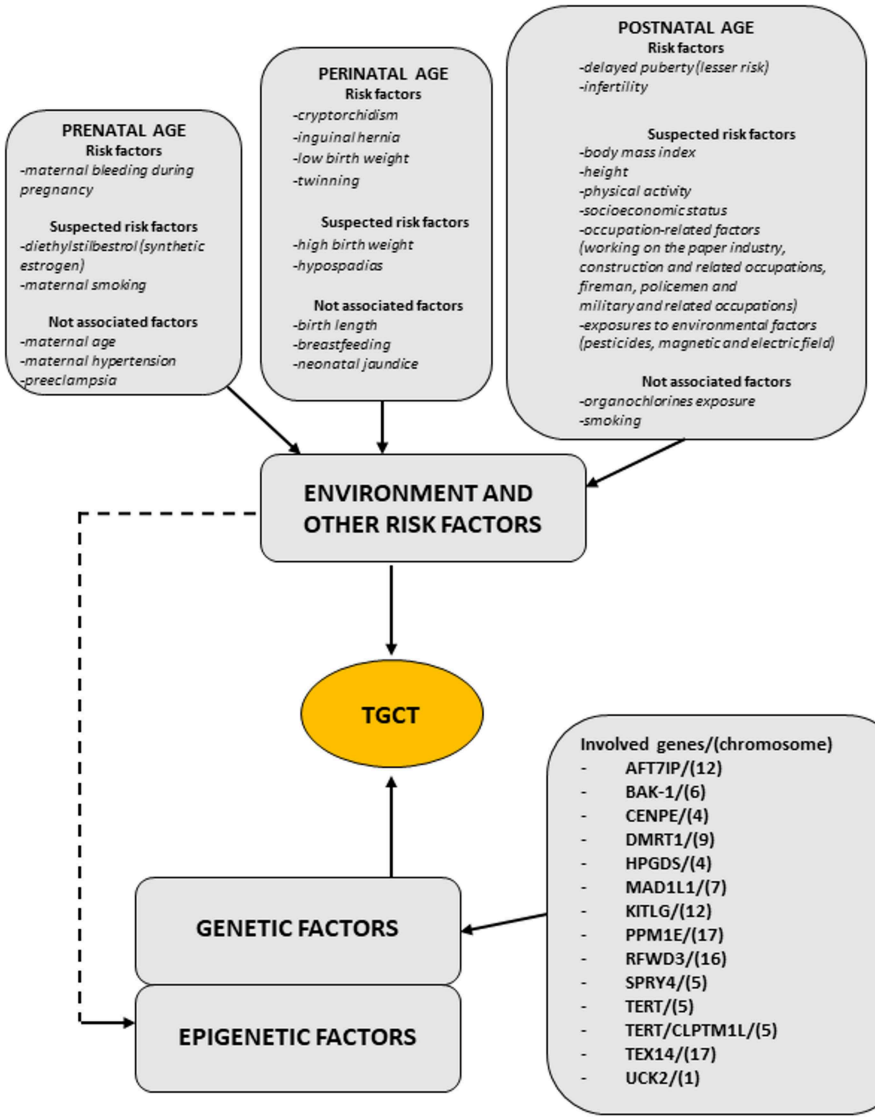

FIGURE 1 | The scheme illustrates the genvironmental risk model showing different etiological factors influencing the development of TGCTs. Suspected risk factors and not associated factors are also listed. Modified from Elzinga-Tinke et al. (7).

reduced or absent. Their presence is hardly diagnosed because of the absence of symptoms. Generally, it is estimated that 70% of GCNIC-positive male subjects will develop TGCT within 7 years (38) with a median age at cancer diagnosis of 35 years.

The most widely accepted hypothesis about GCNIS origin states that they are germ cells in which an arrest of the development has occurred for an abnormal signals or inability to respond to correct signals. The cells continue to express their pluripotency markers, do not differentiate and remain quiescent until puberty. In the quiescent period, GCNIS cells could accumulate chromosomal aberrations that affect genes involved in proliferation and differentiation that lead them to an uncontrolled and potentially malignant growth (39) in coincidence with puberty, when growth signals and hormones produced by Sertoli and Leydig cells induce GCNIS to proliferate.

Previous studies on chromosomal aberrations in invasive seminoma and non- seminoma neoplasms demonstrated that 80–100% of these tumors and GCNIS cells adjacent to cancer exhibited a gain of the short arm of chromosome 12 (or smaller parts thereof) (40) usually in the form of an isochromosome, i(12p) chromosome (41).

This event suggests that gain of 12p could play a key role for TGCTs to acquire invasive ability given that GCNIS cells, that are relatively distant from the cancerous zone, normally do not present short arm of chromosome 12 gain.

In fact, the chromosomal region corresponding to 12p contains genes that could be associated to TGCT development,

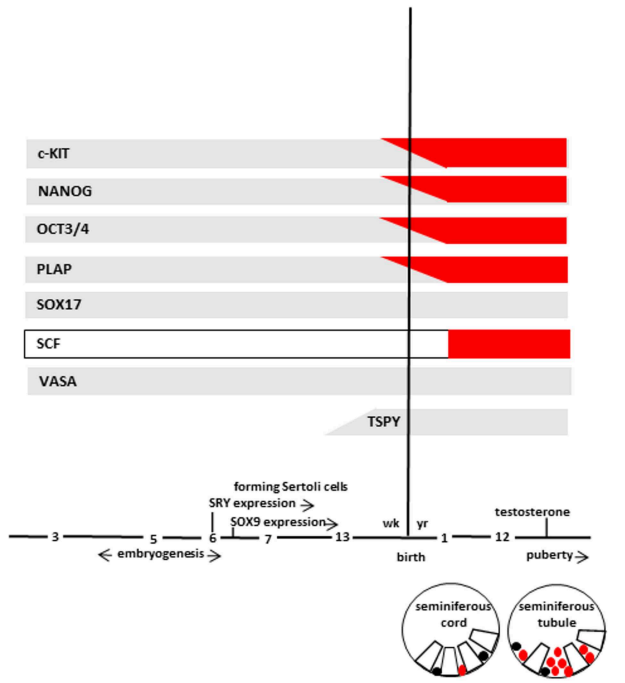

FIGURE 2 | The scheme represents marker expression from early embryogenesis to puberty during the normal (gray bar) and impaired (red bar) testicular development leading to GCNIS. Germ cells (in different stages of maturation), and GCNIS cells are represented as black or red circles, respectively. Sertoli cells are represented as open white boxes. Modified from Elzinga-Tinke et al. (7).

such as NANOG, STELLA, GDF3, and Cyclin D2 (CCND2). In particular, NANOG, STELLA, and GDF3 are pluripotency-related genes, and play an important role in embryonic stem cell self-renewal, whereas CCND2 is involved in the cell cycle regulation. These genes could similarly induce pluripotency in GCNIS (42).

Histologic and biomolecular studies demonstrated several likeness among TGCT and their precursor GCNIS cells. For example, pluripotency markers such as OCT3/4 and NANOG (43–45) are expressed in a similar way by PGCs, fetal gonocytes and GCNIS. In addition, GCNIS cells exhibit several features of PGCs and gonocytes such as the co-expression of OCT3/4 and SOX17 protein (46, 47). Moreover, high c-KIT gene expression was detected in GCNIS similarly to PGCs and fetal gonocytes but not in the adult spermatogonia (43). Similarly, an upregulated c-KIT expression was described in atypical fetal gonads thus strengthening the idea that germ cell transformation and altered testicular development might be strictly associated (48) (**Figure 2**).

Interestingly, GCNIS cells share mRNA/miRNA profiles similar to immature germ cells, and exhibit global CpG methylation erasure. This lack of epigenetic memory is a common feature of PGCs and pluripotent cell types (49).

However, even though PGCs express various biomarkers of pluripotency, they are normally unipotent to produce gametogenic stem cells, so differing from GCNIS cells that exhibit pathologic functional pluripotency.

Taken together, all these findings have led to the hypothesis that GCNIS is the intermediary cell between an arrested and transformed PGC or gonocyte during embryonic/fetal development and TGCT (50).

TABLE 1 | The table summarizes the expression of different markers in the three different stages of germ cell differentiation from PGC to spermatogonium.

Germ cell type	Marker
Gonocyte	OCT3/4 NANOG c-KIT PLAP
intermediate cell	OCT3/4 low positive or negative PCNA
Spermatogonium	MAGE4A VASA TSPY

Future Perspectives About Diagnostic Markers

Diagnosis for TGCTs is greatly based on detecting serum markers such as alfa- fetoprotein, beta-human chorionic gonadotropin, and lactate dehydrogenase but only 60% of all patients show elevations of these markers (51).

Testicular biopsy is the best current diagnostic test for detecting TGCT, even if it is burdened with false negative outcomes due to the non-random distribution of transformed cells throughout the gonad (52). New approaches are necessary to identify GCNIS before testicular cancer appearance, given that these cells can leave the testis and enter the semen where they could be detected by revealing specific markers. However, for some of the assayed markers as OCT3/4, MAGE-A4, and NY-ESO-1 a relatively low sensitivity was demonstrated (53–55).

Recently, a cell surface receptor TDGF-1 (CRIPTO) was identified in blood serum of patients where GCNIS and several tumor cell subtypes were found (56). Therefore, CRIPTO expression could be a useful serum marker for detection of testicular cancer.

Other recent studies showed that undifferentiated and potentially malignant cells could be detected *in vivo* thanks to identification of specific miRNAs (57).

In particular, miRNAs from miR-371–373 (mapped to chromosome 19) and miR- 302–367 (mapped to chromosome 4) family members are upregulated in all TGCT and elevated values could be detected in the serum, regardless of pediatric or adult age, gonadal or extragonadal localization or tumor subtype (seminomas, yolk sac tumors, or embryonal carcinomas) (58).

It's noteworthy that these miRNAs are not up-regulated in other tumor types or disorders. In perspective, detection of high levels in liquid biopsies of well-defined set of embryonic miRNA, such the **two** above mentioned "clusters," might be useful in diagnosis, prognosis and disease management of testicular malignant TGCTs given their association with undifferentiated and potentially malignant cells (59).

A more recent study based on microarray gene expression profiling and gene methylation datasets, suggests that hypomethylation-high expressed genes such as CSF1R, PTPRC, and MMP9, could be involved in TGCT (60).

CSF1R, a cell-surface protein, and PTPRC, a member of the protein tyrosine phosphatase (PTP) family, regulate several cellular activities such as cell growth, differentiation, and tumor transformation (61).

Moreover, this study demonstrates that TGCT tissue samples show up-regulated levels of MMP9, a class of enzymes involved in the degradation of the extracellular matrix.

About this, another recent study shows that activin/TGFbeta signaling within Sertoli cells of GCNIS tumors lead to increased levels of MMP2 and MMP9 metalloproteinases (18) thus strengthening the idea that Sertoli cells have an important role in supporting TGCT development. Indeed, the breakdown of the epithelial barrier by MMPs may contribute to tumor progression, thus allowing the neoplastic germ cell to move into the interstitium.

Overall, seems that higher levels of CSF1R, PTPRC, and MMP9 are related to shorter survival time of TGCT patients, suggesting that they may be involved in TGCT development.

In perspective, these genes could be useful biomarkers for diagnosis, treatment and prognosis evaluation of TGCT, constituting potential therapeutic targets for this type of cancer.

Finally, we must not overlook the fact that dysregulation between somatic and germ cells may support the formation of GCNIS cells, as demonstrated by the role of activin/TGFbeta signaling in promoting an environment advantageous for TGCT onset and progression.

CONCLUSIONS

Testicular cancer onset and development is caused by a mix of genetic, epigenetic and environmental factors. Most TGCT tumors are curable even in advanced stages thanks to cisplatin-based chemotherapy. However, side effects and complications may occur in patients treated with chemotherapeutic agents and in some cases relapse or treatment resistance may occur.

Further studies will be aimed to both develop less toxic therapies and directly target the neoplastic cells, thus overcoming the resistance to chemotherapy.

Currently, an open testicular biopsy, helpful in specific group of patients (men with atrophic testes, infertility, cryptorchidism or suspicious ultrasound), is the sole way to diagnose GCNIS as other early detection methods for TGCT are not available so far.

Obviously, for screening purposes, sensitive and specific non-invasive early detection method are necessary.

Even if genetic and environmental factor of risk (prenatal, perinatal, and postnatal) were considered able to influence the onset of GCNIS, their role in the pathogenesis of TGCT is insufficient to identify an at risk population.

Even if many cytoplasmic and nucleus markers (such as OCT3/4, NANOG, etc.) have been assessed in semen, none of these is a valid marker for GCNIS.

Instead, the detection of specific TGCT's miRNAs (miR-371∼373 and miR-302/367) in semen could be considered a promising non-invasive marker of GCNIS being highly overexpressed both in serum (in all TGCT) and in semen. In addition, MMP9, CSF1R, and PTPRC genes could be useful biomarkers for diagnosis, treatment and prognosis evaluation of TGCT.

In conclusion, improving our knowledge on the molecular mechanisms controlling GCNIS origin and malignant transformation to TGCT, might be useful to develop a noninvasive screening method for population at increased risk for TGCT.

AUTHOR CONTRIBUTIONS

TB, IA, and GL idealized the paper and wrote the first draft. FM and RC participated in literature research and paper writing. All author listed have made intellectual contribution to the work and approved the final version.

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CASE REPORT
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First Case of Mature Teratoma and Yolk Sac Testis Tumor Associated to Inherited MEN-1 Syndrome

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Introduction: Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominantly inherited endocrine tumor syndrome characterized by the development of cancer in various endocrine organs, particularly in the pituitary, parathyroid and pancreas. Moreover, in some cases, also non-endocrine tumors can be diagnosed, developing atypical phenotypes.

Case report: We report herein the clinical history of a patient affected by MEN-1 syndrome who developed atypical features for this disease. The patient's clinical history started in August 2015 when he was referred, at the age of 23 years, to the Emergency Department of our Hospital for the occurrence of progressive asthenia, weakness, tremors and syncope. The biochemical test documented hyper-calcemia and severe hypoglycemia. The patient was referred to our Neuroendocrine Tumor and Pituitary Unit and he was diagnosed with pancreatic insulinoma, hypercalcemic hyperparathyroidism, and a prolactin secreting pituitary adenoma. The MEN-1 syndrome was suspected and genetic tests for mutation of *menin* resulted positive for the pathogenic variant c1548dupG. In January 2016, the patient was diagnosed with intratubular germ cell neoplasia, consisting of a mature teratoma and yolk sac tumor and he underwent a right orchiectomy.

Conclusion: This is the first case report showing the clear association of MEN-1 syndrome with yolk sac tumors and teratomas, as in our case, the c1548dupG represents a pathogenic variant rather than a SNP. This case suggests the opportunity of an accurate evaluation of the testis particularly in young MEN-1 affected patients and that a prompt screening for neoplastic disease should involve all the endocrine glands.

Keywords: menin, SNP, neuroendocrine tumor, insulinoma, hyperparathyroidism

INTRODUCTION

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominantly inherited endocrine tumor syndrome characterized by tumor development in various endocrine organs (1, 2). MEN syndromes are infrequent inherited disorders in which more than one endocrine gland develops noncancerous (benign) or cancerous (malignant) tumors or grows excessively without forming tumors. MEN1 disease is a consequence of the *MEN1* gene mutation (3–5). The *MEN1* gene synthetizes the protein menin, that acts as a tumor suppressor, as confirmed by microsatellite analysis conducted on cancerous tissues of MEN1 patients (6, 7). The protein menin inhibits the cell proliferation through the interaction with histone-modifying enzymes, with transforming growth factor β1 (TGF-β) signaling and Wnt/β-catenin pathways and with several transcription factors (such as nuclear factor κB (NF-κB), peroxisome proliferator-activated receptors (PPARγ), and vitamin D receptor (VDR) (8). Moreover, menin can act by destroying pro-proliferative factors such as insulin-like growth factors I and II (IGF-I and IGF-II) and parathyroid hormone-related protein (PTHrP) (8). MEN-1 syndrome can present as a familial form (more common) or sporadic form. Specific gene mutations can be identified in 70–95% of cases (3–9). The most commonly diagnosed tumors in MEN-1 syndrome involve the parathyroid glands in around 95% of cases, endocrine pancreatic-gastroenteric tract in around 40% of cases and the anterior pituitary gland, in around 30% of cases (10, 11). The first presentation of MEN1, in up to 85% of patients, is a parathyroid tumor; in other cases, the first manifestation may be prolactinoma or an insulinoma (12). Other tumors can occur in MEN-1 syndrome such as adrenocortical and thyroid tumors, meningiomas, angiofibromas, collagenomas, lipomas and gastric, thymic, and bronchial carcinoids (13–19). Notably, MEN-1 syndrome can show a very variable phenotype (9). We report herein the clinical history of a patient affected by MEN-1 syndrome who developed atypical features for this disease. This feature is peculiar as it has never been described in literature. A written informed consent was obtained from the patient for the publication of this case report and any potentially-identifying images/information.

CASE REPORT

The patient's clinical history started at the age of 15 years, when he was diagnosed for minor epilepsy. The patient's actual clinical history started in August 2015 when he was referred, at the age of 23 years, to the Emergency Department of our Hospital for the occurrence of progressive asthenia, weakness, tremors and syncope. The biochemical test documented hypercalcemia and severe hypoglycemia. The glycemic value was 27 mg/dL. The patient was treated with a glucose infusion with symptoms reduction. In September 2015, the patient was admitted to our Neuroendocrine Tumor and Pituitary Unit, to perform a 72 h fasting test for a possible insulinoma. After 7 h fasting, the patient was symptomatic for hypoglycemia. The glycemic plasma value resulted as 20 mg/dL, insulin as 18.6 microIU/mL, C-peptide as 1.7 ng/mL. Again symptoms diminished following the glucose infusion. Additionally, blood tests documented a primary hyperparathyroidism with hypercalcemia (Calcium: 11.7 mg/dL, PTH: 134.5 pg/mL) and hyperprolactinemia (PRL: 220 ng/mL).

The abdominal contrast computerized tomography (CT) documented the presence of four hyper-vascular focal lesions, of <1 centimeter and localized at the pancreatic body and tail, which were suggestive for neuroendocrine tumors (NET) (**Figure 1**). A Gallium-68 labeled somatostatin receptor PET-CT an showed uptake in 3 nodules in the pancreas (**Figure 2**). Cytological findings of the endoscopic ultrasound-guided fine needle aspiration of the larger pancreatic tumor was consistent with a G2 neuroendocrine tumor, with positive immunohistochemistry for chromogranin A, synaptophysin, CDX2 and a Ki67 proliferation index of 4%. Based on the patient's clinical history, immunohistochemistry was performed for insulin and resulted positive in tumor cells. The patient underwent a thyroid and parathyroid ultrasound which resulted negative for both thyroid nodules and hyperplastic parathyroid. The parathyroid scintigraphy however showed two hyper-functioning parathyroid glands. A pituitary contrasted magnetic resonance evidenced the presence of a small pituitary adenoma with a maximum size of 8 millimeters. Consequently, the patient initiated a prophylactic treatment with diazoxide (at the starting dosage of 25 mg/daily with a subsequent dose titration up to 75 mg/daily) to prevent a potential hypoglycemia crisis, with long acting somatostatin analogs (SSA: Lanreotide Autogel 120 mg/monthly) for the pancreatic NET and with a dopamine agonist (cabergoline 0.5 mg half table twice a week) for the micro-prolactinoma.

In the family history, the patient's sister underwent successful neurosurgery to remove a pituitary prolactinoma at the age of 18 years.

According to the patient's clinical assessment and family history, a MEN-1 syndrome was suspected. Genetic testing for the mutation of *menin* resulted positive for the pathogenic variant c1548dupG, in heterozygosis.

All of the patients' first-degree relatives were tested for MEN-1 syndrome, after signing informed consents. **Figure 3** shows the index pedigree.

According to the multidisciplinary decision of the Neuroendocrine tumor (NET) board, the patient first received a total parathyroidectomy before the scheduled sub-total pancreatectomy treatment.

In November 2015, a total parathyroidectomy and a thymectomy were performed. The dosage of intra-operatory serum PTH showed a progressive reduction, from the initial value of 191.3 pg/mL to the final value of 20 pg/mL. The patient was treated with calcitriol without any occurrence of hypocalcemia. The histological examination documented a diffuse hyperplasia of all four removed parathyroid glands in the absence of thymic neoplasia/hyperplasia and only initial adipose thymic involution. One month after surgery, serum PTH concentration was of 5 pg/mL (range 14–72).

In January 2016, the patient was referred with right testicular swelling. The alpha-fetoprotein serum level was 43 ng/mL (<9). A testis ultrasound documented a hypoechoic and hyper-vascularized nodule. The patient underwent a testicular nodule resection. The histological examination showed an

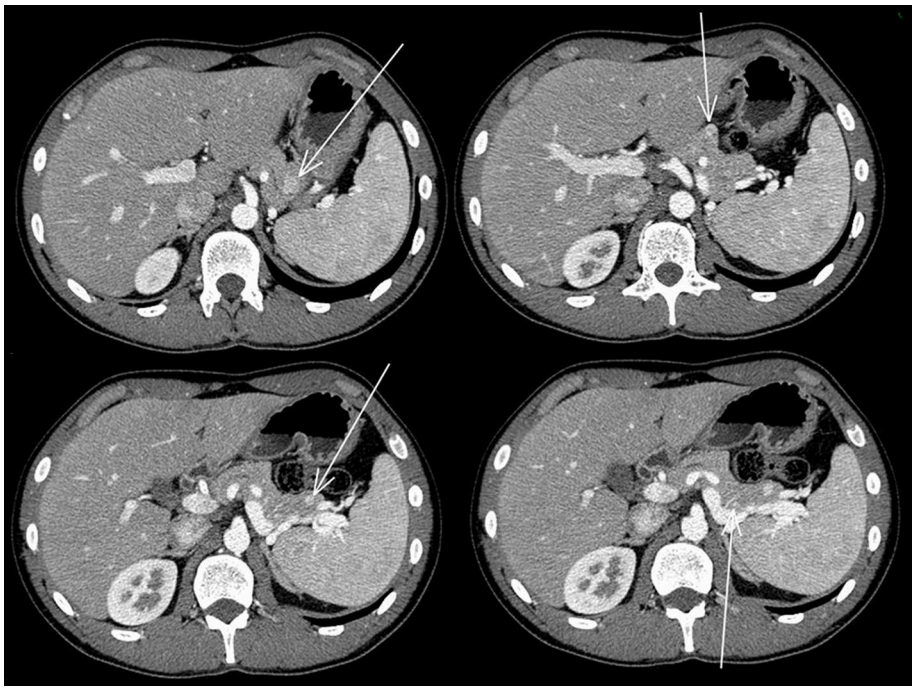

FIGURE 1 | Abdominal contrasted TC scan showed the four pancreatic neuroendocrine tumors that are indicated with the arrows.

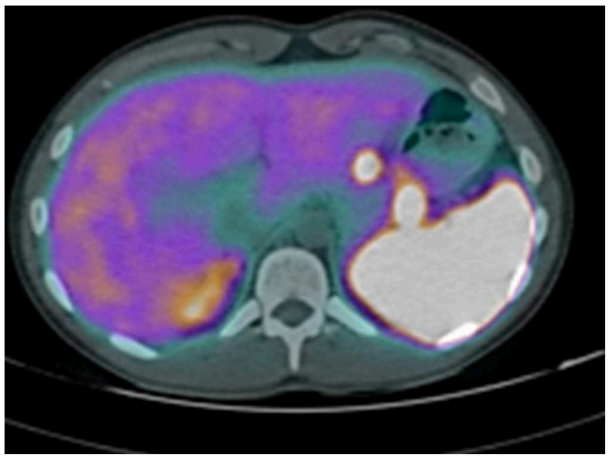

FIGURE 2 | Gallium-68 labeled somatostatin receptor PET-CT showed uptake in 3 Gallium-68 labeled somatostatin receptor PET-CT showed an uptake in 3 nodules in the pancreas.

intratubular germ cell neoplasia (IGCNU), consisting of a mature teratoma and yolk sac tumor, with signs of pre-invasive lesion, such as presence of peri-neoplastic, placental alkaline phosphatase (PLAP) and CD117 positive seminiferous tubules, with basal nuclei and abundant clear cytoplasm (**Figure 4**). According to the presence of germ cell neoplasia, in March 2016, a right orchiectomy was conducted, following germ cell cryopreservation. No neoplastic cells were detected at the histological examination of the resected testis. The post-surgery total body CT and 18F-FDG PET/CT were negative for metastasis 6 months later.

In February 2017, the patient underwent a sub-total pancreatectomy. Treatment with diazoxide was withdrawn.

The histological examination proved the presence of four pancreatic NETs: two of the pancreas body and the other two at the pancreas tail. All the lesions showed a positive immunohistochemistry for chromogranin A and synaptophysin and one only was positive for insulin. The higher mitotic index was 7 per 10 high-power fields with the Ki67 proliferation index ranging from 4 to 8%. These lesions were diagnosed as three non-functioning G2 NET and one insulinoma G2 NET. No other histological alterations were identified in the endocrine and in the exocrine residual pancreas.

At present the patient is in good clinical condition, in the absence of disease recurrence or adverse event such as diabetes mellitus or episodes of hypocalcemia or hypoglycemia. He is still on treatment with SSA and DA, with normal prolactin values. Hormonal replacement therapy with testosterone analogs was not prescribed, given the absence of referred symptoms and according to the laboratory assessment.

Blood test, abdominal CT, Gallium-68 labeled somatostatin receptor PET-CT and pituitary MR are periodically scheduled at our Neuroendocrine Tumor and Pituitary Unit.

DISCUSSION

To our knowledge, this is the first MEN1 patient who also developed an intra-tubular germ cell neoplasia of the testis. It is well-known that endocrine glands are very sensitive to the

development of noncancerous or cancerous lesions. In a recent study by Wautot et al. (20) the menin expression (detected as a 68 KDalton protein) was demonstrated in the brain cortex, the kidney, the pituitary, the testes, the thymus and in the thyroid, providing a rationale for the high risk of neoplasia development at these sites in *MEN1* patients.

We can speculate that the germline c1548dupG pathogenic variant could also play a role in the onset of this peculiar tumor of the testis. In this regard, we also tried to evaluate the status of *MEN1* copy number (CNV), however and unfortunately, we could not assess CNV since our method is set for germline blood-derived fresh DNA rather than on somatic formalin-fixed paraffin-embedded (FFPE) DNA (data not shown). As reported, MEN1 gene encodes for menin that act as tumor suppressor, as confirmed by microsatellite analysis conducted on cancerous tissues of *MEN1* patients. Therefore, although in the absence of a direct evidence, we cannot exclude the relationship between *MEN1*-mutations and testis tumor development. In addition, the c.1548dupG (p.Lys517Glufs; rs761695866) is very well-established as a pathogenic variant and reported as very rare within the population (Varsome Database). The absence of *MEN1* mutations reported for yolk sac and mature teratoma testis tumor (the tumor described in this report), within the ATLAS genome and COSMIC databases may well be due to the rarity of this tumor histotype. The testis tumor affecting our patient was classified as a non-germinomatous germ cell tumor (NGGCTs) (21) or as a type II testicular germ cell tumor (22). This group of testis neoplasia typically occurs in the third and fourth decade of life and includes seminoma, embryonal carcinoma, teratoma, yolk sac tumor, choriocarcinoma, and mixed germ cell tumors (22). All type II testicular germ cell tumors develop from a pre-invasive lesion called intratubular germ cell neoplasia unclassified (IGCNU), defined as malignant germ cells confined to the seminiferous tubules, which usually lack normal spermatogenesis (22).

Similarly, teratomas derive from pluripotent cells (23) and can be differentiated in mature and immature, according to the differentiation grade of tissue within the tumors. Fully differentiated neuroectodermal, mesodermal and endodermal elements are detected in mature teratomas. Instead, embryonic elements deriving from any or all of the three germinal cell layers are typically detected in immature teratomas (23). Teratomas are commonly located in gonads, anterior mediastinum, retroperitoneum, and sacrococcygeal region but can also involve atypical organs, such as the pituitary gland (24).

Similar to other neoplasia in the testis, several factors were suggested as being involved into the onco-genesis, such as genetic disorders and a history of cryptorchidism or testis dysgenesis. Genetic studies have suggested an association between testis oncogenesis and mutations of several genes. In particular, since 2009 there are new genetic insights starting from two testicular germ cell tumors (TGCT)- genome wide association studies (GWAS), followed by several additional TGCT-GWAS (25). In these studies some SNPs with significant associations were identified in or near the genes KITLG (KIT ligand), SPRY4 (sprouty 4: sprout-related, EVH1 domain containing 2), BAK1 (BCL2-antagonist/killer 1), DMRT1 (doublesex and mab-3-related transcription factor 1), TERT (telomerase reverse transcriptase), ATF7IP (activating transcription factor 7 interacting protein), HPGDS (hematopoietic prostaglandin D synthase), MAD1L1 (mitotic arrest deficient-like 1), RFWD3 (ring finger WD domain 3), TEX14 (testis expressed 14), and PPM1E (protein phosphatase, Mg2+/Mn2+ dependent, 1E) (25).

We underline the fact that the Elzinga-Tinke et al. review paper does not associate *MEN1* gene pathogenic variants to the etiopathogenesis of TGCT, so this is the first case report showing a clear association with yolk sac tumors and with teratomas. Furthermore, all the above mentioned GWAS studies identified only SNPs within the called genes. In our case, however, the c1548dupG represents a pathogenic variant rather than a SNP variant. This data can further support the association between our peculiar phenotype with the genotype.

This case report confirms that the early diagnosis of MEN-1 syndrome, along with appropriate screening and prompt therapeutic management of MEN-1 related neoplasia, can improve prognosis, particularly in cases of pancreatic NET, as shown in our previous experience (26, 27). In fact, in most cases, MEN-1 related tumors are diagnosed for local mass effects or for symptoms due to the overproduction of hormones (12). Although MEN-1 related tumors are usually benign, an aggressive behavior, with high risk of malignancy, as for carcinoid tumors and gastrinomas can occur (28). Consequently, in individuals with two or more MEN1-related tumors and in first-degree family members, a genetic test for MEN-1 syndrome should be offered (29). In addition, patients with the genetic diagnosis of MEN-1 syndrome should also be offered an appropriate screening and follow-up for all MEN-1 related tumors.

According to our experience and clinical practice, the integration of diagnostic modalities can improve the sensibility of each diagnostic test allowing an earlier and effective diagnosis. In particular, in our case the integration of neck ultrasound and parathyroid scintigraphy allowed the diagnosis of primary hyperparathyroidism. The sensitivity of ultrasonography is 76–87% with a positive predictive value of 93–97% and a diagnostic accuracy of 88% (30). By converse, 99 mTc-sestamibi scintigraphy has a higher sensitivity (90%) and accuracy (97.2%) then ultrasound (30). However, the concordance between scintigraphy and ultrasound is nor reached in all cases (30). On the same line CDX2 immunohistochemistry was conducted on the diagnostic cytological specimens to confirm the digestive source (and namely pancreatic) origin of tumor cells. CDX2 protein expression was reported positive in a percentage of pancreatic neuroendocrine tumors (31, 32). Similarly, PDX1, a transcription factor, was identified in metastatic NET of gastro-intestinal and pancreatic origin (31, 32).

This case suggests the opportunity of an accurate evaluation of testis particularly in affected young MEN-1 patients. Others neoplasia such as thyroid and breast tumors can be detected in patients affected by MEN1 syndrome and required an appropriate screening (33). However, as these neoplasms are common also in the general population and since the role of *MEN1* gene in the thyroid and breast cancers is uncertain, the association of thyroid and breast tumors and *MEN1* is considered incidental (33).

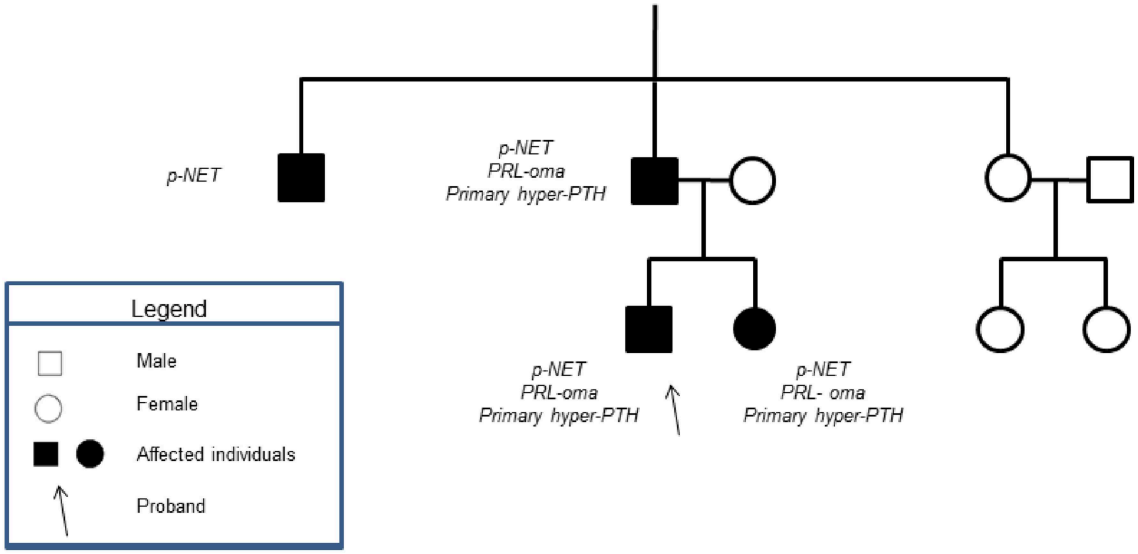

FIGURE 3 | Showed the patient's family tree. None of the patient's male relatives had history of testicular mass.

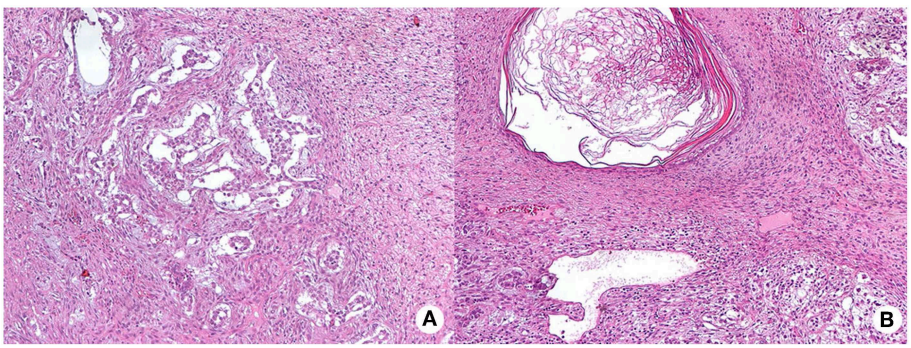

FIGURE 4 | Hematoxylin and eosin (HE) staining of testis intra-tubular germ cell neoplasia (200X magnification) composed by the yolk sac tumor, with mainly anastomosing channels that focally expand to form variably sized cysts lined by primitive tumor cells with varying amounts of clear, glycogenated cytoplasm (mycrocistic or reticular pattern, panel **A**) and of the mature teratoma, with different type of mature tissue such as squamous epithelium with keratinization (panel **B** shows a dermoid cyst).

In conclusion, this unique case report suggests that a prompt screening for neoplastic disease should involve all the endocrine glands (not only pituitary, parathyroids, and pancreas), in patients diagnosed for MEN-1 syndrome, in order to have the opportunity and the benefit of an early diagnosis of neoplasia.

DATA AVAILABILITY

No datasets were generated or analyzed for this study.

ETHICS STATEMENT

This study represents a case report. All the procedures in this case were conducted according to guidelines and according to clinical practice. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics committee of Fondazione Policlinico Gemelli, Rome and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. A written informed consent was obtained from the patient for the publication of this case report and any potentially-identifying images/information.

AUTHOR CONTRIBUTIONS

SC and EC wrote the manuscript. EC, PC, and AC conducted the genetic analysis. GS, ER, and BM conducted the oncological management and follow-up. MM conducted the pathological examination of testis

tumor. GR conducted the pathological examination of the neuroendocrine tumors. GG and DM conducted the clinical diagnosis of testis tumor. LD, AB, SC, AG, and AP conducted the endocrinological diagnosis and follow-up. All the authors reviewed and approved the manuscript version.

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SYSTEMATIC REVIEW
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Role of Viral Infections in Testicular Cancer Etiology: Evidence From a Systematic Review and Meta-Analysis

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The most represented histotype of testicular cancer is the testicular germ-cell tumor (TGCT), both seminoma and non-seminoma. The pathogenesis of this cancer is poorly known. A possible causal relationship between viral infections and TGCTs was firstly evoked almost 40 years ago and is still a subject of debate. In the recent past, different authors have argued about a possible role of specific viruses in the development of TGCTs including human papillomavirus (HPV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), Parvovirus B-19, and human immunodeficiency virus (HIV). The aim of this present review was to summarize, for each virus considered, the available evidence on the impact of viral infections on the risk of developing TGCTs. The review was reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. We included all observational studies reported in English evaluating the correlations between viral infections (HPV, CMV, EBV, Parvovirus B19, and HIV) and TGCTs. The methodological quality of studies included in the meta-analysis was evaluated using a modified version of the "Newcastle–Ottawa Scale." Meta-analyses were conducted using the "Generic inverse variance" method, where a pooled odds ratio (OR) was determined from the natural logarithm (LN) of the studies' individual OR [LN (OR)] and the 95% CI. A total of 20 studies (on 265,057 patients) were included in the review. Meta-analysis showed an association with TGCTs only for some of the explored viruses. In particular, no association was found for HPV, CMV, and Parvovirus B-19 infection (p = ns). Conversely, EBV and HIV infections were significantly associated with higher risk of developing TGCTs (OR 7.38, 95% CI 1.89–28.75, $p = 0.004$; OR 1.71, 95% CI 1.51–1.93, $p < 0.00001$). In conclusion, we found adequate evidence supporting an oncogenic effect of HIV and EBV on the human testis. Conversely, available data on HPV and TGCTs risk are conflicting and further studies are needed to draw firm conclusions. Finally, current evidence does not support an effect of CMV and Parvovirus B-19 on testicular carcinogenesis.

Keywords: viral infections, testicular cancer, human papillomavirus, human immunodeficiency virus, cytomegalovirus, Epstein–Barr virus, Parvovirus B-19

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INTRODUCTION

Testicular cancer (TC) is the most common solid tumor affecting males between 20 and 40 years old and accounting for approximately 1–1.5% of all cancers in men (1, 2). In the last decades, its incidence showed a progressive increase, particularly in some regions of Europe and Northern America (3–5). It is a real variegate cancer, characterized by several histological patterns, comprising germ-cell tumors and non-germ-cell tumors. The former group is the most common and it is further subdivided into two histologic subtypes, namely, seminomas and non-seminomas (6).

Many risk factors have been proposed for TGCTs (7, 8) including cryptorchidism, genetics, and substances of abuse (i.e., drugs, smoke, and hormones). In addition, it is well known that some testicular lesions mimicking a testicular tumor are due to infectious pathology, especially in immunosuppressed patients. Nevertheless, the possible causal relationship between viral infections and TGCTs is still a subject of debate.

Despite the exposure to some viruses that have been certainly associated to other cancer types in males [Epstein–Barr virus (EBV) for Burkitt lymphoma, human immunodeficiency virus (HIV) for Kaposi's sarcoma, hepatitis B virus (HBV) and hepatitis C virus (HCV) for hepatocellular carcinoma, and human papilloma virus (HPV) for penile, oropharyngeal, and anal cancers], few studies evaluated the possible implications of viral infection in the pathogenesis of TGCTs. Curiously, most of the viruses involved in sexually transmittable disorders have an age-related prevalence that coincides with that observed in TGCTs. Moreover, the characteristic long latency and persistence in the host of several viruses could induce a long-term dysregulation of the cell cycle able to induce the cancer development. Again, some studies demonstrated the prevalence of viral DNA/RNA directly in tissue specimens from testicular cancer (9–11). Finally, it has been reported that EBV, as well as other DNA viruses, encodes a protein able to inactivate p53, a mechanism that is able to reduce apoptosis in tumor cells (10, 12).

In this meta-analysis, we aimed to summarize the whole body of literature exploring the correlation between TGCTs and viral infections by HPV, HIV, cytomegalovirus (CMV), EBV, and Parvovirus B-19, with the purpose of clarifying the possible role of these viruses in the pathogenesis of this condition.

MATERIALS AND METHODS

Study Design

This is a systematic review and meta-analysis of published data. The review was reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (13).

Ethical Approval

As this study was a systematic review and meta-analysis of published data, formal ethical approval was not required.

Search Strategy

Electronic databases (Sciencedirect, Medline, Scopus, Embase, the Cochrane library, Clinicaltrials.gov, EU Clinical Trials Register and World Health Organization International Clinical Trials Registry) were searched until 1st February 2019 (without date restriction).

Key search terms were as follows: virus OR viral infection OR viral disease OR human papillomavirus OR HPV OR Cytomegalovirus OR CMV OR Epstein–Barr virus OR EBV OR Parvovirus B19 OR human immunodeficiency virus OR HIV OR acquired immunodeficiency syndrome OR AIDS AND testicular cancer OR testicular neoplasm OR testicular tumor. The electronic search and the eligibility of the studies were independently assessed by two of the authors (AG and FM).

Inclusion Criteria

We included all studies evaluating the correlations between viral infections (i.e., HPV, CMV, EBV, Parvovirus B19, and HIV) and TGCTs. All observational studies (retrospective and prospective cohort studies, case and control series) reported in English were eligible. Testicular cancer was defined as the demonstration of testicular cancer cells at histopathological examination.

Study Selection and Data Extraction

Two authors (AG and AV) independently assessed the inclusion criteria and study selection. Disagreements were discussed with a third reviewer (CF).

Data extraction was performed by five independent investigators (AA, GA, FM, MG, and UV). When studies involved a subgroup of patients considered negligible for the endpoints of meta-analysis (e.g., patients affected by non-testicular cancer), the authors provided only a qualitative data extraction. A manual search of reference lists of studies was performed to avoid missing relevant publications. One author (AV) reviewed the selection and data extraction process. The results were then compared, and any disagreement was discussed and resolved by consensus.

Risk of Bias

Two reviewers (AV and AG) independently judged the methodological quality of studies included in the meta-analysis using a modified version of the "Newcastle–Ottawa Scale" (14). Quality of studies was evaluated in five different domains: "*sample representativeness,*" "*sampling technique,*" "*ascertainment of viral infection,*" "*quality of description of the population and confounders,*" and "*incomplete data on cancer histology*" (**Table S1**). According to the total number of points assigned, each study was judged to be at low risk of bias ($\geq$3 points) or high risk of bias (<3 points). Any discrepancies concerning the author's judgments were referred to a third reviewer (CF) and resolved by consensus.

Statistical Analysis

Odds ratios (ORs) and proportions were calculated with MedCalc 18.5 (MedCalc Software, Seoul, 158-051, Korea). For meta-analysis, Review Manager (RevMan) Version 5.1 (The Nordic Cochrane Centre, The Cochrane Collaboration, 2011) was used. Statistical analysis was conducted using the "Generic inverse variance" method, where a pooled OR was determined from the natural logarithm (LN) of the studies' individual OR [LN (OR)] and the 95% CI. The SE for the LN (OR) was calculated from the

95% CI using the formula: SE = [LN (upper CI limit) – LN (lower CI limit)]/3.92, according to the Cochrane Reviewers' handbook (15). Statistical heterogeneity was assessed by I^2 statistics. The pooled estimates were reported graphically with Forest plots. Meta-analyses were conducted separately for each virus (HPV, EBV, CMV, Parvovirus B-19, and HIV). Sources of statistical heterogeneity were investigated by subgroups and sensitivity analyses (by serially excluding each study or study subgroups basing on methodological quality judgments).

RESULTS

Study Selection

Starting from 198 selected abstracts, we evaluated 163 full texts regarding infection of interest in subjects with testicular cancer. Because of confounding conditions (infection after diagnosis of TC infection during or after radio and/or chemotherapy, animal studies, reviews, and case reports), 138 studies were excluded. Finally, a total of 25 studies were included in the present meta-analysis: 4 for HPV, 8 for EBV, 5 for CMV, 5 for Parvovirus B19, and 3 for HIV (**Figure 1**).

Included Studies

Characteristics of included studies are summarized in **Table 1**. We included in the review a total of 19 studies. Four studies provided information for two viruses (19, 21–23) and one for three (24) viruses, reaching the number of 25. Studies embedded a total of 285,878 subjects. Most of the studies were case–control except for one case series, for both HPV and EBV (19) and one survey for HPV (18). All studies on HIV were cohort studies (31–33). Different techniques to diagnose viral infections were used. Most studies used PCR or detection of serum antibodies. Six studies used immune-histochemistry (IHC), four used *in situ* hybridization (ISH), one used fluorescent *in situ* hybridization (FISH), and one used immunofluorescent staining (IFS).

Assessment of the Risk of Study Bias

In **Table 2**, the criteria used to assess the risk of study bias are reported.

- *Sample representativeness:* All but six studies (10, 19, 24, 25, 27, 30) were judged at low risk of bias for sample representativeness.
- *Sampling technique*: Eleven studies (16, 17, 20–22, 24, 26, 29, 31–33) had adequate sampling strategy (random or consecutive). Other studies did not provide data.
- *Ascertainment of viral infection:* One study (20) was judged at high risk of bias because the diagnosis of viral infection was based on self-administered questionnaires. The remaining studies were at low risk of bias.
- *Quality of description of the population and confounders:* Only three studies were considered at low risk of bias (17, 20, 28). Other studies did not provide adequate description of the study population and/or confounders.
- *Incomplete data on histology*: All but two studies (17, 31) provided adequate data on cancer histotypes.
- *Overall study quality*: In summary, pooling of scores for each domain resulted in five studies to be at high risk of bias (10, 19, 25, 27, 30). The remaining studies were at low risk of bias (16–18, 20–24, 26, 28, 29, 31–33).

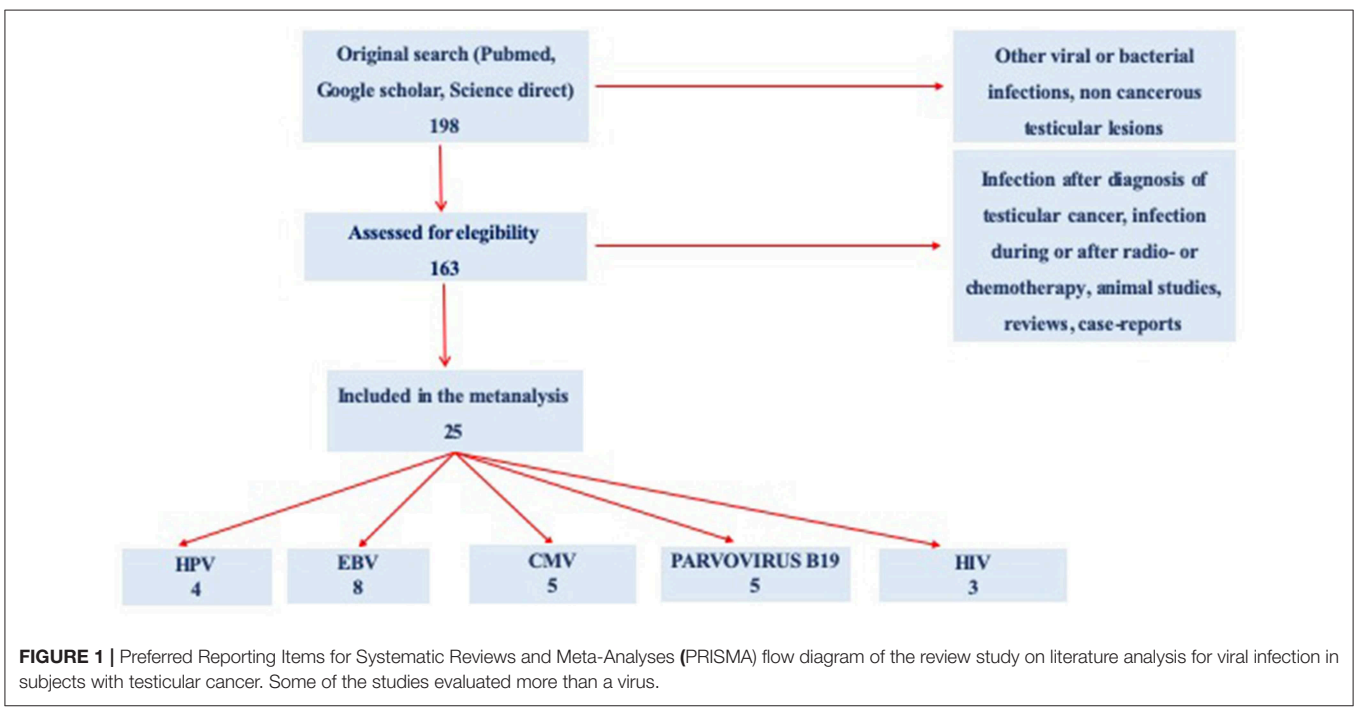

FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses **(**PRISMA) flow diagram of the review study on literature analysis for viral infection in subjects with testicular cancer. Some of the studies evaluated more than a virus.

TABLE 1 | General features of included studies.

Virus	References	Country	Setting	Population	Method	Measure	Result (95% CI)
HPV	Bertazzoni et al. (16)	Italy	Case–control	Cases 61 Controls 23	PCR	Percentage	0% Cases 0% Controls
HPV	Garolla et al. (17)	Italy	Case–control	Cases 155 Controls 84	PCR and FISH	Percentage	9.7% Cases 2.4% Controls
HPV	Strickler et al. (18)	USA	Survey	Total 87 (TC 39)	ELISA	Percentage	5% of TC
HPV	Rajpert-De Meyts et al. (19)	Denmark	Case series	Cases 19 Controls 1	PCR and IHC	Number of cases	0% Cases 0% Controls
EBV	Moss et al. (20)	USA	Case–control	Cases 173 Controls 217	Interview	OR	0.6 (0.3–1.1)
EBV	Algood et al. (21)	USA	Case–control	Cases 56 Controls 30	Serology	Percentage	80% Cases 30% Controls
EBV	Akre et al. (22)	Norway	Case–control	Cases 81 Controls 242	Serology	OR	2.74 (0.62–12.12)
EBV	Shimkage et al. (10)	Japan	Case–control	Cases 27 Controls 25	PCR, IHC, and IFS	Percentage	100% Cases 0% Controls
EBV	Heinzer et al. (23)	Germany	Case–control	Cases 53 Controls 51	Serum antibodies	OR	6.93 (0.8–59.8)
EBV	Gray et al. (24)	Switzerland	Case–control	Cases 39 Controls 12	PCR	Percentage	0% Cases 0% Controls
EBV	Rajpert-De Meyts et al. (19)	Denmark	Case series	Cases 19 Controls 1	PCR, IHC, and ISH non-radioactive	Proportion	6/19
EBV	Fend et al. (25)	Austria	Case–control	Cases 32 Controls 5	PCR and ISH non-radioactive	Percentage	12.5% Cases 0% Controls
CMV	Mueller et al. (26)	Sweden	Case–control	Cases 117 Controls 100	Serology	R.R.	2.0 (1.1–3.6)
CMV	Akre et al. (22)	Norway	Case–control	Cases 81 Controls 242	Serology	OR	1.08 (0.6–1.94)
CMV	Algood et al. (21)	USA	Case–control	Cases 56 Controls 10	Serology	Percentage	50% Cases 10% Controls
CMV	Heinzer et al. (23)	Germany	Case–control	Cases 47 Controls 47	ISH and Serum antibodies	Percentage	4.2% Cases 0% Controls
CMV	Gray et al. (24)	Switzerland	Case–control	Cases 39 Controls 12	PCR	Percentage	0% Cases 0% Controls
Parvo B19	Polzc et al. (27)	USA	Case–control	Cases 23 Controls 7	PCR and IHC	Percentage	73.9% Cases 85.7% Controls
Parvo B19	Gray et al. (24)	Switzerland	Case–control	Cases 39 Controls 12	PCR	Percentage	85% Cases 0% Controls
Parvo B19	Ergunay et al. (28)	Turkey	Case–control	Cases 56 Controls 66	PCR	Percentage	5.4% Cases 0% Controls
Parvo B19	Tolfvenstam et al. (29)	Norway	Case–control	Cases 77 Controls 238	PCR, Serology and IHC	OR	1.03 (0.6–1.77)
Parvo B19	Diss et al. (30)	UK	Case–control	Cases 20 Controls 10	PCR and IHC	OR	1.01 (0.59–1.72)
HIV	Dihr et al. (31)	India	Cohort	Total 251 (5 TC)	Serology	PIR	2.5 (1.04–6.05)
HIV	Goedert et al. (32)	USA	Cohort	Total 268.950 (217 TC)	Serology	SIR	1.7 (1.5–1.9)
HIV	Grulich et al. (33)	Australia	Cohort	Total 13.067 (10 TC)	Serology	SIR	1.46 (0.7–2.69)

FISH, fluorescent in situ hybridization; IHC, immune-histochemistry; IFS, immunofluorescent staining; ELISA, enzyme-linked immunosorbent assay; PIR, proportioned incidence ratio.

Synthesis of Results

Human Papilloma Virus

A total of four studies evaluated the correlation between HPV infection and TGCTs. The pooled sample of patients analyzed was 430, of whom 274 were affected by TGCTs and 156 were healthy controls (**Figure 2**). In two studies, the search for HPV was conducted on histological sections from testicular tissue using PCR (16, 19). In the remaining two studies, the diagnosis of HPV infection was achieved by detection of serum antibodies (18) and by evaluating sperm infection by FISH and PCR (17).

Pooling of results did not show an association between HPV infection and TGCTs (OR 2.79, 95% CI 0.84–9.29,

TABLE 2 | Authors' judgment of study quality according to the "Modified Newcastle–Ottawa Risk of Bias Scoring System."

References	Sample representativeness	Sampling technique	Diagnostic accuracy	Confounders description	Cancer histology	Total score	Risk of bias
Bertazzoni et al. (16)	*	*	*		*	4	LOW
Garolla et al. (17)	*	*	*	*		4	LOW
Polzc et al. (27)			*		*	2	HIGH
Moss et al. (20)	*	*		*	*	4	LOW
Algood et al. (21)	*	*	*		*	4	LOW
Akre et al. (22)	*	*	*		*	4	LOW
Shimkage et al. (10)			*		*	2	HIGH
Heinzer et al. (23)	*		*		*	3	LOW
Gray et al. (24)		*	*		*	3	LOW
Rajpert-De Meyts et al. (19)			*		*	2	HIGH
Fend et al. (25)			*		*	2	HIGH
Mueller et al. (26)	*	*	*		*	4	LOW
Ergunay et al. (28)	*		*	*	*	4	LOW
Tolfvenstam et al. (29)	*	*	*		*	4	LOW
Diss et al. (30)			*		*	2	HIGH
Strickler et al. (18)	*		*		*	3	LOW
Dihr et al. (31)	*	*	*			3	LOW
Goedert et al. (32)	*	*	*		*	4	LOW
Grulich et al. (33)	*	*	*		*	4	LOW

*The symbol * indicate the presence of the criterion considered in the table.*

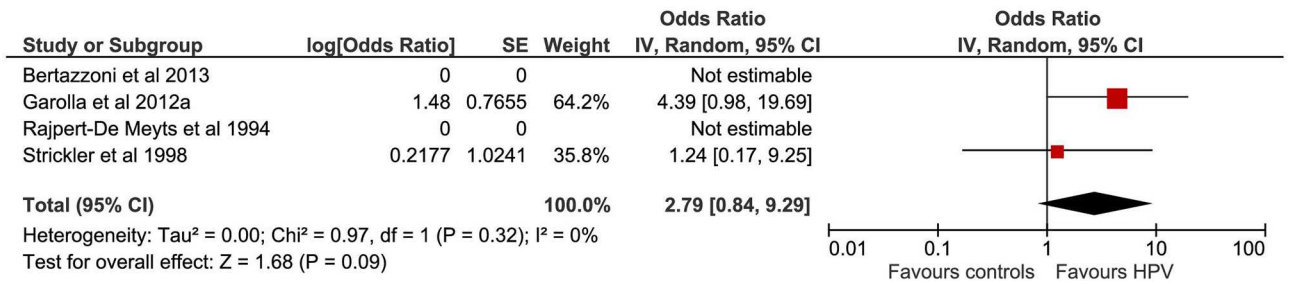

FIGURE 2 | Forest plot: Human papilloma virus (HPV) and testicular cancer.

$p = 0.09$, $I^2 = 0\%$). Sensitivity analysis did not provide statistical changes to aggregate results. Subgroup analysis was not feasible.

Epstein–Barr Virus

A total of eight studies evaluated the correlation between EBV infection and TGCTs. The pooled sample of patients analyzed was 1,063, of whom 480 were affected by TGCTs and 583 were controls (**Figure 3**). In four studies, the search for EBV was conducted on histological sections from testicular tissue by using ISH, IFS, and PCR (10); PCR (24); and PCR, IHC, and on-radioactive ISH (19, 25). In a single study, the history of EBV infection was evaluated by telephone interview (20). In the remaining three studies, EBV diagnosis was achieved by detection of serum antibodies (21–23).

Pooling of results did not show an association between EBV infection and TGCTs (OR 4.78, 95% CI 1.01–22.64, $p = 0.05$), with high degree of statistical heterogeneity ($I^2 = 84\%$). The exclusion of the study by Moss et al. from meta-analysis resulted in a significant association between EBV and TGCTs (OR 7.38, 95% CI 1.89–28.75, $p = 0.004$, $I^2 = 59\%$). Subgroup analysis based on the methods for EBV determination (serology vs. testicular tissue analysis vs. interview) found a significantly higher risk of TGCTs in those patients with a positive serology (test for subgroup differences: $\chi^2 = 24.1$, $p < 0.00001$). The proportion of seminomas among EBV+ patients (at serology) with a diagnosis of TGCTs was 51.69% (95% CI 44.01–59.32%).

Cytomegalovirus

A total of five studies evaluated the correlation between CMV infection and TGCTs (**Figure 4**). The pooled sample of patients analyzed was 751, of whom 340 were affected by TGCTs and 411 were controls. In a single study, the search for CMV was conducted on histological sections from testicular tissue by PCR (24). In four studies, CMV diagnosis was achieved by detection

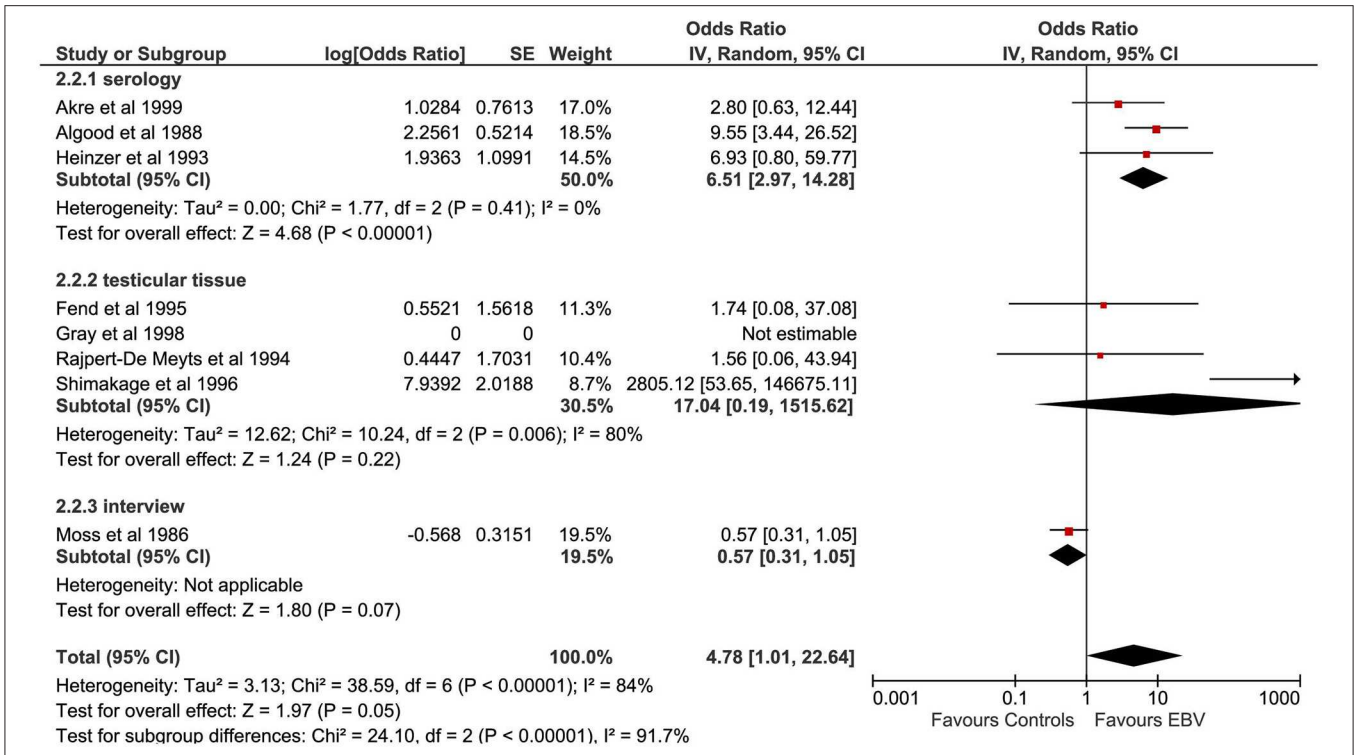

FIGURE 3 | Forest plot: Epstein–Barr virus (EBV) and testicular cancer.

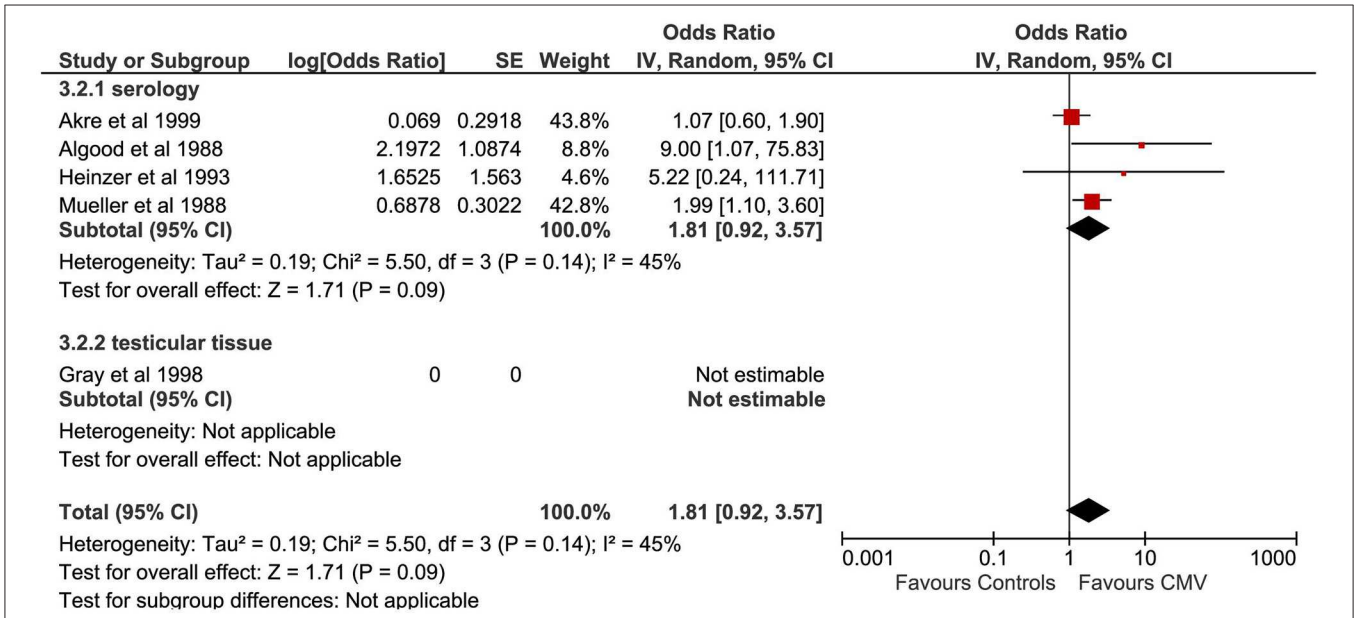

FIGURE 4 | Forest plot: Cytomegalovirus (CMV) and testicular cancer.

of serum antibodies (22, 23, 26). One study used indirect immunofluorescence assay (21).

Pooling of results did not show an association between CMV infection and TGCTs (OR 1.81, 95% CI 0.92–3.57, $p = 0.09$), with moderate statistical heterogeneity ($I^2 = 45\%$). The exclusion of the study by Akre et al. from the meta-analysis resulted in a significant association between CMV and TGCTs (OR 2.38, 95% CI 1.24–4.53, $p = 0.009$) and reduced the heterogeneity

($I^2 = 4\%$). The proportion of seminomas among CMV+ patients (at serology) with a diagnosis of TGCTs was 50.47% (95% CI 42.95–57.97%). Subgroup analysis was not feasible.

Parvovirus B-19

A total of five studies evaluated the correlation between Parvovirus B-19 infection and TGCTs (**Figure 5**). The pooled sample of patients analyzed was 548, of whom 215 were affected by TGCTs and 333 were controls. In a single study, the search for Parvovirus B-19 was conducted by detection of serum antibodies (29). In four studies, Parvovirus B-19 diagnosis was achieved by analyzing samples of testicular tissue by PCR and IHC (27, 30) or only PCR (24, 28).

Pooling of results did not show an association between Parvovirus B-19 infection and TGCTs (OR 1.85, 95% CI 0.37–9.15, $p = 0.45$), with substantial statistical heterogeneity ($I^2 = 73\%$). The serial exclusion of each single study through sensitivity analysis as well as subgroup analysis did not modify the results of the primary analysis.

Human Immunodeficiency Virus

A total number of three cohort studies (31–33) evaluated the correlation between HIV infection and testicular cancer (**Figure 6**). The pooled sample of patients analyzed was 282,268 HIV+, of whom 232 were affected by TGCTs. In all patients, the diagnosis of HIV was confirmed by serology.

Pooling of results showed a significant association between HIV infection and TGCTs (OR 1.71, 95% CI 1.51–1.93, $p < 0.00001$), with low heterogeneity ($I^2 = 0\%$). The serial exclusion of each single study through sensitivity analysis did not modify the results of the primary analysis. The proportion of seminomas among HIV+ patients with a diagnosis of TGCTs was 75.17% (95% CI 69.01–80.66%).

DISCUSSION

Testicular cancer is the most common neoplasm affecting males between 20 and 40 years old and accounting for approximately 1–1.5% of all cancers in men (1, 2). It embraces several histotypes of cancer, classified into the two main groups of seminomas and non-seminomas by the World Health Organization (6).

The pathogenesis of TGCTs is poorly known (34). Genetic factors play an important role in the development of this disease, as demonstrated by the modified expression of specific genes in testicular cancer cells (34, 35). Moreover, the exposition to different environmental agents, such as pesticides and non-steroidal estrogens (i.e., diethylstilbestrol), can increase the risk of developing this neoplasm (35, 36). Additional risk factors correlated to the onset of TGCTs are cryptorchidism, Klinefelter's syndrome, congenital abnormalities, and infertility (2, 8).

Recent efforts in oncological and virological research have brought to light the oncogenic potential of different virus species (37). It is now estimated that ∼10% of worldwide cancers are attributable to viral infections, with the vast majority (85%) occurring in the developing world (37, 38). A possible causal relationship between viral infections and TGCTs was firstly evoked almost 40 years ago. Newell et al. (39) postulated a "viral theory" starting from the evidence of a similar geographical and age distribution of TGCTs and classical Hodgkin's lymphoma. In Hodgkin's lymphoma, the malignant Reed–Sternberg cells display a monoclonal profile where EBV DNA and RNA have been clearly identified (40, 41). Differently, data about EBV DNA and RNA within TGCT cells are few and the etiopathogenetic role of EBV in testicular carcinogenesis is still a matter of debate.

In the recent past, different authors have argued about a possible role of other oncogenic viruses in the development of TGCTs, including CMV, HIV, HPV, and Parvovirus B-19. The aim of this present review was to summarize the available evidence on the impact of viral infections on the risk of developing TGCTs.

Main Findings and Interpretation

A total of 19 studies (10, 16–33) were included in this present systematic review and meta-analysis.

The correlation between HPV infection and TGCTs was evaluated by four studies on 430 patients (16–18, 31). Statistical analysis failed to demonstrate a statistical correlation between HPV infection and increased risk of TGCTs ($p = 0.09$). Notably, there was high between-studies heterogeneity in terms of methodology, potentially limiting drawing firm conclusions from the data.

HPV is one of the most common sexually transmitted viruses (42). It is particularly common in a young sexually active population and its prevalence is closely related to sexual behavior (3). During infection, HPV gains access to the interior of the cells, exerting a direct control on the proliferation and apoptosis of host cells (43). Once inside the cell, HPV DNA can transition from an episomal to a host genome integrated form, thus regulating cell genome transcription. Two specific HPV genes, namely, E6 and E7, are highly conserved among oncogenic HPV genotypes (44, 45). These genes can promote cellular transformation and alter the pathways related to the immune response, leading to carcinogenesis in a plethora of human tissues including vulva, vagina, penis, anus, head, neck, and oropharyngeal cavity (46, 47). In the testis, HPV is capable of directly infecting the male gametes, resulting in reduced fertility due to increased sperm DNA fragmentation and aneuploidy. It is thought that HPV is attached to the spermatozoa in two distinct sites along the equatorial region of the spermatozoon's head, similarly to other viruses infecting the sperm (42, 48).

Nevertheless, concurrently with new insights about infertility causes and treatments (49, 50), we must stress that the majority of recent studies on HPV in males have focused on the impact of viral infection on fertility, oocyte fertilization rate, and miscarriage rate in assisted reproduction technologies (ARTs) (51, 52). Conversely, the data on the association between HPV and TGCTs are scanty. Given the well-known oncogenic potential of HPV and considering its tropism for testicular tissue, the role of this virus in testicular carcinogenesis cannot be excluded. Future good-quality evidence is still needed to clarify the issue.

The association between EBV infection and TGCTs was investigated by eight studies on 1,063 patients (10, 19–25). While pooling of results from all studies did not show an association

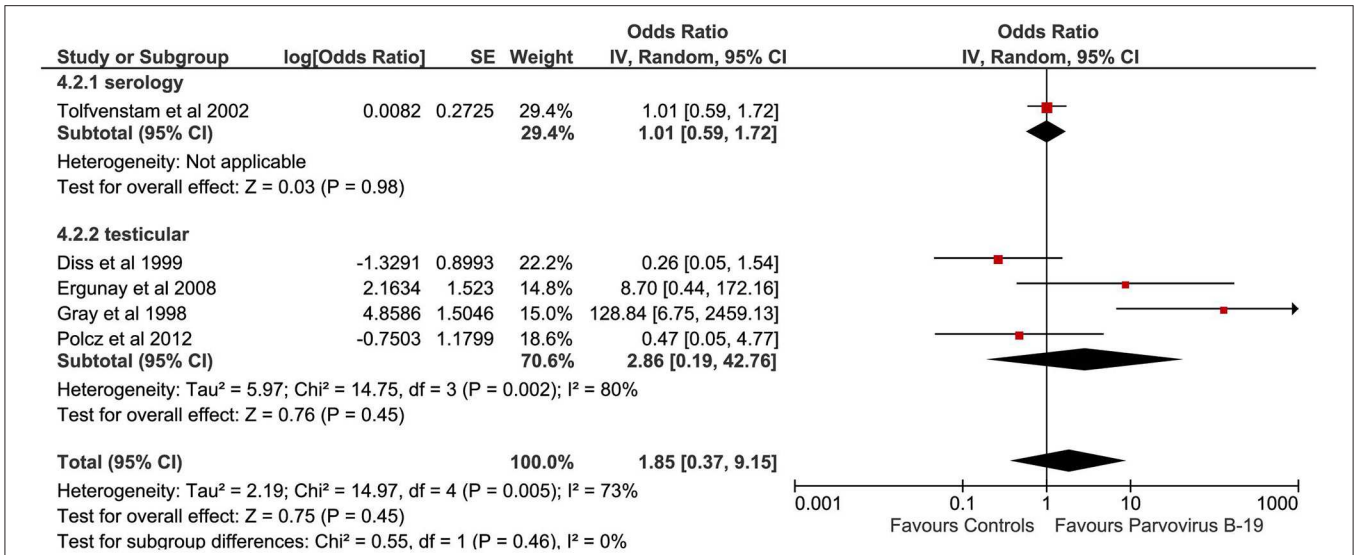

FIGURE 5 | Forest plot: Parvovirus B-19 and testicular cancer.

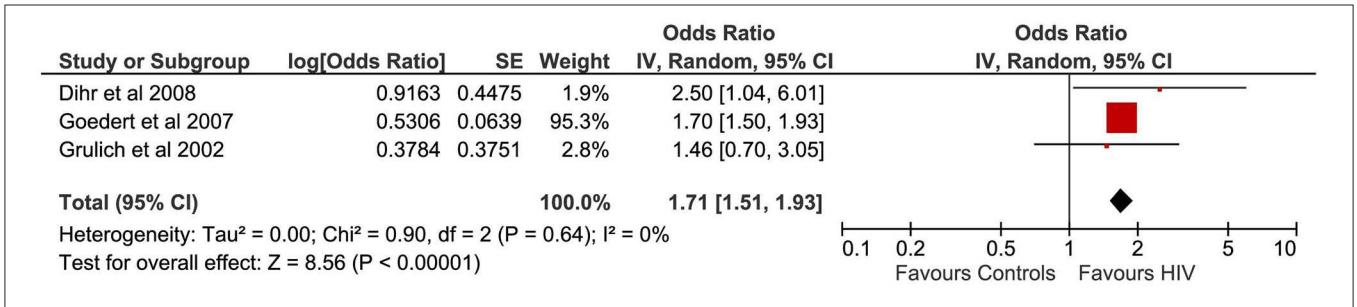

FIGURE 6 | Forest plot: Human immunodeficiency virus (HIV) and testicular cancer.

between EBV infection and TGCTs ($p = 0.05$), the exclusion of a single study from meta-analysis (20) resulted in a significant association between EBV and TGCTs ($p = 0.004$). Importantly, the study by Moss et al. (20) was at high risk of detection bias (i.e., the infection was investigated through a telephone interview), potentially distorting the final effect estimates from meta-analysis. Additionally, subgroup analysis (based on the methods for EBV determination) found a significant higher risk of TGCTs in those patients with a positive serology ($p < 0.00001$), further confirming the association between history of EBV infection and TGCTs onset.

EBV was the first virus shown to cause cancer in humans (53). Besides the well-known association between EBV and Burkitt lymphoma (discovered by Michael Anthony Epstein and Yvonne Barr in 1964) (54), this virus was found to be associated with many other lymphoid, epithelial, and mesenchymal cancers (55). EBV can promote carcinogenesis in both immune-competent hosts and immune-compromised patients (i.e., those who have undergone organ transplantation or who are under immune-suppressive treatments) (53, 56). The mechanisms of EBV-induced carcinogenesis rely on extensive methylation of the host genome, which promotes viral propagation and cellular transformation. The most common oncogenic DNA modifications associated with EBV are phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) mutations, extreme DNA hypermethylation, and amplification of the Janus activated kinase2 (JAK2) (57, 58).

Regarding EBV and TGCTs, there is an epidemiological correlation between these two entities. The incidence of infectious mononucleosis (including EBV-correlated orchitis) is higher in Europe and North America, similar to that of TGCTs. Interestingly, both infectious mononucleosis and TGCTs mainly occur in adolescents, suggesting that testicular differentiation is a factor increasing the susceptibility for both EBV infection and testicular carcinogenesis. (10) Moreover, nasopharyngeal carcinoma (which is linked to EBV) and TGCTs have some common characteristics, including age peak incidences in adolescents and chemoresponsiveness to cisplatin (59). Therefore, this present review found adequate evidence supporting a role for EBV in TGCTs development.

The correlation between CMV infection and TGCTs was investigated by five studies, including 751 patients (19, 21–24, 26). While pooling of results did not show an association between CMV infection and TGCTs ($p = 0.09$), the exclusion of

a single study (22) from meta-analysis resulted in a significant association between CMV and cancer ($p = 0.009$). Notably, the methodological quality of the study by Akre et al. (22) was fair. Therefore, based on available data, the correlation between CMV and TGCTs cannot be sustained.

CMV is a ubiquitous herpes virus that leads to a lifelong persistence (60). The prevalence of CMV infection is extremely high in the general adult population (from 50 to 100%) and the virus is not considered to be oncogenic (61). Probably, due to the high prevalence of the infection, a larger sample of patients would be required to show (or repudiate) any correlation between CMV and TGCTs.

Actually, even if CMV can lead to dramatic complications in immunocompromised individuals, murine experiments repeatedly failed to demonstrate a clear ongogenic activity for this virus (62, 63). In this regard, some authors postulated that CMV may contribute to oncogenesis by "hit-and-run" mechanisms, namely, by inducing human cell transformation and successively disappearing by malignant cell clones (64, 65). However, this theory is not adequately supported by scientific data and the oncogenic potential of CMV is still obscure.

A total of five studies (on 548 patients) evaluated the correlation between Parvovirus B-19 infection and TGCTs (24, 27–30). Pooling of results failed to demonstrate an association between Parvovirus B-19 infection and TGCTs ($p = 0.45$). Additionally, subgroup and sensitivity analyses did not modify the results of the primary analysis, confirming their robustness. Parvovirus B-19 is the only parvovirus known to be pathogenic for humans (66). The virus exhibits a particular tropism for erythroid cells and can rarely cause dramatic complications in humans (67, 68). In immunocompetent hosts, the virus can cause acute, generally self-limiting clinical manifestations including the fifth disease in children and acute polyarthritis in adults (66). In immunosuppressed hosts (including pregnant women), Parvovirus B-19 may cause severe complications including glomerulonephritis, vasculitis, peripheral neuropathies, myocarditis, fulminant hepatic failure, and aplastic anemia (69, 70). There is no robust evidence supporting the role of this virus in human cells oncogenesis, even if a recent study showed a possible correlation with thyroid cancer (71). However, available data do not support the role of Parvovirus B-19 in the etiology of TGCTs.

Three studies, performed on 282,268 patients, evaluated the correlation between HIV infection and TGCTs (31–33), showing a significant association between these two entities ($p < 0.00001$). The results were robust and displayed a low statistical heterogeneity ($I^2 = 0\%$).

The correlation between HIV-induced immunodeficiency and increased cancer risk has long been known (72). In immunocompetent people, the immune system has the ability to suppress oncogenic viruses and exert a continuous surveillance for malignant cells. These biological functions can fail when the immune system is impaired by HIV infection (73, 74). Therefore, HIV infection may promote testicular carcinogenesis mainly through indirect effects on the regulation of cell proliferation and apoptosis (72). Conversely, a direct effect of HIV on proto-oncogen expression in the human testis has not been demonstrated.

Interestingly, the majority of men dying of AIDS have hypospermatogenesis, spermatogenic arrest, or a Sertoli-cell-only testicular histology (75, 76). These histological changes are typically found in patients with TGCTs, supporting the theory that men with HIV may display a premalignant testicular atrophy. This premalignant condition may be due to the general debilitating effects of HIV rather than due to specific HIV-related mechanisms. To support this hypothesis, those patients effectively treated with antiretroviral drugs have a decreased incidence of testicular atrophy and TGCTs (77, 78). Therefore, we can conclude that HIV is associated with a significant higher risk of TGCTs, but effective antiretroviral therapy may considerably attenuate the risk of suffering from this condition.

Strength and Limitations

The present meta-analysis comprehensively evaluates the impact of viral infection on TGCTs risk. We planned sensitivity and subgroup analysis in order to reduce bias related to study heterogeneity. Moreover, we created a modified Newcastle–Ottawa scoring system (*ad hoc*) in order to provide a methodological quality judgment for each study that may help readers in a proper interpretation of the study findings. However, our results are considerably limited by the small number of patients included in specific comparisons, heterogeneity in the study designs and methods, poor methodological quality of some studies (the majority were retrospective studies), and some concerns about the ascertainment of viral infection. In particular, while some studies used sensitive techniques to test viral infections, other studies, like those ones on HIV, based their results on the detection of serum antibodies. Moreover, only few studies reported the presence of other risk factors for the development of testicular cancer. Therefore, even if a relationship between specific viruses and testicular cancer was detected by the present meta-analysis, causation cannot be established.

CONCLUSIONS

We found a possible correlation between specific viruses and testicular cancer, but the evidence was insufficient to establish causality. The correlation between HIV and increased risk of TGCTs is supported by good-quality evidence despite being based on serum antibody titers. Similarly, the evidence suggesting a link between EBV and TGCTs is fair.

Regarding the correlation between CMV and TGCTs, available data are conflicting and further studies are needed to draw firm conclusions. Moreover, poor evidence supports the lack of correlation between Parvovirus B-19 and a meaningful risk of TGCTs. Finally, data about the possible relationship between HPV and TGCTs are inconsistent, but its oncogenic potential for male gonadal tissue cannot be excluded; thus, future good-quality studies are warranted.

DATA AVAILABILITY

No datasets were generated or analyzed for this study.

AUTHOR CONTRIBUTIONS

FM, UV, MG, AA, and GA performed the medline for article search. A manual search of reference lists of studies was performed to avoid missing relevant publications by FM. AG and AV independently assessed the inclusion criteria and study selection. Disagreements were discussed with the third reviewer CF. Data extraction was performed by two independent investigators AG and FM. AV reviewed the selection and data extraction process. The results were then compared, and any disagreement discussed and resolved by consensus of all authors. The draft was written by FM, AV, and AG.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00355/full#supplementary-material

Table S1 | Risk of bias evaluation. Quality of studies included in the meta-analysis was evaluated using five different domains through a modified version of the "Newcastle–Ottawa Scale" (14).

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The Warburg Effect Is Associated With Tumor Aggressiveness in Testicular Germ Cell Tumors

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Testicular Germ Cell Tumors (TGCTs) are a rare group of neoplasms and the most common solid malignancy arising in young male adults. Despite the good response of these tumors to platinum-based chemotherapy, some patients are refractory to treatment and present poor clinical outcomes. During carcinogenesis and tumor development, cancer cells reprogram energy metabolism toward a hyper-glycolytic phenotype, an emerging hallmark of cancer. This phenomenon, known as the Warburg effect or aerobic glycolysis, involves overexpression of metabolism-related proteins, like glucose and monocarboxylate transporters, pH regulators and intracellular glycolytic enzymes. The metabolic profile of TGCTs is very little explored and, recently, this metabolic rewiring of cancer cells has been associated with aggressive clinicopathological characteristics of these tumors. The overexpression of monocarboxylate transporter 4 (MCT4) in TGCTs has been pointed out as a poor prognostic factor, as well as a promising therapeutic target. As a result, the main aim of the present study was to evaluate the prognostic value of key metabolism-related proteins in TGCTs. The immunohistochemical expressions of CD44 (as a monocarboxylate transporter chaperone), glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX), hexokinase II (HKII) and lactate dehydrogenase V (LDHV) were evaluated in a series of 148 adult male patients with TGCTs and associated with clinicopathological parameters. In addition, paired normal tissues were also evaluated. The sample included 75 seminoma and 73 non-seminoma tumors. GLUT1 and CD44 expression was significantly increased in malignant samples when compared to paired normal samples. Conversely, HKII and LDHV expressions were significantly decreased in malignant samples. Concerning the clinicopathological values, CAIX expression was significantly associated with disease recurrence, while HKII expression was significantly associated with aggressive characteristics of TGCTs, including higher staging and non-seminoma histology. In conclusion, this study brings new insights on the metabolic characteristics of TGCTs, showing alterations in the expression of proteins related with the Warburg effect, as well as associations of the hyper-glycolytic and acid-resistant phenotype with aggressive clinicopathological parameters.

Keywords: immunohistochemistry, metabolic reprogramming, testicular germ cell tumors, testicular neoplasms, Warburg effect

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INTRODUCTION

Testicular germ cell tumors (TGCTs) are the most frequent solid malignancies arising in young male adults (1, 2) and show an increase in incidence throughout the last decades, especially in Europea-descendent men (1, 3, 4). Divided into two major histological types, homogeneous seminoma and heterogeneous non-seminoma tumors (1), TGCTs tend to have a good response to platinum-based chemotherapy, with seminomas presenting more favorable outcomes in comparison to non-seminoma tumors (2). Despite the high rates of cure—over 90% in patients with early diagnosed disease (independent of histological type) (1, 5)—about 10–20% are refractory to treatment and present unfavorable clinical outcomes (6, 7).

The major mechanisms involved in the development of TGCTs are copy number variations (aneuploidies) and some recurrent somatic mutations. The isochromosome 12p is present in almost all tumors and is considered a marker for TGCTs. In fact, the underlying mechanisms involving isochromosome 12p in the development of TGCTs are still unclear, but there is enough evidence that implicate this alteration as an earlier triggering event, leading to invasiveness growth and malignization. Furthermore, the most frequently mutated driver oncogenes found in seminomas are *KIT* and *KRAS*, with 25–30 and 5–10% mutation frequencies, respectively (2, 4). Besides that, there is still a lack of information in understanding the complex heterogeneity of TGCTs, which highlights the importance of the discovery of different oncogenic events involving these tumors to optimize treatment and management. In this context, the recently described hallmark of cancer of deregulation of cellular energetics is gaining additional attention in the last years and should be considered as a possible relevant biological mechanism in TGCTs (8, 9).

During carcinogenesis and tumor development, cancer cells reprogram energy metabolism toward a hyper-glycolytic phenotype, even in the presence of high oxygen levels. This phenomenon, known as the Warburg effect or aerobic glycolysis, leads to a higher production of lactate than the normal metabolic phenotype, which relies mostly on oxidative phosphorylation (10–12). To fuel all the energy required and avoid intracellular acidification and apoptosis, cancer cells upregulate some key proteins, like glucose and monocarboxylate transporters, pH regulators and intracellular glycolytic enzymes (13). In comparison to oxidative phosphorylation, glycolysis is not an energetic efficient pathway but is a faster way to provide energy, metabolic intermediates, and biochemical building blocks, essential for anabolic reactions, and thus enhancing the aggressive characteristics presented by malignant cells (14). As demonstrated by many studies (15, 16), the overexpression of metabolism-related proteins plays an important role in the development and maintenance of the malignant phenotype of a vast majority of tumors. In this context, these metabolic players have been pointed out as prognostic factors and can be explored as promising therapeutic targets.

Although studies evaluating the implication of metabolic rewiring in the development and progression of TGCTs are lacking, there is evidence that these tumors present a highly glycolytic behavior, mainly attributed to their elevated levels of glucose consumption as demonstrated by ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) studies (17–20). Furthermore, when comparing malignant and benign samples, a recent study (21) shows that TGCTs overexpress monocarboxylate transporter 4 (MCT4) and its chaperone CD147. Additionally, the upregulation of monocarboxylate transporter 1 (MCT1), MCT4 and CD147 has been associated with aggressive clinicopathological characteristics of these tumors, while MCT4 overexpression was associated with a worse prognosis, with patients presenting a shorter overall and event-free survival. Other studies show that TGCTs have an increase in the expression of glucose transporter 3 (GLUT3) protein (22), which is often found overexpressed by malignant neoplasms (23, 24).

Since metabolic reprogramming in human tumors need to be further exploited, the study of different metabolism-related proteins may offer a better understanding about their role, relevance, and implication in the biological characteristics and the complex heterogeneity of TGCTs. In this context, CD44, a chaperone for proper localization and activity of MCT1 and MCT4 in the plasma membrane, glucose transporter 1 (GLUT1), the pH regulator carbonic anhydrase 9 (CAIX), as well as hexokinase II (HKII), responsible for the irreversible glucose phosphorylation in the earlier steps of glycolysis and lactate dehydrogenase V (LDHV), the isoenzyme with higher affinity for pyruvate, that catalyze the conversion of pyruvate into lactate, arise as key players in the metabolic reprogramming of cancer cells (13, 16, 25, 26).

Therefore, regarding the emerging role of metabolic rewiring in tumors, as well as the biological complexity and the absence of studies considering this context in TGCTs, the aims of this study were to evaluate the expression of CD44, GLUT1, CAIX, HKII, and LDHV in TGCTs and normal samples, using tissue microarrays (TMAs), and to associate the expression with clinicopathological data to determine whether these proteins have some biological and/or prognostic value.

MATERIALS AND METHODS

Case Selection and Clinicopathological Information

The series included 148 formalin-fixed paraffin embedded adult TGCTs samples, retrieved from the Pathology Department of Barretos Cancer Hospital, from 2007 to 2013. Only primary tumors, prior to chemotherapy, were selected. Additionally, paired normal samples were collected and analyzed when available ($n = 66$ for CD44, $n = 59$ for GLUT1, $n = 78$ for CAIX, $n = 87$ for HKII and $n = 84$ for LDHV; the different number of normal samples analyzed for each protein is related to sample loss as a result of block sectioning). The clinicopathological data included age, date of diagnosis, histological types, grading, staging (TNM), presence of vascular invasion, International Germ Cell Cancer Collaborative Group (IGCCCG) stratification risk (63), and dates of surgery, chemotherapy, recurrence, progression and death. Patients' mean age was 32.3 years (ranging

TABLE 1 | Clinicopathological characteristics of adult testicular germ cell tumor patients.

	n (%)
Histological type	
Yolk sac	1 (0.7)
Choriocarcinoma	2 (1.4)
Embryonal carcinoma	8 (5.4)
Immature teratoma (grade I)	2 (1.4)
Mixed teratoma	19 (12.8)
Seminoma	75 (50.7)
Mixed germ cell tumor	41 (27.7)
Stage at diagnosis	
I	76 (51.3)
II	33 (22.3)
III	30 (20.3)
IS	7 (4.7)
IGCCCG stratification risk	
Low	43 (29.1)
Intermediate	17 (11.5)
High	7 (4.7)
Chemotherapy	
BEP	61 (41.2)
EP	14 (9.4)
Other	4 (2.7)
No chemotherapy	65 (43.9)
Status—post treatment	
Alive and disease free	128 (86.5)
Alive and in treatment	5 (3.4)
Cancer related death	12 (8.1)
Death from other causes	3 (2.0)

BEP, bleomycin, etoposide, platinum; EP, etoposide, platinum; Other, carboplatin.

from 18 to 73 years) and most of them were caucasian (62.2%). Detailed information on the clinicopathological data of the sample is depicted in **Table 1**. This study was approved by the Ethics Committee on Research of Barretos Cancer Hospital (number 541235).

TMA Construction and Immunohistochemistry

TMAs were constructed for the immunohistochemical reactions. All the cases were reviewed by an experienced pathologist (ECAS) for diagnostic confirmation and demarcation of tumor areas for TMA cores. Each TMA contained sample cores of 1.0 mm diameter from all histological subtypes and corresponding normal tissues, in triplicate. Liver, kidney and placenta were used as controls for TMA orientation.

Immunohistochemistry for GLUT1 and CAIX was performed using a streptavidin-biotin-peroxidase complex (Ultravision Detection System: Large Volume Anti-Polyvalent, HRP, Lab Vision Corporation, Fremont, CA), according to manufacturer's instructions and as previously described (27). Immunohistochemistry for CD44 was performed using a biotin-free principle (ADVANCE HRP, Dako, Carpinteria, CA), according to manufacturer's instructions. For HKII and LDHV, the reactions were performed using an avidin-biotin-peroxidase complex principle (R.T.U. VECTASTAIN Kit, Vector Laboratories, Burlingame, CA), according to manufacturer's instructions. Details on antigen retrieval and each antibody used are described in **Table 2**. For visualization, slides were incubated with 3,3′-diamino-benzidine (Liquid DAB+ Substrate Chromogen System, Dako, Carpinteria, CA), according to manufacturer's instructions, then counterstained with hematoxylin and permanently mounted. As positive controls, placenta was used for GLUT1, normal gastric mucosa for CAIX and squamous cell carcinoma of oral cavity for CD44, HKII, and LDHV. Negative controls were available in the same tissue sections used as positive controls.

Immunohistochemical Evaluation

TMAs and whole sections were scored semi-quantitatively for extension of expression in cancer cells as follows: 0: no immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5–50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as follows: 0: negative; 1: weak; 2: intermediate; and 3: strong. The final score was defined as the sum of both parameters (extension and intensity) and grouped as negative (score 0–2) and positive (score 3–6), as previously described (28, 29). Only protein expression in plasma membrane was considered for CD44, GLUT1, and CAIX analysis, while for HKII and LDHV only cytoplasmic expression was considered for further analysis. TMAs were evaluated by two experienced pathologists independently (ECAS and CS-N). Discordant cases were reviewed and scored in consensus.

Statistical Analysis

Data collected was analyzed using IBM SPSS Statistics software (version 23.0, IBM Company, Armonk, NY). During immunohistochemical evaluation, loss of tumor representativity in TMA cores as well as whole core loss was observed, influencing the final number of cases used for statistical analysis. Frequency of protein expression in normal and malignant tissues was compared using McNemar's test, while comparison with clinicopathological data was analyzed using Pearson's chi-square test and Fisher's exact test, according to the sample's characteristics. Overall survival was defined as the time from the date of primary diagnosis to last follow-up or death. Event-free survival was defined as the time from the primary diagnosis to the event date (recurrence, disease progression or death). None of the patients presented secondary tumors until the last follow-up. For survival models, only stage II and III patients were considered for further analysis. Overall and event-free survival curves were constructed using Kaplan-Meier's method and the data compared with log-rank test. Multivariate analysis by Cox proportional hazards regression model was used to determine independent predictors of survival. Independent variables were analyzed by univariate analysis, followed by multivariate analysis of all variables that reached a $p < 0.2$ at univariate analysis. For all tests, the level of significance established was 5% (significant results if $p < 0.05$).

TABLE 2 | Detailed aspects of immunohistochemistry.

Protein	Antigen retrieval	Antibody	Clonality	Dilution, incubation time, and temperature
CD44	Citrate (0.01 M, pH = 6.0), 98°C, 20 min	MCA2726 AbD Serotec	Monoclonal (156-3C11)	1:2000, 2 h, RT
GLUT1	Citrate (0.01 M, pH = 6.0), 98°C, 20 min	ab15309 Abcam	Polyclonal	1:500, 2 h, RT
CAIX	Citrate (0.01 M, pH = 6.0), 98°C, 20 min	ab15086 Abcam	Polyclonal	1:2000, 2 h, RT
HKII	EDTA (1 mM, pH = 8.0), 98°C, 20 min	ab104836 Abcam	Monoclonal (3D3)	1:1000, 2 h, RT
LDHV	EDTA (1 mM, pH = 8.0), 98°C, 20 min	ab101562 Abcam	Monoclonal (EPR1564)	1:6000, 2 h, RT

RT, room temperature.

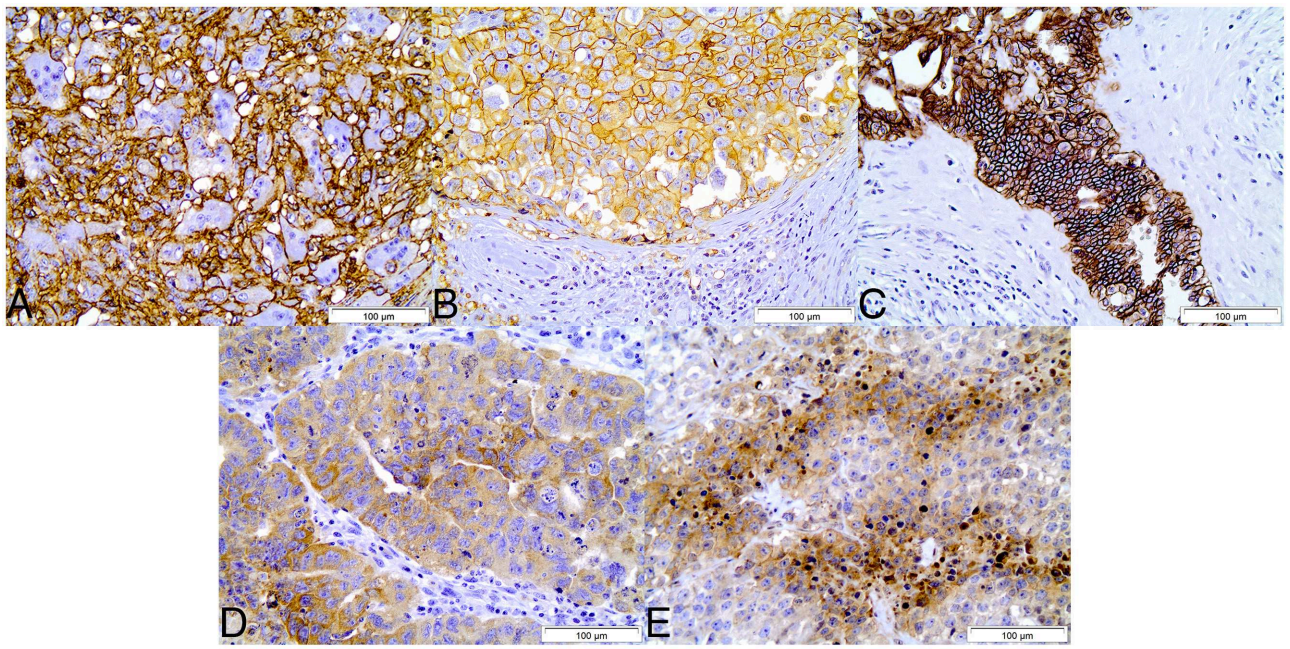


FIGURE 1 | Immunohistochemical expression of CD44 **(A)**, GLUT1 **(B)**, CAIX **(C)**, HKII **(D)**, and LDHV **(E)** in adult testicular germ cell tumors. CD44, GLUT1, and CAIX show plasma membrane staining, while HKII and LDHV show cytoplasmic staining. Magnification: 400x.

RESULTS

Expression of CD44, GLUT1, CAIX, HKII, and LDHV in Testicular Germ Cell Tumors and Paired Normal Tissues

Immuhistochemical evaluation of adult testicular germ cell tumors showed that expression of CD44, GLUT1 and CAIX was mostly exclusively found in plasma membrane. Regarding the expression of HKII and LDHV in tumor samples, both proteins were mostly detected in cytoplasm. According to the results observed in tumors, paired normal tissues showed similar expression patterns, with CD44, GLUT1 and CAIX frequently found in plasma membrane and HKII and LDHV in cytoplasm (**Figure 1**).

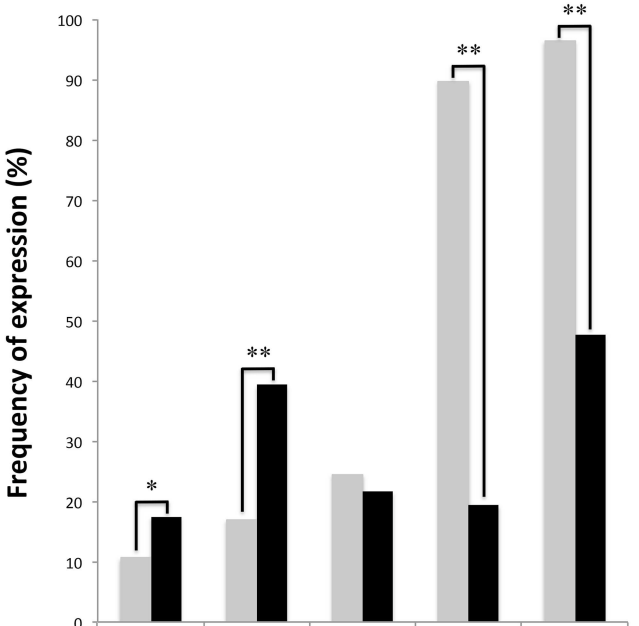
Comparison of protein expression between tumor samples and paired normal tissues showed a significantly increased expression of CD44 and GLUT1 in tumor samples (p =0.004 and $p < 0.001$, **Figure 2**). Conversely, HKII and LDHV expression was significantly decreased in malignant samples ($p < 0.001$ and $p < 0.001$, **Figure 2**).

Clinicopathological Significance of Metabolism-Related Proteins

The associations between the expression of the metabolism-related proteins and the clinicopathological data of the patients are shown in **Table 3**. CAIX expression presented a significant association with recurrence ($p = 0.024$), while HKII showed a statistically significant association with non-seminoma tumors ($p < 0.001$) and higher stages ($p = 0.019$). CD44, GLUT1 and LDHV showed no significant associations with clinicopathological parameters.

Survival Analysis

Kaplan-Meier analysis for the expression of metabolism-related proteins showed no significant associations with overall and event-free survival (data not shown). The predictive prognostic



	CD44	GLUT1	CAIX	HKII	LDHV
Normal	10.6	16.9	24.4	89.7	96.4
Malignant	17.5	39.3	21.6	19.4	47.6

FIGURE 2 | Plasma membrane expression of GLUT1, CAIX, and CD44 and cytoplasmic expression of HKII and LDHV in adult testicular germ cell tumors and correspondent normal testicular tissues. McNemar's test was used to assess differences of expression frequency between tumor and normal tissue. $^{*}p = 0.004$, $^{**}p < 0.001$.

values of the proteins and clinicopathological parameters for overall survival and event-free survival were analyzed by means of Cox proportional hazards regression models (**Tables 4**, **5**, respectively). Univariate analysis revealed predictive prognostic values for stage III and IGCCCG intermediate and high risk stratifications in overall and event-free survival. Multivariate analysis showed that IGCCCG high risk classification was an independent predictor for overall and event-free survival (HR: 9.987 $p = 0.034$ and HR: 11.061 $p = 0.014$, respectively). None of the analyzed proteins presented a prognostic value in TGCTs patients.

DISCUSSION

Our study showed an increase in the expression frequency of GLUT1 and CD44 in adult testicular germ cell tumors. In a previous study, the authors showed that MCT4 and CD147 expression was increased in TGCTs patients (21). Taken together, these results suggest a metabolic reprogramming of malignant cells toward a hyperglycolytic and acid-resistant phenotype in TGCTs.

The high frequency of GLUT1 expression is in agreement with the natural behavior of cancer cells as high glucose consumers. In fact, glucose transporters need to be overexpressed to fuel part of the metabolic reprogramming required by malignant cells to produce energy and intermediates for anabolism, as well as regulate redox state (30). This metabolic reprogramming demands an increased uptake of glucose, mainly provided by GLUT1 (31). In addition, CD44 expression is related to the overexpression of MCT4 previously described in TGCTs (21), suggesting that CD44 and MCT4 work together in lactate efflux (25), favoring the Warburg effect.

Regarding the association of metabolism-related proteins expression with clinicopathological characteristics of TGCTs, our study showed a significant association between CAIX status and recurrence, as well as HKII positive expression with aggressive clinicopathological parameters (non-seminoma tumors and higher staging). CAIX, a hypoxia marker, exerts a pH control regulation, which contributes to the acid-mediated cancer cell invasiveness when overexpressed (32–35), and has been associated with a worse prognosis in a variety of tumors (34). Also, the relation of CAIX expression with recurrence in TGCTs corroborates the role of CAIX in stimulating the invasion and aggressive phenotype of malignant cells and this association had been demonstrated by two studies (36, 37). In the firsty study (36), the authors show that CAIX serum levels was higher in TGCTs metastatic patients when compared to healthy controls, and that CAIX serum levels are significantly associated with intratumoral CAIX expression. However, CAIX serum levels do not demonstrate association with clinicopathlogical data, neither a prognostic value in clinical outcomes. According to the second study (37), the authors show a significant increase in the expression of CAIX in TGCTs samples compared to paired adjacent normal samples. This result, not reached by our study, probably can be explained by the numerical sample difference between the two works. Moreover, the authors show that CAIX positive expression was significantly associated with a worse progression-free survival, predominatly founded in patients with metastatic disease, which is in agreement with the association of CAIX positive expression with recurrence, demonstrated in the present study. Furthermore, the significant association of HKII positive expression with non-seminoma tumors and higher stages demonstrated by our study is in accordance with the role of this protein in providing energy for tumors, leading to disease progression and poor outcomes. Regardless of the lack of information about HKII expression in TGCTs patients, several studies associated HKII overexpression with aggressive characteristics and worse outcomes in different types of neoplasms, such as breast (38–40), cervical (41), colorectal (42), glioblastoma (43), liver (44, 45), lung (39), pancreatic (46, 47) and prostate (48). Indeed, HKII is described as one of the main proteins responsible for mediating the Warburg effect in cancer cells. This protein binds to the voltage dependent anion channel (VDAC) in mitochondria outer membrane, keeping the channel in an open state, gaining direct access to ATP generated intra-mitochondrially for glucose phosphorylation (49). This is in accordance with *in vitro* studies demonstrating the role of VDAC-bound HKII in supporting the Warburg effect (50). Additionally, other activities have been attributed to HKII in cancer metabolism context, which favors the aggressive phenotype of malignant cells, including the production of antioxidant molecules,

TABLE 3 | Association of CD44, GLUT1 and CAIX membrane expression and cytoplasmic expression of HKII and LDHV with clinicopathological characteristics of adult testicular germ cell tumors.

	CD44			GLUT1			CAIX			HKII			LDHV		
	n	**Positive (%)**	***p***	***n***	**Positive (%)**	***p***	***n***	**Positive (%)**	***p***	**n**	**Positive (%)**	***p***	***n***	**Positive (%)**	***p***
Histology			0.850			0.265*			0.367			**<0.001**			1.000
Seminona	54	24 (44.4)		68	63 (92.6)		59	23 (39.0)		73	13 (17.8)		73	64 (87.7)	
Non-seminomatous	60	25 (41.7)		72	70 (97.2)		66	32 (48.5)		71	34 (47.9)		72	63 (87.5)	
T stage			1.000			0.241*			0.465			0.363			0.064
T1	65	28 (43.1)		81	75 (92.6)		71	34 (47.9)		82	29 (35.4)		82	76 (92.7)	
T2+T3+T4	46	20 (43.5)		55	54 (98.2)		52	21 (40.4)		58	16 (27.6)		59	48 (81.4)	
N stage			0.701			0.238*			0.585			0.100			0.075
N0	61	27 (44.3)		79	73 (92.4)		71	29 (40.8)		81	21 (25.9)		81	67 (82.7)	
N1+N2+N3	51	20 (39.2)		58	57 (98.3)		52	24 (46.2)		60	24 (40.0)		61	57 (93.4)	
M stage			0.802			0.605*			0.209			0.457			0.738*
M0	94	40 (42.6)		114	107 (93.9)		104	42 (40.4)		120	37 (30.8)		119	103 (86.6)	
M1	18	7 (38.9)		24	24 (100.0)		19	11 (57.9)		22	9 (40.9)		24	22 (91.7)	
Stage			0.704			0.117*			0.715			**0.019**			0.078
I	54	24 (44.4)		71	65 (91.5)		63	26 (41.3)		73	17 (23.3)		73	60 (82.2)	
IS+II+III	57	23 (40.4)		66	65 (98.5)		59	27 (45.8)		68	29 (42.6)		69	64 (92.8)	
Vascular invasion			1.000			1.000*			0.693			0.428			0.236*
No	74	32 (43.2)		94	89 (94.7)		84	38 (45.2)		95	33 (34.7)		95	87 (91.6)	
Yes	34	15 (44.1)		39	37 (94.9)		37	15 (40.5)		42	11 (26.2)		43	36 (83.7)	
IGCCCG stratification risk			0.800*			1.000*			0.642*			0.470*			1.000*
Low	37	17 (45.9)		39	38 (97.4)		36	15 (41.7)		43	19 (44.2)		42	38 (90.5)	
Intermediate	15	5 (33.3)		17	17 (100.0)		15	8 (53.3)		17	6 (35.3)		17	16 (94.1)	
High	3	1 (33.3)		7	7 (100.0)		5	3 (60.0)		6	4 (66.7)		7	7 (100.0)	
Recurrence			0.551			0.598*			**0.024**			0.236*			0.219*
No	98	43 (43.9)		117	110 (94.0)		106	41 (38.7)		123	36 (29.3)		122	105 (86.1)	
Yes	12	4 (33.3)		16	16 (100.0)		14	10 (71.4)		15	7 (46.7)		16	16 (100.0)	
Progression			0.633*			1.000*			0.678*			0.662*			1.000*
No	58	25 (43.1)		64	63 (98.4)		59	28 (47.5)		68	24 (35.3)		68	60 (88.2)	
Yes	4	1 (25.0)		7	7 (100.0)		6	2 (33.3)		6	3 (50.0)		7	7 (100.0)	

**Pearson's Qui-square test; Fisher's exact test. Significant results ($p < 0.05$) are depicted in bold.*

TABLE 4 | Prognostic factors for overall survival in adult testicular germ cell tumors.

	Univariate analysis				Multivariate analysis			
	n	**HR**	**95% CI**	***p***	***n***	**HR**	**95% CI**	***p***
Histology								
Seminoma	26	1	-	-				
Nonseminomatous	37	0.980	0.340–2.826	0.971				
Stage								
II	33	1	-	-	33	1	-	-
III	30	5.166	1.436–18.587	**0.012***	28	1.404	0.198–9.967	0.735
Vascular invasion								
No	35	1	-	-				
Yes	24	1.628	0.525–5.050	0.399				
IGCCCG stratification risk								
Low	37	1	-	-	37	1	-	-
Intermediate	17	4.344	1.036–18.215	**0.045***	17	3.442	0.485–24.410	0.216
High	7	13.189	3.101–56.088	**<0.001***	7	9.987	1.190–83.822	**0.034**
CD44 plasma membrane								
No	31	1	-	-				
Yes	20	1.040	0.293–3.693	0.951				
CAIX plasma membrane								
No	28	1	-	-				
Yes	24	0.963	0.294–3.161	0.951				
HKII cytoplasm								
No	36	1	-	-				
Yes	25	1.896	0.637–5.646	0.250				

*HR, hazard ratio; CI, confidence interval. *Variables that reached $p < 0.2$ in univariate analysis. Significant results ($p < 0.05$) are depicted in bold.*

direct protection of mitochondria against redox stress (anti-apoptotic effect) and facilitation of autophagy under starvation. Moreover, HIF-1α stimulation by AKT and mTORC1 has been described as the mechanism mainly responsible for HKII upregulation (51).

In contrast to GLUT1 and CD44 expression, a decreased frequency of HKII and LDHV expression in tumor samples, compared to normal testicular samples, was observed in the present study. Due to insufficient information available in the literature about the expression of HKII in normal testis, a search in the Human Protein Atlas (HPA) database, which integrates the protein expression profiles of 44 normal human tissues to RNA sequencing data of 32 out of these 44 tissue types (52), was conducted. HPA data showed that HKII RNA and protein expressions were correlated and more pronounced in male normal tissues, like testis and epididymis, corroborating our results. Also, a study done by Postic and collaborators (53), showed a relation between hexokinases and glucose transporters isoforms. Using rat models, the authors demonstrated that, during embryonic development, HKI and GLUT1 were the major isoforms expressed and related to energy production. After weaning, with the acquisition of insulin-sensitivity tissues, there is a switch in both isoforms, with HKII and GLUT4 participating mostly in energy production. These results suggest the important role of HKII in normal tissues after embryonic development for energy production. Our results are in accordance with this previous data as malignant testicular tissues tend to resemble the hypoxic and undifferentiated embryonic tissues (54, 55), therefore showing higher expression of GLUT1 but lower expression of HKII when compared to normal tissues. Regarding LDHV expression, Dodo and collaborators (56) found that LDHV was co-expressed with LDHX, the major LDH isoform present in testis, which has been found in different animals, including humans (57, 58). Curiously, LDHX mice knock-out presented an ablation of LDHV expression, not demonstrated by wild type mice, resulting in reduced energy production through glycolysis and impaired fertilization (56). Also, another study described that LDHV human transgene expression was able to restore LDHX expression in testis and sperm of LDHX knock-out mice, also restoring sperm motility and fertilization capacity (59). Taken together, these results suggest an important role of LDHV in aerobic glycolysis presented by normal testis, indicating that this protein is required to establish proper physiologic conditions for fertilization.

The natural behavior of cancer cells in reprogramming their metabolism, with a heavier reliance in aerobic glycolysis, provides a solid field for the development of anticancer therapies. Across the decades, different glycolytic inhibitors have been tested in pre-clinical studies and clinical trials, trying to kill cancer cells by pharmacological inhibition of glucose consumption and achieve therapeutic selectivity. Pelicano and co-workers (60)

TABLE 5 | Prognostic factors for event-free survival in adult testicular germ cell tumors.

	Univariate analysis				Multivariate analysis			
	n	HR	95% CI	*p*	*n*	HR	95% CI	*p*
Histology								
Seminoma	26	1	-	-				
Nonseminomatous	37	1.274	0.502-3.238	0.610				
Stage								
II	33	1	-		33	1	-	-
III	30	5.515	1.823–16.681	**0.003***	27	1.517	0.277–8.317	0.631
Vascular invasion								
No	35	1	-	-				
Yes	24	1.121	0.426–2.953	0.817				
IGCCCG stratification risk								
Low	37	1	-	-	37	1	-	-
Intermediate	17	4.380	1.280–14.983	**0.019***	17	3.222	0.203–6.954	0.176
High	7	16.230	4.483–58.758	**<0.001***	6	11.061	1.627–75.201	**0.014**
CD44 plasma membrane								
No	31	1	-	-				
Yes	20	1.238	0.429–3.577	0.693				
CAIX plasma membrane								
No	28	1	-	-				
Yes	24	1.457	0.542–3.917	0.456				
HKII cytoplasm								
No	36	1	-	-	35	1	-	-
Yes	25	1.929	0.744–5.003	0.177*	25	2.015	0.738-5.500	0.172

*HR, hazard ratio; CI, confidence interval. *Variables that reached $p < 0.2$ in univariate analysis. Significant results ($p < 0.05$) are depicted in bold.*

discuss about the use of three potent hexokinase inhibitors: 3-bromopiruvate (3-BP), 2-deoxyglucose (2-DG), and lonidamine. The major inhibitory mechanism of these compounds was the blockage of glucose phosphorylation, mediating the uncoupling of HK from mitochondria, leading to a rapid depletion of cellular ATP. Additionally, *in vitro* studies show a relevant therapeutic effect of the anti-hyperglycemic drug metformin in glycolytic addicted tumors, through the inhibition of HK function (38, 61). Finally, a recent study showed the efficacy of the antifungal drugs—ketoconazole and posaconazole—in glioblastoma cells, with selective inhibition of HKII. Using *in vitro* and *in vivo* models, the authors showed that both drugs were able to increase survival of mice and decrease cell proliferation and tumor metabolism (62). Importantly, both drugs are enrolled in an early phase I clinical trial in high grade gliomas (clinicaltrials.gov—NCT03763396). Despite the promising efficacy of the use of glycolytic inhibitors for glucose addicted tumors, TGCTs show a more complex biology. Our results demonstrate that HKII and LDHV have a role in normal testis, suggesting the importance of these markers in fertilization. Indeed, the use of HK inhibitors in TGCTs patients may lead to an increase in cytotoxicity and even infertility.

The present study, together with the study done by Silva and co-workers (21), corroborates that TGCTs present a switch in cellular metabolism toward a hyper glycolytic and acid-resistant phenotype, mainly associated with a worse prognosis. Additionally, HKII appears as a marker of tumor aggressiveness, bringing new insights about the metabolic characteristics of these tumors. Although our results showed a bone fide characterization of TGCTs metabolism, further studies are warranted to achieve a better understand, especially about HKII role in testicular malignancies, providing new evidences for future therapeutic strategies.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was approved by the Ethics Committee on Research of Barretos Cancer Hospital (number 541235). Written informed consent was waived as this study was considered a minimal-risk study.

AUTHOR CONTRIBUTIONS

MB performed immunohistochemical reactions, statistical analysis, and wrote the manuscript. MZ performed immunohistochemical reactions. ES and CS-N analyzed histological sections and performed the immunohistochemical

evaluations. FC and LL aided in the study design and discussion of the results. CP was responsible for the study design, contributed to the discussion of the results, organization, and review of the manuscript. All authors read and approved the manuscript.

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REVIEW
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Testicular Cancer: Genes, Environment, Hormones

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Testicular cancer (TC) represents one of the most peculiar clinical challenges at present. In fact, currently treatments are so effective ensuring a 5 years disease-free survival rate in nearly 95% of patients. On the other hand however, TC represents the most frequent newly diagnosed form of cancer in men between the ages of 14 and 44 years, with an incidence ranging from <1 to 9.9 affected individuals per 100,000 males across countries, while the overall incidence is also increasing worldwide. Furthermore, cancer survivors show a 2% risk of developing cancer in the contralateral testis within 15 years of initial diagnosis. This complex and multifaceted scenario requires a great deal of effort to understand the clinical base of available evidence. It is now clear that genetic, environmental and hormonal risk factors concur and mutually influence both the development of the disease and its prognosis, in terms of response to treatment and the risk of recurrence. In this paper, the most recent issues describing the relative contribution of the aforementioned risk factors in TC development are discussed. In addition, particular attention is paid to the exposure to environmental chemical substances and thermal stress, whose role in cancer development and progression has recently been investigated at the molecular level.

Keywords: suscepibility genes, temperature, endocrine discruptors, disorders of sex development, GWAS

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INTRODUCTION

With an overall annual incidence of nearly 1% among all newly diagnosed cancers in males, testicular cancer (TC) represents the most common tumor in men at the age ranging between 14 and 44 years, which is considered to be the fully working/reproductive age (1, 2). Before the 1970s, the mortality rate for TC was extremely high due to the metastatic degeneration of the disease, whilst the only two treatments to contain the risk of relapse were the retroperitoneal lymph node dissection, associated or not with radiotherapy. Thereafter, the development of an effective chemotherapy changed the "rules of the game." In fact, the current multidisciplinary approach to the treatment of TC, comprising surgery and adjuvant chemo- or radiotherapy, results in a 5 years survival rate of >95%. As a consequence, TC is now considered as a model for a curable cancer (3).

In spite of these indisputable progresses, TC still presents multi-leveled challenges that should not allow our guard to be lowered:

- Epidemiological evidence: the annual incidence of TC has doubled over the past 40 years with an increasing trend over time, particularly in Caucasian males (4). Indeed, data from the African and Asian continents show an incidence lower than one case every 100,000 males, whilst Scandinavian countries report the highest rate of newly affected individuals worldwide (from 9.4 to 9.9 males every 100.000 males). This trend by ethnicity is further confirmed by data from United States where TC is found more frequently in white males compared to African Americans (1.2 vs. 6.9 affected individuals per 100.000 males, respectively) (5, 6).
- Clinical evidence: previous history of TC, even if properly treated and monitored, represents a major risk factor for a second contralateral cancer. The overall risk for a secondary TC is approximately 5% within 5 years from diagnosis, and in most cases presenting within 2 years from the first diagnosis (7). In this regard, primary testicular size and the degree of invasion of the rete testis are considered two major prognostic factors for relapse (8, 9).
- Therapeutic evidence: in spite of the aforementioned effectiveness, the treatment of TC itself is frequently associated with an increased risk of developing long lasting adverse effects like infertility, hypogonadism, metabolic/cardiovascular derangements, and osteoporosis, which actually represent the most relevant life threatening consequences of the TC therapy (10–12).

For all these reasons, the identification of pathogenic mechanisms and risk factors involved in testicular carcinogenesis still represent topics of extremely high clinical interest. Indeed, the probability of developing TC is the result of a combination of a number of factors that can be distinguished, in general terms, into genetic, environmental, and hormonal factors.

GENES

The fact that TC development relies on genetic factors is widely acknowledged. Despite the fact that 90% of males affected by TC have no previous familiar cases of this disease, population-based studies in the late 1990's-early 2000's showed that having a brother with an history of TC increases the risk of the disease from 8- to 10-fold, compared to the general male population. On the other hand, having a father affected by TC increases the relative risk for the male child from four- to 6-fold (13–15). In 2002, a pioneering study on a population-based registry, evaluating 9.6 million individuals from the nationwide Swedish Family-Cancer Database, attempted to distinguish between the respective genetic, pure-environmental and childhood-environmental contribution to the development of cancers, essentially based on epidemiologic considerations (16). Interestingly, TC resulted as one of the most associated neoplasms with genetic factors (25%), right after the thyroid (53%), and endocrine glands in general (28%). In addition, a recent study on a population-based registry, evaluating monozygotic and same-sex dizygotic twin individuals disclosed an esteemed familiar risk of heritability for TC of nearly 40% with a significant portion, however, attributable to shared environmental conditions (17).

In spite of the clear evidence supporting the genetic background in TC development, the availability of reliable studies providing qualitative and quantitative data about the genetic basis of familial TC still represents a major challenge. In 2006, a linkage study on 237 pedigreed families, with a history of one or more cases of TC, identified six regions of interest on chromosomes 2p23, 3p12, 3q26, 12p13-q21, 18q21-q23, and Xq27 as susceptibility loci. However, further widenings showed that no single locus accounted for the majority of the familial aggregation observed in TC, suggesting at the same time a major role of multiple susceptibility loci with singular weaker effects (18). To this regard, significant advances have been provided by the availability genome wide association studies (GWAS) that, since the mid-2000's, progressively increased the number of susceptibility loci with a predicted effect on TC development (19–24). In a recent GWAS and meta-analysis, comprising more than 5,500 cases and 19,000 controls from northern Europe, (25) identified and confirmed 44 independent TC risk loci (19 newly discovered and 25 previously reported). Interestingly, through a complex *in situ* chromosome conformation-capture analysis in TC cells, a tentative model of chromatin interactions between predisposition SNPs and target genes was performed, identifying three possible pathogenic mechanisms. In particular, 10 of the risk loci contained genes associated with the transcriptional regulation of cell development such as *GATA4* and *GATA1* genes. These are transcription factors involved in the specification and differentiation of postnatal testicular development, whose risk alleles polymorphisms have been previously associated with tumor progression (26–31). A significant association was also found for *PRDM14* and *DMRT1* genes, involved in germ cell specification-sex determination, and the *SALL4* gene through the disruption of the *POU5F1* binding motif (32–35), the latter associated with the maintenance of pluripotency in embryonic stem cells (36). In addition, five TC risk loci were associated with candidate genes with roles in microtubule and chromosomal assembly, particularly the *TEX14* gene, involved in kinetochore-microtubule assembly in the testicular germ cells (37–39), the *WDR73* gene, encoding a key protein for microtubule organization during interphase (40), and the microtubule assembly–related genes *PMF1*, *CENPE,* and *PCNT* (41–44). Furthermore, three TC risk loci subtended a major role of KIT–MAPK signaling, in agreement with recent evidence showing the *KIT* gene as a major somatic driver for TC development (24). In clinical terms, the 44 identified risk loci for TC accounted for 34% of the father-to-son familiar risk for TC development, whilst the top 1% genetic risk at a polygenic risk scores model had a relative risk of 14% and a 7% lifetime risk of developing TC (25). However, this pattern is likely to be widened, thanks to the increasing number of GWAS successively issued. A very recent meta-analysis of five available GWAS, including the X-chromosome, identified further 12 risk loci associated with TC, highlighting the possible involvement of additional cell pathways in TC, such as germ cell development and pluripotency through the *TFCP2L1* and *ZFP42* genes, the kinetochore function through the *ZWILCH* gene, the response to DNA damage through the

TIPIN gene and the mitochondrial function through the *TKTL1* and *LHPP* genes (45).

From this brief summary, it is clear that the pathogenesis of TC relies on a wide spectrum of genetic factors. Recently, a great deal of interest has been sparked by the role of gene copy number variations (CNVs) in cancer development and, particularly, in TC (46–48). In this regard, our group recently investigated the involvement of the *E2F1* gene CNVs as a TC risk factor (49). As a member of the E2F protein family, E2F1 is a transcription factor that regulates the transition of the cell cycle from the G1 phase to the S phase, through an interaction with the retinoblastoma tumor suppressor (RB) protein (50–52). Deregulation of E2F1-pRB binding increases the access of E2F1 to E2F1-binding target genes, containing the E2F-binding site, and this is thought to increase the susceptibility of tumor development (53). Importantly, experimental overexpression of E2F1 in carcinoma cell lines has been associated with increased cell proliferation through the mTOR signaling pathway (54). Interestingly, in our study group of the 261 patients with an history of testicular germ cell tumors and the 165 controls, we found duplications of the *E2F1* gene only in TC patients with a global prevalence of 6.5 percent. This was associated with the increased expression of the E2F1 protein only in tumor tissue specimens obtained from those patients harboring three copies of E2F1, whilst surrounding non tumor-tissue showed both lower E2F1 protein expression and downstream-mTOR phosphorylation (49). These results are highly suggestive of an involvement of E2F1 CNVs in TGCT susceptibility through the Akt/mTOR signaling pathway.

It should also be noted that several risk factors clinically associated with TC development, largely rely on genetic factors. Cryptorchidism, the failed descent of the testis in the scrotum through the inguinal canal during the embryonic life, affects 2–9% of boys born full term and is associated with an almost 9-fold increased risk of TC, compared to the general population (55, 56). The migration process of the embryonal testis can be functionally divided into two sequential phases: the trans-abdominal phase and the inguino-scrotal phase (57). Data from animal models disclosed that each phase is finely regulated by specific factors. In particular, the transabdominal migration of the testis depends primarily on insulin-like peptide 3 (INSL3) and its receptor, RXFP2 (58–60), whilst the inguino-scrotal phase largely depends on androgens signaling (61, 62). Genetic screening in cryptorchid boys showed, respectively, a 2 and 4% prevalence of mutations in the *INSL3* and *RXFP2* genes, more frequently in bilateral forms, whilst there is less agreement for a causative role of polymorphic variants (63–65). Interestingly, there is poor association between mutations of the *AR* gene and isolated cryptorchidism since the prevalence in cryptorchid males is generally lower than 2% (63, 66). In addition, expansion sites in the first exon of the *AR* gene, also known as poly CAG and GGN repeats, are acknowledged as a modulator of AR transactivation activity but their causative role in undescended testis is still under debate (67, 68).

With regards to cryptorchidism, one of the most relevant causes of this clinical condition is a chromosomal alteration such as Klinefelter syndrome (KS), affecting ∼1 in every 700 men (69). KS patients typically present with small testes, infertility, high levels of gonadotrophins and testosterone (T) at the lower levels of normality, whilst cryptorchidism presents nearly six times more frequently than in the general male population (69). The existing literature relating KS and TC, principally Leydig cell tumor, is quite abundant and mainly rely on case reports, however no conclusive association has been provided by the few available epidemiological studies on larger cohorts (63, 70–84). Hence, further studies are required to clearly identify the relative risk of TC associated to KS.

Other genetic causes of isolated cryptorchidism are ascribed to mutations of the AMH gene or its receptor in the persistent müllerian duct syndrome described below (85, 86). In addition, hypospadias, the urethral malformation during embryonal penis development, is also considered a risk factor for TC (87). In particular, hypospadias accounts for about 10% of familial clustering, whilst the estimated heritability of this disease ranges from 57 to 77% (88, 89).

ENVIRONMENT

The identification of direct environmental causes of TC development represents a problem with higher complexity. In fact, most of the acknowledged tumorigenic physical or chemical agents act indirectly through the disruption of the hormonal circuits regulating testis function, or by influencing the function of susceptibility genes (90). However, according to available literature, exclusive environmental risk factors for TC can be formally distinguished into four main classes: microbiological, mechanical, chemical, and physical.

Microbiological

Epidemiological data in 2002 estimated viral infections to be the causative role of ∼12% of cancers worldwide (91). In particular, the pathogenic role of infectious agents in testis tumors has been hypothesized since the late 1980s. Based on epidemiological similarities between Hodgkin's disease and TC, Algood et al. (92) investigated the possible causative role of early exposure to the Epstein-Barr virus (EBV), through the evaluation of antibodies to the EBV capsid antigen, in a small group of patients with an history of stage I germ cell tumors of the testes, receiving surveillance after orchiectomy (92). Interestingly, 80% of patients showed elevated titers for anti EBV antibodies compared to the control subjects, strongly linking cancer disease to previous viral exposure. In 1994 (93) further investigated the detection of EBV-DNA in testis specimens from patients with testicular germ cell tumors, including preinvasive carcinoma *in-situ*. A weak positivity for EBV DNA was detected in only six out of the 20 samples but none of the specimens showed a positive staining at either anti EBV-immunohistochemistry or *in situ*-hybridization techniques, ruling out a direct involvement of EBV and rather suggesting a putative growth-stimulating role of EBV-transformed lymphocytes infiltrating in testis stromal tissue (93). In 2013 Yousif et al. (94) aimed to quantify the possible association between viral infections and TC through a meta-analysis. Interestingly, serological markers of exposure to EBV, Cytomegalovirus, and Parvovirus B19 were associated with TC with pooled odd ratios (OR) of respectively, 4.80, 1.85, and 2.86.

Particularly for Human-immunodeficiency virus (HIV), authors first identified a pooled OR of 1.79 (94). This evidence was subsequently confirmed by several studies showing a relative risk ranging from 0.7 to 3.1 [reviewed in Hentrich and Pfister (95)]. However, as for EBV, a clear mechanistic model explaining the association between TC and HIV is currently under debate.

Mechanical

Despite being poorly acknowledged among typical risk factors for TC, mechanical, and particularly traumatic events on the testis, are considered a causal factor of this disease. Indeed, the experimental model of intra-testicular hematoma induced by injection of an autologous blood equivalent in rat testis was associated with a significant and long-lasting alteration of the testis structure, such as the reduction of the overall testis volume and the reduction of the seminiferous epithelium size. All these features resulted in altered testis function such as the under-representation of the germ cell population within the seminiferous tubule, altered sperm parameters, and a trend toward lower testosterone levels (96). Based on this profound alteration of the testis' functional architecture, the degeneration into cancer is rather intuitive, particularly in those situations of prolonged though subclinical testis trauma. In spite of this simple model, available evidence linking testis trauma to cancer are sparse and/or non-conclusive.

An interesting study from Dusek et al. (97) aimed to quantify the contribution and mutual interactions of very different types of potential risk factors for TC through the administration of standardized questionnaires to patients recruited in two Czech cancer centers, compared to healthy and age-matched controls (97). Interestingly, in addition to acknowledged risk factors like cryptorchidism and testis atrophy, a significant association was found for testicular trauma resulting with a nearly doubled risk for TC compared to the controls.

Another example of this model is represented by prolonged testis-micro traumas in patients practicing sports. In fact, testicular derangements such as testicular torsion, epididymitis, and testicular tumors are frequently observed by medical sport physicians (98). Coldman et al. (99) showed that cycling, particularly during teenage years, was associated with an almost doubled risk for TC even after correction for confounding factors like cryptorchidism or inguinal hernia. Also, horse-riding resulted in a nearly 3-fold greater risk for TC, which remained substantially unaltered after correction for confounding factors, whilst no significant association was reported for motorcycling or soccer (99). However, subsequent studies failed to identify a significant association between these sports with TC, suggesting further investigation (100, 101).

Chemical

Available data on chemicals exerting a direct role as a risk factor for TC mainly derive from occupational studies. Particular attention has generally been drawn by the exposure to heavy metals in extracting and processing plants. Heavy metals, most frequently absorbed as organometallic compounds, are known to accumulate in tissues, both disrupting their biological functions and representing long-term reservoirs, resulting in prolonged exposure to metal pollutants (102). In particular, transition series-metals like cadmium (Cd), mercury, and cobalt are acknowledged as carcinogens from several experimental studies performed in both animal and cell models (103–105). However, a direct association between Cd exposure and TC is still under ascertainment. In 2011, an epidemiological study for cancer incidence was performed in the Kempen area across the Dutch-Belgian border, featuring the very long activity of cadmium and zinc smelters. Compared to the control population, identified through regional population-based cancer registries, environmental exposure to Cd showed an increased risk for female lung cancer, male and female bladder cancer and prostate cancer but not TC (106). Similarly, another study focused on the north-east Belgium area investigated the ∼17 years incidence of cancers, finding an overall increased risk of doubling the 24-h urinary cadmium excretion, however no significant association with TC was documented (107). On the other hand, previous studies performed on metal workers in the Hannover region of Germany showed a nearly doubled risk of developing TC compared to the aged matched healthy controls, but no single chemical emerged at significant levels from the association analysis (108). In addition, Norwegian metal workers working with ferrosilicon and silicon furnaces showed a more than doubled incidence of TC compared with the estimated incidence in the general Norwegian population according to the age and historical period (109).

Another class of environmental chemicals associated with TC is pesticides, as depicted by epidemiological studies disclosing an increased incidence of TC in agricultural employees (110–112). However, two great meta-analysis in 1992 and 1998, respectively, failed to recognize a significant risk due to the exposure to pesticides in farmers (113, 114). To this regard, opportune distinctions should be made since substantial difference exists among countries in terms of chemicals, formulations, and regulatory principles (90). Furthermore, great differences in terms of toxicological effects of the different molecule classes is likely to exist in humans. In fact, organochlorines pesticides are supposed to act as endocrine disruptors (see below) whilst pyrethroids are likely to exert a direct effect on the cell cycle (115, 116).

Physical

Among those physical risk factors theoretically associated with an increased risk of TC, such as the exposure to ionizing radiations, ultraviolet light and electrical work, the most clinically valued is exposure heat stress. As depicted by the external location of male genitalia, the proper germ cell maturation within the seminiferous tubule is maintained at 2–8°C below the body core temperature (117). Systematic exposure of the testis to over-physiological temperatures has been associated with several, and generally reversible, testis derangements such as a reduced sperm count, motility, mitochondrial function, and even altered sperm membrane composition (118, 119). As for other environmental factors, the possible association between heat exposure and TC was investigated through occupational studies. Early studies in 1995 performed on TC patients and healthy age-matched

controls, revealed that occupational exposure to high or extreme temperatures was associated with an adjusted OR of respectively, 1,2 and 1,7, suggesting external temperature as an independent risk factor (120). A subsequent study in 2001 confirmed that the standardized incidence ratio for TC in fire fighters was 3,0 with no increased risk from any other cause of death (121). However, exposure to milder heat stress like showering and bathing was not associated with any significant risk for TC (122).

HORMONES

Exactly like other endocrine tissues in the body, the testis is both the target and the source of hormones strictly linked in a feedback-loop regulating pathway (123). In particular, the activity of the hypothalamus/pituitary/gonadal axis takes place from the early phases of embryo development, regulating testis descent and, the adequate location within the scrotal sac and the proper spermatogenic and endocrine functions, whose systemic effects are well-known (124). The early disruption of this hormonal circuit reverberates on testis function in adult life and represents a major risk factor for TC. Indeed, a tentative mechanistic model of the neoplastic transformation of germ cells has been developed by Rajpert-De Meyts et al. (125). This hypothesis is based on the strict similitude between primordial germ cells and gonocytes with tumor cells of the carcinoma *in situ* (CIS), verified at the molecular level by the shared expression of genes involved in pluripotency and proliferation, such as *NANOG*, *STELLAR, DPPA-5*, *GDF3*, *K-RAS,* and *CCND2* (126–129). In this context, the delayed development of germ cells, associated with long-term maintenance of embryonic genes, would represent a key event for the subsequent degeneration into cancer cells (130, 131). The disruption of the hormonal milieu of germ cells would then result in misleading signals altering the cell phase-switch toward mitosis and meiosis, with the consequent risk of a neoplastic transformation in adult life (125).

The most studied model of hormonal risk factor for TC is the disorders of sex development (DSD) in 46, XY males, frequently associated with androgen-insensibility syndrome (AIS), further distinguished into complete (CAIS), partial (PAIS), or mild (MAIS) forms (132). As can be guessed from the name of this pathological condition, its clinical characteristics range from a female phenotype of CAIS, in spite of an XY karyotype and normal androgen production, to severe under masculinization in PAIS, such as female external genitalia or hypospadias or micropenis, or male infertility and/or gynecomastia in MAIS. A general feature of the different forms of AIS is the altered function of the androgen receptor, resulting in a resistance to androgens as activating ligands. Genetic variants of the *AR* gene are commonly acknowledged as being causative of AIS. In particular, 95% of CAIS are associated with inactivating mutations of AR. In PAIS however, mutations of the *AR* gene are detected in <25% of patients whilst a complementary causative role has been ascribed to genetic variants of the protein and cofactors that concur to the AR signaling pathway, such as the deficiency 17β-hydroxysteroid dehydrogenase (17β-HSD), a key enzyme in steroidogenesis (133, 134). Also, persistent müllerian duct syndrome (PMDS) is a form of disorder of sex differentiation in 46, XY males caused by an inactivating mutation of the gene for AMH/MIS (45% of cases) or its type II receptor (39% of cases) (135). PMDS patients present genotypically and phenotypically as males with unilateral or bilateral cryptorchidism and/or an inguinal hernia at infancy (136). In general, DSD, and in turn AIS, are associated with and increased risk of TC with an estimated overall prevalence of nearly 5.5 percent, ranging from 0.8% in CAIS-associated DSD, 15% in PAIS to 17% in 17β-HSD deficiency (137, 138). Despite the fact that TC in PMDS has been described in several case reports, association studies on large cohorts are actually not available (139–145). Of note, testis retention in the abdomen represents an association with DSD and represents by itself a risk factor of TC as previously discussed. Interestingly, the association between TC and cryptorchidism has been documented in DSD from both AR mutation and PMDS. Thus, a major pathogenic role of DSD-associated testis retention in TC cannot be ruled out (146, 147).

Intriguingly, available data on derangements of the upstream hypothalamus/pituitary/gonadal axis showed an inconsistent association with TC. In particular, activating and inactivating mutations of the LR-receptor (LHR) gene are causative of DSD forms like isosexual precocious puberty in boys and Leydig cell hypoplasia, respectively. The latter presents variably from normal appearing female external genitalia to hypergonadotropic hypogonadism with microphallus and hypoplastic male external genitalia (148, 149). However, few available data documented TC only in patients with isosexual precocious puberty, particularly for testicular interstitial cell tumor observed in a 9 years old boy (with no available genetic screening at the time of the analysis), and two cases of activating mutations of the *LHR* gene reporting testicular seminoma in adult life (150–152).

TESTIS CANCER: SOMETHING IN BETWEEN GENES, ENVIRONMENT, AND HORMONES

As for the majority of oncological diseases, TC is the result of a complex interaction among the aforementioned genetic, environmental, and hormonal risk factors. To this regard, testicular dysgenesis syndrome (TDS) is probably the most reliable and clinically adherent model that describes the actual pathogenesis of TC (153).

The concept of TDS derives from the observation of the worldwide increasing incidence of a cluster of male urinary-genital alterations such as infertility, cryptorchidism, hypospadias and, indeed, TC. All these clinical conditions share the common feature of originating during fetal life, during which the impaired function of Sertoli and/or Leydig cells alters the proper testis function from germ line development to hormone production. The combination of these conditions results in a range of clinical consequences in adult age,

spanning from infertility, to disorders of sexual development and cryptorchidism that, in turn, are risk factors for TC (154). In addition to the already mentioned genetic factors, there is a general agreement according to which the failure of testis nourishing cells is due to environmental factors, such as the exposure to chemical pollutants with endocrine disrupting activities (155). This hypothesis was originally suggested by the wide geographical variation observed for TDS symptoms in different countries. The most striking evidence, suggesting the possible influence of anthropogenic factors, was surely the differential prevalence of TDS-related disorders observed in two nations at equal latitude and industrialization, respectively, Denmark, with "higher" prevalence of TDS symptoms, and Finland with "lower" prevalence of TDS symptoms (156–159).

The mechanistic hypothesis of the endocrine disruption in TDS relies on two main pathways. The first one is the estrogen hypothesis, consisting of the exposure to chemicals with estrogenic properties that exert a central disruption of the hypothalamus/pituitary/gonadal axis, reducing in turn T release from the testis. Diethylstilbestrol (DES) is a clear example of an endocrine disruptor with estrogen action. Being an estrogen receptor agonist, DES was frequently prescribed to pregnant women during the 50s−60s in order to relieve abortions and pregnancy-related complications. However, males born from DES-treated mothers showed an increased incidence of epididymal cysts, altered sperm parameters, cryptorchidism, and TC (160–162). Similar to DES, the common plastic additive bisphenol A (BPA) was also acknowledged as a partial estrogen agonist (163). Exposure to BPA in males has been associated with increased levels of prolactin, estradiol, and the sex hormone-binding globulin level (164). Furthermore, higher levels of BPA in semen were associated with signs of a direct impairment of spermatogenesis such as poor sperm count and function (165).

Another suggested mechanism of endocrine disruption in males is the anti-androgen activity, typically exerted by phthalates plasticizers (166). Data obtained in cell and animal models are highly suggestive of direct influence of phthalates compounds on the endocrine function of Leydig cells, particularly impairing T and INSL3 production (167–169). Accordingly, increased prevalence of reduced anogenital distance (AGD), cryptorchidism, hypospadias, and other genital-urinary disorders were observed in male subjects from mothers exposed to this class of chemicals (170).

The list of substances with acknowledged or possible endocrine disrupting effects is continuously increasing and the exposure to these environmental agents is currently considered the major causative factor of the increasing incidence of TC throughout the last decades. However, equal exposure to the same disruptor is not univocally associated with the same phenotype of TDS, highlighting the role of the genetic background in the establishment of the susceptibility to genital-urinary disorders, in general, and to TC in particular (171). An interesting example of how genetic and environmental factors interact, determining a clinical phenotype, has been provided by our group in a very recent investigation focused on the *E2F1* gene (172). As cited above, altered *E2F1* expression has been significantly associated to several testis disorders such as spermatogenic impairment, cryptorchidism, and TC, particular in those cases of increased gene expression related to supernumerary gene copy numbers (49). Interestingly, through the use of an engineered NTERA-2 cl.D1 cell model, cultured at over-physiological temperature, the *E2F1* gene expression was up-regulated in a temperature- and gene-copy number- dependent manner. Altogether, these results suggest that the clinical condition associated with abnormal E2F1 expression, due to copy number variation, can be worsened even more by other concomitant environmental conditions, such as heat stress or an history of cryptorchidism, with a likely impact on TC development.

TABLE 1 | Genetic factors and related mechanism associated to testis cancer.

Gene	Mechanism	References
GATA4, GATA1	Specification and differentiation of postnatal testicular development	(25–31)
PRDM14, DMRT1	Germ cell specification-sex determination	(25, 32–35)
SALL4	Disruption of POU5F1 binding motif; maintenance of pluripotency in embryonic stem cells	(25, 36)
TEX14, WDR73, PMF1, CENPE, and PCNT	Microtubule and chromosomal assembly; kinetochore-microtubule assembly; microtubule organization during interphase; microtubule assembly–related genes	(25, 37–44)
KIT	KIT–MAPK signaling	(25)
FCP2L1, ZFP42	Germ cell development and pluripotency	(45)
ZWILCH	Kinetochore function	(45)
TIPIN	Response to DNA damage	(45)
TKTL1, LHPP	Mitochondrial function	(45)
E2F1	Copy number variation	(49)
INSL3, RXFP2	Cryptorchidism	(55, 56, 63–65)
AR, 17β-HSD	Cryptorchidism; steroidogenesis; disorders of sex development	(55, 56, 63, 66–68, 133, 134, 137, 138)
AMH, AMH type II receptor	Cryptorchidism; disorders of sex development	(85, 86, 139–145)
LHR	Steroidogenesis	(150–152)

CONCLUSIONS AND FUTURE PERSPECTIVES

TC is the most prevalent tumor disease in male subjects at reproductive age, showing a progressive increase throughout the last four decades. The current model that better explains this trend, based on clinical and experimental evidence, relies on the increased exposure to environmental factors, particularly chemical pollutants with endocrine disrupting activity, that alters the major hormonal axis that drives testis development and function from gestational age. The susceptibility to these alterations further depends on genetic factors that strongly justify the strong familiarity of TC (**Table 1**).

A very recent field of investigation that aims to integrate genetic and environmental factors on the risk for TC is epigenetics, namely the inheritance of genetic factors that do not rely on the variation of the genetic sequence but rather on the regulation of gene expression trough DNA methylation and histone modification. Very recent investigations showed that DNA from tumor cells display significant hypomethylation compared to normal germ cells, evidence likely due to the over-expression of de-methylating factors that are generally suppressed after fetal germ cell development (173). It is a shared opinion that environmental factors largely govern the balance between methylating and de-methylating factors (174).

Finally, it should be noted that in spite of the high sensitivity of TC to chemotherapy, explaining the good prognosis of treatment, there is still a large population of patients suffering from drug resistance or inefficient treatment settings among chemotherapy, radiotherapy, or surveillance (175). In this regard, some pioneering studies have succeeded in identifying genetic markers of good responses or tolerability to therapeutic agents, thus improving the overall outcome of treatments. This is the case for the identification of genetic variants in the *SLC16A5* gene, which has been significantly associated to ototoxicity induced by cisplatin (176).

In conclusion, the availability of novel strategies of investigation are of paramount importance to clarify the key aspects of TC development, progression and therapy, in order to further improve the prevention and treatment of a highly curable disease with an unexplained increasing diffusion.

AUTHOR CONTRIBUTIONS

AG conceived the manuscript. IC, MG, and IŠ performed bibliographic research. CF supervised manuscript draft. LD wrote most of the manuscript.

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REVIEW
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Proteomics for the Identification of Biomarkers in Testicular Cancer–Review

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A large number of biomarkers have been proposed for the diagnosis of testicular cancer, representing putative molecular targets for anticancer treatments. However, no conclusive data have been provided. Proteomics represents a research field recently developed. It evaluates the large-scale analysis of the full protein components of a single cell, of a specific tissue, or of biological fluids. In the last decades, proteomics has been applied in clinical fields, thanks to modern technology and new bioinformatic tools, to identify novel molecular markers of diseases. The aim of this review is to argue the findings of recent studies in the discoveries of putative prognostic and diagnostic markers of testis cancer by proteomic techniques. We present here a panel of proteins identified by proteomics which might be used after validation for early detection and the prognostic evaluation of testicular tumors. In addition, the molecular mechanisms revealed by these proteomic studies might also guide the development of novel treatments in future.

Keywords: testis cancer, proteomics, biomarker, germ cell tumor, proteins

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INTRODUCTION

Testicular germ cell tumors (TGCT) is the most frequent cancer occurring in young men. TGCTs are classified into two subgroups: non-seminoma and seminomas germ cell tumors. Seminomas rate for 50% of testicular cancer and non-seminoma germ cell tumors rate for 40% of testicular cancer. The remaining 10% of testicular cancer is associated with tumors and they usually include both seminoma and non-seminoma components (1). The difference in the diagnosis between seminoma and non-seminoma is fundamental for the objective of treatment and of prognosis.

TGCTs originate from transformed gonocytes or undifferentiated spermatogonia, but the pathogenesis of TGCT remains unexplored (2). To date, accessible markers for the diagnosis and follow-up aftercare include a-fetoprotein (AFP), ßHCG and LDH. However, AFP and ßhCG have high specificity (90%) but often relatively low sensitivity. For this reason tumor markers alone are not able to detect many recurrences, indeed in about 40% of men with disease recurrence the levels of these markers are usually "normal." The LDH appears to have poor diagnostic performance (3). Therefore, the discovery of novel clinical biomarkers would clearly help the early detection and the monitor of the disease.

The diagnosis of "cancer" can be challenging. In addition to histopathological interpretation and immunohistochemical stains for confirming the precise cell of origin (4), more recently global gene expression profiling has been applied in order to facilitate the treatment decisions and prognosis. One key application for patients with the primary disease is precise prognosis, which helps to divide patients into different risk groups and select both treatment and monitoring strategies. Usually, the prognosis is based on clinical parameters such as age and tumor stage. Recently, considerable attempts have been made to incorporate molecular information in the staging process for accurate prognosis. Different tumors are classified in accordance to similar gene expression patterns. They represent a "molecular signature," composed by several tens or hundreds of genes, in particular with regard to cell morphology or tissue characteristics. Global gene expression is most easily measured using cellular RNA; on the other hand, protein expression profiling provides a more dynamic view, offering additional informations on protein-protein interactions, post-trasductional modifications and finally on protein abundance (5).

Testicular cancer has been mainly studied at a genetic level. However, proteomics represents a promising technology that could allow novel insight into the disease at the molecular level to increase the understanding of their function. In the present molecular era proteomic is evaluated as a crucial point in personalized medicine to identificate specific target proteins for the pathophysiological state. Moreover, modern bioinformatic analysis offers information about the involvement of the proteins in the biological pathways of the tumor (6). Proteomics analysis of testicular tumor compared to normal testicular tissue may create a platform for enhanced understanding of differentially expressed proteins which might represent potential biomarkers for cancer. Protein expression profiling is a powerful tool in clinical practice, particularly in identifying cancer biomarkers to help the diagnosis and to choose a personalized treatment and monitoring of patients.

In the field of testicular tumors, particularly, the studies are few and outdated. Currently the main problem in the identification of protein markers is the small number of proteins which have been associated to TGCT.

PROTEOMIC TECHNOLOGIES APPLIED IN TGCT: FEATURES AND PERFORMANCES

Technological advances in proteomics have improved sensitivity and multiplexing ability of the method, as well as the possibility of identifying protein interactions. These advances can be of help to understand the molecular mechanisms involved in TGCT. The use of proteomics technologies offers an appealing approach to the identification and development of new tools to be used in clinical practice, identified by the simultaneous comparison of hundreds or thousands of proteins. The development and use of performant sample preparation techniques together with the increasing availability of proteomics technologies and recent technical advances in mass spectrometry (MS) enable identification and quantification of proteins involved in the diseases, although they are expressed at low abundance (7).

Up to now the most common proteomics technologies applied in the studies about TGCT include "gel-based" proteomics such as 2D-PAGE and 2D-electrophoresis associated with mass spectrometry (MS). SELDI-TOF and High-performance liquid chromatography (HPLC) associated with tandem mass spectrometry (MS/MS) moreover have been used in two studies. One additional proteomic study was performed in serum by SELDI-TOF.

Separation Techniques

2D Gel Electrophoresis

2D gel electrophoresis detaches proteins consistent with their isoelectric point. A second, independent separation step is then performed, dependent on mass (molecular weight), using sodium dodecyl sulfate polyacrylamide gel electrophoresis. This 2D-PAGE method can be performed to develop samples at high resolution and on a large scale. It might represent a primary screening method to form hypotheses and to guide further researches. 2D gel electrophoresis is the most frequently performed technique for proteomic analysis, although some limitations which are related to protein solubility (i.e., membrane proteins not easily solubilized), poor protein separation in the initial pH gradient (too basic or too acidic proteins) and low and high molecular weight (8, 9).

To better identify the separated proteins, proteases may be used to digest bands or spots obtained 2D-electrophoresis. The smaller fragments are then ionized and analyzed by mass spectrometry (MS).

High-Performance Liquid Chromatography (HPLC)

HPLC is used with the aim to obtain a complete protein recovery, including small basic and hydrophobic types (9). In fact, protein separation is based on some specific protein properties (hydrophobicity, surface charges, specific amino acid sequences) (10). Connecting HPLC with a mass spectrometer it is possible to obtain the rapid separation and the comprehensive identification of components of a complex protein mixture, with the aim to deeply analyze a proteome.

Proteomic Analysis

Mass Spectrometry (MS)

MS-based techniques can be used to study complex protein mixtures previously fractionated by electrophoresis or HPLC. This technology provides precise mass values by the measurement of the mass-to-charge ratio (m/z) of the ions generated from the peptides and the proteins. In recent years a significant technical improvement of mass spectrometers has been observed. Modern mass spectrometers, such as time of flight (TOF), Fourier transform ion cyclotron resonance (FT- ICR) and Orbitrap detectors provide extremely accurate masses of analytes (11). Makarov invented the Orbitrap in 1999, as a mass analyzer that couples high resolution with high mass-accuracy, a significant m/z range and a high dynamic range (12, 13). The high mass-accuracy of the Orbitrap significantly

contributes to the amount of acquired data and the number of analytic approaches that can be connected to MS (14).

SELDI TOF-MS

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI TOF-MS) has been used widely in biomarker discovery because its sensitivity. It requires moreover only a small amount of protein for analysis. This method decreases the sample complexity by studying proteins with a specific chemical property that allows them to be adsorbed in a known surface (e.g., a charged, hydrophobic, or functionalized affinity surface). The non-bound protein population is removed by extensive washing. The adsorbed sample is ionized and analyzed (15).

PROTEOMICS OF TGCT TISSUE

Table 1 summarizes the proposed putative markers which have been identified in TGCT tissue.

For the first time in 2006 Zimmermann et al applied proteomic analysis to neoplastic germ cell tissue (16). This strategy has been applied to identify differences in the proteomes obtained by non-neoplastic and neoplastic tissues. Protein extracts have been analyzed by 2DPAGE combined with mass spectrometry. The comparative study permitted the identification and detection of quantitative differences of expressed proteins between seminoma and non-seminoma tissue.

Glutathione S-transferase (GSTs) M3 protein has been downregulated in seminoma tissues. GSTs detoxification enzymes partecipate in the pathogenesis of cancers. GSTM3 is a critical GSTs variant and previous evidences showed that GSTM3 polymorphism is associated with an increased risk to develope a cancer (20, 21). A lot of studies previously investigated the association of GSTM3 gene polymorphism with the risk to develop a lung cancer. Furthermore, a reduction in GST protein level was earlier reported in human TGCT (22), but the M3 isoform has not been previously identified in TGCT. Only proteomics permitted the identification of this specific isoform, as possibly associated with testis cancer. The M3 homodimer is a specific isoform specifically identified in the brain and in the testis (23). The GSTM3 gene is polymorphic and GSTM3 polymorphisms control the enzyme activity by the modulation of substrate binding (24). Distinct polymorphisms of GST-M3 enzyme are associated with a higher risk for TGCT formation (16). In particular, the study published in 2009, performed on a large population of patients who survived TGCT, reported that GSTP1 genotype influences the risk of developing a TGCT (25). M3 and P1 polymorphisms of GSTM3 represent promising markers for predicting the risk of TGTC formation.

In 2009 Leman *et al.* used a proteomics analysis to discover a pattern of proteins related with nuclear changes in seminoma cells (17). The changes in the form and in the size of the nucleus are trademarks of the cancer cell. Proteomics approach focused to mark a specific pattern of proteins related with the specific alterations in the nuclear structure of seminoma cells. Using high-resolution two dimensional gels, four nuclear matrix proteins have been identified in seminomas, but not in the normal tubules and three of these four proteins are part of gamma-tubulin complex component 6 (GCP6).

The γ-tubulin complex is a large multiprotein complex which plays an essential role in microtubule nucleation at the centrosome. C-tubulin ring complex (c-TuRC) is a key component of the centrosome which nucleates microtubules. GCP6 is localized in the pericentriolar material, a protein matrix composed by protein complexes involved in centrosome-associated functions (i.e., microtubule nucleation). Moreover, GCP6 is needed for centriole duplication and Polo-like kinase4 (Plk4)-induced centriole over-duplication. GCP6 interacts in fact with Plk4 and it is phosphorylated by the same Plk4. Therefore, controlling centriole numbers GCP6 preserves cells to have the correct number of centrosomes and cilia. Excessive number of centrioles lead to tumorigenesis in flies (26) and has been associated with chromosomal instability in humans (27).

Another specific protein for seminomas is Cyclin-dependent kinase 10 (CDK10). CDK10 is a Cdc2-related kinase and is a key element in the advance from the G2 to M phase transition of the cell cycle. Two isoforms of CDK10 have been documented: HCDK10-1 and HCDK10-2 (17). CDK10 is highly represented in colorectal cancer where it takes part in the suppression of apoptosis and in the stimulation of tumor growth *in vitro* and *in vivo*. The modulation of CDK10 expression in colorectal cancer indicates that CDK10 is involved in cell growth and it is associated with a reduction in chemosensitivity. Finally it inhibits apoptosis through the upregulation of the expression of Bcl-2 (28). Both the CDK10 isoforms are expressed in the nuclear matrix of the seminomas, supporting the role of CDK10 in the cell cycle regulation that may induce testicular cancer.

In normal testis moreover seven specific nuclear matrix proteins have been detected, which are absent in seminoma tissue. Some of these proteins are testis specific: Y177 encoded-like protein 4, cytokeratins, glutamine synthetase, and StAR-related lipid transfer protein 7 (StarD7).

StarD7 has been reported for the first time as related with nuclear matrix and it is part of the family of StAR1-related lipid transfer (START) proteins. These proteins are involved in lipid transport, metabolism and signaling (17). Overexpressed StARD7 gene has been specifically linked with colorectal cancer. StarD7 protein upregulation has been documented in differentiating cytotrophoblast suggesting that StarD7 might have a function in throphoblast differentiation through phospholipids uptake and transport (29). The loss of StarD7 protein in JEG-3 cells alters ABCG2 multidrug transporter level, cell migration, cell proliferation, and differentiation marker expression. StarD7, expressed in normal testis but not in seminoma tissue, might have a role in maintaining the differentiated form of the normal cells. The loss of StarD7 might induce cancer development.

The testicular microenvironment is a unique environment. Spermatogenesis and tumorigenesis at testicular level in humans are two biological processes which have got numerous similarities between them. It is very important to know the

TABLE 1 | Proteins identified through proteomics as putative markers in TGTC tissue.

Protein	Gene	Molecular function (Gene Ontology)	Expressed in TGTC	Biological function in TGTC	Reference
Glutathione S-transferase (GSTs) M3 protein	GSTM3	Protein binding	Downregulated in seminoma	Polymorphism of GSTM3 predict higher risk of TGCT development	Zimmermann (16)
Gamma-tubulin complex component 6	GCP6	Microtubule binding	Identified in seminomas but not in the normal tubules	Role for microtubule nuclation at the centrosome. Excessive number of centrioles have been associated with chromosomal instability.	Leman (17)
Cyclin-dependent kinase 10	CDK10	Protein binding; kinase activity	Identified in seminomas but not in the normal tubules	Involved in cell cycle regulation	Leman (17)
StAR-related lipid transfer protein 7	STARD7	Lipid binding	Absent in seminomas	Involved in metabolism and signaling. It might play a role in mantaining the differentiated form of the normal cells.	Leman (17)
Mab-3 doublesex- and Related Transcription Factor 1	DMTR1	Molecular function	Over-expressed in mixed germ cell-sex cord stromal tumore and spermatocytic tumor	Controller of mitotic proliferation of germ cells	Liu (18)
Piwi-Like RNA Mediated Gene silencing 1	PIWIL1	Protein and mRNA binding	Specific protein of TGCT	Involved in RNA silencing during translational activity. It improves DNA methylation.	Liu (18)
Transmembrane (C-Terminal) Protease, Serine 12	TMPRSS12	Serine-type endopeptidase activity	Specific protein of TGTC	Previously reported in prostate and liver cancer. Unknown biological mechanism.	Liu (18)
p21-activated kinase 4	PAK4	Protein binding	Overexpressed in embryonal carcinoma	Involved in cell protections from apoptosis	Castillo (19)

common mechanisms between the two biological processes. In 2013 Liu *et al.* performed an extensive study for tumor marker identification by proteomic of testicular tissues. Using 2D-high performance liquid chromatography (HPLC)–MS/MS (LTQ Orbitrap Velos hybrid mass spectrometer) the Authors identified 7,346 proteins in testis tissue with normal spermatogenesis (18).

The protein data were confirmed by immunohistochemistry and by comparison with previously published data from the Human Testis Proteome Database. These date have been confirmed by using of a GWAS study, using associated SNPs in case of differential expression of these proteins. Among these testicular proteins, six novel cancer/testis gene transmembrane protease have been characterized: serine 12 (TMPRSS12), tubulin polymerisation promoting protein family member 2 (TPPP2), protease serine 55 (PRSS55), double-sex and mab-3 related transcription factor 1 (DMRT1), piwi-like RNA-mediated gene silencing 1 (PIWIL1), and hemogen (HEMGN). The last four proteins have been proposed to exert a central role in spermatogenesis and cancer development, although they still haven't known specific functions in testis (18).

The Mab-3 doublesex- and Related Transcription Factor 1 (DMTR1) has been associated to prostate cancer. It is a controller of mitotic proliferation in germ cells, thanks to a specific zinc finger structural motif which is associated with cell cancer proliferation. A recent study confirmed these results in the human testis; nuclear expression of DMRT1 was reported in spermatogonia, but not in primary spermatocytes that have entered meiosis 1 or in more mature germ cells. DMRT1 expression was confirmed in the germ cells of testicular mixed germ cell–sex cord stromal tumor (MGC-SCST) and spermatocytic tumor but not in those of seminoma. For this reason the germ cells of MGC-SCST are related to spermatogonia, which espress DMRT1. The strong expression of DMRT1, together with the

absence of TCLF5 in the germ cells of both MGC-SCST and spermatocytic tumor, suggests a premeiotic origin for both tumors (30).

The second protein is Piwi-Like RNA Mediated Gene silencing 1 (PIWIL1) which, together with the allelic variant rs10773777, was also detected in prostate cancer cases. PIWIL1 was also detected by proteomic approach as a specific protein of TGCT. This protein might play a pivotal function in RNA silencing during the modulation of tranlational activity. Previous studies reported that PIWIL1 plays an important role in cancer development, improving DNA methylation. Several cancer-germline genes have been defined to stimulate PIWIL1 as a part of oncogenic pathways involved in cell proliferation (31). More recently PIWIL1 expression was demonstrated in spermatocytes and spermatids. Up to 70% of TGCT samples express PIWIL1, which is not normally expressed in premeiotic germ cells. These evidences support that in many germ tumors is present an aberrant expression of PIWIL1. The enhanced expressions of piwil2 was found in seminomas and has not been reported in testicular non-seminomas tumors (31).

The third protein is Transmembrane (C-Terminal) Protease, Serine 12 (TMPRSS12). It was previously related with colorectal cancer for the variant rs11169552 (32). This protein has been demonstrated to be expressed in spermatids and spermatocytes (33). The Tubulin Polymerization-Promoting Protein Family Member 2 (TPPP2) and its variant rs1952524 was linked to liver cancer (34). Another protein is the Protease Serine 55 (PRSS55) along with the variant rs4404875, which has been mainly identified in Leydig and Sertoli cells and it is is associated with prostate and ovarian cancer (35). Finally Hemogein (HEMGN), which regulates proliferation and differentiation in hematopoietic cells, has been associated with thyroid cancer (36). Among the 300 proteins expressed in human testis, only 22.7% are TGCT-related proteins, of which only 65 proteins have been evaluated, indicating that other candidate proteins exist, although not still studied, The functional analysis of only 65 out 300 proteins might depend to still low sensitive methods in the field of proteomics. The six proteins previously cited might represent moreover important targets for personalized therapy in this kind of neoplasy (18).

In a very recent study, Castillo *et al.* identified 174 phosphorylated kinases in human testis by metal oxide affinity chromatography using TiO2 combined with LC-MS/MS. Protein phosphorylation is involved in the modulation of cell cycle, cell growth, cell differentiation and cell death. Two kinases have been studied in the testis phosphoproteome as candidates for further studies by immunodetection procedures. Immunodetection has been specifically used to study the potential function of cyclin dependent kinase 12 (CDK12) and p21-activated kinase 4 (PAK4) in testicular tissue. The in silico protein-protein interactions have been studied, and a functional analysis in a human embryonal carcinoma cell line has been performed. PAK4 is localized in human spermatogonia. Its function in preventing the activation of caspase is well-known. In embryonal carcinoma it has been observed that PAK4 protects cells from apoptosis. PAK4 inhibitors might represent an interesting pharmacological target for novel drugs modulating behavior of testicular cancer (19).

PROTEOMICS OF TCGC IN SERUM

Protein markers that distinguish cancer patients by healthy controls can be moreover searched in serum.

A single serum proteomic study regarding testis cancer was carried out. In 2010, Strenziok et al utilizing Surface-enhanced laser desorption ionization time-of-Xight mass spectrometry (SELDI-TOF MS) identified the protein profiles of TGCT patients that are different in a highly significant degree from normal subjects (37). CM10 ProteinChip® array identified 138 peaks in a mass range of 3,800–10,000 Da that might repredent a "molecular fingerprint" to differentiate tumor serum sample from non-tumor serum samples. The spectra of proteins have been investigated by the proteomic platform "proteomic.net." Five peaks have been verified by CM10 ProteinChip® (6.48, 6.84, 8.15, 8.17, 8.92 kDa). There was no single peak that could discriminate the group of seminoma vs. control subjects, so a cluster classifications has been performed.

For statistical analysis, an artificial intelligence learning algorithm used three different bioinformatics methods to develop the training set for the decision trees, support vector machines, and neural networks and to differentiate between the two groups. Decision tree analyses developed the most powerful classifier with 89.4% specificity and 91.5% sensitivity (CM10 ProteinChip®, 95% confidence interval of 82.6–95.5%).

In this study the authors after the first step in investigation separating cancer from healthy controls with the definition of protein profiles of TGCT patients that differ in a highly significant degree from normal controls; the protein identification of peak masses was not necessary to differentiate cancer patients from healthy subjects.

Validation of these results may permit proteomic profiling to become a useful tool especially for aftercare follow up.

CONCLUSIONS

In the past 20 years molecular biomarker identification achieved importance in the field of personalized medicine, aimed to identify a cancer in the early stages and to develop novel therapeutical approaches. According to these premises, the discovery of novel specific markers would help the management of patients with testicular cancer.

Proteomics has proven to represent a promising platform for identifying biomarkers linked to testicular cancer.

The conventional tumor markers AFP, hCG, and LDH have demonstrated value in the clinical management of testicular malignant TGCT. However, their limitations in sensitivity and specificity prevent more universal application, especially in patients with seminoma. Tissue and serum biomarkers show exciting promises in the identification of markers for the

diagnostics of TGCT. Furthermore, no proteomic studies have been permorfed aimed to detect markers of TGCT in semen, although it represents a promising source of putative biomarkers in different clinical situations (38–40).

Proteomic identification of TGCT-related proteins will allow to validate candidate markers for early detection and the prognostic evaluation of testicular tumors. In addition, the molecular mechanisms revealed by these proteomic studies might also guide the development of novel treatments in future.

AUTHOR CONTRIBUTIONS

DM and GG: conceptualization and writing—original draft preparation. DM and FP: literature analysis. FV and AP: writing—review and editing. AP: supervision.

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Germ Cell Neoplasia *in situ* (GCNIS) in Testis-Sparing Surgery (TSS) for Small Testicular Masses (STMs)

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Purpose: The testis-sparing surgery (TSS) is surgical technique accepted for small testicular masses (STMs). Frozen section examination (FSE) is an essential assessment at the time of TSS. The aim of this study is to measure the maximum distance of the foci of ITGCN from STMs.

Methods: In our hospital between June 2010 and October 2017 a total of 68 patients with STM underwent a TSS. All the testis specimens were totally embedded and processed via the whole-mount method and a diagnosis of germ cell tumor with GCNIS were made. The distance between STMs and GCNIS were calculated by two pathologists directly on the slides considering for the third dimension the number of the paraffin blocks in which the foci of GCNIS were found.

Results: The STMs were classic seminoma in 62 out 68 cases, embryonal carcinoma in 4 cases, while in 2 case a diagnose of mixed germ cell tumor were made. The size of the STMs was between 0.5 and 2 cm and the foci of GCNIS were observed in seminiferous tubules very closed to SMTs or as skip lesions in the surrounding testicular parenchyma, dispersed in normal testis. In 48 out of 68 cases (70.5%) foci of GCNIS were at the distance from SMTS of 1.5 cm or below and in 60 out of 68 cases (88%) at the distance of 2 cm or below The distance of GCNIS from the STMs was not related to the histological subtype of the germ cell tumor, while there is a linear correlation between size of the STMs and the distance of foci of GCNIS ($p = 0.0105$; $r = 0.9167$).

Conclusion: Our data showed that foci of ITGCN were not observed beyond 2.5 cm from the STM. In particular we demonstrated that exist a linear correlation between size of STMs and distance of the foci of GCNIS from STMs ($p = 0.0105$; $r = 0.9167$). In conclusion mapping the tissue around the tumor not randomly but in targeted areas could reduce the false negative biopsies of the testis with GCNIS, increasing the radicality of the TSS procedure.

Keywords: testis, small testicular mass, seminoma, intratubular germ cell neoplasia, PLAP

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INTRODUCTION

Malignant germ cell tumors appear clinically as palpable masses and radical orchiectomy is considered the standard surgical treatment for these lesions (1). The recent use of high-frequency ultrasonography has led to an increase in detection of incidental small testicular masses (STMs) defined as non-palpable, <25 mm in diameter, intrascrotal masses (2). The testis-sparing surgery (TSS) is an accepted surgical technique for STMs. Since most STMs are benign, unnecessary orchiectomy is to be considered too "costly" from a hormonal and reproductive point of view (3–5). Organ-sparing surgery has numerous advantages from an endocrine point of view in avoiding hypogonadism (6). Orchiectomy in fact causes a reduction in testosterone levels, which in turns represents a risk factor for cardiovascular diseases (7), osteoporosis (8), and lower urinary tract syndrome (LUTS) (9). As a consequence, patients who underwent orchiectomy often require life-long androgen replacement therapy and have a higher risk of low bone mineral density (10) and LUTS (11). Furthermore, preservation of fertility is another issue in young patients affected by testicular tumors. Moreover, it has been reported that unilateral orchiectomy has disruptive effects on overall spermatogenesis (4). Liu et al. reported no significant changes in terms of sperm concentrations and motility in 11 patients with TSS history for benign testicular tumors (12).

The indications for TSS are still controversial, especially for patients with normal contralateral testis. According to the German Cancer Study Group, TSS can be considered only for selected patients with malignant tumors in solitary testis or with a bilateral tumor with a lesion diameter <2 cm and no invasion of rete testis, with normal preoperative serum (LH) levels (13).

Moreover in 2011, the update of the EAU Guidelines considered TSS as an alternative surgical treatment only for patients with synchronous bilateral testicular tumors, metachronous contralateral tumors or for lesions in patients with solitary testis, and normal preoperative testosterone levels if the volume of the tumors represents <30% of the testicular volume.

To avoid secondary surgery, the urologist needs to know the quality of the resected small tumor and the surrounding tissue. The diameter of the mass is an important parameter for TSS; several studies demonstrated that in the case of non-palpable, symptomatic masses with a diameter of <2 cm, TSS represents the best surgical management because the prevalence of benign histology is ∼80% (14–19).

Multiple biopsies of the surrounding tissue, in order to evaluate the presence of microscopic areas of tumor infiltration, is performed after the enucleation of the mass. The presence of foci of GCNIS in the multiple biopsies surrounding the small testicular mass (STM), at frozen section examination (FSE), could help the pathologist discern benign from malignant neoplasms, when a non-conclusive diagnosis of STMs is made. In fact, the presence of GCNIS near the mass seems sufficient for the diagnosis of malignant testicular germ cell tumors (TGCTs) (20–25).

In case of the diagnosis of TGCTs, adjuvant approaches such as radiotherapy, chemotherapy (one cycle of carbolatin AUC7 in case of seminoma) or surveillance must be considered.

TABLE 1 | Main clinical features of the patients (continuous variables are expressed as mean ± DS).

		Min-max
NST (*n.*)	6/68	
ST (*n.*)	62/68	
Age (years)	37 ± 11	(25-60)
Tumor size (mm)	13.7 ± 4.3	(5-20)
Testis volume (ml)	20.8 ± 8.5	(5-30)
FSE distance (mm)	16.1 ± 3.4	(10-24)
Cryptorchidism	27/68	
Infertility	21/68	
Chemical exposition	1/68	

The goal of our study is to define the maximum distance of the expected presence of foci of GCNIS from malignant STMs, in order to provide the surgeon with significant information during the intraoperative procedures of TSS.

MATERIALS AND METHODS

Sixty-eight patients with a STM <2 cm and with a volume <30% of the testicular volume, underwent TSS in our hospital between June 2010 and October 2017. All patients had a testicular mass <2 cm at inspection and a scrotal high frequency ultrasound (US); no physical or radiological signs of malignancy or metastases were observed.

During surgical procedures the FSE of the STMS and multiple biopsies of the surrounding testicular tissue were performed. An operative ultrasound was applied to permit the surgeon to measure and perform the biopsies at the right distance.

As a result of the diagnosis, a traditional radical orchiectomy was performed in case of diagnosis of GCT and/or GCNIS identification in FSE, in order to ensure the best oncological result.

All the diagnosis made on frozen section biopsies were confirmed at histological examination after immune-histochemical staining with PLAP and CD117.

All testis specimens were totally embedded and processed via the whole-mount method and a final pathology reports of germ cell tumors with GCNIS were made (26, 27). For the diagnosis of the GCNIS we isolated the areas with histological features of GCNIS in the whole mount section. A confirmative immune-histochemical staining with PLAP and CD117 was performed.

The distance between STMs and GCNIS was calculated by two pathologists (LML and FP) on the whole mount section, directly on the slides, considering the third dimension for the number of paraffin blocks in which the foci of GCNIS were found. Each paraffin block was 5 mm in thickness.

For the immune-histochemical studies, the avidin-biotin-peroxidase complex method was performed on the paraffin sections, applying a technical procedure previously reported, using a commercially available kit (Dako LSAB2, Dakopatts, Glostrup, Denmark) and the following commercially available monoclonal antibodies: PLAP, CD117 (28, 29).

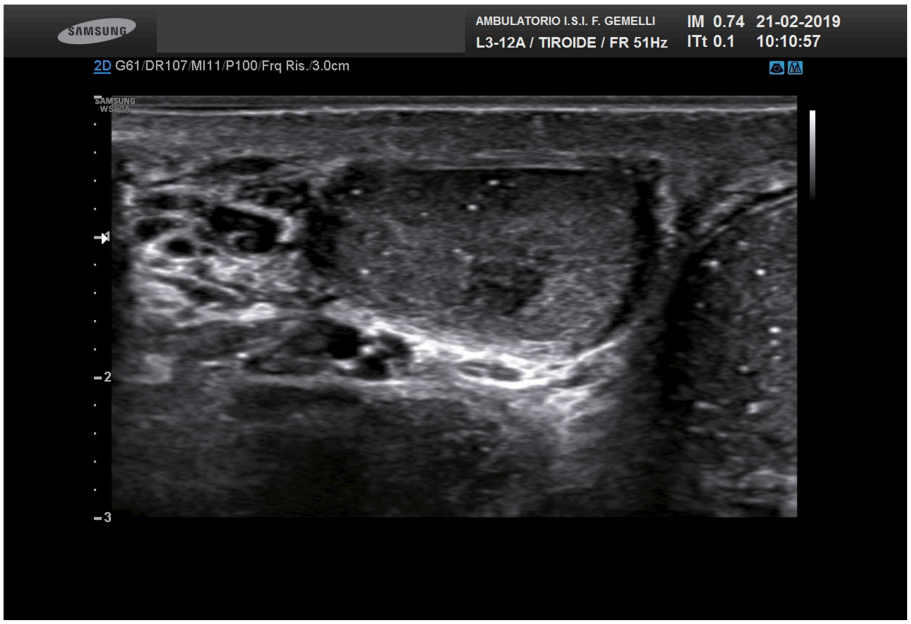

FIGURE 1 | Ultrasound image of STM.

Statistical analysis was performed using GraphPad Prism (version 5, La Jolla, CA) or MedCalc (version 10.2, Ostend, Belgium) software. Continuous variables normally distributed were reported as the mean and standard deviation (SD). A comparison of continuous variables normally distributed was performed using the Student's *t*-test (U-paired or Mann-Whitney *t*-test). A comparison of categorical variables between the two groups was performed by the Chi-square statistic or using the Fisher exact test when appropriate. All *p*-values are considered statistically significant when $p < 0.05$.

RESULTS

The main clinical features of the patients, including the main risk factors for TGCTs, are reported in **Table 1**. **Figure 1** shows an ultrasound image explicative of a STM.

Classic seminoma was found in 62 out of 68 cases with STMs, embryonal carcinoma was found in four, while a mixed germ cell tumor was found in two (seminoma, embryonal carcinoma, and yolk sac tumor). No histological signs of neoplastic invasion of rete testis or necrosis were observed.

The size of the STMs was between 0.5 and 2 cm and the foci of GCNIS were observed in seminiferous tubules very close to SMTs or as skip lesions in the surrounding testicular parenchyma. In 24 cases, the foci of GCNIS were at a distance of 1.5 cm or below from SMTS and in 31 cases at a distance of 2 cm or below. GCNIS was not detected in 13 FSE biopsies.

The distance of GCNIS from the STMs was not related to the histological subtype of the germ cell tumor, while there was a linear correlation between the size of the STMs and the distance of foci of ITGCN ($p = 0.0105$; $r = 0.9167$; **Figure 2**).

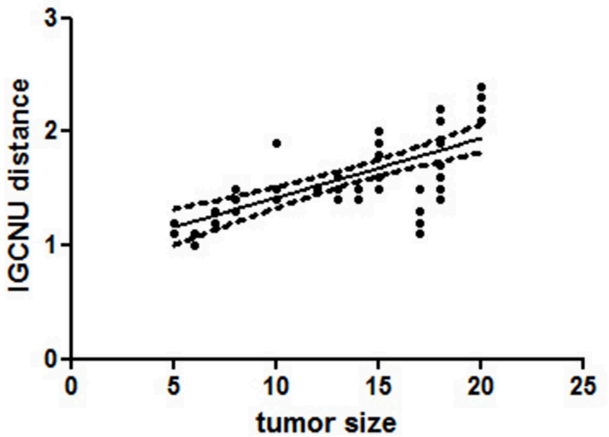

FIGURE 2 | A linear regression analysis performed to evaluate the significant association between different variables [tumor size and distance of foci germ cell neoplasia *in situ* (GCNIS) showed a linear correlation between size of the small testicular masses (STMs) and the distance of foci of GCNIS ($p = 0.0105$; $r = 0.9167$)]. All *p*-values are considered statistically significant when $p < 0.05$.

In detail, foci of GCNIS have been found within 2 cm from the STMs with a diameter ≤1.5 cm. GCNIS have been found up to 2.5 cm from the neoplastic lesion, when tumor size ranged from 1.5 cm up to 2 cm, We did not observe foci of GCNIS beyond 2.5 cm from the STMs (**Figures 3A–C**).

DISCUSSION

The use of an ultrasound in the primary evaluation of patients with infertility and with local scrotal symptoms has led to high

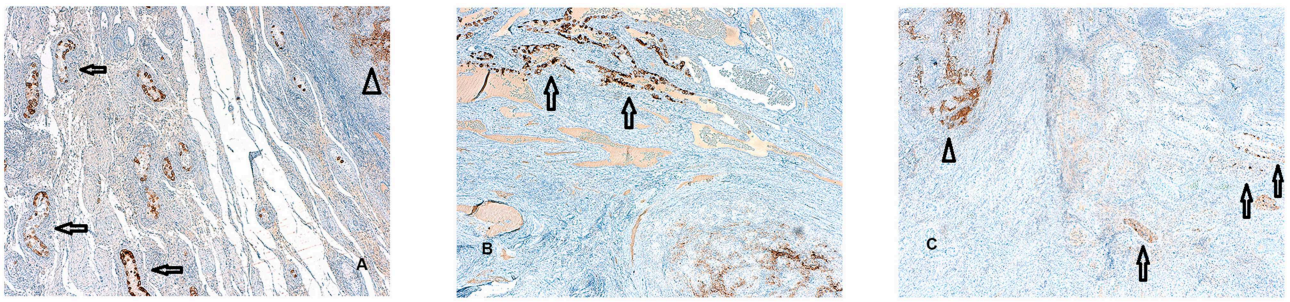

FIGURE 3 | (A–C) Foci of germ cell neoplasia *in situ* (GCNIS) identified by PLAP immunostaining surrounding the small testicular mass (STM) diagnosed as seminoma. A focal rather than random distribution of the GCNIS in testicular tissue adjacent to TGCTS was evident (PLAP immunostaining magnification 5X). This foci of GCNIS (arrows) are separated form the STM (arrows head) by tubular testis without the sign of intratubular germ cell neplasia and in all the cases examined,foci of GCNIS were not observed beyond 2,5 cm from the STM.

incidence in the early detection of small, mostly benign, testicular masses (3). According to the literature, testis sparing surgery is a safe and feasible procedure for patients presenting a benign small testis mass. It has been demonstrated that more than two thirds of asymptomatic testicular masses <2 cm are pathologically benign (13, 16, 17, 19). Although TSS is a controversial approach (30), it is justified in highly selected clinical scenarios.

In our study we performed TSS and FSE in a population of selected patients with STMs. When a diagnosis of malignant tumors was made after TSS, the orchiectomy was performed in our study. However, several papers demonstrated that the surveillance of STMs is an option along with testis sparing surgery (30).

In our study, FSE of the primary lesion and the surrounding tissue was performed to determine the nature of the testicular mass and to evaluate the radicality of the surgical procedure (3, 31). FSE has gained increasing accuracy, after initial skepticism about its diagnostic power. FSE represents a useful tool, especially with the aim to discriminate between benign or malignant testicular neoplasms when a histological diagnosis of STM is not conclusive (16, 20–22, 25). Sometimes in fact, the FSE of the STM is unable to discriminate between benign and malignant tumors, especially when the STM is very small, when the material for the FSE is poor or when the tissue resected is affected by artifacts, caused by technical frozen procedures (18, 32, 33). In these cases, identifying foci of GCNIS near the STM may help pathologists to classify STM as a germ cell tumor. In fact, previous data have been reported demonstrating that foci of GCNIS were found in up to 82% of tissues surrounding a germ cell tumor and that the risk of progression to invasive malignancy is 50 and 70% at 5- and 7-years follow-ups, respectively (13, 34).

FSE in surrounding tissue is a very accurate procedure. It has been reported in fact that the false-negative biopsies for the diagnosis of GCNIS has been found in only 0.5% of the biopsies (35). The majority of false negative biopsies are caused by sampling errors when a biopsy is not taken from a representative area.

Our data showed that foci of GCNIS have not been observed beyond 2.5 cm from the STM. To provide detail, we demonstrated the linear correlation between the size of STMs and the distance of the GCNIS foci from STMs. For small masses with a diameter up to 1.5 cm, GCNIS was observed up to 2 cm from the tumor. When the tumor diameter was more than 1.5 cm, GCNIS foci was detected up to 2.5 cm from the tumor. These results are in accordance with GCNIS-mapping studies that reported a focal rather than random distribution of the GCNIS in testicular tissue adjacent to TGCTs (36, 37).

Despite the limitations of this study, since it is a retrospective study analyzing TSS with targeted biopsies, it is the first study providing data on the correct distance to perform FSE in TSS from. Further studies are needed to provide confirmation in total orchiectomies and namely to analyze the linear correlation between the size of GCT and the distance from GNIS in orchiectomy samples. These studies will provide clearer evidence to support partial orchiectomy.

Moreover, targeted testicular biopsies performed at the right distance from STMs and measured by intraoperative US, might reduce the false negative rates in the diagnosis of GCNIS, thus supporting pathologists in detecting clinical situations at risk for malignant TGCTs.

The knowledge of the distance, from STM beyond which we did not find GCNIS, could help surgeons during intraoperative procedures, suggesting the best site at which to perform the testicular biopsies for the FSE.

In conclusion, the histological characterization by FES of the tissue around tumors in targeted areas could reduce false negative biopsies of testis with GCNIS. This approach may improve the efficacy of radical excision through the TSS procedure, reducing the risk of leaving residual cancer during TSS and subsequently of the disease recurrence.

ETHICS STATEMENT

All the procedures described in this paper are included in the consensus statement of our hospital that the patients signed when a surgical approach is recommended. The FSE of the STMS, the multiple biopsies of the surrounding testicular tissue and the processing of the neoplastic nodule via the whole-mount method

are routinary processes in our department of pathology as well as the immunohistochemical staining with PLAP and CD117 performed in order to make a correct histopathological diagnosis. For this reasons, we believe that an ethical review process was not required for this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Protein Expression of PTTG-1, OCT-4, and KLF-4 in Seminoma: A Pilot Study

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Seminomas are the most frequent kind of testicular germ cell tumors (TGCTs), accounting for 50% of tumor diagnosis in young men, whereas non-seminomas account for 40% and mixed forms for 10% of cases. It is currently supposed that TGCTs evolve from a pre-invasive stage of carcinoma *in situ* (CIS). Octamer-binding transcription factor 4 (OCT4) is essential for self-renewal of stem cells. It is considered as a major regulator of cell pluripotency. Prior studies have shown that seminoma expresses OCT4. Transcription factor Krüppel-like factor 4 (KLF4) has moreover associated with embryonic stem cell maintenance. Finally, we previously demonstrated the expression of PTTG1 in CIS and seminomas. In this pilot study, we compared the combined expression of PTTG1 with KLF4 and OCT4 in seminoma, in order to validate our hypotesis that PTTG1 marks a specific population of stem cells in neoplastic tissue, strictly related with tumor. Formalin-fixed and paraffin-embedded testicular tissues by 5 patients who underwent an orchidectomy for seminoma have been collected and immunofluorescence analysis was performed using antibody rabbit monoclonal PTTG-1 and mouse monoclonal OCT4 or mouse monoclonal KLF4 antibody. In seminoma we observed that tumor cells strongly express OCT-4 in all seminomas and in the intratubular areas of seminoma. Expression of KLF-4 was observed in many tumor cells. PTTG1 marks some specific OCT4- and KLF4-positive tumor cells, mainly localized at the periphery of the neoplasm. In the intertubular infiltration areas nests of cells expressing both OCT4/KLF4 and PTTG1 have been observed. This is the first identification of a cell population in seminoma characterized for being OCT4, KLF4, and PTTG1 positive cells in seminoma, associated with cancer invasiveness. Further investigation is needed to elucidate if a functional abrogation of PTTG1 might be used in order to offer new therapeutic approaches in the clinical workout of seminoma.

Keywords: testis cancer, seminoma, PTTG-1, OCT-4, KLF-4, infiltration

INTRODUCTION

Seminomas are the most frequent type of testicular germ cell tumors (TGCTs), accounting for 50% of cases in young men, whereas non-seminomas account for 40% and mixed forms for 10% of cases (1). Despite the high prevalence of TGCTs, the molecular mechanisms associated with their development have not been still completely clarified.

It is currently supposed that TGCTs evolve from a pre-invasive stage of carcinoma *in situ* (CIS) (2).

CIS are macroscopically distinct cells that are located on the basement membrane of the seminiferous tubules in the testis and have specific morphological features more similar to embryonic germ cells than spermatogonial stem cells (3). CIS are considered the precursors of seminomas since they both histologically resemble primordial germ cells (PGCs) and gonocytes and have a positive staining for c-kit and PLAP.

For instance, the oncogene c-kit, which encodes for a transmembrane tyrosine kinase receptor, is highly expressed in TGCTs. C-kit has as its specific ligand the stem cell factors and it is required for normal development of germ cells (4, 5). c-kit is highly expressed in seminomas and teratomas (6).

Placental alkaline phosphatase (PLAP) is moreover considered a widely used marker for TGCTs (7).

Apart from the well-known markers (i.e., PLAP and c-kit), previous studies have been carried out to identify new molecular markers for TGCTs.

Octamer-binding transcription factor 4 (OCT4) is a homeobox transcription factor that is essential for self-renewal of stem cells. It is considered as a major regulator of cell pluripotency (8). Importantly, it has been implicated in tumorigenesis of primordial germ cells. Prior studies demonstrated the expression of OCT4 in seminoma (9).

Transcription factor Krüppel-like factor 4 (KLF4) is strongly expressed in postmeiotic spermatids and in Leydig cells, but has been not reported in spermatogonia (10). KLF4 is involved in embryonic stem (ES) cell maintenance (11, 12). Simultaneous depletion of Klf4, Klf2, and Klf5 lead to ES cell differentiation, confirming the critical role of KLF4 in the maintenance of ES cell pluripotency and selfrenewal. Moreover, KLF4 was used, associated with other transcriptional factors, to induce pluripotency in differentiated cells (13). Finally, KLF4 was expressed in mouse spermatogonial stem cells shortly after withdrawal from the stem cell niche (14) in addition to pluripotent cells derived from human testis.

Previous data reported that altered levels of Pituitary-tumor-transforming-gene 1 (PTTG1) are expressed in pre-cancer lesions, suggesting that PTTG1 has a role in human tumorigenesis (15). We previously examined firstly the expression of PTTG1 in CIS and seminomas (16). In CIS, only isolated cells express PTTG1. Furthermore, in the peripheral area of seminoma, PTTG1 was mostly detected as localized in the nucleus, whereas in the central nucleus of seminoma, PTTG1 was mainly expressed in cytoplasm. Moreover, in the zones of seminoma infiltration we demonstrated the presence of clusters of PTTG1-positive cells. We hypotyzed that PTTG1 marks a population of neoplastic cells, both in CIS and in seminoma, so linking CIS to seminoma carcinogenesis. Interestingly, no differences have been observed in the expression of PTTG1 in foci and micronodules of seminoma, so that we hypothesized that when the tumor has a small size, in the early stage of the carcinogenesis, PTTG-1 expression is homogeneously distributed. On the contrary, with the increasing tumor size, this subgroup of nuclear PTTG1-positive cells move from the center to the periphery of the tumor, and it might be associated with neoplastic infiltration of surrounding tissue. PTTG1 in fact is known to play an important role in tumor infiltration and neoplastic angiogenesis. PTTG1 expression in neoplastic cells on the tumor infiltration area and in the intertubular spaces may reflect this property important for tumor cells in invading surrounding tissues and inducing neoplastic angiogenesis.

In this pilot study, we compared the combined expression of PTTG1 with KLF4 and OCT4 in seminoma, in order to validate our hypothesis that PTTG1 could mark a specific subset of neoplastic stem cells, strictly related with tumor.

MATERIALS AND METHODS

The study was conducted in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from each patient.

Formalin-fixed and paraffin-embedded testicular tissues by 5 patients who underwent an orchidectomy for seminoma were collected at the Department of Surgical Pathology Fondazione Policlinico "A. Gemelli" from 2014 to 2017. The age of the patients ranged between 25 and 70 years with a median of 36.

After deparaffinization tissues slides were rehydrated using a graded alcohol solution. Antigen retrieval was performed in 10 mM citrate buffer at pH 6.0 for 10 min in microwave oven. After allowing to cool room temperature, slides were washed twice in distilled water for 2 min and sequentially rinsed once in PBS for 5 min.

To confirm the diagnosis of seminoma we evaluated the co-expression of PLAP and c-kit. For detection of PLAP the monoclonal antibody 8A9 (Dako, Hamburg, Germany; dilution 1:50) was chosen because staining with this antibody is more sensitive compared with other PLAP antibodies.c-KIT was detected by rabbit polyclonal antibody (c-KIT antibody from Dako; dilution 1:100). All primary antibodies were incubated overnight at 4°C. Immunoreactions were visualized by means of the avidin–biotin–complex (ABC method) using AEC (3-amino-9-ethylcarbazol) as chromogen on an immunostainer (Techmate 500; Dako).

Sections have been then incubated a room temperature with primary antibody rabbit monoclonal PTTG-1 (Securin, clone EPR3240, abcam, Cambrige, UK, 1:500 for 1 h). The PTTG-1 were visualized using the highly cross-adsorbed, Alexa Fluor 488-conjugated goat anti-Rabbit IgG secondary antibody (TermoFisher Scientific, USA, 1:1000 for 1 h). After the slides were rinsed in PBS and subsequently were incubated with mouse monoclonal OCT4 (clone OTI9B7, Novus Biological, UK 1:50 for 30 min) or with mouse monoclonal KLF4 (clone CL5785, Novus Biological, 1:1000 for 30 min). This antibodies were visualized using the highly cross-adsorbed, Alexa Flour 594-conjugated goat anti-Mouse IgG secondary antibody (TermoFisher Scientific, 1:1000 for 1 h). Slides are rinsed in PBS, mounted in

TABLE 1 | Clinical and ultrasound characteristics of the patients.

Patient *n*.	Age	History of cryptorchidism	Infertility	Testis size	Ultrasound characteristics	Tumor size	TMN
1	25	X		10 ml	Hypoecoic single area; testicular microlithiasis	3 × 2 × 2 cm	T1M0N0
2	25			16 ml	Multiple hypocoic areas	2.5 × 3 × 3.5 cm	T2M0N0
3	26		X	20 ml	Hypoechoic single nodule with hyperechoic isles	3 × 2.5 × 3 cm	T2M0N0
4	36	X	X	12 ml	Iso-hypoechoic with hyperechoic striae	2.5 × 1.5 × 2 cm	T1M0N0
5	70	X		10 ml	Hypoecoic single area	2.5 × 2 × 2 cm	T2M0N0

Vectashield (H-1000, Vector Laboratories, Peterborough, UK) and double immunofluorescence slides were visually examined under immunofluorescence microscopy using an Olympus BX41 fluorescence microscope (Olympus, Although Center, Valley;PA, USA) and digital images were captured using and attached Olympus DP71 digital camera with the x40 objective.

RESULTS

Clinical and ultrasonographic informations are reported in **Table 1**.

All seminomas demonstrated the co-expression of PLAP and c-kit.

We moreover observed that tumor cells strongly express OCT-4 in all seminoma cells and in the areas of intratubular seminoma (**Figure 1A**). Expression of KLF-4 was observed in many tumor cells (**Figure 1B**). PTTG1 marks some specific OCT4-(**Figure 1A**) and KLF4-positive (**Figure 1B**) tumor cells, mainly localized at the periphery of the neoplasm.

In the intertubular infiltration areas nests of cells expressing both OCT4/KLF4 and PTTG1 (**Figures 2A,B**, respectively) have been observed.

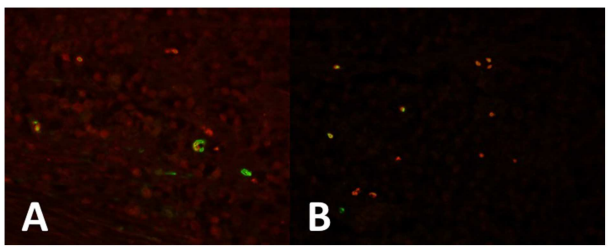

FIGURE 1 | (A) Seminoma expresses strong OCT4 expression in all cells (red). PTTG1 marks some specific OCT4-positive (green) tumor cells, mainly localized at the periphery of the neoplasm. **(B)** KLF-4 is expressed in some tumor cells (red). PTTG1 marks some specific KLF4-positive tumor cells (green).

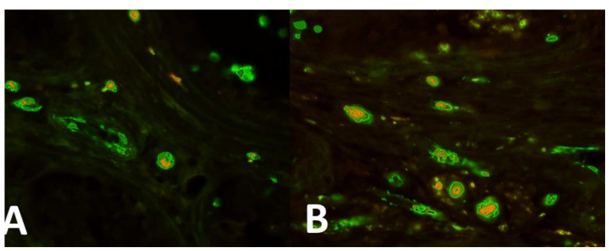

FIGURE 2 | (A) Nests of cells expressing both OCT4 (green) and PTTG1 (yellow) in the intertubular infiltration infiltration area. **(B)** Areas nests of cells expressing both KLF4 (green) and PTTG1 (yellow) in the intertubular infiltration infiltration area.

DISCUSSION

PTTG1 is a securin protein involved in facilitating sister-chromatid separation in metaphase (17) and exerts a crucial role during the mitosis by defending the chromosomal stability (18). In normal testicular tubules PTTG1 has been identified in normal spermatocytes and spermatids, suggesting that PTTG-1 may play a pivotal function in spermatogenesis in testis, and specifically in differentiation and survival of germ cells (16). PTTG1 in moreover an oncogene since its overexpression induces aneuploidy (19) and stimulates tumor formation, as previously reported in pituitary, thyroid, breast, uterine, ovarian, lung and colon tumors (20–24). In malignant tumors, previous data demonstrated the association between PTTG1 levels, tumor angiogenesis and metastasis (24). Moreover, high expression of PTTG1 is associated with greatly aggressive tumor and with the onset of metastasis (25).

PTTG1 has been demonstrated to contribute to cell migration, invasion and angiogenesis by induction of MMP-2 secretion and expression (26). In lung cancer has been demonstrated the concomitant overexpression of PTTG1 or MMP9. Xu et al. demonstrated that the co-expression of PTTG1 and MMP9 is associated with tumor cell migration and proliferation (27). PTTG1 might also play a pivotal role in inducing tumor angiogenesis in seminoma mainly through the regulation of the expression of many angiogenic factors, including bFGF, VEGF and IL-8 (28–30).

In this study we confirmed that some cells in seminoma express PTTG1 and demonstrated that it is a sub-population of tumor stem cells OCT4- and KLF4- positive cells.

Germ cells, in the early phases of embryonic development, are reserved as primordial germ cells, in order to escape the signals involved in the differentiation of the somatic cells of the embryo (31). Primordial germ cells preserve so their undifferentiated state, as demonstrated by the expression of a lot of stem cell markers, including OCT4, NANOG, stage-specific embryonic antigens and tumor rejection antigens (2, 32). The spermatogonial stem cells (SSC) can moreover be reprogrammed in cell culture to recover a pluripotent state.

These findings demonstrate that the SSC are in a relatively primitive developmental state, permitting the reconversion into an early embryonic, pluripotent state without any genetic modification. Molecular studies demonstrated that SSC occasionally functionally go away from the control of their niche, which naturally restricts their developmental potency to normal spermatogenesis and also regulates proliferation. If the stem cell niche fails to control the proliferation of gonocytes or SSC, a transformation of germline stem cells is supposed to occur, thus resulting in a CIS (33, 34). This early neoplasia can subsequently produce a seminoma or an embryonal carcinoma (35).

Furthermore, we have reported in intertubular infiltration areas the presence of nests of cells expressing both OCT4/KLF4 and PTTG1. Malik reported that PTTG1 is associated with cell angiogenesis, migration and invasion. PTTG1 in fact induces expression and secretion of MMP-2 (26). The functional blocking of PTTG1 may induce suppression of tumor growth and metastasis development, by the down-regulation of MMP-2 expression. Moreover, it is known that PTTG1 over-expression is associated with the secretion and expression of MMP-2. Previous data in HUVEC cells have been reported demonstrating that MMP-2 regulates cell migration, invasion, and endothelial tubule formation. All these data may bring to the conclusion that PTTG1 serves as one of the principal controller of MMP-2 and that some of the oncogenic effects of PTTG1 are mediated through the regulation of expression of MMP-2 (29). In neoplastic cells the expression of PTTG1 localized in the intertubular spaces, associated with OCT4 and KLF4 expression, suggest us that PTTG1 mark a specific subpopulation of SSC, characterized by high invasivity. The expression in seminoma stem cells of PTTG1 may permit them to invade surrounding tissues and to lead to neoplastic angiogenesis.

This study presents some limitations, which are represented by the limited number of clinical cases investigated and the absence of control cases (i.e., non-germ cell tumors).

CONCLUSIONS

This pilot study provides the first identification of a cell population in seminoma characterized for being OCT4, KLF4, and PTTG1 positive cells in seminoma, associated with cancer invasiveness. Further investigation are needed to extend the number of clinical cases investigated, to analyze the co-localization of PTTG1 with MMP2, MMP9, VEGF and to clarify if a functional abrogation of PTTG1 might represent a novel therapeutic approaches in the clinical management of seminoma.

DATA AVAILABILITY

All datasets for this study are included in the manuscript/supplementary files.

ETHICS STATEMENT

Institutional Scientific Board of International Scientific Institute Paul VI approved the study. This study was conducted in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from each patient.

AUTHOR CONTRIBUTIONS

DM and FP: conceptualization. DM and GGr: literature analysis. MM, TC, and FM performed immunofluorescence analysis. DM and GGr: writing—original draft preparation. GGu and AB: writing—review and editing. AP: supervision.

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