TESTIS CANCER: GENES, ENVIRONMENT, HORMONES

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TESTIS CANCER: GENES, ENVIRONMENT, HORMONES

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Testicular cancer (TC) is the most common cancer in males aged 20-40 years, with a worldwide incidence of 7.5 per 100,000, but the rates vary considerably between countries and ethnic groups and there is evidence also for an increasing incidence in last decades. About 95% of all TCs are represented by testicular germ cell tumors (TGCTs), which include seminoma and non-seminoma histological types.

It is generally assumed that the development of TGCT is under endocrine control. In particular, unbalanced androgen/estrogen levels and/or activity are believed to represent the key events for TGCT development and progression. Furthermore, recent evidence has suggested genetic association of TGCT with variations in genes involved in hypothalamic-pituitary-testicular axis and steroidogenic enzymes. This recent evidence expands the current knowledge on the role of genetic contribution in testicular cancer susceptibility, and supports the hypothesis that variations in hormone metabolism genes might change the hormonal environment implicated in testicular carcinogenesis. Therefore, hormonal carcinogenesis is an important and controversial area of current research in TGCT, and further attention is given to genetic factors influencing hormone-related cancer risk.

The genetic component to TGCT is in general strong. In fact, although environmental factors clearly contribute to TGCT development (and probably to its increasing incidence in some geographical areas), the proportion of TGCT susceptibility accounted for by the genetic effects is estimated at 25%. TGCT has high familial risks compared with most other cancer types that are generally no more than two-fold: brothers of individuals with TGCT have an 8- to 12-fold increased risk of disease, and sons of affected individuals have a 4- to 6-fold increased risk. Despite this strong familial relative risk, early results from linkage studies identified a limited relationship with genetic factors, suggesting that TGCT is a genetically complex trait.

However, more recently, four genome-wide association studies (GWAS) from the UK and USA have reported association of TGCTs with six new loci (KITLG, SPRY4, BAK1, DMRT1, TERT, and ATF7IP). The strongest association for TGCT susceptibility was found for SNPs in KITLG (ligand for the membrane-bound receptor tyrosine kinase KIT) gene with a greater than 2.5-fold increased risk of disease per major allele, which is the highest reported for any cancer to date. These studies are being now replicated by other researches and attention is given to the relationship between these genetic variations, TGCT risk and frequently associated anomalies of the reproductive tract, such as cryptorchidism and infertility.

Finally, over the past few decades, TCGT research has focused also on external environmental causes acting mainly as endocrine disrupters of androgen and oestrogen pathways, even during the foetal development of the testis. It is well known that the testicular dysgenesis syndrome (TDS) hypothesis, proposed ten years ago, suggests that disturbed testicular development in fetal life may result in one or more of four disorders postnatally, named cryptorchidism, hypo-spadias, poor semen quality, and TGCT. These four disorders are therefore considered as one clinical entity and are linked together by epidemiological and pathophysiological relations. The relative contribution of genetics and environment in TGCT development, and the interactions between endocrine disruptors and variations in genes involved in hormonal carcinogenesis is therefore another interesting area of research.

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Testis cancer: genes, environment, hormones

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Testicular cancer (TC) is the most common cancer in males aged 20–40 years, with a worldwide incidence of 7.5 per 100,000, but the rates vary considerably between countries and ethnic groups and there is evidence also for an increasing incidence in last decades. About 95% of all TCs are represented by testicular germ cell tumors (TGCTs), which include seminoma and non-seminoma histological types. It is generally assumed that the development of TGCT is under endocrine control. In particular, unbalanced androgen/estrogen levels and/or activity are believed to represent the key events for TGCT development and progression.

Furthermore, recent evidence has suggested genetic association of TGCT with variations in genes involved in hypothalamic–pituitary–testicular axis and steroidogenic enzymes (1). This recent evidence expands the current knowledge on the role of genetic contribution in TC susceptibility, and supports the hypothesis that variations in hormone metabolism genes might change the hormonal environment implicated in testicular carcinogenesis. Therefore, hormonal carcinogenesis is an important and controversial area of current research in TGCT, and further attention is given to genetic factors influencing hormone-related cancer risk. The genetic component to TGCT is in general strong. In fact, although environmental factors clearly contribute to TGCT development (and probably to its increasing incidence in some geographical areas), the proportion of TGCT susceptibility accounted for by the genetic effects is estimated at 25%. TGCT has high familial risks compared with most other cancer types that are generally not more than twofold: brothers of individuals with TGCT have an 8- to 12-fold increased risk of disease, and sons of affected individuals have a 4- to 6-fold increased risk (2). Despite this strong familial relative risk, early results from linkage studies identified a limited relationship with genetic factors, suggesting that TGCT is a genetically complex trait. However, more recently, genome-wide association studies (GWAS) have reported association of TGCTs with new loci (such as those in KITLG, SPRY4, BAK1, DMRT1, TERT, and ATF7IP genes). The strongest association for TGCT susceptibility was found for SNPs in KITLG (ligand for the membrane-bound receptor tyrosine kinase KIT) gene with a >2.5-fold increased risk of disease per major allele, which is the highest reported for any cancer to date (3, 4). These studies are being now replicated (5) and attention is given to the relationship between these genetic variations, TGCT risk, and frequently associated anomalies of the reproductive tract, such as cryptorchidism and infertility (6). Finally, over the past few decades, TCGT research has focused also on external environmental causes acting mainly as endocrine disrupters of androgen and estrogen pathways, even during the fetal development of the testis (1). It is well known that the testicular dysgenesis syndrome (TDS) hypothesis, proposed 10 years ago, suggests that disturbed testicular development in fetal life may result in one or more of four disorders postnatally, named cryptorchidism, hypospadias, poor semen quality, and TGCT (7). These four disorders are therefore considered as one clinical entity and are linked together by epidemiological and pathophysiological relations. The relative contribution of genetics and environment in TGCT development, and the interactions between endocrine disruptors and variations in genes involved in hormonal carcinogenesis is therefore another interesting area of research.

The aim of this Research Topic is to give an update on the most controversial issues in research areas of TGCT, as well as original contributions giving novel hypotheses and understandings on the pathogenesis and progression of TGCT. Both clinical and basic researches are reported and many questions are addressed. One of these deals with analysis of anthropometric measures and risk of TGCT (8), an obscure matter that this study clarified, showing that TC is positively associated with height and negatively associated with body mass index, indirectly suggesting that modifications in hormonal factors and food intake during childhood and puberty might influence susceptibility to cancer. The effects of endocrine disruptors during *in utero* and/or neonatal development have been a matter of discussion and areas of uncertainty exists. One of these is the possible epigenetic modification following exposure to these molecules and Vega and colleagues review this topic showing how chromatin modifications, which can affect testicular physiology might increase susceptibility to TGCT and the potential molecular pathways involved in these alterations in the context of environmental exposures (9).

Not only TGCT development and progression but also treatment outcome and risks of complications from cisplatin chemotherapy seem to be genetically determined. This is well documented by Fung and colleagues, who showed for the first time that a polymorphism in *ARVCF* gene influences TGCT outcome, carriers of risk allele being exposed to a higher risk of refractory TGCT after initial chemotherapy (10). Furthermore, patients who received chemotherapy had a higher risk of developing semen HPV infection (11), potentially exposing these subjects to HPV-related disorders and worse fertility prognosis.

Physiological and genetic aspects of primordial germ cells, gonocytes, and spermatogenic stem cells transformation are also interesting research areas. One report reviewed the current knowledge on the biology of the postnatal germ cell development (12), another one looked for possible modifications, caused by copy number variations, in genes involved in cell migration of primordial germ cells (13), and Brokken and colleagues showed that polymorphisms in the aryl hydrocarbon receptor repressor gene (that modulates the effects of environmental pollutants) are associated with progression of TGCT to invasive forms (14).

Finally, this Research Topic reports review on the association between cryptorchidism and TGCT (15, 16), clarifying that, regardless of age at orchidopexy, unilateral vs. bilateral forms, or position of undescended testes, patients with history of cryptorchidism are at higher risk of developing TGCT (15), and suggesting that changes in the spermatogonial stem cell self-renewal and differentiation in cryptorchid testes might be an important pathway leading to TGCT (16).

Taken together, the articles presented in this Research Topic report comprehensive review and original articles dealing with most recent research fields in the pathogenesis, progression, and clinical associations of TGCT.

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Case-control study of anthropometric measures and testicular cancer risk

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The etiology of *testicular* germ cell tumors (TGCTs) is poorly understood. Recent epidemiological findings suggest that, TGCT risk is determined very early in life, although the available data are still conflicting. The rapid growth of the testes during puberty may be another period of vulnerability. Body size has received increasing attention as possible risk factor for TC. To clarify the relation of body size and its anthropometric variables to TGCT risk, the authors analyzed data from 272 cases and 382 controls with regard to *height* (cm), weight (Kg), and body mass index (BMI; kg/m^2). Overall, participants in the highest quartile of height were more likely to be diagnosed with TGCTs than participants in the lowest quartile of height, OR 2.22 (95% confidence intervals (CI): 1.25–3.93; adjusted; $p_{trend} = 0.033$). Moreover, histological seminoma subgroup was significantly associated with tallness, very tall men (>182 cm) having a seminoma TGCT risk of OR = 2.44 (95% confidence intervals (CI): 1.19–4.97; adjusted; $p_{trend} = 0.011$). There was also a significant inverse association of TGCT with increasing BMI ($p_{trend} = 0.001$; age-adjusted analysis) and this association was equally present in both histological subgroups. These preliminary results indicate that testicular cancer (TC) is inversely associated with BMI and positively associated with height, in particular with seminoma subtype. Several studies have reported similar findings on body size. As adult *height* is largely determined by high-calorie intake in childhood and influenced by hormonal factors at puberty, increased attention to postnatal exposures in this interval may help elucidate the etiology of TGCTs.

Keywords: testicular cancer, height, weight, body mass index, body size

INTRODUCTION

Testicular cancer (TC) is the most common type of malignancy in men aged 15–40 years (Dieckmann and Pichlmeier, 2004). The incidence of TC has more than doubled worldwide over the last 40 years in several populations (Purdue et al., 2005; Bray et al., 2006). The reasons of this rise are not entirely clear (McGlynn and Cook, 2009).

Evidence of incidence peak of TC among young adults suggests that causal factors operate very early in life (Bray et al., 2006). One theory attributes it to the increase in endogenous estrogen levels during prenatal life and/or later exposures to various occupational and environmental estrogenic chemicals, termed endocrine-disrupting chemicals (EDCs) (Toppari et al., 1996; Sharpe, 2003; Cook et al., 2011; Giannandrea et al., 2011). According to this hypothesis, these exposures are also risk factors for other male reproductive disorder, such as cryptorchism and impaired sperm quality (Skakkebaek et al., 2001; Storgaard et al., 2006). Recently, it has been suggested that post-natal exposures might increase the risk of developing TC, and that secretion of sex hormones, which are involved in the growth and the development of the testicle, might be instrumental in TC progression (James, 2010; Trabert et al., 2011).

One possible risk factor that has received increasing attention is body size, which is determined by hereditary, nutritional, and hormonal factors (Dieckmann and Pichlmeier, 2002). Height, weight, and body mass index (BMI) have been examined in a number of studies, with mixed results (Lerro et al., 2010). Several studies have reported inverse associations of TC with BMI (Petridou et al., 1997; Bjørge et al., 2006; Lerro et al., 2010). A number of studies have reported that increased adult height may be a risk factor for TC, thereby suggesting that factors related to adult height may also be related to risk of these tumors (Richiardi et al., 2003; Dieckmann et al., 2008; Lerro et al., 2010). One explanation for the height association may be that better childhood nutrition increases both adult height and risk of TC. This hypothesis is supported by epidemiological observations of constantly increasing incidence of TC since the beginning of the twentieth century with the only major interruption in this trend occurring for men born during World War II or immediately thereafter, when food availability had been dramatically reduced (Moller, 1989; Aschim et al., 2005; Giannandrea, 2009).

To clarify the relation of anthropometric variables to TGCT risk and to one another, we analyzed data from 321 cases and 465 controls enrolled at our laboratory with regard to height (cm), weight (Kg) and body mass index (BMI; kg/m^2).

MATERIALS AND METHODS

Participants among 321 consecutive patients affected by TC and 465 controls were originally selected at the Laboratory of Seminology, Semen Bank, Department of Experimental

Medicine, University of Rome "La Sapienza," Rome, Italy. Controls were recruited among healthy men who underwent to seminal analyses in order to ascertain their fertility status before getting married. We excluded men with conditions possibly associated with impaired sperm quality, and/or with a history of neoplastic pathologies to provide the most suitable control group. Only men aged 20–55 years at diagnosis were eligible for the study. Inclusion was restricted to this age range as normal height growth is complete between 16 and 20 years, while there is a characteristic decline in height growth in adulthood. Some of the controls who underwent to seminal analyses were men affected by severe obesity according to WHO classification (WHO, 2000), and therefore, this group was excluded from both cases and controls in order to avoid the confounding effect of this condition on our study variables.

Only patients with tumors of germ cell origin were included in the analyses. We categorized the cases into the two major histopathologic subgroups: seminomas or non-seminomas, which include embryonal carcinomas, endodermal sinus tumors, teratomas, choriocarcinomas, or germ cell tumors of mixed morphology. Our study population was therefore finally restricted to a total of 272 cases and 382 controls, according to the selection criteria described above.

Statistical analyses were performed by use of a SPSS statistical package. All tests were two sided, with $p < 0.05$ defined as statistically significant. Logistic regression models were calculated to obtain odds ratios (OR) and 95% confidence intervals (CIs) with adjustment for the potentially confounding factors, height, weight, and age at diagnosis (Armitage et al., 2001). ORs, confidence limits, and p values were adjusted for age because of imbalance of age distribution between cases and controls.

Height, weight, and BMI were categorized into quartiles on the basis of the distribution among the entire study group. Categories were 20–29 years, 30–34 years, 35–39 years, and ≥40 years with respect t to age; ≤173 cm, 174–178 cm, 179–182 cm, and >182 with respect to height. BMI [weight (in kilograms) divided by height (in meters) squared] was calculated for each subject and categorized into slim (BMI $<23.15\,\mathrm{kg/m^2}$), normal weight (BMI 23.16–$24.97\,\mathrm{kg/m^2}$), overweight (BMI 24.98–$27.4\,\mathrm{kg/m^2}$), and obese (BMI $\geq27.4\,\mathrm{kg/m^2}$). Tests for trend were performed by use of the categorized variables.

RESULTS

The descriptive tabulation of anthropometric measures distribution of cases and controls is shown in **Table 1**. The mean ages of the non-seminoma cases was lower than the mean age of the seminoma cases (30.6 years, standard deviation (SD): 6.0 vs. 34.1 years, SD: 5.9; $p < 0.05$). It is well-acknowledged that non-seminoma peak at younger age than seminoma. Therefore, the risk of TC decrease with increasing age at diagnosis, with non-seminomas having a more significant inverse relationship with age ($p_{\mathrm{trend}} < 0.001$) than seminomas ($p_{\mathrm{trend}} = 0.021$) (**Table 2**). The relations of anthropometric variables and TC risk are shown in **Table 2**. In the multivariate analysis, BMI was inversely associated and height was positively associated with risk of TC (**Table 2**).

Overall, participants in the highest quartile of height were more likely to be diagnosed with *testicular* germ cell tumors (TGCTs) than participants in the lowest quartile of height, OR 2.22 (95% confidence intervals (CI): 1.25–3.93; adjusted; $p_{\mathrm{trend}} = 0.033$). Moreover, histological seminoma subgroup was significantly associated with tallness, very tall men (>182 cm) having a seminoma TGCT risk of OR = 2.44 (95% confidence intervals (CI): 1.19–4.97; adjusted; $p_{\mathrm{trend}} = 0.011$). The multivariate-adjusted odds ratio for the highest quartile group of BMI relative to the lowest was 0.42 (95% CI: 0.24–0.75; $p_{\mathrm{trend}} = 0.01$), with no difference by histological subtype (**Table 2**). There was also a significant inverse association of TGCT with increasing weight ($p_{\mathrm{trend}} = 0.001$; age-adjusted analysis) and this association was equally present in both histological subgroups.

DISCUSSION

These preliminary results indicate that TC is positively associated with height, in particular with seminoma subtype, and inversely related with BMI. Therefore, the present study indicated an excess of tall men in the cohort of patients with TC. To our knowledge, this study is the first to explore the relationship between tallness and the risk of TC from a southern European country. Thus far, the majority of studies on tallness in relation to the risk of TC were conducted in US and Nordic countries (Akre et al., 2000; McGlynn et al., 2007; Dieckmann et al., 2008; Cook et al., 2010; Lerro et al., 2010). Measures of adult height, as well as the incidence rate of TC, resulted both elevated among Scandinavian populations (Richiardi et al., 2003; Purdue et al., 2005).

Table 1 | Descriptive statistical analysis of anthropometric measures (Mean ± SD).

	Patients (*n*)	Age (years)	Height (cm)	BMI	Weight (Kg)
Controls (C)	382	35.7 ± 7.1	177.6 ± 6.5	24.7 ± 2.0	78.1 ± 8.7
All TC (TC)	272	32.4 ± 6.2	177.8 ± 6.3	23.8 ± 2.3	75.5 ± 9.0
Seminoma (S)	138	34.1 ± 5.9	177.5 ± 6.1	24.0 ± 2.5	75.7 ± 9.4
Nonseminoma (NS)	134	30.6 ± 6.0	178.2 ± 6.5	23.6 ± 2.2	75.3 ± 8.6
TC vs. C		b	a	a	a
S vs. C		a	a	a	a
NS vs. C		b	a	a	a

[a] *Not significant.*

[b] *p < 0.05.*

Table 2 | Relation of age, height, weight, and body mass index (BMI) to testicular germ cell tumor risk by histology.

Variable	Controls (n. 382)		All TC (n. 272)				Seminoma (n. 138)				Nonseminoma (n. 134)			
	No.	%	No.	%	OR	95% CI	No.	%	OR	95% CI	No.	%	OR	95% CI
AGE (YEARS)														
20–29	56	15.1	82	30.7	1.0	Referent	29	21.2	1.0	Referent	53	40.8	1.0	Referent
30–34	93	25.0	85	31.8	0.63	0.38, 1.06	42	30.7	0.59	0.32, 1.08	43	33.1	0.76	0.35, 1.66
35–39	129	34.7	70	26.2	0.33	0.20, 0.56	47	34.3	0.41	0.22, 0.77	23	17.7	0.25	0.12, 0.52
≥40	94	25.3	30	11.2	0.24	0.13, 0.56	19	13.9	0.41	0.21, 0.81	11	8.5	0.14	0.06, 0.30
						p trend < 0.001				p trend = 0.021				p trend < 0.001
HEIGHT (cm)														
≤173	108	28.3	68	25.0	1.0	Referent	32	23.2	1.0	Referent	36	26.9	1.0	Referent
174–178	98	25.7	81	29.8	1.62	0.99, 2.66	50	36.2	1.88	0.99, 3.54	31	23.0	1.37	0.73, 2.54
179–182	93	24.3	54	19.9	1.37	0.83, 2.25	23	16.7	1.03	0.61, 1.98	31	23.0	1.77	0.92, 3.40
>182	83	21.7	69	25.4	2.22	1.25, 3.93	33	23.9	2.44	1.19, 4.97	36	26.9	1.99	0.96, 4.12
						p trend = 0.033				p trend = 0.011				p trend = 0.025
BMI														
≤23.15	84	22.0	110	40.4	1.0	Referent	54	39.1	1.0	Referent	56	41.8	1.0	Referent
23.16–24.97	121	31.7	71	26.1	0.92	0.52, 1.63	33	23.9	1.47	0.76, 2.84	38	28.4	0.46	0.18, 1.15
24.98–27.40	128	33.5	65	23.9	0.79	0.44, 1.39	32	23.2	1.34	0.69, 2.60	33	24.6	0.35	0.14, 0.87
>27.40	49	12.8	26	9.6	0.42	0.24, 0.75	19	13.8	0.60	0.32, 1.14	7	5.2	0.24	0.10, 0.59
						p trend = 0.001				p trend = 0.004				p trend = 0.004
WEIGHT (Kg)														
≤72	94	24.6	102	37.5	1.0	Referent	52	37.6	1.0	Referent	50	37.3	1.0	Referent
73–80	157	41.0	93	34.1	0.56	0.30, 1.03	42	30.4	0.81	0.39, 1.66	51	38.0	0.33	0.13, 0.82
81–87	78	20.4	51	18.7	0.56	0.31, 1.00	27	19.5	0.97	0.48, 1.96	24	17.9	0.27	0.11, 0.64
>87	53	13.8	26	9.5	0.27	0.13, 0.52	17	12.3	0.44	0.20, 0.96	9	6.7	0.14	0.05, 0.38
						p trend = 0.001				p trend = 0.021				p trend = 0.001

OR adjusted for all continuous variables in the table; significance of p value for trend < 0.05.

Though differences in genetic backgrounds among Scandinavian and southern European populations exist, these preliminary findings imply that possible postnatal exposures may play an important role in determining risk of TC. As adult height is largely determined during the first 2 years of life, it may be postulated that high calorie nutrition after birth could have a role in TC pathogenesis (Frankel et al., 1998; McGlynn et al., 2007; Giannandrea, 2009). In addition, as final adult height is strongly dependent on testis sex-steroids (Veldhuis et al., 2005) a correlation between height and TC risk may suggest that androgen secretion during puberty might be involved in TC progression. One further height-related factor may be insulin-like growth factor (IGF) and the IGF pathway, which is also involved in the control of spermatogenesis (Gunnell et al., 2001; Giovannucci et al., 2004). Increased serum IGF-1 levels were associated with increased height has also been reported in several studies (Chia et al., 2008).

Height has been positively associated with risk of TC in the majority of studies in which it has been examined (Akre et al., 2000; McGlynn et al., 2007; Dieckmann et al., 2008; Cook et al., 2010; Lerro et al., 2010). Akre et al. (2000) found a statistically significant increased risk of TC associated with greater height, and this association was particularly evident in men with seminomas. Dieckmann et al. (2008) found that very tall men (>195 cm) carried a TC risk of OR: 3.35 (95% confidence intervals (CI): 2.88–3.90; adjusted). In the STEED Study, increased height was significantly related to risk of TC (OR: 1.85; CI 95%: 0.71, 1.32) (McGlynn et al., 2007; Lerro et al., 2010).

A number of studies have so far reported an inverse relationship between BMI and TGCTs, although the available results are still conflicting, with some studies reporting null findings (Petridou et al., 1997; Bjørge et al., 2006; Lerro et al., 2010). A recent meta-analysis conducted by Lerro et al. (2010) has produced a tentative evidence that TC risk was inversely associated with BMI, with a summary OR of 0.92 (95% CI: 0.86–0.98; $p = 0.011$). The biological reasons of this negative association are likely to be more complex of those contemplated to explain the association of TC with tallness. Some authors have suggested that lower BMI found in previous studies could also be a mathematical artifact as this association may be related to an excess

of tall men in TC cohorts rather than to leanness of the patients (Dieckmann and Pichlmeier, 2002). This is due to the fact that height is squared in the denominator of the BMI-formula, lowering the numerical calculation of MBI in very tall men at hight TC risk despite normal weight. However, our study shows additional significant inverse association of TGCT with increasing weight ($p_{\text{trend}} = 0.001$), therefore confuting, at least in part, this suggestion.

Body size is primarily determined by hereditary, nutritional, and hormonal factors (Dieckmann and Pichlmeier, 2002). The hormonal determinants can be divided into growth hormones and sex hormones. Most previous studies on postnatal risk factors for TC have focused on endogenous sex hormones. Obesity is inversely associated with both total testosterone and sex hormone-binding globulin (Akre et al., 2000). Furthermore, obese men have increased levels of both estradiol and estrone coming mostly from extraglandular conversion of androgen precursors (Akre et al., 2000; Dieckmann and Pichlmeier, 2002).

Since testicular germ cell cancer is probably initiated *in utero*, postnatal hormones are likely to function as promoters. These hormones may continue to exert their effect in advanced stages of tumor development at time of puberty when the testicles grow and develop rapidly.

Adult stature can be considered a proxy of childhood nutrition, although stature is also determined by genetic and hormonal factors. It has been suggested that the trend of increasing adult height and the increasing TC incidence are biologically interconnected with improved nutrition in early life.

In summary, we have found a lower risk of TC among men with high BMI. Further investigation of this inverse relationship may be warranted, for which the present results provide only limited support. Furthermore, tallness was positively associated with risk of TC. The biologic mechanism suggested is the promotion of this cancer by sex hormones and/or growth hormones, such as growth hormone and/or IGF-I.

FUNDING

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Epigenetic: a molecular link between testicular cancer and environmental exposures

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In the last decades, studies in rodents have highlighted links between *in utero* and/or neonatal exposures to molecules that alter endocrine functions and the development of genital tract abnormalities, such as cryptorchidism, hypospadias, and impaired spermatogenesis. Most of these molecules, called endocrine disrupters exert estrogenic and/or antiandrogenic activities. These data led to the hypothesis of the testicular dysgenesis syndrome which postulates that these disorders are one clinical entity and are linked by epidemiological and pathophysiological relations. Furthermore, infertility has been stated as a risk factor for testicular cancer (TC). The incidence of TC has been increasing over the past decade. Most of testicular germ cell cancers develop through a pre-invasive carcinoma *in situ* from fetal germ cells (primordial germ cell or gonocyte). During their development, fetal germ cells undergo epigenetic modifications. Interestingly, several lines of evidence have shown that gene regulation through epigenetic mechanisms (DNA and histone modifications) plays an important role in normal development as well as in various diseases, including TC. Here we will review chromatin modifications which can affect testicular physiology leading to the development of TC; and highlight potential molecular pathways involved in these alterations in the context of environmental exposures.

Keywords: testicular cancer, physiology, epigenetic, endocrine disrupters

TESTICULAR CANCERS

Testicular cancer (TC) is a rare solid tumor which account for 1% of cancers in men. However, it is the most common cancer in men in their 20s and 30s (Ziglioli et al., 2011). It is a real sanitary problem as it affects men during their reproductive years. Interestingly, the development of TC has been associated with urogenital abnormalities (Olesen et al., 2007). TC development has been associated with cryptorchidism, hypospadia, and hypofertility. Indeed, epidemiological studies argue for an increased risk of testicular germ cell tumor in males who suffer for fertility troubles (Burns et al., 2010).

EPIDEMIOLOGY

In developed countries, the prognosis for TC is excellent, with a 5-year survival rate >95%. Despite it represents the most frequent solid cancer in young men, it seems a quite rare pathology (Richardson et al., 2012). Thus all these data has led to consider testicular tumors as a curable neoplasm. It has to be noted that there are some geographic and racial variation in the risk of TC development (Richiardi et al., 2004). Indeed, in US, Hispanic men had the largest increase in incidence rates for non-seminomas (Townsend et al., 2010). Next to this, the genetic predisposition has been suggested as men with familial history of germ cell TC (TGCC) have a 4- to 10-fold increased risk to develop also such tumor (Greene et al., 2010). The analysis of this genetic risk seems to be associated with an autosomal recessive mode of inheritance that must result

of the combined effects of several alleles. It was estimated that 25% of TC susceptibility account for by genetic effects (Turnbull and Rahman, 2011). If TCs are quite rare in African men, the morbidity and mortality of TC is quite high in developing countries (Ugwumba and Aghaji, 2010). These data highlight the impact of poverty and paucity of resources to detect and treat cancers. In that line, it has been reported that socioeconomic position has an impact on the risk of TC development as well as the survival rate (Richardson et al., 2012).

The main way to identify TC as soon as possible is autopalpation by men to look at increased size or the appearance of "hard" testis (Umeh and Chadwick, 2010). After identification of the anomalies, biopsies will help to characterize the type of TC. Then imaging will also play a key role to determine the lymphatic extension and potential metastasis (Brunereau et al., 2012).

There are several kinds of TCs that need to be characterized by the physician using anatomopathological approach. There are several kinds depending on cell type at the origin of the development of TC (van de Geijn et al., 2009); thus there are intratubular germ cell neoplasia (IGCN), atypical germ cells with maturation arrest (MA), pseudolymphovascular invasion, real lymphovascular invasion in germ cell tumors, macroscopic Sertoli cell nodules, and Sertoli cell tumors. However, we can thus distinct germ cell tumors versus non-germ cell tumors. The most common TC are germ cell one which represent 95% of these tumors. There are two main types described which are seminoma and non-seminomatous

germ cell tumors, with a third one which is the spermatocytic seminoma (Mannuel et al., 2012).

Here we will focus this review on germ cell cancers. Their incidence has been reported to be continuously increasing in the last decades in men of different industrialized countries.

Regarding the two major groups of testicular germ cell cancers which are the seminomas and non-seminomas, each represent approximately 50% of germ cell tumors. It is interesting to note that around 15% of TGCC contain a mix of seminoma and non-seminomatous tumors (Aschim et al., 2006).

Seminomas are managed with orchiectomy and surveillance or radiotherapy in stage I and chemotherapy in more advanced stages (Warde et al., 2011). On the other side, non-seminomas which consist of several histologic types, such as choriocarcinoma, teratoma, yolk sac tumor, and embryonal cell carcinoma, each with different tumor marker profile, are less sensitive to radiation and are frequently treated with chemotherapy (Warde et al., 2011).

GENETIC ASSOCIATION

The development of TGCC is associated with many chromosomal abnormalities (Summersgill et al., 1998). Indeed, TGCC are aneuploid; with non-seminomatous tumors and seminoma being respectively hypotriploid and hypertriploid. The major association is with the gain of material from the chromosome arm 12p in both seminoma and non-seminomatous tumors (Atkin and Baker, 1982). The analyses of these chromosomal reorganizations lead to the characterization of potential candidates involved in the TGCC pathology. Among them, there is amplification of the KRAS and Cyclin d2 genes (Rodriguez et al., 2003). They are associated with malignant transformation and proliferation. Next to these genes, there are also those implicated in the cell pluripotency such as Stella and Nanog (Clark et al., 2004).

The strongest association for TGCC susceptibility is for single nucleotide polymorphisms (SNPs) at the 12q22 within the *kit-ligand* gene (Kanetsky et al., 2009). It is correlated with a 2.5-fold increased risk of disease. This gene has been involved in several aspect of primordial germ cell (PGC) development. Indeed, it seems to act on PGC migration and survival (Gu et al., 2009). These impacts might rely on the downstream target KRAS which then activate the p110 catalytic subunit of the PI3K pathway which in turn through AKT pathways will act on proliferation, survival, and migration (Sasaki et al., 2003). KRAS could also mobilize the MAPK pathways reinforcing its impact on proliferation, survival, and migration processes.

Next to this, as testicular physiology is under the control of the endocrine functions mainly through the activity of androgen and estrogen receptors, it was deeply studied if polymorphisms of genes involved in hormonal metabolism could be associated with a higher risk of TCs. Even if some reports are contradictory, the studies focused on the androgen receptor (AR), the estrogen receptors and genes involved in either synthesis or degradation of the hormones.

Regarding the estrogen receptors, it was demonstrated that polymorphisms in ERα are associated with azoospermia (Romerius et al., 2011) and are more likely to be associated with the risk of seminoma and metastasis (Brokken et al., 2012); whereas polymorphisms in ERβ are more likely to be link to altered

spermatogenesis (Aschim et al., 2005) and with risk of TGCC. New to this, polymorphisms in 17-β hydroxydehydrogenase-4 which convert androgen and estrogen to weaker hormones were associated with TGCC (Chia et al., 2010; Ferlin et al., 2010). In these metabolic pathways, polymorphisms in cytochrome P450 Cyp-1A1 gene, encoding a hormone-metabolizing protein, were identified and inversely correlated with TC. Their effects were more or less severe regarding the different polymorphisms suggesting that it may contribute to susceptibility to TGCC development (Figueroa et al., 2008; Kristiansen et al., 2011).

In this hormonal context, one of the most studied genes in regards to polymorphisms is the AR. The AR gene has two polymorphic regions in exon-1 with CAG codon encoding for glutamine and GCN which encode for glycine. Changes in the length of these polymorphic trinucleotide repeats, (CAG) and/or (GGN), lead to altered transactivation of the AR which has been shown to play a role in several forms of endocrine cancer such as prostate cancer. Regarding TC some studies are a bit contradictory showing either or not link with increased risk of TGCC (Rajpert-De Meyts et al., 2002; Garolla et al., 2005). However, it appears that the increased risk of seminoma was associated with the shorter CAG repeat length. This suggests that an increased AR transactivation may be involved in the development of seminoma and/or progression of carcinoma *in situ* (CIS) to seminoma (Davis-Dao et al., 2011). It is also demonstrated that the combination of altered number in repeat for both CAG and CGC is important for the correlation with TC. Indeed, Garolla et al. (2005) showed that the combination of CAG (20 repeats) and GGC (17 repeats) was more frequent in patient with TGCC (8 versus 1.7% in control patients).

Like most of the cancer pathologies, TCs seem to be the results of either genetic and environmental factors. It has been stated that TCs derive from a precocious lesion, the CIS of the testis, also known as IGCN or testicular intraepithelial neoplasia (TIN; Sonne et al., 2008). This lesion deserves great attention, because the diagnosis of CIS may lead to a precocious diagnosis of TCs. Usually, the diagnosis of CIS is incidental.

If there is a consensus on the fact that the process of the TC pathology may found its origin during embryonic life of the individual, it can also be suspected that there might also be some other events participating to its appearance. Indeed, it seems quite a long process that TC occurs in the 20s or 30s of life when first event took place in fetal life. Thus it could be hypothesized that there might be a second hit at puberty, like hormone burst that could provoke the definitive occurrence of TC. This clearly highlights the importance of the microenvironment. A study on patients with testicular maldescent shows that there is around a two-fold increased risk to develop TGCC if the orchidopexy is performed after the age of 13, compare to men who had the operation before their 13 (Walsh et al., 2007). Thus placing the testes in the scrotum before puberty decreases the incidence of TGCC; suggesting that intra-abdominal location at puberty promotes testicular tumorigenesis. Gene expression profiling carried out on TGCC samples demonstrate marked differences between the histological subtypes of TGCC. This reflects stages and patterns of differentiation. It also supports a model of differentiation from IGCN to seminomas or embryonal carcinomas. Gene expression patterns and

genomic imprinting analyses in TGCC show similarity to PGCs and gonocytes (Ziglioli et al., 2011).

ENVIRONMENTAL ISSUES

THE TESTICULAR DYSGENESIS SYNDROME HYPOTHESIS

In 1993, Skakkebaek and colleagues proposed that various disorders of male reproductive health, namely cryptorchidism, hypospadias, subfertility, and testicular germ cell tumor, derived from perturbations of embryonal programing and gonadal development during fetal life (Sharpe and Skakkebaek, 1993). Thus they defined it as testicular dysgenesis syndrome (TDS; Skakkebaek et al., 2001). The anomalies may lead to early symptoms, such as hypospadias and undescended testis, as well as late effects such as testis cancer and infertility. The most frequent abnormality due to TDS may be impaired spermatogenesis.

A fetal origin is obvious with regard to two symptoms of TDS: hypospadias and cryptorchidism. Moreover, studies suggested that the precursor cells of testis cancer, CIS testis, are similar to fetal gonocytes. The accepted etiology for germ cell cancer suggests that developmental arrest of fetal germ cell differentiation is a main event leading to persistence of gonocytes, which in turn develop into CIS. The causes remain unknown, although disturbances in the microenvironment provided by the Sertoli and Leydig cells may play a major role. Moreover, spermatogenesis is strictly controlled and depends on a succession of signals provided by the local environment (Skinner et al., 1991; Verhoeven, 1992; Jégou, 1993).

Among all the studies on testicular functions, there are several windows of time that must be critical for its development. Next to the importance of the fetal development, it appears that puberty must be an important timing. This is when hormone levels will reach optimal concentrations leading to the secondary sexual characters. This will also coincide with the appearance of the TGCC, as men are affected between 15 and 35 years old.

Hormones play major impacts on testicular functions throughout the life of the individuals. Indeed, testis is a key target for androgen and estrogen actions. These hormonal sensitivities have been studied for decades (Verhoeven et al., 2010). The role of testosterone is evident in patients with complete androgen insensitivity syndrome (Sultan et al., 1993).

Moreover, testicular descent occurs in two phases: the transabdominal phase, which animal studies suggest depends on the insulin-like hormone 3 (INSL3) produced by interstitial Leydig cells (Kaleva and Toppari, 2005; Ferlin et al., 2006, 2009). It has been demonstrated that the expression of the Insl3 gene is under the control of the estrogen signaling pathway (Cederroth et al., 2007). Then, the inguinoscrotal phase is dependent on androgens.

Androgens play a crucial role in the control of spermatogenesis. Molecular details have been discovered using transgenic mice invalidated (knock-out, KO) for AR either in the testis or in different testicular compartments. Such mice have low testosterone levels with altered expression of steroidogenic enzymes, even Leydig cell mass is altered (Wang et al., 2009). AR is involved in autocrine action of testosterone on Leydig cells. Testosterone deficiency is responsible for spermatogenesis arrest due to altered Sertoli functions (Wang et al., 2009).

In mouse ERα have been demonstrated to be involved in the maturation of the spermatozoa (Lubahn et al., 1993). These KO mice present an excess of fluid which increases the pressure within the seminiferous tubules and leads to the germ cell loss (Eddy et al., 1996; Hess et al., 1997). Surprisingly, the ERβ-KO mice show no testicular phenotype (Couse and Korach, 1999).

The deficient male mice for Cyp19 (Cyp19-KO), encoding for the enzyme responsible for the aromatization of testosterone into estrogens, develop normally and their genital tract is anatomically similar to that of the wild-type (Robertson et al., 1999). Males are fertile; however, some of Cyp19-KO males exhibit an altered spermatogenesis by the age of 5 months (Robertson et al., 1999). By the age of 1 year, all males develop abnormal spermatogenesis with a blockage of germ cell maturation at the spermatid stage. This is due to an increase in apoptotic rates when compared to the wild-type animals. The observation of abnormal acrosome development in the Cyp19-KO mouse suggests that acrosome biogenesis could be estrogen-dependent (Robertson et al., 1999). Interestingly, estradiol have been demonstrated to play a role as a survival factor for the human germ cells (Pentikäinen et al., 2000), and also is beneficial for sperm motility (Carreau and Hess, 2010). Moreover, next to these data, deleterious effects of numerous endocrine disruptors on sperm count and male genital tract (cryptorchidism, hypospadia, and infertility) have been documented (Iguchi et al., 2001; Sikka and Wang, 2008) particularly in the context of *in utero* and/or neonatal exposures.

Imbalanced equilibrium between the estrogen and androgen levels *in utero* is hypothesized to influence TC risk. Thus, alterations in genes involved in the action of sex hormones may contribute to variability of an individual's susceptibility to TC. Mutations in testosterone pathway genes may alter the level of testosterone *in vivo* and hypothetically the risk of developing TC (Kristiansen et al., 2012). In regard with the hypothesis of the TDS and the known impact of steroids on testicular development and functions, it has been hypothesized that endocrine-disrupting chemicals could play a role in these pathologies.

ENDOCRINE DISRUPTERS

The endocrine disrupters (EDs) are compounds which may be of industrial or natural origin and which act to dysregulate steroid function and metabolism. They produce their effects by mimicking, antagonizing, or altering endogenous steroid levels (androgens or estradiol, E2) *via* changing rates of their synthesis or metabolism and/or expression or action at receptor targets.

The question of environmental endocrine disruption has been a topic of public concern for many years and remains high on the scientific agenda. Indeed the number of chemical used is constantly increasing in developed countries, it is supposed that humans and animals can be exposed to a growing number of contaminants which can accumulate in their bodies and may have adverse consequences for health. Large range of chemicals (banned or still in use) have been characterized as EDs. Among these EDs, highly produced, Bisphenol A (BPA; Rubin, 2011) is present in plastics, including beverage and food storage containers and in the ink used for thermal paper receipts. Some individuals have also been exposed to contaminants with adverse effects originating from medical use (diethylstilbestrol, DES; Bullock et al., 1988),

or dietary habits (phytoestrogens; Naciff and Daston, 2004). More surprisingly, in China, human excretions were suspected to be the major contributor of estrogens in municipal wastewater (Zhou et al., 2012).

Moreover, studies have made correlations between elevated levels of phthalates in urines of pregnant women and lower masculinization of their progeny (Suzuki et al., 2012). EDs have also been detected in the maternal milk (Hines et al., 2009).

Regarding the urogenital development and more particularly testis, the impacts of EDs have been quite well described on human and rodent models (Volle et al., 2009; Toppari et al., 2010; Desdoits-Lethimonier et al., 2012). This is mainly true for hypospadia, cryptorchidism, and infertility; but the link with TGCC remain to be defined.

It has been demonstrated that EDs with estrogenic activity lead to a decrease of expression of steroidogenic genes which by the end result in lower testosterone concentrations (Joensen et al., 2008). However, the mechanisms seem to differ between species. This highlights the difficulties to find good experimental models. Indeed, one will claim that human cells will not react as murine cells; and other may assume that cell lines or explants tissues might not totally react as in the body. Indeed, endocrine systems are really complex and need to be analyzed in regard of the complexity of integrative physiology.

The effect of such EDs in human pathology is quite difficult to establish. It is really difficult to have standardized cohort between the studies, which is due to the history of patients (environmental and genetic factors). This heterogeneity leads to inconsistent studies which do not allow definitive conclusions on the potential involvement of EDs in TGCC.

It has been demonstrated that there was a six-fold increase in the risk of seminoma among plastic workers exposed to polyvinyl chloride (PVC; Ohlson and Hardell, 2000). However, Hardell et al. (2004a) did not found association between PVC exposure and TC.

Another study from Hardell et al. (2006) show that chlorinated biphenyls (PCBs) could play a role during fetal exposure in the etiology of TC as case mothers were identified to have significantly higher concentrations of these PCBs. However, regarding PCBs, no different risk pattern could be demonstrated for seminoma and non-seminomatous TC (Hardell et al., 2003, 2004b).

However, several studies have demonstrated some effects of these molecules on risk of TGCC (Hardell et al., 2003, 2004b, 2006; McGlynn et al., 2009). In their study McGlynn et al. (2009) associated PCBs with a decrease risk of TGCC development. Different PCBs are either associated with decrease risk of either seminoma or non-seminoma. In the same line of evidence, it was demonstrated that in patients with seminoma there was differences in concentrations of different PCBs (Purdue et al., 2009). Some PCBs (44, 49, and 52) were found to be lower accumulated in patients with seminoma compare to congeners; whereas other PCBs (99, 138, 153, 167, 183, and 195) were significantly higher accumulated in these patients. This suggest that rather than specific concentration of one or another PCB, it may be the relative concentrations of multiple one that might be relevant to established clear correlation with the risk of TC.

Moreover, evidences suggest that exposures to pesticides could be risk factor of TC development. Indeed, a recent study reported a significant association between TC development and use of insecticides such as organochlorine pesticides, namely dichlorodiphenyldichloroethylene (p,p′-DDE) isomer and hexachlorobenzene (Giannandrea et al., 2011).

Next to this, as TC development seems to be either associated with genetic predisposition and or environmental exposure, it might be of interest to analyze the potential combination of such anomalies. Consistent with this hypothesis, as polymorphisms in AR and some organochlorine pesticides have been associated to risk of TGCC development, and that some of these organochlorine pesticides present anti-androgenic activities, Biggs et al. (2008) have studied the potential interaction of AR polymorphisms and exposure to p,p′-DDE and the association with TC risk. According to their study, they were not able to demonstrate any association between p,p′-DDE exposure and TC risk, either or not in combination with (CAG)n length. However, a point to take in consideration is that p,p′-DDE concentration was measured in adult patients, but as TC is supposed to found its origin in fetal life, this must be the exposition in this period of life that must be significant to define such association. However, it will be difficult for instance to establish such correlation as we do not have access to fetal/neonatal blood of these patients. However, it could be though to perform some clinical trials to collect blood of new borns and make assay for pesticides concentrations. Then it will be necessary to follow these boys and see if they will further develop TC, and perform correlation with fetal/neonatal concentrations of pesticides.

ED and insulin-like-3

Next to their steroidogenic function, Leydig cells during development express the insulin-like-3 gene which is responsible for gubernaculum maturation (Ivell and Anand-Ivell, 2011). In human, INSL3 is produced by prenatal, neonatal, and adult Leydig cells to various extents (Ivell and Anand-Ivell, 2011). INSL3 production seems to be dependent on the state of Leydig cell differentiation, and is stimulated by the long-term trophic effects mediated by luteinizing hormone (LH; Toppari et al., 2007). This finding clearly helps in understanding the complete process of testis descent. The role of the INSL3 on testis descent was highlighted by the fact that mouse KO for the gene encoding *Insl*3 results in cryptorchidism (Adham and Agoulnik, 2004). Moreover, animal model of ED exposures demonstrated that INSL3 production is sensitive to estrogenic or anti-androgenic compounds. This clearly suggests that maternal exposure to EDs during pregnancy can result in cryptorchidism, a factor that predispose to TC development.

ED and steroidogenesis

This link is of particular importance. Indeed, if CIS have been described in boys at birth (Jacobsen and Henriques, 1992), TCs appear in young men between 15 and 35 years old, suggesting that puberty and probably the increase of hormone concentrations must be key events.

The impact of EDs on steroidogenesis has been demonstrated for several decades now. EDs have been described to inhibit critical cellular functions involved in steroidogenesis, such as transport of cholesterol into the mitochondria, the expression of steroidogenic genes or the activity of these enzymes (Vanparys

et al., 2012). Indeed, it has been demonstrated that exposure of adult rats to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCCD) led to lower production of testosterone in response to human choriogonadotropin (hCG), relative to testes from control rats (Kleeman et al., 1990). The addition of pregnenolone is able to restore normal testosterone secretion. This highlights that the impact on steroid synthesis might results from altered cholesterol synthesis or mobilization to the mitochondria (Moore et al., 1991). TCDD was also observed to reduce the number and size of the Leydig cells (Johnson et al., 1994). Moreover, the use of primary culture of Leydig cells also demonstrate that TCDD represses *Cyp11a1* expression, through the alteration of the ability of hCG to increase intracellular cAMP levels (Lai et al., 2005).

The impact on the animal phenotypes will depend on the age at exposure. Thus fetal/neonatal exposure will lead to altered internal genital organs. Indeed, experimental models have shown that disruption of the androgen signaling results in feminization of external genitalia (Nagahama et al., 2004). In animal models, exposures to low concentrations of EDs are able to alter testosterone synthesis without any effect of feminization. However, the most interesting thing is that even after neonatal exposure, the adult testosterone concentrations are decreased suggesting long-term impact that is specific to this particular window of exposure (Volle et al., 2009). This has been clearly highlighted as the exposition of adult animal lead to transitory testosterone decrease, associated with temporary germ cell death. In human, majority of the results relies on epidemiological studies and correlative data (Woodruff, 2011). However, several studies using testicular explant models have tried to characterize the impact of EDs on human Leydig cells (Desdoits-Lethimonier et al., 2012). By the end, all these data led to the idea that fetal cells must be more sensitive than adult Leydig cells.

Regarding the signaling pathways involved, and as main regulators of the expression of steroidogenic genes, a reduced activity of the cAMP-mediated PKA pathway would also be expected to reduce the mobilization of cholesterol by cholesterol hydrolases. However, at the molecular level, it has been demonstrated in mouse that neonatal exposure to EDs with estrogenic activity leads to a decrease of testosterone synthesis through ER receptors and that the orphan nuclear receptor SHP must be a key intermediary for this effect (Volle et al., 2009). Indeed, the SHP KO mice seem to be less sensitive to estrogenic EDs than their wild-type littermates. It was also demonstrated that the impact of SHP on steroidogenesis is due to the repression of either expression and/or activity of the nuclear receptors Sf-1 and Lrh-1 (Volle et al., 2009). It is to note that SHP was not involved in early post-natal decrease of testosterone production induced by estrogenic EDs. This highlights that the involved molecular pathways affected by EDs are multiple and complex.

ED and germ cell differentiation

Spermatogenesis, leading to spermatozoa formation, is a complex process with multiple steps involving mitosis, meiosis, and spermiogenesis. It takes place in the seminiferous tubules. There, germ cells are organized from the base of the tubule to the lumen. Germ cells are supported by the nursing Sertoli cells, which extend from the base to the lumen of the seminiferous tubules. Efficient spermatogenesis also relies on the integrity of tight junctions between the Sertoli cells which form the blood–testis barrier (BTB; Cheng and Mruk, 2012). BTB has many functions in the testis such as maintaining a particular immune context and also the control of the flow of nutrients and growth factors that are required for the development of germ cells.

The key role of retinoids in the differentiation process of germ cells was highlighted by two studies demonstrating stra8 as a key factor (Bowles et al., 2006; Koubova et al., 2006). It also involved the timely regulation of the gene encoding for cyp26b1, an enzyme responsible for retinoid degradation. Indeed, CYP26B1 in Sertoli cells acts as a masculinizing factor to arrest male germ cells in the G0 phase of the cell cycle and prevents them from entering meiosis (Li et al., 2009). This induction of retinoid pathway to induced entry in meiosis seems to be inhibited by the nuclear receptor SHP, a co-repressor of the retinoid acid receptors RAR (Volle et al., 2007). In the opposite manner, the FGF9 signaling pathway acts to determine germ cell fate to enter meiosis (Boisen et al., 2001).

EPIGENETIC AND TESTICULAR CANCERS
TRANSGENERATIONAL EFFECTS: ROLE OF EPIGENETIC MODIFICATIONS
Epigenetic refers to changes of DNA information without any change of the sequences. It relies on histones post-transductional modification (acetylation, methylation. . .) or of DNA methylation levels. Thus, inheritance of these information requires germline transmission of epigenetic patterns between generations. The epigenetic programing of the germline occurs during embryonic development in a sex-specific manner (Western, 2009). These processes are crucial for reproductive functions, as most of mouse models with specific germline invalidation for gene responsible of DNA or histones modifications led to sterile animals (Peters et al., 2001; La Salle et al., 2007). Regarding the male germline, it becomes imprinted following sex determination. Thus after puberty when spermatogenesis is fully functional, there are also specific epigenetic modifications along the different steps of germ cell differentiation from spermatogonial precursors up to testicular spermatozoa. Indeed, it has been demonstrated that throughout the different steps of spermatogenesis, germ cells have a dynamic of the epigenetic modifications. This is highlighted by the changes in the expression levels of the enzymes involved in these modifications. If DNA methyltransferases (Dnmts) are mainly expressed in the spermatogonia, histone methyltransferases (HMTs) are mainly expressed at the spermatocyte levels (Godmann et al., 2009). Then other histone modifications such as hyperacetylation of the H4 histone play a key role in the removal of histones and their replacement by protamines during spermatogenesis (Dhar et al., 2012). Such epigenetic modifications have also been demonstrated to be associated with infertility as for example reduced expression of *Dnmt3b* in patients with spermatogenic arrest (Adiga et al., 2011).

If the EDs cannot alter the DNA sequence, there are numerous of studies demonstrating that they can impact the epigenome. Indeed, environmental factors can alter the epigenetic programing which will impact the development of the offsprings. Interestingly, it has been demonstrated in the last decade that these effects could also potentially impact the subsequent generations

(Huang et al., 2011; Schoevers et al., 2012). Such effect was first demonstrated for the exposure with vinclozolin, a compound with anti-androgenic activities (Anway et al., 2005). Thus exposure during embryonic gonadal sex determination can alter the male germline epigenetics (e.g., DNA methylation). The epigenetic mechanism involves the alteration of DNA methylation in the germline that appears to transmit transgenerational adult onset disease, including spermatogenic defects and cancer (Anway et al., 2005). At the molecular level, the authors identify by global transcriptomic approach that the expression of genes involved in these processes such as the Dnmt or responsible for histone modifications was affected by vinclozolin treatment (Anway et al., 2005). All these data suggest the main involvement in transgenerational transmission.

Potential link between epigenetic and TGCT?

These modifications in epigenetic programing by EDs are of particular interest as they have been suspected to be responsible of the increased rate of pathologies related to the TDS such as TCs. This potential association between ED and epigenetic in TGCT development is highlighted by the fact that recently, number of studies have demonstrated the modified epigenetic profile of tumor cells compare to "normal" cells (Okamoto and Kawakami, 2007; Brait et al., 2012). Indeed, it appears that tumor cells show an epigenetic pattern similar to undifferentiated spermatogonial stem cells (Almstrup et al., 2010). Indeed, CIS cells have a permissive and fetal-like chromatin structure (Almstrup et al., 2010). This is consistent with the fact that CIS, precursor of TGCT have already been identify in testis of embryos.

DNA methylation.

DNA methylation is an important event during germ cell development. These enzymatic modifications of DNA rely on Dnmts. Among them, expression profiles of DNMT3a and DNMT3l suggest that they might act during pre-natal germ cell development for the establishment of *de novo* methylation. On the other side, DNMT1 and DNMT3b rise shortly after birth in the male (Godmann et al., 2009). It was thus hypothesized that these two Dnmts must be involved in the maintenance of methylation patterns in proliferating spermatogonia.

The use of mouse models invalidated for genes encoding these enzymes highlights their crucial involvement during spermatogenesis. Dnmt3a$^{-/-}$ males show a greatly reduced number of spermatocytes (Yaman and Grandjean, 2006; La Salle et al., 2007). This suggests a major involvement for progression through meiosis. In the same line, the males invalidated for Dnmt3L participate to the acquisition of DNA methylation at paternally imprinted regions, unique non-pericentric heterochromatic sequences, and interspersed repeats, including transposable elements. Moreover, Dnmt3L$^{-/-}$ males present alterations of meiotic process leading to spermatogenesis arrest, and spermatocytes apoptosis (Webster et al., 2005). As Dnmt3L expression is restricted to gonocytes, the presence of defects in later stages suggests alteration of processes required for completion of spermatogenesis.

The major role of these epigenetic alterations has been demonstrated in carcinogenesis. Indeed, it has been shown that DNA methylation is associated with repression of tumor suppressor gene expression. This epigenetic process is one of the most studied

in the research field, and has been recognized as a major mechanism during TGCC progression (Manton et al., 2005; Ellinger et al., 2009). Moreover, the DNA methylation pattern seems to correlate with histological features of the different types of TGCC. Undifferentiated TGCC (seminomas, IGCN unclassified, and gonadoblastomas) are hypomethylated, whereas more differentiated TGCC (teratomas, yolk sac tumors, and choriocarcinomas) show a higher degree of methylation. Embryonal carcinomas show an intermediate pattern. Thus such parameters could be used to discriminate between seminoma and non-seminoma (Brait et al., 2012).

Such impact involved modifications of genes encoding for enzymes involved in DNA methylation. Consistent with cell type origin of TGCC, the Dnmts are mainly expressed in fetal testis and in the more undifferentiated cell type (spermatogonia) during adult normal spermatogenesis.

Regarding cancers, Dnmt1 was not expressed in seminoma, but upregulated in embryonal carcinoma (Omisanjo et al., 2007). In contrast, the expression of *Dnmt3a* was found up-regulated in TGCC compared to non-tumor testicular tissues (Yamada et al., 2004). The expression pattern of *Dnmt3b* has been deeply studied and show that it could be used as a predictive marker for relapse of stage I seminomas (Arai et al., 2012). Lastly, *Dnmt3l* was overexpressed in the non-seminoma tumors (Minami et al., 2010).

These changes in Dnmts expression will lead to main alteration of transcriptomic profile between tumors and normal tissues (Alagaratnam et al., 2011). Among them, two targets are of particular interest as they are genes of pluripotency. Indeed, early fetal germ cells and undifferentiated germ cell tumors have in common the expression of pluripotency markers such as the transcription factors *Nanog* and *Oct3/4*. Regarding *Nanog* promoter, it was found hypomethylated in spermatogonia and hypermethylated in sperm (Nettersheim et al., 2011). This selective repression might reflect that the cells need to suppress pluripotency in order to prevent malignant transformation. Finally, methylation of CpGs in the *Nanog* promoter in germ cell tumors and derived cell lines correlated to differentiation state. In the same line, the study *Oct3/4* showed that seminoma and embryonal carcinoma were hypomethylated (De Jong et al., 2007).

Histone methylation.

Many enzymes are involved in histone methylation with specific or redundant level of methylation. These differences rely on the modified histone, targeted amino-acid residues and the number of methyl groups that are added to histone. These modifications are performed by several members of the HMT family. On those involved in spermatogenesis, the Suv39h1 and suv39h2 mediate histone H3 di and/or trimethylation at lysine 9 (Schotta et al., 2003). They are involved in meiosis as double KO mice present defects in male meiosis and highly pronounced apoptosis of stage IV spermatocytes during the transition from mid to late pachytene (O'Carroll et al., 2000; Peters et al., 2001).

G9a, a mammalian HMTase, is a candidate for H3-K9 methylation in non-heterochromatic loci (Tachibana et al., 2007). G9a is essential for early embryonic development and plays a dominant role in H3-K9 methylation of euchromatin. Its role was highlighted as mice lacking G9a are sterile, with germ cells undergoing

apoptosis during the pachytene stage (Tachibana et al., 2007). Interestingly, it was demonstrated that G9a was a target of retinoid signaling pathway, a key regulator of germ cell differentiation, and that it was inhibited in the context of estrogenic exposure leading to the increased germ cell apoptosis induced by EDs (Volle et al., 2009).

Another HMT is the enhancer of zeste 2 (EZH2) which trimethylate histone H3 at the lysine 27 (Chang and Hung, 2012). It was demonstrate that during spermatogenesis, EZH2 is restricted to round spermatids in the perinuclear acrosome region (Lambrot et al., 2012). This localization is concomitant with the dramatic epigenetic reorganization that occurs during spermiogenesis leading to an extreme compaction of the chromatin.

DNA methylation, histone methylation are epigenetic modifications functioning in transcriptional control and have been implicated in the deregulation of gene expression in cancer. As mentioned above, the CIS cells present epigenetic profile similar to ES cells. Such properties of chromatin have been associated with a high transcriptional and proliferative activity. This is due to lower levels of DNA methylation and of histone methylation (H3K9me2 and H3K27me3; Almstrup et al., 2010). This is consistent with a lower expression of the gene encoding for the EZH2 in the TGCC compared to normal testis tissues (Hinz et al., 2010). This suggests that in TGCC EZH2 does not exert its often assumed oncogenic properties during malignant transformation and progression. High EZH2 levels in normal testicular tissue and the inverse association of its expression levels with the severity of spermatogenic failure point to its potential value as a molecular marker for spermatogenic defects and may indicate an important physiological role of EZH2 during intact spermatogenesis.

It has been suggested that histone H2A and H4 arginine 3 dimethylation might be a mechanism by which IGCNU and seminoma maintain the undifferentiated state; while loss of these histone modifications (Eckert et al., 2008) could be involved in the somatic differentiation observed in non-seminomatous tumors.

In healthy testis, the distribution of histone H3 methylation was dependent on the developmental stage of spermatogenic cells and in non-seminoma, histone H3-K4 and K9 methylation was detected in all histological subtypes (Lambrot and Kimmins, 2011). This suggested that histone H3-K4 and K9 methylation could be associated with abnormal gene expression in non-seminoma. Histone modifications determine epigenetic patterns of gene expression with methylation of histone H3 at lysine 4 (H3K4), and are often associated with active promoters. LSD1/KDM1 is a histone demethylase that suppresses gene expression by converting dimethylated H3K4 to mono- and unmethylated H3K4 (Wang et al., 2011). Interestingly, LSD1 protein level is highly elevated in pluripotent cancer cells and in human testicular seminoma tissues that express *Oct3/4* (Wang et al., 2011).

Histone acetylation.   This is another histone modification that must also lead to gene repression. These modifications are performed by specific enzymes so-called histone deacetylase (HDCA). Such post-translation modifications are implicated in normal spermatogenesis (Fenic et al., 2008). Indeed, H3K9ac was shown in spermatogonia, spermatocytes, elongating spermatids, and ejaculated spermatozoa of fertile and infertile men (Steilmann et al., 2011). In spermatogonia, the stainings for H3K9ac, H3K18ac, and H3K23ac were strong. Then spermatocytes, the stainings for H3K9ac, H3K18ac, H3K23ac, and H3K4me3 were reduced in the preleptotene to pachytene stage, but in diplotene stage the stainings for H3K18ac, H3K23ac, and H3K4me3 seemed to become intense in later stages (Song et al., 2011a).

The main involvement of histone acetylation during spermatogenesis is the hyperacetylation of histone H4 during spermiogenesis (Dhar et al., 2012). This signal seems to play crucial role for removal of histones and their replacement by protamines, which is key feature for nucleus condensation, and thus formation of spermatozoa. There are multiple members classified in different subfamilies. Interestingly, choriocarcinomas showed generally high expression for all three class I HDAC isoforms (Gryder et al., 2012). However in contrast with other types of tumors, no diagnostic or prognostic values for HDAC1–3 in TGCC could be inferred (Fritzsche et al., 2011).

Impact of TGCC treatment.   Some testis tumors are treated using cisplatin (Koychev et al., 2011). Recent report analyzed the impact of such treatment on the integrity of spermatozoa chromatin in rats (Maselli et al., 2012). As expected, the cisplatin treatment lead to susceptibility of DNA to denaturation and the number of strand breaks were significantly increased in mature sperm. After a recovery period, it was noted that mature sperm did not show significant DNA damages. However, the protamination level of the sperm of these animals was significantly decreased. This was associated with an up-regulation of the histones H1.2, H4, H2A1, and H2B1A. This suggests long-term effect of cisplatin treatment that could have consequences for progenies even after the arrest of cancer cure.

Small non-coding RNA.   Next to these well studied epigenetic processes, it also appears that microRNA and small RNA play important roles in both germ cell differentiation and transmission to subsequent generations (He et al., 2009; Suh and Blelloch, 2011; Buckley et al., 2012). Indeed, achieving the correct spatial and temporal expression of germ cell-specific genes is fundamental to the production of spermatozoa (Song et al., 2011b). Notably, for the regulation of genes involved in the repression of protein translation is central to many embryonic processes, and is particularly active during spermatogenesis.

The miRNA and siRNA are generated by the nuclear RNase III enzyme Drosha and the cytoplasmic RNase III enzyme Dicer (Papaioannou and Nef, 2010). The involvement of these particular RNA has been highlighted by the generation of mouse models invalidated for genes encoding Drosha or Dicer (Korhonen et al., 2011; Wu et al., 2012). These invalidations led to sterility due to disrupted spermatogenesis characterized by depletion of spermatocytes and spermatids leading to oligoteratozoospermia or azoospermia. miRNAs mostly act by destabilizing target mRNAs or inhibiting their translation. Next to this, the PIWI-interacting RNAs (piRNAs) are predominantly expressed in the germ cell lineage (Kibanov et al., 2012). The analyses on this particular class of RNA suggest that they have a potential role in epigenetic

regulation of cell polarization. Moreover piRNA seem to be involved in silencing of transposon expression.

Many miRNA and siRNA have been implicated in the different steps of spermatogenesis. Among them, miR-449 and miR-34 seems to have common targets on the E2F signaling pathway which is mainly involved in the regulation of male germ cell development (Bao et al., 2012). Mir-17-92 (Mirc1) cluster and Mir-106b-25 (Mirc3) cluster miRNAs were suggested to cooperate in regulating spermatogonial development (Tong et al., 2012). The overexpression of miR-184 was demonstrated to promote the proliferation of a germ cell line, GC-1spg (Wu et al., 2011). It was also shown that transient inhibition of miR-21 in SSC-enriched germ cell cultures increased the number of germ cells undergoing apoptosis (Niu et al., 2011).

Interestingly, several miRNAs are unique to testis. Regarding the involvement of miR199a-5p was highlighted by the study showing an inverse relationship between miR-199a-5p and embryonal carcinoma antigen podocalyxin-like protein 1 (PODXL) expression (Cheung et al., 2011). This suggests that PODXL must be a downstream effector mediating the action of miR199a-5p. This is of particular interest as PODXL, an anti-adhesive protein, is expressed in aggressive tumors.

Next to this, miR-371-373 and miR-302 clusters are overexpressed in malignant TGCC (Novotny et al., 2012). It downregulates mRNAs involved in biologically significant pathways involved in cellular senescence induced by oncogenic stress.

Among other examples, miRNA-383 expression is downregulated in the testes of infertile men with MA (Lian et al., 2009). These results suggest that it functions as a negative regulator of proliferation, in part, through inactivation of the pRb pathway. Thus an abnormal expression of miRNA-383 may potentiate the connections between male infertility and testicular germ cell tumor.

Therapy issues. A percentage of tumors are resistant to cisplatin treatment. It seems to be associated with the high cytoplasmic expression of p21. Interestingly, it was demonstrated that there is an inverse association between cisplatin resistance and the expression of Oct4 and miR-106b (Koster et al., 2010). Thus, it was suggested that modulation of the Oct4/miR-106b/p21 pathway could open new perspectives in the treatment of chemoresistant TC.

CONCLUSIONS/PERSPECTIVES

The increasing incidence of reproductive tract diseases and particularly TC, during the last decades, is of concern. Indeed, even if it is a well curable disease with a good 5-year survival rate, it affects men during the time of their reproductive life (between 15 and 40), suggesting that it may affect both fertility and also health of the progeny. It cannot be exclude that germ cells generated at the beginning of carcinogenesis could transmit altered DNA material, due to genetic, epigenetic perturbation. This higher rate of appearance for TC cases is supposed to be associated with exposure to environmental chemicals. This also need to be deeply studied as in our modern society, men are exposed to an increasing amount of chemicals. This suggests that the incidence of TC could be even more important in the future decades. All these potential consequences point out the importance to study the involved mechanisms in appearance, and progression of TGCC. This means that we have to better understand the etiology for such cancers.

In long-term perspective, an increased knowledge of genetic, epigenetic, and gene expression patterns correlated with data of anatomopathology will lead to a better definition and understanding of the pathology. It also suggests that there is a need to analyze patients in a case by case approach in order to identify genetic, epigenetic alterations, and modifications of gene expression patterns. This will help to propose personalized therapy that would probably help in improving survival rate and life quality of survivors and avoid relapse.

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Chemotherapy refractory testicular germ cell tumor is associated with a variant in Armadillo Repeat gene deleted in Velco-Cardio-Facial syndrome (*ARVCF*)

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Introduction: There is evidence that inherited genetic variation affects both testicular germ cell tumor (TGCT) treatment outcome and risks of late-complications arising from cisplatin-based chemotherapy. Using a candidate gene approach, we examined associations of three genes involved in the cisplatin metabolism pathway, *GSTP1*, *COMT*, and *TPMT*, with TGCT outcome and cisplatin-induced neurotoxicity. **Materials and Methods:** Our study population includes a subset of patients ($n = 137$) from a genome-wide association study at the University of Pennsylvania that evaluates inherited genetic susceptibility to TGCT. All patients in our study had at least one course of cisplatin-based chemotherapy with at least 1 year of follow-up. A total of 90 markers in *GSTP1*, *COMT,* and *TPMT* and their adjacent genomic regions (± 20 kb) were analyzed for associations with refractory TGCT after first course of chemotherapy, progression-free survival (PFS), overall survival (OS), peripheral neuropathy, and ototoxicity. **Results:** After adjustment for multiple comparisons, one Single nucleotide polymorphism (SNP), rs2073743, in the flanking region (± 20 kb) of *COMT* was associated with refractory TGCT after initial chemotherapy. This SNP lies within the intron region of the Armadillo Repeat gene deleted in Velco-Cardio-Facial syndrome (*ARVCF*). The G allele of rs2073743 predisposed patients to refractory disease with a relative risk of 2.6 (95% CI 1.1, 6.3; $P = 0.03$). Assuming recessive inheritance, patients with the GG genotype had 22.7 times higher risk (95% CI 3.3, 155.8; $P = 0.04$) of developing refractory disease when compared to those with the GC or CC genotypes. We found no association of our candidate genes with peripheral neuropathy, ototoxicity, PFS and OS. **Discussion:** This is the first study to suggest that germline genetic variants of *ARVCF* may affect TGCT outcome. The result of this study is hypothesis generating and should be validated in future studies.

Keywords: *GSTP1*, *COMT*, *TPMT*, *ARVCF*, testicular germ cell tumor, refractory disease, neuropathy, ototoxicity

INTRODUCTION

Testicular germ cell tumor (TGCT) is the most common solid malignancy that affects men between the ages 15 and 35 (National Cancer Institute, 2011). Cisplatin-based chemotherapy is the standard treatment for patients with metastatic disease and is highly effective with a 5-year survival rate that approaches 95% (Jemal et al., 2008). Since its introduction in the 1970s, there is a large and growing population of long-term survivors with TGCT. However, cisplatin-based chemotherapy causes peripheral neuropathy and ototoxicity in approximately 20–30% of long-term TGCT survivors and risk of these adverse events increases with higher cumulative dose of cisplatin (Fung and Vaughn, 2011).

Germline genetic variation affects both TGCT outcome and late-complications arising from cisplatin-based treatment. Single nucleotide polymorphisms (SNPs) of the bleomycin hydrolase (de Haas et al., 2008) (*BLMH*) and the high plasminogen-activator inhibitor 1 (de Haas et al., 2010) (*PAI-1*) genes have been shown to impact survival among TGCT patients (de Haas et al., 2008, 2010). Variants in glutathione *S*-transferase pi (*GSTP1*), which encodes an enzyme that detoxifies chemotherapeutic agents by conjugating reactive electrophiles to glutathione (Mannervik et al., 1985), appear to be important in the development of long-term peripheral neuropathy and ototoxicity in adult TGCT patients (Oldenburg et al., 2007a,b). Similarly, SNPs of thiopurine *S*-methyltransferase (*TPMT*) and catechol *O*-methyltransferase (*COMT*), which encodes enzymes that metabolize thiopurine drugs (Weinshilboum, 2006) and catecholamine containing chemical via methylation (Weinshilboum, 2006), respectively, predispose the pediatric population to increased risk of hearing loss (Ross et al., 2009) after cisplatin therapy.

In an attempt to validate and extend previous findings regarding effects of germline genetic variation on cancer outcome and treatment-related toxicities via their effects on drug metabolism (Coate et al., 2010), we used a candidate gene approach to investigate the associations of *GSTP1*, *COMT*, and *TPMT*, three genes involved in the cisplatin metabolism pathway, with treatment outcome and cisplatin-induced neurotoxicity. We hypothesized that genetic variants in *GSTP1*, *COMT*, and *TPMT* that confer higher intracellular concentration of cisplatin or its metabolites are associated with lower rates of TGCT recurrence and higher incidence of cisplatin-related neurotoxicity. To test this hypothesis, we evaluated a selected panel of SNPs in *GSTP1*, *COMT*, and *TPMT* with cancer outcome, ototoxicity, and peripheral neuropathy in an existing cohort of men with TGCT who have received at least one course of cisplatin-based chemotherapy.

MATERIALS AND METHODS

STUDY POPULATION

Our study cohort is derived from a subset of TGCT case subjects enrolled into an ongoing case-control study designed to evaluate inherited genetic susceptibility to TGCT. Details of the parent case-control study have been described previously (Kanetsky et al., 2009, 2011) In brief, case subjects were recruited from a network of hospitals, including the Hospital of the University of Pennsylvania (HUP), University of Pennsylvania Cancer Network, Fox Chase Cancer Center, and also from the Pennsylvania and New Jersey State Cancer Registries. Only men between ages 18 and 50 years with pathologically confirmed TGCT were included, and those with human immunodeficiency virus, Klinefelter's or Down syndrome were excluded. All enrolled patients with TGCT who underwent cisplatin chemotherapy at HUP were potentially eligible for the current study. A total of 137 patients had at least one course of cisplatin-based chemotherapy, at least 1 year of follow-up since initiation of cisplatin-based chemotherapy and at least one clinical consultation with an oncologist at HUP and are included in the final analytic cohort.

The study was approved by the institutional review board at the University of Pennsylvania. All patients gave written informed consent.

GENOTYPING FOR SNPs OF *GSTP1*, *COMT*, AND *TPMT*

All study participants had previously been genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 platform; details of genotyping efforts and quality control measures have previously been reported (Kanetsky et al., 2009, 2011). A total of 90 SNP markers with genotype call rates $\geq$ 90% that mapped to the genomic regions ($\pm$20 kb) of *COMT, TPMT*, and *GSTP1* (43, 44, and 3 markers, respectively) were used for analysis. A complete listing of annotated SNP markers are available from the authors by request.

TREATMENT RESPONSE ASSESSMENT

A complete review of medical records was performed to abstract information regarding treatment response, patient demographics, tumor characteristics, and treatment information. Monitoring for treatment response included both serologic [alpha fetal protein (AFP) and beta human chorionic gonadotropin (β-HCG)] and radiographic studies (plain radiography, computed tomography, or magnetic resonance imaging).

We evaluated three treatment efficacy endpoints: (1) refractory disease after first course of chemotherapy, (2) progression-free survival (PFS), and (3) overall survival (OS). Clinical remission was defined as disappearance of all tumors by radiographic methods and normalization of both AFP and β-HCG level for at least 4 weeks after completion of first course of chemotherapy. Cancer recurrence was defined as initial clinical remission with subsequent development of disease evidenced by radiographic imaging or serology. Refractory disease was defined as persistent radiographic or serologic evidence of disease after initial course of chemotherapy.

NEURO- AND OTO-TOXICITY ASSESSMENT

We collected information regarding cisplatin-related peripheral neuropathy and ototoxicity from patients using a self-administered mailed questionnaire instrument. We adapted 12 of the 15 questions from the Michigan Neuropathy Screening Instrument (MNSI) (Moghtaderi et al., 2006) for assessment of peripheral neuropathy. For ototoxicity, we used 10 questions from an existing screening survey of hearing problem at the National Institute on Deafness and other Communications Disorders (Fung and Vaughn, 2011) with inclusion of one additional question about the presence of tinnitus.

In addition to mailing the questionnaire to all 137 patients in our study cohort, we distributed it on site to patients ($n = 31$) who had routine clinic visits at the Hospital of the University of Pennsylvania from July 2010 to June 2011. To improve the response rate, we sent reminder letters to non-responders 8 weeks after initial mailing of the questionnaire. At 12 weeks, phone calls were made to all remaining non-responders.

Patients were defined as having peripheral neuropathy if their MNSI score was two or greater. The threshold was based on the results of a MNSI validation study in subjects with diabetic peripheral neuropathy (Feldman et al., 1994). Subjects were defined as having ototoxicity if they answered "yes" to three or more of the 11 questions.

STATISTICAL ANALYSIS

We summarized the baseline characteristics of our population using means and standard deviations for continuous variables (age at diagnosis, age at initiation of chemotherapy, initial cisplatin dose, and cumulative life-time cisplatin dose) and proportions for categorical variables [genetically inferred race from GWAS, histology, International Germ Cell Consensus Classification (IGCCC), primary site of tumor, and type of chemotherapy]. We compared the baseline characteristics between cohorts with and without completed toxicity questionnaires using Fisher's exact test for categorical variables and *t*-test for continuous variables. We used logistic regression models to investigate associations between baseline characteristics and peripheral neuropathy, ototoxicity, and refractory disease after first course of chemotherapy. We conducted a time to event analysis using a Cox proportional hazards model to assess the effects of baseline covariates on PFS and OS.

We summarized the major and minor allele frequencies of each SNP marker and determined if their genotypic distributions were in Hardy–Weinberg equilibrium.

We studied associations of SNP markers with endpoints assuming co-dominant, recessive, and dominant genetic models using Pearson's chi-squared tests. We accounted for multiple comparisons by adjusting the *p*-values of these associations using the method of Benjamini and Hochberg (1995). We used multivariable logistic regression models to assess associations of genetic markers with peripheral neuropathy and ototoxicity, after adjusting for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and cumulative dose of cisplatin. To assess the association between each SNP and refractory disease after initial chemotherapy, we used multivariable logistic regression models with adjustment for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and dose of cisplatin during first course of chemotherapy. Since refractory disease accounts for less than 10% of all treatment outcomes, estimates from the logistic regression model are interpreted and reported as relative risks.

To assess the association between each SNP and PFS or OS, we used Cox proportional hazards models. In these time-to-event models, patients contributed to the analyses from the initiation of chemotherapy until date of last follow-up or death in the OS model with inclusion of cancer recurrence as an additional censoring event in the PFS model. In the PFS model, we adjusted for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and dose of cisplatin during first course of chemotherapy. In the OS model, we adjusted for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and cumulative dose of cisplatin. We report hazard ratios (HR) and corresponding 95% CI from the Cox models.

All statistical analyses were performed using Stata 12 (StataCorp LP, Texas, 2011).

RESULTS

BASELINE CHARACTERISTICS OF PATIENTS

Baseline characteristics of all 137 patients and the subset of 66 patients (48.2%) who completed the toxicity questionnaire are given in **Table 1**. As expected, whites account for the majority of patients in both groups (86.8% in the entire cohort and 93.9% in the questionnaire subgroup) and the mean age at TGCT diagnosis is similar (31.0 years and 32.7 years, respectively). Tumor characteristics are similar between the groups: the predominant histology is non-seminoma (81.7% in the entire cohort and 75.8% in the questionnaire subset), the primary site of presentation is the testicle (89.0% and 90.9%, respectively), and the majority of tumors are classified as good prognosis at initiation of chemotherapy by the IGCCC criteria (68.6% and 80.3%, respectively). For both groups, bleomycin, etoposide, and cisplatin (BEP) is the most common initial chemotherapy regimen (62.0% of the entire group and 59.1% of the questionnaire subset) and the mean cumulative cisplatin dose (mg/m^2) is comparable among them (410.9 and 404.5, respectively).

In general, patients who completed the toxicity questionnaire are similar to the non-responders. There are no differences in terms of histology, primary site of TGCT, types of initial chemotherapy, or cumulative cisplatin dose administered ($P > 0.05$). However, those who completed the questionnaire were older at the time of diagnosis of TGCT ($P = 0.03$) and hence at initiation of chemotherapy ($P = 0.02$). A larger proportion of non-responders were non-whites (19.7% versus 6.1%, $P = 0.02$) and also had poor risk disease (32.4% versus 12.1%, $P = 0.01$) when compared to those who completed the questionnaire.

PERIPHERAL NEUROPATHY AND OTOTOXICITY

Among the 66 patients who completed the toxicity questionnaire, 25 (37.9%) and 30 (45.5%) of them reported peripheral neuropathy and ototoxicity, respectively (**Table 2**). Patients who were diagnosed with TGCT at an older age were more likely to report developing peripheral neuropathy ($OR = 3.1$, 95% CI 1.5, 6.4 per 10 year increase in age) as were patients with higher cumulative life-time cisplatin dose ($OR = 1.6$, 95% CI 1.1, 2.2 per 100mg/m^2 increased in dose). Those with non-seminoma ($OR = 0.3$, 95% CI 0.1, 0.8) and BEP ($OR = 0.3$, 95% CI 0.1, 0.95) as initial treatment had statistically significantly reduced risk of peripheral neuropathy compared to those with seminoma or treatment with etoposide and cisplatin (EP), respectively. Regarding ototoxicity, only higher cumulative life-time cisplatin dose ($OR = 1.5$, 95% CI 1.1, 2.1 per 100mg/m^2 increased in dose) and total cisplatin dose ($OR = 2.1$, 95% CI 1.03, 4.3 per 100mg/m^2 increased in dose) during initial chemotherapy increased this risk significantly.

TREATMENT OUTCOME

In our cohort of 137 patients, 11 (8.0%) of them had refractory disease after initial course of chemotherapy and 126 (92%) of them achieved clinical remission. Among those with remission, 30 (23.8%) of them experienced disease relapse after a mean follow-up time of 46.8 months, with a total of eight deaths within the entire cohort. **Table 3** shows that extra-gonadal primary ($RR = 9.7$, 95% CI 2.5, 37.3), intermediate/poor risk cancer ($RR = 28.2$, 95% CI 3.5, 228.7), and initial therapy with etoposide/ifosamide/cisplatin (VIP) ($RR = 135.0$, 95% CI 6.7, 2733.6) were statistically significantly associated with refractory disease. Patients with non-seminoma ($HR = 3.4$, 95% CI 1.03–10.9), extra-gonadal primary ($HR = 3.5$, 95% CI 1.7, 7.3), intermediate/poor risk cancer ($HR = 4.1$, 95% CI 2.2, 7.7), initial VIP ($HR = 27.7$, 95% CI 7.9, 96.7), and higher total cisplatin dose during initial chemotherapy ($HR = 1.5$, 95% CI 1.01–2.3 per 100mg/m^2 increased in dose) all had worse PFS. Patients with extra-gonadal primary ($HR = 5.5$, 95% CI 1.3, 22.9), intermediate/poor risk cancer ($HR = 17.7$, 95% CI 2.2, 143.5), VIP as initial chemotherapy ($HR = 255.7$, 95% CI 12.2, 5367.1), and higher cumulative life-time cisplatin dose ($HR = 1.6$, 95% CI 1.2, 2.1 per 100mg/m^2 increased in dose) had worse OS.

ASSOCIATIONS OF VARIANTS IN *COMT, TPMT,* AND *GSTP1* WITH PERIPHERAL NEUROPATHY, OTOTOXICITY, AND TREATMENT OUTCOMES

We found statistically significant associations of four SNP markers (rs4646316, rs4380755, rs5008499, and rs6591256) in or near *COMT*, *TPMT*, and *GSTP1* with neuropathy and five SNP markers (rs3788306, rs12189790, rs17420046, rs6938294, and rs6912842) with ototoxicity at the nominal *p*-value of 0.05.

Table 1 | Baseline characteristics of patients.

		All patients ($n = 137$)		Patients with completed toxicity questionnaire ($n = 66$)		Patients without completed toxicity questionnaire ($n = 71$)		*p*-value[a]
DEMOGRAPHICS								
Race	White	119	86.8%	62	93.9%	57	80.3%	*0.02*
	Black	5	3.7%	0	0.0%	5	7.0%	
	Hispanic	4	2.9%	0	0.0%	4	5.7%	
	Asian	5	3.7%	3	4.6%	2	2.8%	
	Other	4	2.9%	1	1.5%	3	4.2%	
Mean age at diagnosis ± SD (years)		31.0 ± 8.5		32.7 ± 8.6		29.5 ± 8.3		*0.03*
CHARACTERISTICS OF TGCT								
Histology	Seminoma	25	18.3%	16	24.2%	9	12.7%	0.12
	Non-seminoma	112	81.7%	50	75.8%	62	87.3%	
Primary site at presentation	Testicle	122	89.0%	60	90.9%	62	87.3%	0.24
	Retroperitoneum	6	4.4%	4	6.1%	2	2.8%	
	Mediastinum	9	6.6%	2	3.0%	7	9.9%	
IGCCC prognosis group[b]	Good	94	68.6%	53	80.3%	41	57.7%	*0.01*
	Intermediate	11	8.0%	5	7.6%	6	8.5%	
	Poor	31	22.6%	8	12.1%	23	32.4%	
	Unknown	1	0.7%	0	0.0%	1	1.4%	
CHARACTERISTICS OF CHEMOTHERAPY								
Type of initial chemotherapy	EP	46	33.6%	26	39.4%	20	28.2%	0.35
	BEP	85	62.0%	39	59.1%	46	64.8%	
	TIP	1	0.7%	0	0.0%	1	1.4%	
	VIP	4	2.9%	1	1.5%	3	4.2%	
	Other	1	0.7%	0	0.0%	1	1.4%	
Mean initial total cisplatin dose ± SD (mg/m²)[c]		345.3 ± 80.4		337.9 ± 80.0		352.1 ± 82.6		0.30
Mean cumulative cisplatin dose ± SD (mg/m²)[d]		410.9 ± 169.2		404.5 ± 166.8		416.9 ± 172.4		0.67
Mean age at initiation of chemotherapy ± SD (year)		31.5 ± 9.1		33.4 ± 9.3		29.7 ± 8.5		*0.02*

SD, standard deviation; TGCT, testicular germ cell tumor; EP, etoposide and cisplatin; BEP, bleomycin, etoposide, and cisplatin; TIP, paclitaxel, ifosamide, and cisplatin; VIP, etoposide, ifosamide, and cisplatin.

[a] *P-values for comparison of baseline characteristics between cohorts with and without completed toxicity questionnaires. Fisher's exact test used for categorical variables and t-test used for continuous variables.*

[b] *IGCCC prognosis group of TGCT at initiation of first course of chemotherapy.*

[c] *Mean total cisplatin dose administered during first course of chemotherapy.*

[d] *Mean cumulative life-time cisplatin dose administered by the end of last follow-up visit.*

Italic values indicate p ≤ 0.05.

However, after correcting for multiple testing, these associations did not retain statistical significance (**Table 4**).

Nine SNPs in or near *COMT*, *TPMT*, and *GSTP1* had a statistically significant association with refractory disease after initial chemotherapy at the nominal *p*-value of 0.05 (**Table 5**). After correcting for multiple testing, we found that recessive inheritance of the G allele of rs2073743 confers increased risk ($RR = 22.7$, 95% CI 3.3, 155.8) of refractory disease after first course of chemotherapy. However, this association did not retain statistical significance after adjustment for race, IGCCC, primary site of disease at diagnosis, type of initial chemotherapy, and total dose of cisplatin during first course of chemotherapy.

The G allele of rs2073743 had a frequency of 22.76% in the cohort and its genotypic distribution was in Hardy–Weinberg proportions ($P = 0.46$). This allele was present in 40.91% of patients with refractory disease compared to 21.14% of those with clinical remission after initial chemotherapy, predisposing patients to refractory disease with a relative risk of 2.6 (95% CI of 1.1, 6.3; $P = 0.03$).

We found statistically significant association of six SNPs (rs1042781, rs2073743, rs2531706, rs5748505, rs6518598, and rs4380755) in or near *COMT*, *TPMT*, and *GSTP1* with PFS and six SNPs (rs366148, rs2239395, rs1858770, rs12158214, rs887200, and rs372534) with OS at the nominal *p*-value of 0.05

Table 2 | Risk factors for development of peripheral neuropathy and ototoxicity of 66 patients who completed the toxicity questionnaire.

	Peripheral neuropathy					Ototoxicity				
	Yes N (%)	No N (%)	OR	95% CI	*P*-value	Yes N (%)	No N (%)	OR	95% CI	*P*-value
AGE AT DIAGNOSIS[a]										
Mean ± SD (year)	37.0 ± 8.7	30.0 ± 7.4	*3.1*	*1.5, 6.4*	*<0.01*	33.6 ± 7.9	31.9 ± 9.1	1.3	0.7, 2.3	0.44
RACE										
White (*n* = 62)	23 (92.0)	39 (95.1)	1.0	–	–	30 (100)	32 (88.9)	1.0	–	–
Non-white (*n* = 4)	2 (8.0)	2 (4.9)	1.7	0.2,12.9	0.61	0 (0.0)	4 (11.1)	1.0	NA	NA
PRIMARY SITE										
Testicle (*n* = 60)	22 (88.0)	38 (92.7)	1.0	–	–	26 (86.7)	34 (94.4)	1.0	–	–
Extra-gonadal (*n* = 6)	3 (12.0)	3 (7.3)	1.7	0.3, 9.3	0.53	4 (13.3)	2 (5.6)	2.6	0.4, 15.4	0.29
HISTOLOGY										
Seminoma (*n* = 16)	10 (40.0)	6 (14.6)	1.0	–	–	9 (30.0)	7 (19.4)	1.0	–	–
Non-seminoma (*n* = 50)	15 (60.0)	35 (85.4)	*0.3*	*0.1, 0.8*	*0.02*	21 (70.0)	29 (80.6)	0.6	0.2, 1.8	0.32
IGCCC PROGNOSIS GROUP										
Good (*n* = 53)	20 (80.0)	33 (80.5)	1.0	–	–	23 (76.7)	30 (83.3)	1.0	–	–
Intermediate/poor (*n* = 13)	5 (20.0)	8 (19.5)	1.0	0.3, 3.6	0.96	7 (23.3)	6 (16.7)	1.5	0.5, 5.2	0.50
TYPES OF INITIAL CHEMOTHERAPY										
EP (*n* = 26)	14 (56.0)	12 (29.3)	1.0	–	–	12 (40.0)	14 (38.9)	1.0	–	–
BEP (*n* = 39)	11 (44.0)	28 (68.3)	*0.3*	*0.1, 0.95*	*0.04*	17 (56.7)	22 (61.1)	0.9	0.3, 2.4	0.84
VIP (*n* = 1)	0 (0)	1 (2.4)	NA	NA	NA	1 (3.3)	0 (0)	NA	NA	NA
INITIAL TOTAL CISPLATIN DOSE[b]										
Mean ± SD (mg/m^2)	360.0 ± 81.6	324.4 ± 73.4	2.0	0.9, 4.1	0.08	360.0 ± 56.3	319.4 ± 88.9	*2.1*	*1.03, 4.3*	*0.04*
CUMULATIVE CISPLATIN DOSE[c]										
Mean ± SD (mg/m^2)	476.0 ± 173.9	361.0 ± 148.1	*1.6*	*1.1, 2.2*	*0.01*	460.0 ± 188.6	358.3 ± 131.7	*1.5*	*1.1, 2.1*	*0.02*

OR, odds ratio; CI, confidence interval; SD, standard deviation; NA, not applicable; EP, etoposide and cisplatin; BEP, bleomycin, etoposide, and cisplatin; VIP, etoposide, ifosamide, and cisplatin.

[a]*Odds ratio per increase of 10 years of age.*

[b]*Odds ratio per increase of 100 mg/m^2 of total cisplatin dose administered during first course of chemotherapy.*

[c]*Odds ratio per increase of 100 mg/m^2 of cumulative life-time cisplatin dose administered by the end of last follow-up visit.*

Bold-italic values indicate p ≤ 0.05.

(**Table 6**). In particular, patients with the recessive GG genotype of rs2073743 had an inferior PFS with a HR of 3.6 (95% CI 1.1, 11.7) when compared to those with the GC or CC genotypes. However, after correcting the *p*-values for multiple testing, none of these associations remained statistically significant.

DISCUSSION

This study examined the association between three genes involved in cisplatin metabolism, *GSTP1*, *COMT*, and *TPMT*, with TGCT outcomes and cisplatin-induced ototoxicity and peripheral neuropathy. We found one SNP (rs2073743) in the flanking region (±20 kb) of *COMT* that was associated with refractory TGCT after initial chemotherapy. This SNP lies within the intron region of the Armadillo Repeat gene deleted in Velo-Cardio-Facial syndrome (*ARVCF*). This is the first study to show that germline genetic variants of *ARVCF* may affect TGCT outcome.

Although there is no functional information for SNP rs2073743, we hypothesize that it may affect disease risk by altering the expression of the *ARVCF* gene. *ARVCF* gene is a member of the p120 catenin family of proteins (Reintsch et al., 2008) and its over-expression has been shown to cause disruption of cell adhesion (Reintsch et al., 2008), which may facilitate cancer progression. A recent study by Lin et al. (2011) reported that the dominant inheritance of the minor T allele of rs5993891 of *ARVCF* is associated with a decrease in prostate cancer specific mortality (*HR* = 0.21, 95% CI 0.07, 0.61). Therefore, it is possible that SNP rs2073743 of *ARVCF* may predispose patients to higher risk of refractory TGCT by inhibition of cell adhesion, which consequently causes more aggressive tumor biology.

In contrast, SNP rs2073743 may be a marker for genetic variations in *COMT* that affect the risk of refractory TGCT by altering the level of *S*-adenosyl-L-methionine (AdoMet) and the concentration of estrogen and its metabolites. *COMT* encodes a methyltransferase that is critical for the metabolism of endogenous catecholamine containing chemicals and catechol drugs with AdoMet being its intermediary metabolite (Weinshilboum, 2006). There is some evidence to suggest that cisplatin may interact synergistically with AdoMet (Ochoa et al., 2009). Ochoa and colleagues found that administration of both AdoMet and cisplatin causes a 3.0–6.2-fold increase in frequency of renal toxicity in mice while cisplatin alone causes only moderate toxicity and administration AdoMet by itself did not result in any nephrotoxicity (Ochoa et al., 2009). Consequently, it is possible that increased activity of the *COMT* enzyme causes a decrease in the concentration of AdoMet, which may in turn lead to decreased cytotoxic activity of cisplatin.

Table 3 | Association of baseline characteristics with testicular cancer treatment outcome of 137 patients in the study cohort.

	Refractory disease					Progression-free survival				Overall survival			
	Yes N (%)	No N (%)	RR	95% CI	P-value	Mean PFS (mo)	HR	95% CI	P-value	Mean OS (mo)	HR	95% CI	P-value
AGE AT DIAGNOSIS[a]													
Mean ± SD (year)	27.0 ± 5.3	31.4 ± 8.7	0.5	0.2, 1.2	0.11	46.8	1.0	0.7, 1.04	1.00	59.0	0.5	0.2, 1.3	0.17
RACE													
White (n = 119)	11 (100)	108 (85.7)	1.0	–	–	48.9	1.0	–	–	61.8	1.0	–	–
Non-white (n = 18)	0 (0.0)	18 (14.3)	1.0	NA	NA	33.2	0.5	0.2, 1.7	0.29	40.8	2.1×10^{-7}	NA	1.00
PRIMARY SITE													
Testicle (n = 122)	6 (54.5)	116 (92.1)	1.0	–	–	48.8	1.0	–	–	56.5	1.0	–	–
Extra-gonadal (n = 15)	5 (45.5)	10 (7.9)	*9.7*	*2.5, 37.3*	*<0.01*	30.8	*3.5*	*1.7, 7.3*	*<0.01*	79.3	*5.5*	*1.3, 22.9*	*0.02*
HISTOLOGY													
Seminoma (n = 25)	0 (0)	25 (19.8)	1.0	–	–	57.7	1.0	–	–	59.0	1.0	–	–
Non-seminoma (n = 112)	11 (100)	101 (80.2)	1.0	NA	NA	44.4	*3.4*	*1.03, 10.9*	*0.04*	59.1	1.9×10^{7}	NA	1.00
IGCCC PROGNOSIS GROUP													
Good (n = 94)	1 (9.1)	93 (73.8)	1.0	–	–	50.1	1.0	–	–	55.7	1.0	–	–
Intermediate/poor (n = 43)	10 (90.9)	33 (26.2)	*28.2*	*3.5, 228.7*	*<0.01*	39.6	*4.1*	*2.2, 77*	*<0.01*	66.5	*17.7*	*2.2, 143.5*	*0.01*
TYPES OF INITIAL CHEMOTHERAPY													
EP (n = 46)	1 (9.1)	45 (35.7)	1.0	–	–	40.5	1.0	–	–	51.9	1.0	–	–
BEP (n = 85)	7 (63.6)	78 (61.9)	4.0	0.5, 33.9	0.20	49.1	1.1	0.6, 2.2	0.75	62.3	2.7	0.3, 22.7	0.38
TIP (n = 1)	0 (0)	1 (0.8)	NA	NA	NA	54.2	NA	NA	NA	54.2	NA	NA	NA
VIP (n = 4)	3 (27.3)	1 (0.8)	*135.0*	*6.7, 2733.6*	*<0.01*	2.9	*27.7*	*7.9, 96.7*	*<0.01*	10.7	*255.7*	*12.2, 5367.1*	*<0.01*
Others (n = 1)	0 (0)	1 (0.8)	NA	NA	NA	315.4	NA	NA	NA	315.4	NA	NA	NA
INITIAL TOTAL CISPLATIN DOSE[b]													
Mean ± SD (mg/m²)	345.5 ± 104	345.2 ± 78.6	1.0	0.9, 1.08	1.00	46.8	*1.5*	*1.01, 2.3*	*0.04*	59.0	2.2	0.9, 5.6	0.10
CUMULATIVE CISPLATIN DOSE[c]													
Mean ± SD (mg/m²)											*1.6*	*1.2, 2.1*	*<0.01*

RR, relative risk; CI, confidence interval; HR, hazard ratio; NA, not applicable; SD, standard deviation; EP, etoposide and cisplatin; TIP, paclitaxel, ifosamide, and cisplatin; BEP, bleomycin, etoposide and cisplatin; VIP etoposide, ifosamide, and cisplatin.

[a] Refractory disease: relative risk per unit increase of 10 years of age; PFS and OS: hazard ratio per unit increase of 10 years of age.

[b] Refractory disease: relative risk per unit increase of 100 mg/m² of total cisplatin dose administered during first course of chemotherapy; PFS and OS: hazard ratio per unit increase of 100 mg/m² of total cisplatin dose administered during first course of chemotherapy.

[c] Refractory disease and PFS: life-time cumulative cisplatin dose is not applicable since status for refractory disease or cancer relapse was ascertained after initial course of chemotherapy only; OS: hazard ratio per unit increase of 100 mg/m² of cumulative life-time cisplatin dose.

Bold-italic values indicate p ≤ 0.05.

Table 4 | Association of genetic variants that reached a nominal *p*-value of 0.05 with peripheral neuropathy and ototoxicity among 66 patients who completed the toxicity questionnaire.

	Gene (SNP)	Genotype	Case N (%)	Control N (%)	Univariate model		Multivariate model[a]	
					Odds ratio (95% CI)	*p*-value	Odds ratio (95% CI)	*p*-value
Peripheral neuropathy	**COMT**							
	rs4646316	Co-dominant genetic model						
		T/T	0 (0)	3 (7.3)	0.1 (0.02, 0.8)	0.03	0.1 (0.01, 0.7)	0.03
		T/C	9 (37.5)	23 (56.1)	0.3 (0.1, 0.9)	0.03	0.3 (0.1, 0.9)	0.03
		C/C	15 (62.5)	15 (36.6)	1.0	–	1.0	–
		Dominant genetic model						
		T/-	9 (37.5)	26 (63.4)	0.4 (0.1, 0.98)	0.05	0.3 (0.1, 0.98)	0.05
		C/C	15 (62.5)	15 (36.6)	1.0	–	1.0	–
	TPMT							
	rs4380755	Co-dominant genetic model						
		T/T	1 (4.3)	0 (0)	8.7 (1.02, 74.1)	0.05	18.9 (1.1, 323.8)	0.04
		T/C	9 (39.1)	8 (21.1)	2.9 (1.01, 8.6)	0.05	4.3 (1.1, 18.0)	0.04
		C/C	13 (56.5)	30 (78.9)	1.0	–	1.0	–
	rs5008499	Co-dominant genetic model						
		T/T	1 (4.5)	0 (0)	15.0 (1.5, 152.0)	0.02	14.4 (0.97, 212.0)	0.05
		T/C	8 (36.4)	6 (15.0)	3.9 (1.2, 12.3)	0.02	3.8 (0.99, 14.6)	0.05
		C/C	13 (59.1)	34 (85.0)	1.0	–	1.0	–
	GSTP1							
	rs6591256	Dominant genetic model						
		G/-	12 (52.2)	30 (76.9)	0.3 (0.1, 0.99)	0.05	0.3 (0.1, 1.1)	0.08
		A/A	11 (47.8)	9 (23.1)	1.0	–	1.0	–
Ototoxicity	**COMT**							
	rs3788306	Dominant genetic model						
		G/-	20 (74.1)	17 (48.6)	3.0 (1.02, 9.0)	0.05	4.9 (1.3 – 18.8)	0.02
		A/A	7 (25.9)	18 (51.4)	1.0	–	1.0	–
	TPMT							
	rs12189790	Co-dominant genetic model						
		T/T	0 (0)	0 (NA)	13.8 (1.1, 90.1)	0.04	20.6 (1.5, 277.1)	0.02
		T/C	13 (43.3)	7 (19.4)	3.2 (1.19.5)	0.04	4.5 (1.2, 16.6)	0.02
		C/C	17 (56.7)	29 (80.6)	1.0	–	1.0	–
		Dominant genetic model						
		T/-	13 (43.3)	7 (19.4)	3.2 (1.1, 9.5)	0.04	4.5 (1.2, 16.6)	0.02
		C/C	17 (56.7)	29 (80.6)	1.00	–	1.0	–
	rs17420046	Co-dominant genetic model						
		T/T	1 (3.3)	2 (5.6)	0.1 (0.0, 0.6)	0.02	0.01 (0.0, 0.3)	0.01
		T/G	1 (3.3)	12 (33.3)	0.2 (0.1, 0.8)	0.02	0.1 (0.03, 0.6)	0.01
		G/G	28 (93.3)	22 (61.1)	1.0	–	1.0	–
		Dominant genetic model						
		T/-	2 (6.7)	14 (38.9)	0.1 (0.02, 0.6)	0.01	0.1 (0.01, 0.4)	0.01
		G/G	28 (93.3)	22 (61.1)	1.0	–	1.0	–
	rs6938294	Co-dominant genetic model						
		G/G	1 (3.3)	2 (5.7)	0.04 (0.0, 0.6)	0.02	0.01 (0.0, 0.3)	0.01
		G/A	1 (3.3)	12 (34.3)	0.2 (0.1, 0.8)	0.02	0.1 (0.03, 0.6)	0.01
		A/A	28 (93.3)	21 (60.0)	1.0	–	1.0	–
		Dominant genetic model						
		G/-	2 (6.7)	14 (40.0)	0.1 (0.02, 0.5)	0.01	0.04 (0.01, 0.4)	0.01
		A/A	28 (93.3)	21 (60.0)	1.0	–	1.0	–
	rs6912842	Dominant genetic model						
		T/-	13 (43.3)	7 (19.4)	3.2 (1.1, 9.5)	0.04	4.5 (1.2, 16.6)	0.02
		G/G	17 (56.7)	29 (80.6)	1.0	–	1.0	–

CI, confidence interval.

[a] *Adjusted for race, IGCCC, primary site of cancer, type of chemotherapy administered, and cumulative life-time cisplatin dose administered.*

Table 5 | Association of genetic variants that reached a nominal *p*-value of 0.05 with refractory disease after initial chemotherapy among 137 patients in the study cohort.

Gene (SNP)	Genotype	Yes N (%)	No N (%)	Univariate model		Multivariate model[a]	
				Relative risk (95% CI)	*p*-value	Relative risk (95% CI)	*p*-value
COMT							
rs2073743	Co-dominant genetic model						
	G/G	3 (27.3)	2 (1.6)	8.8 (1.2, 65.8)	0.04	4.7 (0.3, 69.4)	0.25
	G/C	3 (27.3)	48 (39.0)	3.0 (1.1, 8.1)	0.04	2.2 (0.6, 8.3)	0.25
	C/C	5 (45.4)	73 (59.3)	1.0	–	1.0	–
	Recessive genetic model						
	G/G	3 (27.3)	2 (1.6)	22.7 (3.3, 155.8)	< 0.01[b]	21.8 (1.01, 470.7)	0.05[c]
	C/–	8 (72.7)	121 (98.4)	1.0	–	1.0	–
rs4646316	Co-dominant genetic model						
	T/T	0 (0)	6 (4.9)	0.01 (0.0, 0.6)	0.03	0.001 (0.0, 0.3)	0.02
	T/C	1 (9.1)	59 (48.0)	0.1 (0.01, 0.8)	0.03	0.03 (0.02, 0.5)	0.02
	C/C	10 (90.9)	58 (47.1)	1.0	–	1.0	–
	Dominant genetic model						
	T/–	1 (9.1)	65 (52.8)	0.1 (0.01, 0.7)	0.02	0.03 (0.001, 0.7)	0.02
	C/C	10 (90.9)	58 (47.2)	1.0	–	1.0	–
rs366148	Dominant genetic model						
	A/–	3 (27.3)	9 (7.3)	4.8 (1.07, 21.1)	0.04	2.6 (0.3, 21.5)	0.4
	G/G	8 (72.7)	114 (92.7)	1.0	–	1.0	–
rs2531706	Dominant genetic model						
	G/–	5 (45.5)	91 (76.5)	0.3 (0.1, 0.9)	0.03	0.2 (0.02, 0.97)	0.05
	A/A	6 (54.5)	28 (23.5)	1.0	–	1.0	–
rs5748505	Dominant genetic model						
	T/–	4 (36.4)	88 (69.8)	0.3 (0.1, 0.9)	0.03	0.2 (0.05, 1.2)	0.09
	C/C	7 (63.6)	38 (30.2)	1.0	–	1.0	–
rs1034564	Recessive genetic model						
	A/A	3 (30.0)	7 (5.8)	7.0 (1.5 33.0)	0.01	4.7 (0.5, 42.8)	0.2
	G/–	7 (70.0)	114 (94.2)	1.0	–	1.0	–
TPMT							
rs12524744	Co-dominant genetic model						
	T/T	0 (0)	0 (0)	20.6 (1.3, 319.3)	0.03	20.1 (0.5, 822.4)	0.10
	T/C	4 (40.0)	16 (12.8)	4.5 (1.2, 17.9)	0.03	4.5 (0.7, 28.7)	0.10
	C/C	6 (60.0)	109 (87.2)	1.0	–	1.0	–
	Dominant genetic model						
	T/–	4 (40.0)	16 (12.8)	4.5 (1.2, 17.9)	0.03	4.5 (0.7, 28.7)	0.10
	C/C	6 (60.0)	109 (87.2)	1.0	–	1.0	–
rs9396834	Co-dominant genetic model						
	C/C	5 (45.4)	15 (11.9)	6.6 (1.1, 40.8)	0.04	5.0 (0.6, 45.0)	0.15
	C/T	3 (27.3)	62 (49.2)	2.6 (1.03, 6.4)	0.04	2.2 (0.7, 6.7)	0.15
	T/T	3 (27.3)	49 (38.9)	1.0	–	1.0	–
	Recessive genetic model						
	C/C	5 (45.5)	15 (11.9)	6.2 (1.7, 22.71)	0.01	4.4 (0.7, 26.1)	0.10
	T/–	6 (54.5)	111 (88.1)	1.0	–	1.0	–
GSTP1							
rs6591256	Co-dominant genetic model						
	G/G	0 (0)	10 (8.7)	0.1 (0.01, 0.9)	0.05	0.04 (0.01, 0.93)	0.05
	G/A	4 (36.4)	67 (58.3)	0.3 (0.1, 0.97)	0.05	0.2 (0.04, 0.96)	0.05
	A/A	7 (63.6)	38 (33.0)	1.0	–	1.0	–

CI, confidence interval.

[a]*Adjusted for race, IGCCC, primary site of disease at diagnosis, type of chemotherapy administered, and total dose of cisplatin received during initial chemotherapy.*

[b]*Corrected p-value for multiple testing: 0.04.*

[c]*Corrected p-value for multiple testing: 0.72.*

Table 6 | Association of genetic variants that reached a nominal *p*-value of 0.05 with progression-free and overall survival among 137 patients in the study cohort.

	Gene (SNP)	Genotype	Mean PFS/OS (mo)	Univariate model		Multivariate model[a]	
				Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
Progression-free survival	**COMT**						
	rs10427871	Co-dominant genetic model					
		G/G	NA	45.7 (2.5, 845.7)	0.01	9469.3 (62.7, 1429366.1)	< 0.01
		G/A	7.7	6.8 (1.6, 29.1)	0.01	97.3 (7.9, 1195.6)	< 0.01
		A/A	48.4	1.0	—	1.0	—
		Dominant genetic model					
		G/-	7.7	6.8 (1.6, 29.1)	0.01	97.3 (7.9, 1195.6)	< 0.01
		A/A	48.4	1.0	—	1.0	—
	rs2073743	Recessive genetic model					
		G/G	43.9	3.6 (1.1, 11.7)	0.03	1.2 (0.3, 4.6)	0.84
		C/-	46.2	1.0	—	1.0	—
	rs2531706	Dominant genetic model					
		G/-	47.5	0.5 (0.3, 0.9)	0.03	0.4 (0.2, 0.8)	0.01
		A/A	42.6	1.0	—	1.0	—
	rs5748505	Dominant genetic model					
		T/-	51.1	0.5 (0.3, 0.95)	0.03	0.5 (0.2, 0.9)	0.02
		C/C	38.3	1.0	—	1.0	—
	rs6518598	Dominant genetic model					
		A/-	51.8	0.5 (0.3, 0.9)	0.02	0.4 (0.2, 0.8)	0.01
		C/C	38.8	1.0	—	1.0	—
	TPMT						
	rs4380755	Recessive genetic model					
		T/T	2.0	61.8 (5.6, 681.9)	< 0.01	273.9 (16.8, 4464.8)	< 0.01
		C/-	49.0	1.0	—	1.0	—
Overall survival	**COMT**						
	rs366148	Co-dominant genetic model					
		A/A	11.2	49.1 (3.4, 716.6)	< 0.01	15.9 (0.3, 880.5)	0.18
		A/G	50.0	7.0 (1.8, 26.8)	< 0.01	4.0 (0.5, 29.7)	0.18
		G/G	61.2	1.0	—	1.0	—
		Dominant genetic model					
		A/-	46.7	8.3 (2.0, 34.8)	< 0.01	4.0 (0.5, 29.7)	0.18
		G/G	61.2	1.0	—	1.0	—
	rs2239395	Dominant genetic model					
		G/-	51.0	7.7 (1.8, 32.3)	0.01	8.9 (1.4, 57.4)	0.02
		T/T	59.6	1.0	—	1.0	—
	rs1858770	Co-dominant genetic model					
		C/C	11.2	30.5 (1.5, 608.1)	0.03	26.7 (0.3, 2150.4)	0.14
		C/A	53.2	5.5 (1.2, 24.7)	0.03	5.2 (0.6, 46.4)	0.14
		A/A	59.8	1.0	—	1.0	—
		Dominant genetic model					
		C/-	48.5	6.6 (1.3, 32.6)	0.02	5.2 (0.6, 46.4)	0.14
		A/A	59.8	1.0	—	1.0	—
	rs12158214	Recessive genetic model					
		T/T	42.4	5.4 (1.04, 27.8)	0.05	NA	NA
		C/-	61.4	1.0	—	1.0	—
	rs887200	Dominant genetic model					
		G/-	46.9	4.1 (1.02, 16.4)	0.05	4.0 (0.8, 18.7)	0.08
		A/A	62.5	1.0	—	1.0	—

(Continued)

Table 6 | Continued

	Gene (SNP)	Genotype	Mean PFS/OS (mo)	Univariate model		Multivariate model[a]	
				Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
Overall survival	**TPMT**						
	rs372534	Co-dominant genetic model					
		G/G	10.5	26.5 (1.8, 389.7)	0.02	4.7 (0.3, 74.3)	0.27
		G/A	54.0	5.2 (1.4, 19.7)	0.02	2.2 (0.5, 8.6)	0.27
		A/A	62.6	1.0	—	1.0	—
		Recessive genetic model					
		G/G	10.5	46.9 (4.2, 528.3)	< 0.01	0.6 (0.03, 12.9)	0.73
		A/-	59.9	1.0	—	1.0	—

PFS, progression-free survival; OS, overall survival; CI, confidence interval; NA, not applicable.

[a] PFS: hazard ratio adjusted for race, IGCCC, type of chemotherapy administered, and cumulative life-time cisplatin dose administered by the end of last follow-up visit; OS: hazard ratio adjusted for race, IGCCC, primary site of cancer, type of chemotherapy administered, and cumulative life-time cisplatin dose administered by the end of last follow-up visit.

TGCTs are thought to develop from totipotent primordial germ cells (Sinisi et al., 2003) and there is evidence to suggest that a combination of high gonadotropin level coupled with unbalanced androgen and estrogen level may be a key event for TGCT development and progression (Garolla et al., 2005). Fotsis et al. (1994) reported that 2-methoxyoestriol, which is an endogenous estrogen metabolite, inhibits angiogenesis and suppresses tumor growth in mice. Since *COMT* degrades catechol metabolites from estradiol and estrone to methylated compounds (Weinshilboum, 2006), genetic variation of *COMT* may be implicated in the development and progression of TGCT. Recently, Ferlin et al. (2010) studied 17 polymorphic markers in 11 genes involved in hormone metabolism and reported that the minor A allele of rs11205 in the 17 β-hydroxysteroid dehydrogenase type 4 gene (*HSD17B4*), which is essential for metabolism of estrone to estradiol, is associated with higher risk of TGCT ($OR = 2.73$, 95% CI 1.47, 7.06). In the same study, however, they found no association of rs4680 of *COMT* with development of TGCT (Ferlin et al., 2010).

Since the association between rs2073743 with refractory disease was no longer statistically significant after adjusting for race, IGCCC, primary site of disease at diagnosis, type of initial chemotherapy, and total dose of cisplatin during first course of chemotherapy, we examined if any of these covariates are associated with rs2073743. Interestingly, we found that patients with the G/G genotype at this marker have higher odds of having both extra-gonadal primary ($OR = 14.63$, 95% CI 2.22, 96.35) and initial chemotherapy with VIP ($OR = 45.00$, 95% CI 2.77, 730.57). However, we found no such association with race, IGCCC, and total dose of cisplatin. Therefore, patients with rs2073742 may have genetic predisposition to more aggressive TGCT at presentation. This may instead explain their increased risk of refractory disease after initial chemotherapy. On the contrary, since VIP is commonly used for treatment of extra-gonadal TGCT, it simply may be a marker for extra-gonadal disease. Finally, our failure to detect any association of rs2073743 with PFS or OS may result from inadequate statistical power due to small sample size ($n = 137$) and the relatively short mean follow-up time of 46.8 months within the context of the excellent 5-year survival rate of TGCT, which approaches 95% (Fung and Vaughn, 2011).

Our study confirms the known established prognostic factors for TGCT, including both IGCCC risk groups and primary site of disease presentation, thus suggesting that our results are internally valid. Based on the original study ($n = 5862$) that validated the IGCCC (Wanderas et al., 2006), the 5-year OS for good, intermediate, and poor risk metastatic GCT are 91%, 79% and 48%, respectively while the 5-year PFS for these respective prognostic groups are 89%, 75%, and 41%. Our study showed slightly better 5-year OS for all risk groups (good risk: 98.7%, intermediate risk: 87.5%, and poor risk: 76.1%) but comparable 5-year PFS except for that of the intermediate risk group (good risk: 80.0%, intermediate risk: 21.8%, and poor risk: 44.1%). These minor differences may be attributed to the smaller sample size of our study cohort. Similarly, our study also confirmed that patients with extra-gonadal primary have worse 5-year OS (testicular: 94.7%, retroperitoneal: 83.3%, and mediastinum 75.0%) and PFS (testicular: 73.0%, retroperitoneal: 22.2%, and mediastinum 33.3%).

Our study confirmed that increased cumulative dose of cisplatin predisposed patients to both hearing loss and peripheral neuropathy. In our study, 82% of patients who received >400 mg/m² of cumulative cisplatin and 38% of those with ≤400 mg/m² self-reported ototoxicity, which were higher than those previously reported by Bokemeyer et al. (1998). In their study, they showed that approximately 20% of TGCT patients treated with ≤400 mg/m² of cumulative cisplatin and 50% of those who received >400 mg/m² developed hearing impairment after a median follow-up of 58 months, which were assessed by pure-tone audiometry (Bokemeyer et al., 1998). These differences may be explained by the more objective method of ototoxicity assessment by Bokemeyer and colleagues.

Peripheral neuropathy occurred in 45.5% of our patients, consistent with other studies which showed that 14–43% of TGCT survivors experience persistent symptomatic peripheral neuropathy after cisplatin-based chemotherapy (Hansen et al., 1989;

Aass et al., 1990; Bissett et al., 1990; Boyer et al., 1990; Bokemeyer et al., 1996; Roth et al., 1988; Petersen and Hansen, 1999; von Schlippe et al., 2001). Our study reported that the odds for peripheral neuropathy increased by 1.55 times per 100 mg/m^2 increase in cumulative cisplatin dose, which is similar to those reported by other studies (Brydoy et al., 2009; Glendenning et al., 2010). For instance, Glendenning et al. (2010) reported that there is a 1.91 times higher odds of developing peripheral neuropathy with each 200 mg/m^2 increase in cumulative cisplatin dose. Similarly, Brydoy et al. (2009) found that there is a higher prevalence of paresthesias following five or more cycles than following one to four cycles of cisplatin-based chemotherapy in TGCT survivors. Since peripheral neuropathy is expected to be more prevalent in an older population, it is not surprising that age was a significant variable in our regression analysis.

GSTP1, COMT, and *TPMT* have been implicated in the development of cisplatin-induced hearing impairment (Oldenburg et al., 2007a,b; Ross et al., 2009) and peripheral neuropathy (Oldenburg et al., 2007a). Ross et al. (2009) demonstrated that rs12201199 of *TPMT* ($OR = 17.0$, 95% CI 2.3, 125.9) and rs9332377 of *COMT* ($OR = 5.5$, 95% CI 1.9, 15.9) are associated with cisplatin-induced hearing loss in children. Regarding *GSTP1*, Oldenburg et al. (2007a,b) found statistically significant associations between rs1695 of this gene with peripheral neuropathy and cisplatin-induced long-term hearing impairment in TGCT survivors. The A > G polymorphism of rs1695 is a missense mutation that leads to amino acid change at codon 105 (Ile > Val) (Oldenburg et al., 2007a,b) and may affect the detoxification capability of this enzyme with chemotherapeutic agents.

In our study, however, we did not find significant associations of ototoxicity and peripheral neuropathy with any of our candidate genes. The following reasons may explain our null results. First, rs12201199 of *TPMT* and rs1695 of *GSTP1* were not included in the Affymetrix Genome-Wide Human SNP Array 6.0 platform and therefore were not examined. Although one SNP of *TPMT* in our study (rs11964408) was in linkage disequilibrium

with rs12201199, its relatively weak linkage with rs12201199, with a correlation coefficient of 0.65, may explain the lack of significant association with peripheral neuropathy. Second, we may have inadequate statistical power due to the small number of patients in our neurotoxicity cohort ($n = 66$) to detect such associations. For instance, the risk for development of ototoxicity with rs9332377 of *COMT* in our study was increased with an odds ratio of 1.29 (95% CI 0.54, 3.11) similar to that of Oldenberg and colleague (Ross et al., 2009). However, this association may not have reached statistically significance ($P = 0.57$) due to the small sample size of our study. Third, the mechanism for development of cisplatin-induced ototoxicity may be different in adults than in children, and therefore genes other than *COMT* and *TPMT* may be involved. Finally, unlike the studies by Oldenburg et al. (2007b) and Ross et al. (2009), patients in our study used a questionnaire instead of audiometric testing to screen for hearing impairment. As a result, misclassification of hearing loss may explain our failure to detect any association with ototoxicity.

The association between SNP rs2073743 of *ARVCF* and refractory TGCT should first be replicated in future studies with a larger number of patients and subsequently validated using a different patient cohort. In particular, studies to elucidate the function of the *ARVCF* gene in the pathogenesis and progression of TGCT may give insight into development of therapeutic options in TGCT treatment. If indeed presence of the G/G of the *ARVCF* gene SNP rs2073743 is associated with refractory TGCT, incorporation of this prognostic factor into the IGCCC may be warranted.

In conclusion, our pilot data suggests that patients with the G/G genotype of the *ARVCF* gene SNP rs2073743 may have increased risk of refractory TGCT after chemotherapy. Although the underlying mechanism for this increased risk of refractory disease is unknown, confirmation of the observed association may have consequences for risk classifications in patients with metastatic disease and may be of use to select patients who will benefit from more aggressive treatment at its onset.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Testicular cancer and HPV semen infection

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Testicular cancer represents the more frequent solid tumor affecting males aged 15–35 years. In the last decades, its incidence showed a progressive increased probably due to genetic and environmental factors. Despite exposure to some viruses such as HIV, HCV, EBV, and HPV is frequently related to cancer development, there are no studies aimed to evaluate the possible implication of viral infections in the pathogenesis of testicular cancer. In this study, we analyzed sperm parameters and prevalence of HPV on sperm in 155 testicular cancer patients at diagnosis (T−1), after orchiectomy (T0) and after 12 months from surgery or from the end of adjuvant treatments (T12). All patients showed a significantly higher prevalence of semen infection than controls (9.5% and 2.4% respectively,) and altered sperm parameters both at T−1 and T0. Considering sperm parameters, at T−1 we observed a reduction of progressive motility, and after orchiectomy patients showed a reduction of sperm concentration and count and a further worsening of motility. Thereafter, patients were assigned to three groups on the basis of medical option after surgery: S = surveillance, R = radiotherapy, and C = chemotherapy +/− radiotherapy. At T12, untreated patients had an improvement of sperm parameters while R group and even more C group had a strong decrease of sperm number ($p < 0.01$ both vs. T0 and S group). Moreover, patients who received radio and/or chemotherapy had a very high prevalence of HPV semen infection (S = 7.7%, R = 30.8%, and C = 61.5%). In conclusion, patients with testicular cancer had frequently altered sperm parameters and higher prevalence of HPV semen infection that were worsened after radio and chemotherapy. Because HPV infection is a risk factor for cancer development and it may further reduce fertility, we suggest screening for HPV in testicular cancer patients at diagnosis and particularly after adjuvant treatments.

Keywords: chemotherapy, human papillomavirus, male infertility, radiotherapy, sexual transmitted diseases, sperm infection, sperm parameters, testicular cancer

INTRODUCTION

Testicular cancer represents a rare pathology, accounting for about 2% of total cancer. However, considering men aged 15–35, it becomes the most common solid malignancy (Siegel et al., 2012; Viatori, 2012). Moreover, many authors demonstrated an increasing secular trend especially in some regions of Europe and North America (Adami et al., 1994; Richiardi et al., 2004; Zoltick, 2011). Many risk factors have been studied as a pre-disposing factor in the development of this cancer, but only for some there is a high level of evidence (Senturia, 1987; Buetow, 1995). Conditions particularly involved are cryptorchidism, previous contralateral testicular cancer, family history and the presence of gonadal dysgenesis (Dieckmann and Pichlmeier, 2004; McGlynn and Trabert, 2012). Although many genital cancers are closely related to Papillomavirus (HPV), to date no study evaluated the possible role of viruses in the pathogenesis of testicular cancer. HPV is one of the most common sexually transmitted infections (STIs), it is particularly common in young sexually active population and its prevalence is closely related to sexual behavior (Dillner et al., 2000). HPV was found, from a long time, in a high percentage of genital benign lesions as warts and condylomata and carcinomas (Gissmann, 1984; Shah and Buscema, 1988).

In particular is well-established the etiologic role of high-risk HPV types in the carcinogenesis of the vulva, vagina, penis, anus, head, neck, and oropharyngeal cavity (Backes et al., 2009; Smith et al., 2009; Guily et al., 2011). Recently, we reported a significant presence of (HPV) DNA in thawed semen samples from patients with testicular cancer who cryopreserved semen (Foresta et al., 2010). This finding was confirmed by other authors showing similar data from patients who banked their sperm because of chemo/radiotherapy (Kaspersen et al., 2011). These data are very important because cryopreserved semen samples, especially when patients undergo therapies that can affect spermatogenesis, are frequently used by assisted reproductive techniques, such as intracytoplasmic sperm injection (ICSI). It has been observed that the bond between virus and sperm is very strong and that conventional washing procedures aimed to select sperm before *in vitro* fertilization (IVF) have low efficacy in eliminating HPV sperm infection (Foresta et al., 2011a; Garolla et al., 2012). In addition, we demonstrated that artificially infected sperm, both transfected with E6/E7 HPV genes and incubated with viral proteins, are able to enter the oocyte, to deliver HPV genome, and that viral genes are then actively transcribed by the penetrated oocyte (Foresta et al., 2011b). Furthermore, a recent study showed that HPV

infected couples undergoing assisted reproduction techniques (ART) cycles experienced an increased risk of pregnancy loss compared to non-infected ones (Perino et al., 2011). In these couples the more predictive factor of early abortion was represented by HPV sperm infection. An important risk factor for HPV infection is the presence of immunosuppression status. Patients with cancer, particularly after adjuvant therapies, as chemotherapy and radiotherapy can be immunosuppressed and therefore at higher risk for this infection (Rasmussen and Arvin, 1982; Bieber et al., 2006; Fallahian et al., 2010). Despite the large body of literature concerning the role of HPV in many cancer types, very little is known about this viral infection and testicular cancer. In the light of these considerations, we evaluated semen parameters and the prevalence of HPV semen infection in a large group of patients with testicular cancer at diagnosis, after orchiectomy and after further 12 months of surveillance or after the end of radiotherapy and/or chemotherapy. In subjects with HPV semen infection, we evaluated also the percentage of infected sperm.

MATERIALS AND METHODS

PATIENTS
Written informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee. We enrolled 155 consecutive patients affected by testicular cancer candidate to unilateral orchiectomy, who attended the Centre for Human Reproduction Pathology for andrological examination. A medical history including previous circumcision, smoking, and sexual behavior was obtained from each patient. As control subjects, we used a group of 84 healthy proven fertile men. All subjects collected semen for standard semen analysis, detection of HPV DNA and fluorescence *in situ* hybridization (FISH) for HPV at diagnosis and 1 month after orchiectomy. Therefore, patients who underwent chemo and/or radiotherapy were reevaluated after 12 months from the end of treatments. Patients who were followed-up just by surveillance repeated the same analysis after 12 months from surgery.

SEMEN PROCESSING
Semen samples were obtained by masturbation after 3 days of sexual abstinence. After liquefaction at room temperature, semen volume, pH, sperm concentration, viability, motility, and normal morphology were determined following World Health Organization guidelines for semen analysis (WHO, 2010). An aliquot was used for further analysis.

HPV DNA DETECTION
The presence of HPV infection was detected by PCR amplification of HPV DNA with SPF10 primers and then genotyped by the INNO-LiPA HPV Genotyping Extra assay (Innogenetics, Gent, Belgium), which can identify the following HPV genotypes: HPV-6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69/71, 70, 73, 74, and 82 (Eklund et al., 2010).

SPERM FLUORESCENCE *in situ* HYBRIDIZATION (FISH) FOR HPV
Samples containing at least 2×10^6 ejaculated sperm were fixed in a methanol-acetic acid solution for at least 1 h at −20°C. To permeabilize, samples were digested with pepsin diluted

1:25,000 in pre-warmed 0.01 mol/L-1 HCl for 10 min at 37°C. Permeabilization of the specimens was stopped with 3–5-min washes in PBS 1×; then samples were dehydrated in 70%, 80%, and absolute ethanol for 2 min and finally air-dried. Samples were then overlaid with 20 mL of hybridization solution (Pan Path, Amsterdam), containing biotin-labeled HPV DNA probe (a mix of total genomes containing the conserved HPV region). Each slide was covered with a glass coverslip, and the edges were sealed with nail polish to prevent loss of the mixture during denaturation and hybridization. After a simultaneous denaturation of cellular target DNA and HPV DNA probe on a heating block for 5 min at 95°C, hybridization was performed by incubating the samples at 37°C overnight in a humidified chamber. Thereafter, the coverslips were carefully removed and the slides were washed in PBS 1× for 10 min. After 15 min incubation at 37°C with the differentiation reagent (Pan Path), the slides were washed three times in PBS 1×. The biotin-labeled HPV probe was detected by incubation with 1:200 streptavidin Texas Red® (Vector Laboratories, Burlingame, CA) for 40 min at room temperature. After detection, the slides were washed twice in PBS 1×/0.01% Triton and then twice in PBS 1× and mounted with a solution containing 4′,6-diamidino-2phenylindole (DAPI) and anti-fade (BioBlue, BioView Ltd., Nes Ziona, Israel). Samples were analyzed using a fluorescence microscope (Nikon ViCo Video Confocal Microscope) equipped with a triple bandpass filter set (FITC, TRITC, DAPI). For each slide, at least 200 spermatozoa were analyzed. Evaluation of nuclear hybridization signals was performed in triplicate by different investigators. In samples that resulted negative during the follow-up, results were confirmed by PCR. When nuclei were completely and homogeneously stained and multiple small spots or single large signals were present, the sperm cells were classified as positive. The method was tested on control slides containing CaSki cells, a human cervical carcinoma cell line with stably integrated and transcriptionally active HPV genomes, which served as a control for the specific probe. Cells smeared on salinated glass slides were fixed with 4% paraformaldehyde in PBS for 10 min. After fixation, cells were subjected to 3–5-min washes in PBS 1× and then dehydrated with 5-min ethanol washes (30%, 60%, and 95%). Cell smears were then air-dried and stored at 4°C until use.

STATISTICAL ANALYSIS
The values shown are the averages of at least three evaluations performed by different operators. Data regarding sperm parameters were presented as mean ± SD while data on semen infection as percentage. Differences between data were determined by two-tailed Student's *t*-test after acceptance of normal distribution with the Kolmogorov-Smirnov test. Comparisons of proportion were performed with a one-sided non-parametric resampling test. *P*-values (two sided) <0.05 were considered statistically significant.

RESULTS
Mean age of patients with testicular cancer was 31.2 ± 5.4 years, not different from control subjects (30.8 ± 4.7 years). In **Table 1** are reported sperm parameters and HPV DNA detection in semen

samples from controls and from patients evaluated before (T−1) and 1 month after orchiectomy (T0). At T−1, patients showed sperm concentration, count, and morphology not different from controls, but a significant reduction of progressive motility ($p <$ 0.05). After orchiectomy, we found a significant reduction of sperm concentration and count (both $p < 0.05$ vs. T−1) and a further reduction of sperm motility compared to T−1 and control subjects ($p < 0.01$). Moreover, sperm volume and morphology were not significantly different in patients compared with controls before and after orchiectomy. Interestingly, the percentage of subjects with HPV infection was higher among patients than controls (9.7% vs. 2.4%). This percentage remained unchanged at T0. After orchiectomy, patients were assigned to three groups based on medical option: S = candidate to surveillance, R = candidate to radiotherapy, and C = candidate to chemotherapy with or without radiotherapy. In **Table 2**, are reported sperm parameters, the percentage of patients with semen infection and percentage of infected sperm observed in the three groups at T0 and after 12 months of surveillance or from the end of radio and or chemotherapy (T12). At T0, considering patients from different groups we found no difference in sperm parameters and prevalence of HPV infection. Six patients were lost during

follow-up (4 from group S, 1 from group R, and 1 from group C), therefore data at T12 are referred to the remaining 149 patients. Considering patients as a whole, the mean values of sperm parameters observed at T12 was not different from that at T0. However, patients who underwent surveillance had a significant increase of sperm number and motility compared to T0 ($p < 0.05$). In contrast, subjects who had radiotherapy showed no increase in sperm number and reduced sperm motility compared to S group ($p <$ 0.05). Moreover, patients who underwent chemotherapy associated or not with radiotherapy, had a reduction of both sperm number and motility vs. T0 vs. patients from groups S ($p <$ 0.05 and $p < 0.01$ respectively). In the three groups, we found no significant differences in both ejaculate volume and normal morphology comparing T12 with T−1 and T0. Considering all patients, HPV semen infection was significantly higher in patients at T12 respect to T0 (34.9% vs. 9.7%). By the comparison of different groups, the prevalence of infection resulted 7.7%, 30.8% and 61.5% in S, R, and C groups respectively. Finally, patients treated by chemotherapy had also a higher percentage of HPV infected sperm compared to T0 and to S (both $p < 0.05$). In **Figure 1** there is an example of FISH analysis for HPV performed in a semen sample from testicular cancer patients.

Table 1 | Sperm parameters and HPV DNA detection in semen samples from control subjects and patients at the time of diagnosis (T−1) and 1 month after orchiectomy (T0).

	Ejaculate volume (mL)	Sperm concentration (million/mL)	Sperm count (million)	Progressive motility (%)	Normal morphology (%)	Positive PCR (%)
Controls ($n = 84$)	3.3 ± 0.8	46.9 ± 11.3	153.0 ± 53.1	53.4 ± 12.1	21.6 ± 6.4	2 (2.4)
T−1 ($n = 155$)	3.2 ± 1.3	40.1 ± 45.2	110.1 ± 125.3	$34.2 \pm 17.7^*$	18.9 ± 8.3	15 (9.7)*
T0 ($n = 155$)	3.1 ± 1.3	$19.8 \pm 16.6^{**, \#}$	$61.9 \pm 45.5^{**, \#}$	$31.2 \pm 15.1^{**}$	18.5 ± 9.3	15 (9.7)*

*$p < 0.05$ vs. control subjects.

**$p < 0.01$ vs. control subjects.

#$p < 0.05$ vs. T−1.

Table 2 | Sperm parameters, HPV DNA detection and FISH for HPV observed in testicular cancer patients 1 month after orchiectomy (T0) and after 12 months (T12) from the end of any treatment.

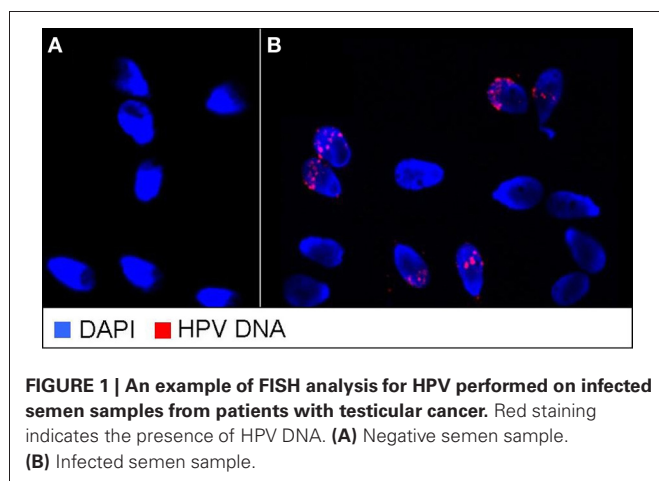| Groups	Ejaculate volume (mL)	Sperm concentration (million/mL)	Sperm count (million)	Progressive motility (%)	Normal morphology (%)	Positive PCR (%)	FISH on sperm (%)
T0							
S ($n = 46$)	2.9 ± 1.3	19.4 ± 14.4	54.6 ± 45.2	30.9 ± 14.1	19.8 ± 9.5	5 (11.1)	22.4 ± 8.3
R ($n = 55$)	3.2 ± 1.5	20.7 ± 19.7	67.1 ± 55.7	33.7 ± 17.6	17.9 ± 9.2	4 (7.2)	24.0 ± 3.7
C ($n = 54$)	3.1 ± 1.1	19.4 ± 15.2	62.7 ± 60.2	29.1 ± 12.9	17.9 ± 9.3	6 (10.9)	25.3 ± 3.8
Total ($n = 155$)	3.1 ± 1.3	19.8 ± 16.6	61.9 ± 45.5	31.2 ± 15.1	18.5 ± 9.3	15 (9.7)	24.0 ± 5.4
T12							
S ($n = 42$)	3.1 ± 1.5	$35.5 \pm 25.7^*$	$94.2 \pm 93.5^*$	$43.7 \pm 18.2^*$	18.6 ± 10.1	4 (7.7)	24.9 ± 3.7
R ($n = 54$)	3.1 ± 1.7	23.8 ± 20.6	69.9 ± 58.7	$30.8 \pm 15.3^{\#}$	18.0 ± 13.7	16 (30.8)$^{*, \#}$	28.7 ± 6.0
C ($n = 53$)	3.3 ± 1.5	$11.1 \pm 9.9^{*, \#\#}$	$24.4 \pm 17.4^{*, \#\#}$	$20.8 \pm 14.2^{*, \#\#}$	17.1 ± 11.2	32 (61.5)$^{**, \#\#}$	$39.6 \pm 8.2^{*, \#}$
Total ($n = 149$)	3.2 ± 1.5	22.6 ± 20.6	61.3 ± 50.5	30.7 ± 18.5	17.8 ± 11.5	52 (34.9)**	29.4 ± 6.9

Data are shown as total and subgrouping patients in: S group = patients who underwent surveillance, R group = patients who underwent radiotherapy, and C group = patients who underwent chemotherapy followed or not by radiotherapy.

*$p < 0.05$ vs. T0.

**$p < 0.01$ vs. T0.

#$p < 0.05$ vs. S.

##$p < 0.01$ vs. S.

FIGURE 1 | An example of FISH analysis for HPV performed on infected semen samples from patients with testicular cancer. Red staining indicates the presence of HPV DNA. **(A)** Negative semen sample. **(B)** Infected semen sample.

DISCUSSION

It is of concern, especially during last years, the increasing incidence of reproductive tract diseases and particularly testicular cancer (Richardson et al., 2012). The latter disease is particularly found among males aged between 15 and 40 years (Siegel et al., 2012; Viatori, 2012) and, although it is a curable disease with a good prognosis, it is a major risk factor for fertility (Jacobs and Vaughn, 2012). In fact, patients with testicular cancer have frequently a concomitant alteration of spermatogenesis already at diagnosis, that can be further affected by chemo or radiation therapies (Schrader et al., 2001; Bieber et al., 2006). In this paper, we considered sperm parameters and prevalence of HPV semen infection in testicular cancer patients pre- and post-orchiectomy. Moreover, after 12 months we evaluated the effect of different medical options on the same parameters. As previously reported (Selice et al., 2011), in affected patients we observed reduced sperm motility at diagnosis that was still present 1 month after orchiectomy together with a significant reduction of sperm number. Interestingly, we found that patients at diagnosis had a significant increase of HPV semen infection compared to controls. This finding could represent a cause of testicular cancer or an effect of this malignancy. Many hypotheses have been raised regarding the possible cause of testicular cancer development, as in utero and pre-pubertal exposure to endocrine didisrupters, genetic predisposition, and environmental conditions. Despite it is well-recognized that some viral infection have a major role in the development of many cancers (EBV, HCV, HPV, HIV etc.), this link has never been proposed for testicular tumors. On the other hand all tumors, including testicular cancer, may induce an impairment of general health exposing patients to infections. Moreover, testicular cancer patients after orchiectomy may undergo to different medical options based on type and tumor staging. Low risk conditions frequently allow just surveillance, while conditions at higher risk are usually treated by adjuvant therapies. Patients from S group had a significant improvement of sperm number and motility. This finding suggests that the residual testis may have a compensatory role after contralateral orchiectomy if patients are not exposed to treatments affecting spermatogenesis. Far from many years, it is well-known that both chemo and radiotherapies deeply impair spermatogenesis and immune system. The results of this study demonstrated that at T12 patients who received radio and even more those who received chemotherapy had altered sperm parameters both vs. pre-treatment vs. surveillance group. The strong association between kind of treatment and sperm alteration is further underlined by the comparison of sperm parameters from different groups at T0. This analysis showed no difference, suggesting that there was no relation between sperm parameters and the degree of malignancy at diagnosis. In the same way, adjuvant treatments resulted strongly related to HPV infection susceptibility. In this study, we found that after surveillance 7% of patients had HPV semen infection rising to 31% and 61% after radio and chemotherapy respectively. Therefore, drug-mediated immunosuppression seems to be strictly related to HPV seminal infection in testicular cancer patients. Finally considering FISH analysis for HPV, beside a trend to increase in R group, we observed a significantly higher percentage of infected sperm in subjects who received chemotherapy. This is the first study evaluating HPV sperm infection in semen samples from patients with testicular cancer. By our findings we can conclude that HPV infection is more prevalent in affected patients than controls. However, as recently suggested by other authors (Bertazzoni et al., 2013), this infection seems not to be cause of testicular cancer, but rather the effect of a status of higher vulnerability induced by the tumor. More and larger studies of follow-up of HPV infected patients will clarify whether or not there is a cause-effect relationship between HPV and testicular cancer or *vice versa*. Moreover, our results demonstrated that treatments frequently used in testicular cancer patients and in particular chemotherapy, are able to strongly increase the prevalence of HPV semen infection, probably through their immunosuppressive action. In conclusion, we suggest testing sperm HPV in patients affected by testicular cancer before and particularly after treatments inducing immunosuppression, because this infection can in turn induce cancer in many sites and further reduce male fertility. Further and larger studies are needed to confirm our findings also in patients affected by other tumors.

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Germ cell development in the postnatal testis: the key to prevent malignancy in cryptorchidism?

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To permit normal postnatal germ cell development, the mammalian testis undergoes a complex, multi-staged process of descent to the scrotum. Failure of any part of this process leads to congenital cryptorchidism, wherein the malpositioned testis finds itself at the wrong temperature after birth, which leads to secondary germ cell loss and later infertility and risk of cancer. Recent studies suggest that neonatal gonocytes transform into the putative spermatogenic stem cells between 3 and 9 months, and this initial postnatal step is deranged in cryptorchid testes. In addition, it is thought the abnormality high temperature may also impair apoptosis of remaining gonocytes, allowing some to persist to become the possible source of carcinoma *in situ* and malignancy after puberty. The biology of postnatal germ cell development is of intense interest, as it is likely to be the key to the optimal timing for orchidopexy.

Keywords: germ cell, gonocyte, spermatogium, testis, cryptorchidism, orchidopexy

INTRODUCTION

The right way to treat undescended testes (UDT) remains to be solved. Although much is known about the development of the testes and sperm cells, there is a missing link in our knowledge. It is not known when and how the progenitors of germ cells transform from neonatal gonocytes into spermatogonia or spermatic stem cells. Recent research suggests that a critical stage of sperm cell development may occur in the early postnatal period. Germ cell development has been studied extensively in the fetus and at puberty, but not in this postnatal window. We will propose in this review that knowledge about early postnatal germ cell development is crucial for the optimal management of UDT.
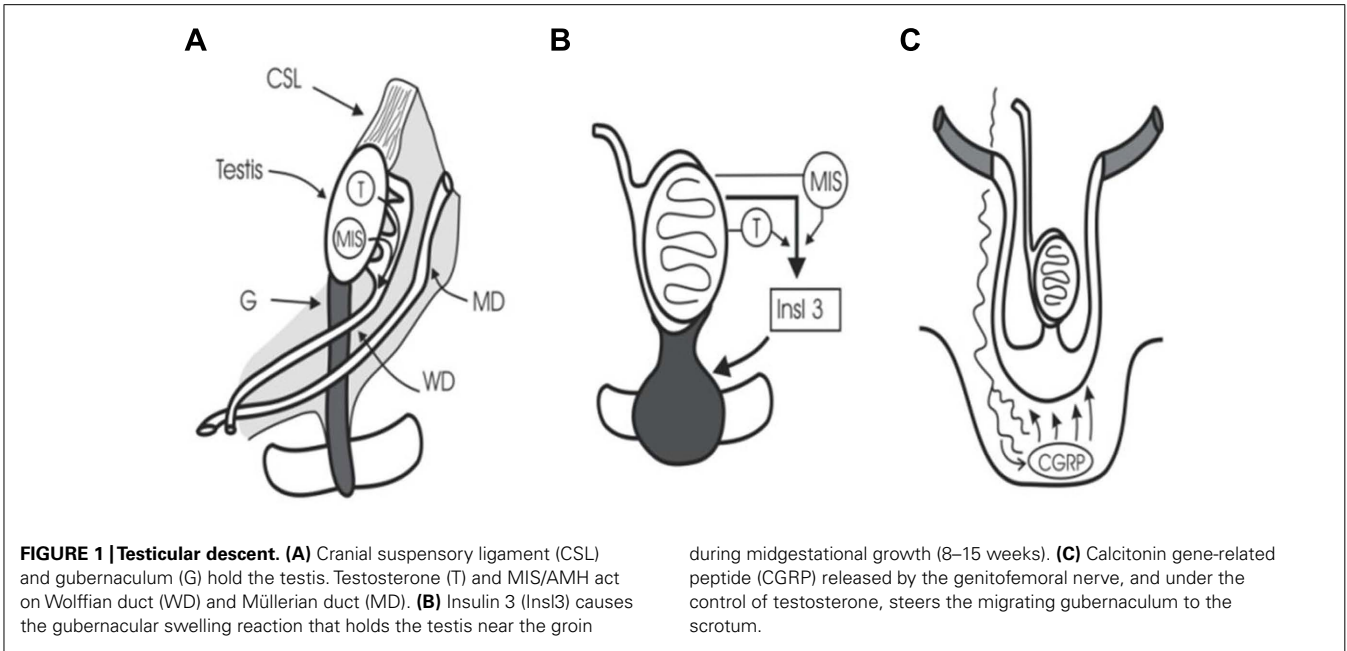
Undescended testes, or cryptorchidism, is a major problem occurring in about 5% of neonatal boys, with surgery to pull the testes down to the scrotum (orchidopexy), currently recommended between 6 and 12–18 months (Ritzen et al., 2007; Hutson et al., 2010). It has been previously shown in numerous studies that cryptorchidism in the past was associated with a 30–60% risk of infertility or lack of germ cells, and a 5- to 10-fold increase in testicular malignancy. Crucial steps in maturation of gonocytes into the sperm cell lineage and of sperm cell development could be occurring in this early postnatal period and could be dependent on low temperature present in the scrotum but lacking in UDT. In addition, massive cell death should occur in non-maturing gonocytes, clearing the testis of any undifferentiated, pluripotential neonatal germ cells, as persisting cells could go on to undergo cell division and become cancerous. The timing of surgery for orchidopexy aims to prevent these problems but lacks scientific evidence. In this review, we examine the evidence for early postnatal germ cell development to determine what controls these steps and also to examine the evidence for early orchidopexy to try and prevent abnormal germ cell development.

NORMAL EMBRYOLOGY AND POSTNATAL DEVELOPMENT IN HUMANS

At 5 weeks of gestation, embryonic gonocytes migrate from the umbilical stalk into the ambisexual gonad. Human sexual development then begins at 7–8 weeks' gestation when the SRY (sex-determining region Y) gene initiates testicular differentiation with Sertoli cell development (Sinclair, 1994). Cords of Sertoli cells surround the newly arrived germ cells and the interstitial cells form Leydig cells and peritubular myoid cells. Müllerian-inhibiting substance (or anti-Müllerian hormone) (MIS/AMH) and testosterone synthesis by Sertoli cells and Leydig cells, respectively, trigger regression of Müllerian ducts and preservation of Wolffian ducts which form epididymis, vas, and seminal vesicles (Wilson et al., 1981; Lee and Donahoe, 1993; **Figure 1**). The intra-abdominal fetal testis descends to the scrotum in a complex, two-stage process that in humans is complete at birth, while in rodents the second phase occurs in the first week after birth (Hutson et al., 1997). The regulation of this two-stage process is not the subject of this review, but interested readers should see some recent reviews (Ivell and Hartung, 2003; Adham and Agoulnik, 2004; Amann and Veeramachaneni, 2007; Bay et al., 2011).

In baby boys, the temperature of the intra-scrotal testes drops to 33°C at birth, with all the testicular enzyme systems readjusting presumably within a few weeks to this new environment (Zorgniotti, 1991). Between 2 and 4 months of age pituitary gonadotropins stimulate a sudden increase in testosterone production which peaks at about 3–6 months, before it wanes and becomes almost negligible until the onset of puberty. This brief
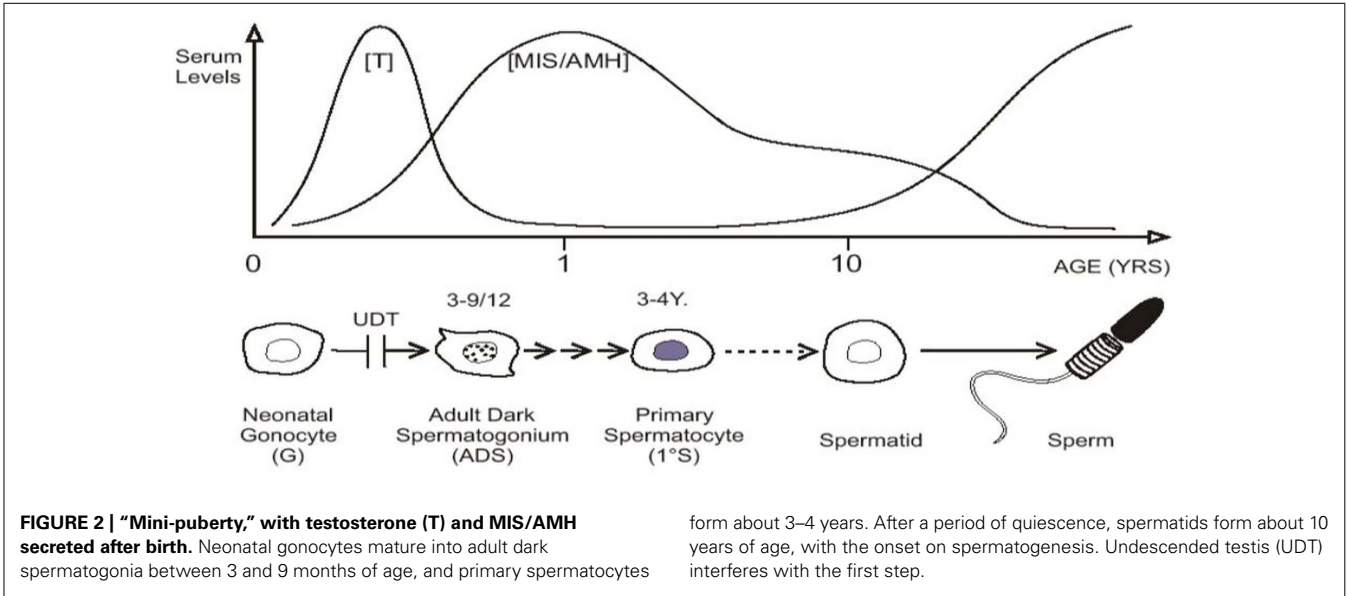
FIGURE 1 | Testicular descent. (A) Cranial suspensory ligament (CSL) and gubernaculum (G) hold the testis. Testosterone (T) and MIS/AMH act on Wolffian duct (WD) and Müllerian duct (MD). **(B)** Insulin 3 (Insl3) causes the gubernacular swelling reaction that holds the testis near the groin during midgestational growth (8–15 weeks). **(C)** Calcitonin gene-related peptide (CGRP) released by the genitofemoral nerve, and under the control of testosterone, steers the migrating gubernaculum to the scrotum.

surge of gonadotropins and androgens is known as "mini-puberty" (Job et al., 1988; Hadziselimovic and Zivkovic, 2007). As androgen levels subside there is a surge in production of MIS/AMH by Sertoli cells, which peaks at 6–12 months, but remains high then until puberty when serum levels fall (Baker et al., 1990; Yamanaka et al., 1991; Aksglaede et al., 2010; **Figure 2**).

ABNORMAL POSTNATAL DEVELOPMENT IN UDT IN HUMANS

Failure of the first or second phase of testicular descent leads to congenital cryptorchidism. The causes of UDT remain largely unknown with some authors suggesting a primary testicular hormonal malfunction (Heyns and Hutson, 1995), while others have suggested a secondary hormonal malfunction, such a hypothalamic–pituitary axis deficiency (Hadziselimovic and Zivkovic, 2007) or a placental failure to produce chorionic gonadotropin (Heyns and Hutson, 1995). Based on twin studies, it has also been suggested that risk factors should be sought in the intrauterine environment and the maternal genes. However, another possibility first suggested in the eighteenth and nineteenth centuries, was that there was an anatomical defect in the mechanism of descent. With the establishment of endocrinology in the early twentieth century, these anatomical concepts were mostly replaced with explanations of hormonal dysfunction, such as described above (Heyns and Hutson, 1995). However, our own research over the last 25 years, along with others, has suggested

FIGURE 2 | "Mini-puberty," with testosterone (T) and MIS/AMH secreted after birth. Neonatal gonocytes mature into adult dark spermatogonia between 3 and 9 months of age, and primary spermatocytes form about 3–4 years. After a period of quiescence, spermatids form about 10 years of age, with the onset on spermatogenesis. Undescended testis (UDT) interferes with the first step.

that anatomical faults may be a common cause of cryptorchidism (Backhouse, 1964). Cryptorchidism is associated with other congenital abnormalities in less than 20% of the cases, but when present then most are often related to abnormalities of the midline and the caudal development field of the body (Cortes, 1998; Jensen et al., 2010; Thorup et al., 2010). Two key observations in favor of this view are the failure to document convincing primary hormonal defects, as well as the realization that the second phase of descent is a very complex anatomical process, that is likely to be prone to subtle anomalies (Amann and Veeramachaneni, 2007; Thorup et al., 2012). This is further supported by the fact that the anomaly most often occurs unilaterally, and in cases where associated Wolffian duct and/or ureteric bud malformations are present, these are predominantly found ipsilaterally (Cortes et al., 1998a).

If something goes wrong with migration of the gubernaculum from the external inguinal ring in the groin across the pubis and into the scrotum, the initially normal postnatal testis is retained deep to the inguinal fat pad, which is an effective insulator, keeping the UDT at 34–37°C (Mieusset et al., 1993), rather than the normal scrotal temperature of 33°C, and this is believed to trigger the progressive postnatal testicular dysfunction, as the postnatal testis function is only optimal at 33°C (Zorgniotti, 1991). The exquisite temperature sensitivity of the testis has been well documented for a long time, but is currently not a fashionable area of research in this era of molecular biology. However, the phenomenon has been solidly proven despite the genetic regulation of it not being fully described. This abnormal postnatal development in the testis also leads to inhibited postnatal androgen production (Raivio et al., 2003) and MIS/AMH production at 6–12 months of age (Yamanaka et al., 1991).

The net effect of cryptorchidism is germ cell loss, leading to infertility (Mengel et al., 1974; Hadziselimovic, 1985). This has been well described in many long-term outcome studies in men with a previous history of cryptorchidism in childhood (Cortes, 1998; Gracia et al., 2000; Cortes et al., 2001; Vincardi et al., 2001). However, the cause for this germ cell loss and its timing have remained elusive until relatively recently. In the 1950s, orchidopexy was recommended in boys aged 10–15 years if the cryptorchid testes failed to descend spontaneously into the scrotum at the onset of puberty (Gross and Jewett, 1956; Vincardi et al., 2001). This recommendation was based on the clear observation that a significant number of cryptorchid testes descended spontaneously at puberty. In addition, it was assumed that germ cell development was totally quiescent in childhood. Indeed, it was thought that the testis was effectively in "suspended animation" until the onset of spermatogenesis in early puberty.

Current long-term follow-up studies of men with a previous history of cryptorchidism show a 5- to 10-fold increase in malignancy risk (Whitaker, 1988; Wood and Elder, 2009). However, it is easy to forget that because of the very long lag-time (i.e., 30–40 years) between intervention and measurement of the outcome, in this case development of testicular malignancy, that the age of orchidopexy in most of these studies in the current literature is about 5–15 years of age. This has led to the widespread view in adult endocrinology and oncology that orchidopexy is unable

to prevent development of cancer in men (Gracia et al., 2000). However, the age for orchidopexy is now much younger, and it remains to be seen whether this will alter outcomes in adulthood. Recent studies actually indicate that operation for cryptorchidism at a young age lowers the risk of testicular cancer in adulthood (Pettersson et al., 2007).

Meanwhile, in pediatrics, it first became apparent in the 1970s that degeneration of the UDT was occurring in early childhood (Mengel et al., 1974). Initially it was noted that there was macroscopic atrophy of the testis in early primary school years leading to the view that maybe orchidopexy should be done in 5- to 6-year-old boys rather than at 10–15 years, based on the assumption that this might prevent the obviously visible development of atrophy. During the 1970s and early 1980s there was histological evidence accumulating of degeneration visible in boys of 2 years of age with UDT, leading to the recommendation in pediatric surgery that orchidopexy might be optimally done in 2-year-old boys (Hadziselimovic et al., 1975). It was then appreciated that there were signs of early degeneration in the testis on electron microscopy at about 12 months of age (Hadziselimovic, 1985; **Figure 3**).
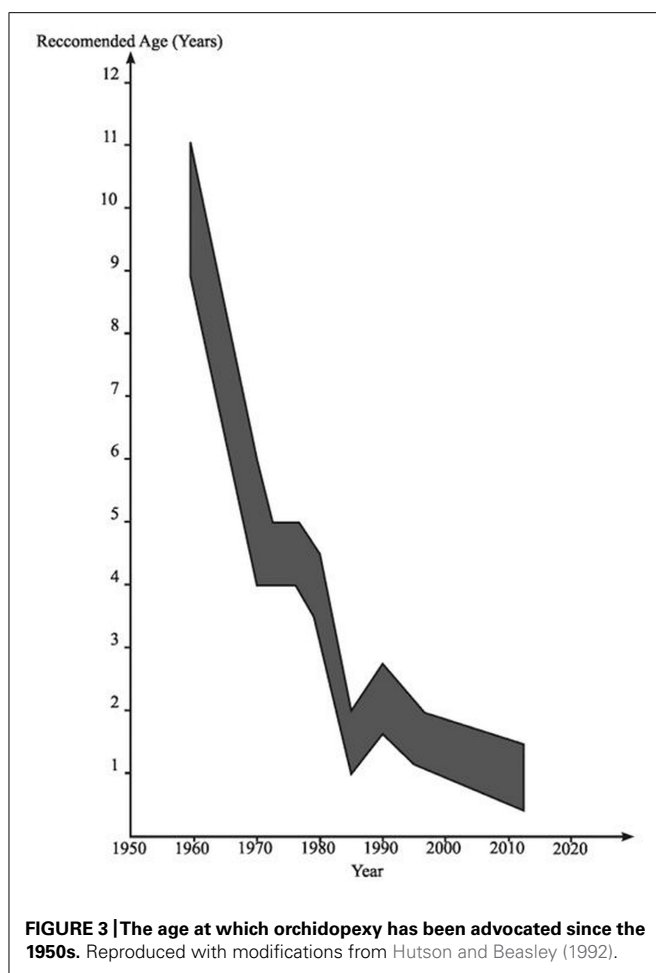
Furthermore, it was reported that all UDT harbored germ cells at the time of birth, but the number of germ cells was decreased in about one-fourth of cryptorchid newborns (Cortes, 1998). Lack of germ cells has been reported from 12, and especially from 18 months of age, and therefore surgery has been recommended before 12 or 18 months of age (Ritzen et al., 2007).

Recent studies have implicated the very first phases (i.e., in the first year) of postnatal germ cell development in the etiology of the subsequent infertility and risk of malignancy. However, we first need to describe normal germ cell development so that the effects of cryptorchidism can be understood in context.

NORMAL GERM CELL DEVELOPMENT

Around 4–6 weeks of gestation within the embryonic urogenital ridge the primitive gonad forms on the anteromedial surface of the middle kidney or mesonephros. Primordial germ cells form from endodermal cells in the caudal edge of the yolk sac stalk, and then migrate into the embryonic celom around the wall of the midgut and into the urogenital ridge, eventually colonizing the ambisexual gonad and differentiating into gonocytes. By 22 weeks of gestation, these primitive germ cells mature into fetal spermatogonia (Hadziselimovic, 1983). Mesenchymal cells in the 7- to 8-week ambisexual gonad surrounding the arriving germ cells form the Sertoli cells with the onset of sexual differentiation to create the testicular cords, inside a basement membrane. Outside the cords some mesenchymal cells form Leydig cells and begin hormone synthesis to produce androgen and insulin-like hormone 3 (INSL3), while others form into myoepithelial cells around the cords.

At birth, the fetal spermatogonia or neonatal gonocytes are located in the center of the spermatic cords. Throughout childhood it appears that the germ cells remained in the center of the cords, and seemed to be in "hibernation" until the onset of spermatogenesis at puberty, as there was little histological change in their appearance or location. However, in recent years, it became apparent that by 3–4 years of age the cell in the center of the

FIGURE 3 | The age at which orchidopexy has been advocated since the 1950s. Reproduced with modifications from Hutson and Beasley (1992).

testicular cords is a primary spermatocyte, rather than a neonatal gonocyte (Hadziselimovic, 1983).

Recent studies show that the neonatal gonocyte migrates between the Sertoli cells to the periphery of the cord between 3 and 9 months of age, or 2 and 6 days of age in mice (Drumond et al., 2001; Huff et al., 2001b), where it comes in contact with the basement membrane. These factors trigger transformation into type-A spermatogonia, which line the basement membrane, displacing the adjacent Sertoli cells. The regulatory factors involved in this transformation are mostly unknown (see below), although platelet-derived growth factors (PDGF) B and D, and also PDGF receptor-beta (PDGFR-β) have been implicated (Basciani et al., 2008, 2010). Inhibition of PDGFR-β tyrosine kinase activity in the first week postnatally in a mouse causes a severe reduction in the proliferation of gonocytes and increases their apoptosis (Basciani et al., 2008, 2010). Type-A spermatogonia mature into type-B spermatogonia, then migrate back into the center of cord again by 3–4 years, to become primary spermatocytes.

Alongside these studies of early postnatal germ cell development have been studies searching for the potential stem cell of spermatogenesis, with the aim of using these stem cells for recolonizing the infertile testis (Brinster, 2007; Waheeb and Hofmann, 2011). Based on morphological criteria, human spermatogonia

have been classified as dark type-A (Ad), pale type-A (Ap), and type-B. Ad-spermatogonia have nuclei showing a homogeneous, dark stainable chromatin and one or more clear cavities of vacuole like appearance (Clermont, 1963). The type-A spermatogonium has become the likely stem cell of spermatogenesis (de Rooij and Russell, 2000; Drumond et al., 2001; Biers and Malone, 2010). Interestingly not all neonatal gonocytes transform into type-A spermatogonia, so that by 1–2 years of age the total germ cell number is less than half that at birth (Hadziselimovic, 1983). The remaining gonocytes are thought to undergo apoptosis, or programed cell death, so that by 2 years of age there are none left in the testis (Huff et al., 2001b). This process can be seen readily in the postnatal rat testis, as signs of apoptosis are rare until day 3, but by day 4 analysis shows that one-third of them are lost (Roosen-Runge and Leik, 2000).
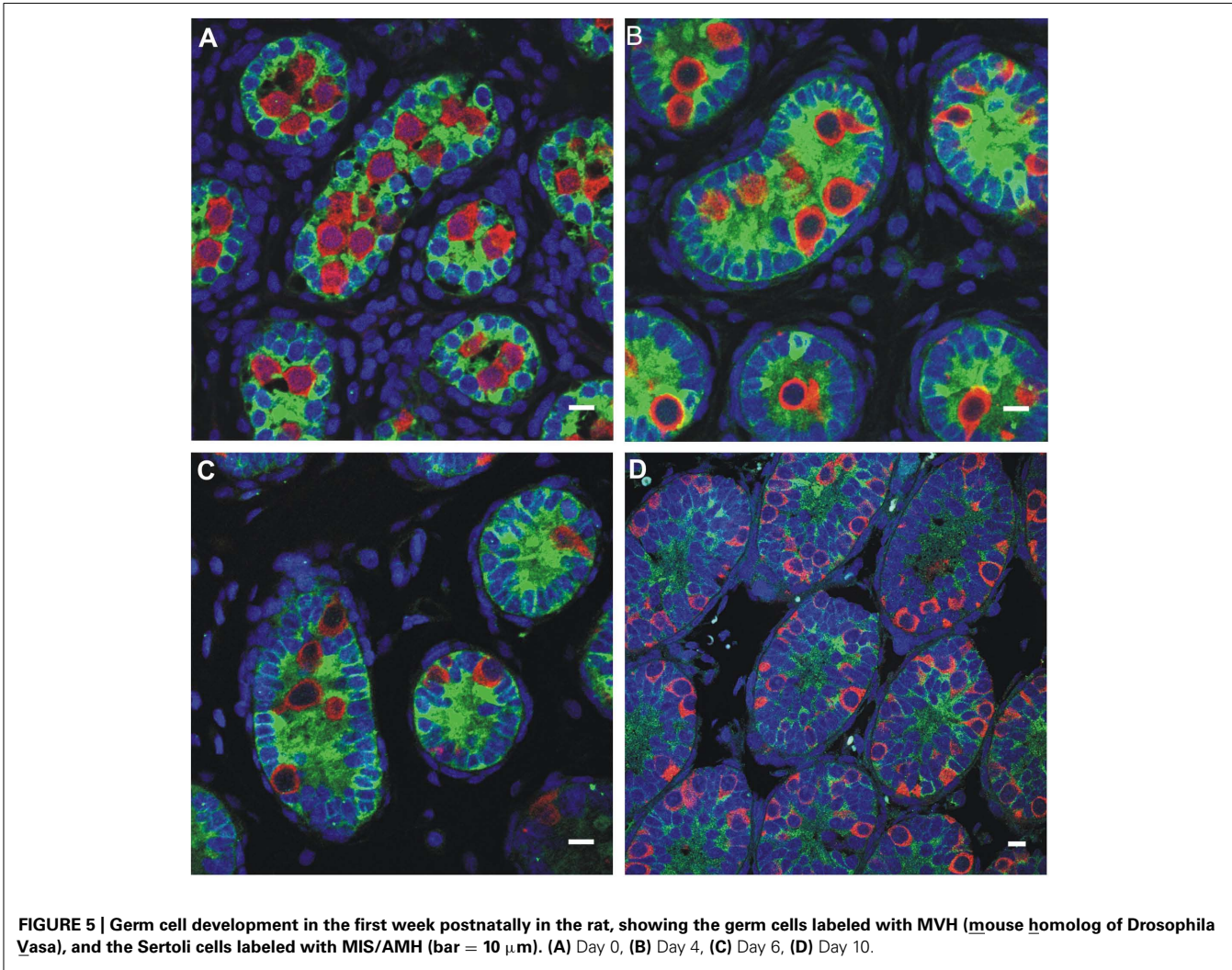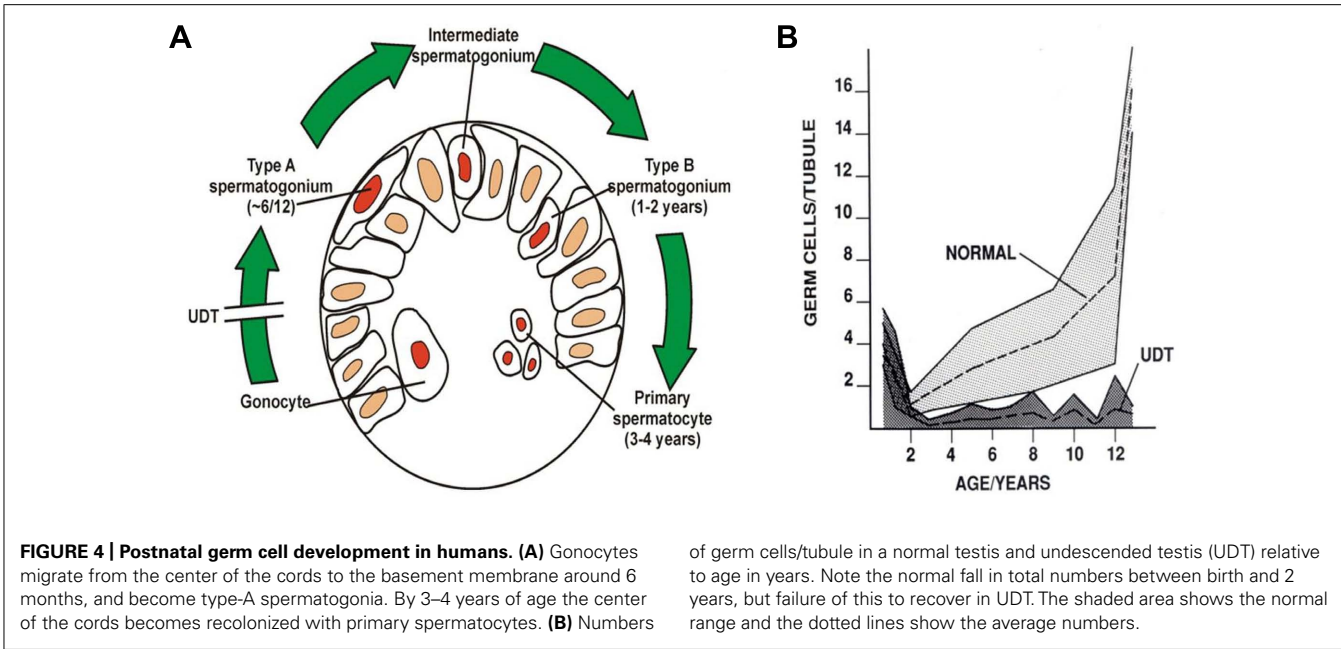
THE SPERMATOGENIC STEM CELL

As neonatal gonocytes seem to differentiate into unipotent stem cells for subsequent spermatogenesis, their change in appearance, location within the cord, and function suggest dramatic changes in germ cellular physiology. These changes are regulated by a precisely coordinated expression of key proteins, some of which are known.

In early embryos, germ cells and pluripotent embryonic stem cells can form any type of cell. However, as development proceeds, the ability of germ cells to form different cells becomes restricted. In testes a subpopulation of germ cells retain the ability to differentiate, but only as unipotent spermatogenic stem cell (SSCs; Hofmann et al., 2005).

Huckins and Oakberg have proposed a widely accepted model for spermatogenic development which is useful to describe here (Huckins, 1971; Oakberg, 1971; de Rooij and Russell, 2000). In this model, single type-A spermatogonia are the putative SSCs, which can self-renew, while paired type-A spermatogonia are differentiating paired daughter cells connected by an intercellular bridge. Paired type-A spermatogonia divide into chains of aligned cells, which then become type A_1, A_2, A_3, and then A_4 spermatogonia. The latter cells (A_4) divide to form intermediate spermatogonia and the type-B spermatogonia. Type-B cells then divide to form primary spermatocytes that enter meiosis (**Figure 4**). All these steps are regulated by growth factors from Sertoli and possibly the peritubular myoid cells (Skinner, 1991; Jegou, 1993). A recent morphological study (Drumond et al., 2001) showed that postnatal development of type-A spermatogonia may occur more rapidly than in mature spermatogenesis.

The type-A spermatogonia and SSCs have been shown recently to express a range of different markers (**Figure 5**). Undifferentiated SSCs express the integrins β-1 and α-6 (Shinohara et al., 1999), and the receptors for glial-derived neurotrophic factor (GDNF), such as GDNF family receptor alpha-1 (GFRα-1) and receptor tyrosine kinase (RET) (de Rooij and Russell, 2000). In addition undifferentiated SSCs express Zbtb-16 (previously known as Plzf; Buaas et al., 2004; Costoya et al., 2004). Once SSCs start to differentiate, they begin expressing a range of different markers, including c-Kit, the receptor for stem cell factor (Besmer et al., 1993; Yoshida et al., 2006). They also express Sohlh1 and Sohlh2, as well as neurogenin 3 (Ballow et al., 2006; Filipponi et al., 2007; Hao et al., 2008). As can be seen from this brief overview, most of what we know about SSCs

FIGURE 4 | Postnatal germ cell development in humans. (A) Gonocytes migrate from the center of the cords to the basement membrane around 6 months, and become type-A spermatogonia. By 3–4 years of age the center of the cords becomes recolonized with primary spermatocytes. **(B)** Numbers of germ cells/tubule in a normal testis and undescended testis (UDT) relative to age in years. Note the normal fall in total numbers between birth and 2 years, but failure of this to recover in UDT. The shaded area shows the normal range and the dotted lines show the average numbers.

FIGURE 5 | Germ cell development in the first week postnatally in the rat, showing the germ cells labeled with MVH (mouse homolog of Drosophila Vasa), and the Sertoli cells labeled with MIS/AMH (bar = 10 μm). (A) Day 0, **(B)** Day 4, **(C)** Day 6, **(D)** Day 10.

comes from studies of adult spermatogenesis or stem cell research, rather than a direct study of the type-A spermatogonium in the postnatal testis.

ABNORMAL GERM CELL DEVELOPMENT

All cryptorchid testes of newborns contain germ cells, although in some testes the number is impaired compared to normal. So significant deranged germ cell development in cryptorchid testes probably begins postnatally, except in the dysgenetic testis in disorders of sex development (DSD). The first step to fail is transformation of gonocytes into type-A spermatogonia, which is delayed or interrupted. Evidence for this disruption is found in the persistence of large numbers of gonocytes in the center of the testicular cords well beyond 6 months of age (**Figure 6**), and a decreased number of type-A spermatogonia (Hadziselimovic et al., 1986; Huff et al., 1989, 1991). After the first year or so the number of spermatogonia decrease, while those that remain are gonocytes with bizarre nuclei (Hadziselimovic, 1983). By 3–4 years of age the abnormality has become even more obvious, with failure of primary spermatocytes to appear (Huff et al., 1989).

The abnormally high temperature of the cryptorchid human testis is considered by most authors to be the cause of this germ cell maldevelopment. Considerable studies have been performed in various animal models showing that heat stress leads to a combination of both indirect and direct effects on the germ cells, causing impaired transformation and maturation as well as inhibited apoptosis. This thermal injury is mediated by reactive oxygen species and certain heat-shock proteins, which damage the germ cells as well as Sertoli cells (Setchell, 1998; Zini et al., 1999; Ivell and Hartung, 2003).

Defective transformation of gonocytes into type-A dark spermatogonia between 3 and 9 months of age correlates with abnormal sperm counts after puberty (Hadziselimovic and Herzog, 2001a; Huff et al., 2001a). In cases of no adult dark spermatogonia, the age of orchidopexy has not affected a poor result for spermatogenesis in adulthood, at least so far

FIGURE 6 | Abnormal persistence of gonocytes beyond 6 months of age in a boy with cryptorchidism. The germ cells are labeled with MVH (mouse homolog of Drosophila Vasa), and the Sertoli cells are labeled with MIS/AMH (bar = 10 μm).

(Hadziselimovic and Herzog, 2001b). The degree of risk of subsequent infertility can be quantitated, as it is almost inevitable if there are fewer than 1% of the number of spermatogonia per tubular cross-section, seen in biopsies from age-matched controls (Cortes et al., 1996). Under normal physiological circumstances, the surge of gonadotropins and testosterone, and possibly other as yet unknown hormones, occurs at 2–3 months of postnatal age. This hormonal surge stimulates the gonocytes in the testis to develop into adult dark spermatogonia. In cryptorchidism there has been reported evidence for a beneficial role of adjuvant gonadotropin-releasing hormone to improve fertility in UDT (Biers and Malone, 2010). It has been questioned as a general recommendation (Hutson, 2010). Recently there have been described different groups of cryptorchid boys, and if the patients at the time of surgery have a decreased number of germ cells and/or inhibin B and no compensatory increase in gonadotropins, then supplementary hormonal treatment may be indicated after early surgery for UDT (Thorup et al., 2012). It has been suggested that antisperm antibodies may be involved in the germ cell loss in cryptorchidism, but there is no evidence for postpubertal antibodies after previous surgery in childhood for cryptorchidism (Mirilas et al., 2003). All these studies suggest that the postpubertal poor fertility is likely to be caused by an abnormally small pool of SSCs, the type- A spermatogonia.

The risk of cancer after cryptorchidism in infancy is less easily correlated with abnormal germ cell development after birth. It has been well described that the risk of developing a testicular germ cell cancer is about 5- to 10-fold higher in men with a previous history of cryptorchidism compared with those with descended testis in childhood (Chilvers et al., 1986; Giwercman et al., 1987; Moller et al., 1998; Wood and Elder, 2009). Testicular germ cell tumors are extremely common, affecting 1% of young men (Rorth et al., 2000).

The common precursor of these tumors is the premalignant carcinoma *in situ* (CIS) cell, which is proposed to be a fetal germ cell that failed to differentiate, and remains, dormant in the testis until after puberty (Skakkebaek et al., 1982). The evidence supporting this view comes from the many similarities between CIS cells and fetal gonocytes, which share many markers and morphological characteristics (Holstein and Korner, 1974; Skakkebaek et al., 1982; Rorth et al., 2000; Rajpert-De Meyts et al., 2003). CIS cells may well originate during fetal development in DSD, where the germ cell physiology is deranged by the underlying genetic anomaly. By contrast, this seems less plausible in cryptorchidism if the maldescent is caused by extraneous mechanical defects. In cryptorchidism, therefore, the CIS cell may arise from the neonatal gonocytes, which are abnormally abundant. The gonocytes have pluripotent stem cell properties, which if they persist until after puberty, with or without mutation, may develop into cancer.

Carcinoma *in situ* cells are more likely to be present where there are specific genetic defects, and there may be developmental arrest (Skakkebaek et al., 1987, 1998; Honecker et al., 2004). Expression profiles of CIS cells and embryonic stem cells are very similar, which supports the view of CIS being a multipotent stem cell (Almstrup et al., 2004). A unifying hypothesis has been proposed to link testicular maldevelopment, testicular cancer, low sperm
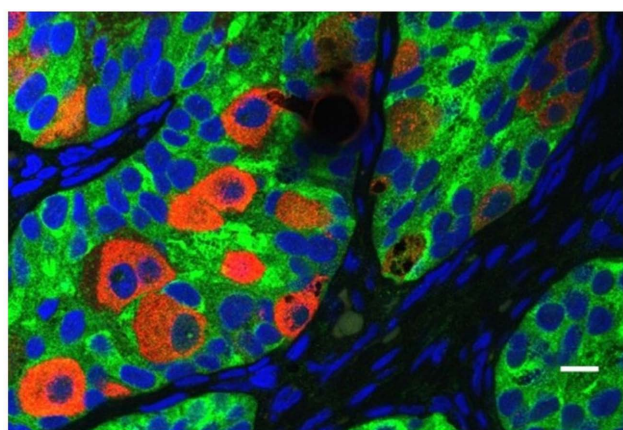
counts, and cryptorchidism, known as the testicular dysgenesis syndrome (TDS; Asklund et al., 2004).

Carcinoma *in situ* is more common in men with previous cryptorchidism, and is documented in 2–3% of adult patients (Giwercman et al., 1987, 1989). However, the prevalence of CIS is higher in those men with macroscopic testicular atrophy (Harland et al., 1998) or those with bilateral cryptorchidism (Swerdlow et al., 1997). Where cryptorchidism is unilateral, the contralateral descended testis has a slightly increased risk of cancer (Forman et al., 1994). Not surprisingly, cancer risk is also increased where testes were intra-abdominal, the genitalia were abnormal or there was a known abnormal karyotype (Cortes et al., 1999).

In a pediatric collective series of six studies, the risk of CIS in 2800 boys with cryptorchidism was 0.36% only (Cortes, 1998). When compared to higher incidence data from adults these pediatric figures support the hypothesis that the characteristics of CIS cells and later testicular cancer develops over time, and are not present from fetal life. Generally CIS in pediatric series is described in later childhood (Cortes et al., 1999). However, in this paper we present a 12-month-old boy with suspected CIS in bilateral cryptorchid testes (**Figure 7**). It has to be emphasized that positive immunohistochemical markers indicating CIS in the adult testis do not have such predictive value when applied to the childhood cryptorchid testis. Positive staining by such markers of persistent fetal germ cells is seen in almost half of the cryptorchid testes of boys younger than 5 years (Thorup et al., 2012). Presence of placental alkaline phosphatase (PLAP)-positive immunohistochemical stained gonocytes in bilateral cryptorchid testes of infant boys indicates the ability of delayed germ cell transformation and a preserved good fertility potential (Cortes et al., 2012). Further research in this field is therefore needed.

It has been suggested that testicular biopsy itself at the time of orchidopexy is a risk factor for postpubertal cancer (Swerdlow et al., 1997). In the review by Swerdlow et al. (1997) only 9% of the operated testes were subjected to biopsy, consistent with a possible selection bias by the surgeon, who may have only picked the more atrophic testes for analysis. This view is supported by a large study of 830 cryptorchid patients having routine biopsy, where there was no correlation between the biopsy itself and subsequent malignancy (Moller et al., 1998).

Some authors have suggested that adolescents with a previous history of cryptorchidism and orchidopexy should be offered a biopsy to identify if CIS is present, before invasion has occurred (Giwercman et al., 1989). Alternative screening methods such as reliable blood or semen analysis are not yet available, although testicular ultrasonography may have a place (Holm et al., 2001). Ultrasonography shows a relationship between microlithiasis and CIS, but the significance of this is uncertain (Lenz et al., 1996), particularly as more recent studies of testicular microlithiasis show that it is quite common in normal adolescents (Goede et al., 2009). CIS is also associated in some patients with cryptorchidism with multinucleated germ cells, consistent with aberrant germ cell development (Cortes et al., 2003).

There is inconclusive evidence that an early age of surgery leads to a reduction in risk of cancer (Pike et al., 1986; Swerdlow et al., 1997; Wood and Elder, 2009). However, because of the long lag-time between treatment and outcome, the age at
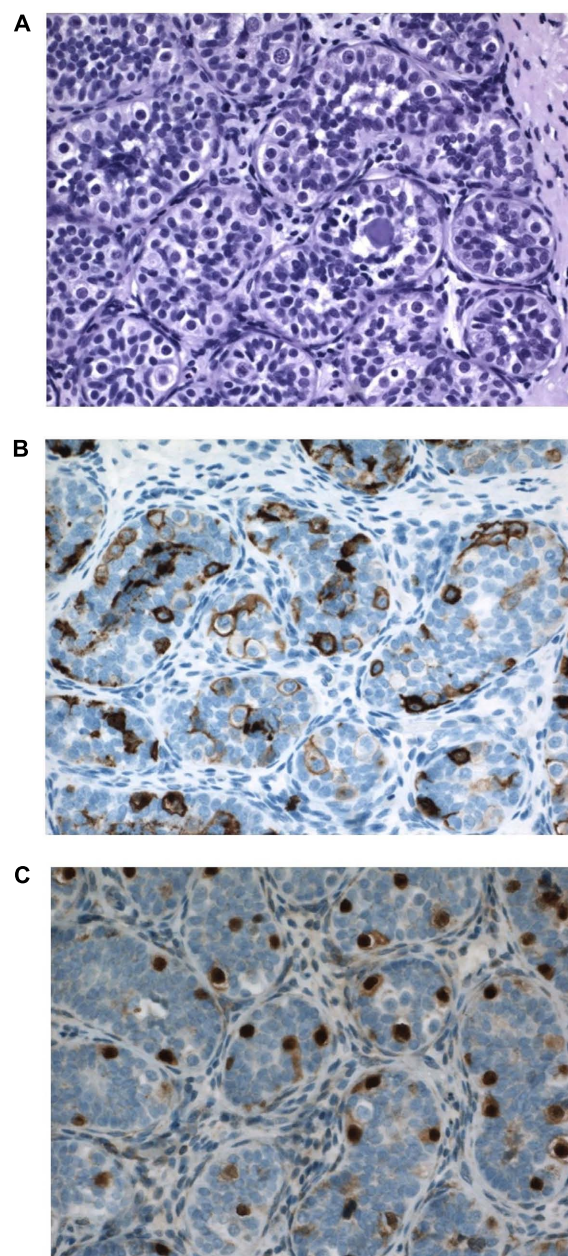
FIGURE 7 | Testicular tissue from a 12-month-old boy with bilateral cryptorchidism. Carcinoma *in situ* (CIS) is suspected. **(A)** H&E staining: Sertoli cells are normal and the germ cell number is at the upper range for age. Some germ cells have two nuclei, and some nuclei have irregular morphology and some nucleolus. A microlith is clearly seen within a tubule. **(B)** Strong PLAP expression seen in all germ cells. **(C)** OCT4 expression in germ cells also in the periphery of tubules, which is not often seen so impressively in most cryptorchid testes at a similar age.

surgery in these follow-up studies averaged more than 2 years old. In the context of the proposal, that early germ cell development is the key, these long-term results are consistent with either no effect of age at all, or with the suggestion that lack of neonatal gonocyte transformation and impaired apoptosis predisposes to malignancy.

One of the problems that has bedeviled the relationship between cryptorchidism and malignancy is the lack of an animal model, as rodents with UDT do not develop testicular tumors. Another problem is that evidence is accumulating that there are some testes that become undescended after birth, and that this group, now known as acquired cryptorchidism, have a different risk of malignancy compared with congenital UDT.

ACQUIRED CRYPTORCHIDISM

After birth, the distance from the inguinal canal to the scrotum increases from 4–5 to 8–10 cm in early adolescence (Smith et al., 1989). This means that the spermatic cord must double in length between birth and puberty (Hutson et al., 2010). We have previously proposed that failure of this elongation is the cause of ascending testes or acquired UDT (Hutson and Hasthorpe, 2005), and this is the likely explanation for many children still having orchidopexy at 5–10 years, despite recommendations for surgery in infancy (Donaldson et al., 1996).

Realization that there are two types of UDT has triggered a re-evaluation of the cause and effect of UDT. First, it is becoming apparent that men who had untreated, acquired UDT have no increased cancer risk, but still have impaired fertility (Cortes et al., 1998b). Secondly, it means that current long-term follow-up studies in adults are likely to contain a mixture of patients with both congenital and acquired UDT (Bonney et al., 2008), making their interpretation difficult.

Investigators have puzzled over why UDT in rodents caused infertility but not cancer, as in humans. We propose that this is the result of differences in timing in when the testis reaches the low-temperature scrotal environment. In humans, the testis is at 33°C from birth, so that congenital UDT has the potential to damage neonatal gonocytes by high temperature. By contrast, rodent testes only reach the scrotum after 12 days of age (Bergh, 1991), which is well after normal gonocyte transformation and/or apoptosis is complete (**Figure 8**).

Undescended testis in rodents, therefore, is actually a model for acquired UDT in humans, with high temperature damaging the SSCs, but with no cancer risk as the gonocytes will have developed or disappeared normally. Once this key difference in timing of testicular descent is understood, extrapolation between rodent models and the human situation is straightforward, as other key processes are the same. Both species groups have a postnatal "mini-puberty" at 3–6 months in humans and 2–4 days in mice, when there is burst of testicular hormones and cytokines which are likely to be involved in gonocyte development. In addition, gonocyte migration from the center of the cords to the basement membrane to form SSCs is similar in both timing and general morphology.

A temperature difference between undescended and normally descended testes in rats is not present until after 12 days of age (Zorgniotti, 1991), which is well after the gonocytes should have transformed normally into SSCs or have undergone apoptosis. If excess persisting gonocytes are the cause of CIS and cancer in cryptorchidism, then none will be present in the testis when the temperature becomes abnormal in rodent UDT, consistent with the lack of cancer in these models. However, the high temperature of the UDT will still damage SSCs leading to subsequent infertility (**Figure 8**).
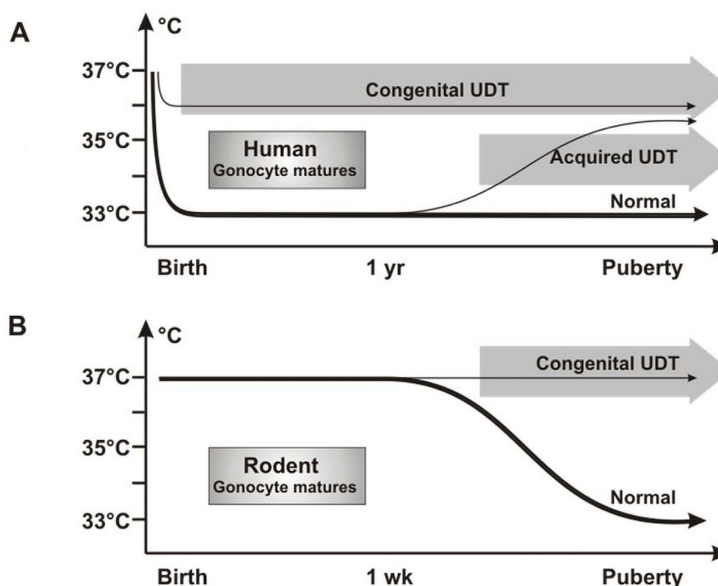

FIGURE 8 | Testicular temperature in humans and rodents.
(A) Human gonocytes mature in the first year when the testicular temperature is 33°C. Congenital UDT have abnormal temperature early, which interferes with gonocyte development. By contrast, acquired UDT only interferes with subsequent survival of stem cells. **(B)** In rodents, gonocytes mature before a change in temperature occurs, so that gonocyte development is unaffected, similar to acquired UDT in humans.

CONCLUDING REMARKS

All the evidence available points to early germ cell maturation being the "missing link" in the disconnection between the timing of orchidopexy and the subsequent risks of both infertility and malignancy. This suggests that infants getting really early operation may have significantly improved outcomes, although this remains hypothetical. Furthermore, in some cases supplementary hormonal treatment may be needed to achieve the normal transformation to adult dark spermatogonia.

The key role of heat in interfering with the signaling that controls both gonocyte transformation and/or apoptosis needs to be reinvestigated fully, as it is likely to lead to changes in clinical management of congenital cryptorchidism.

The recognition of acquired UDT as a real clinical entity has not only informed our understanding of the possible management in children, but also provided an insight into why rodent animal models of cryptorchidism do not demonstrate testicular cancer. In both circumstances, the abnormal temperature affects the testis well after gonocyte maturation or apoptosis is complete, so that secondary heat shock depresses stem cell function alone, leading to infertility.

The next few years should help to shed light on these new developments further, so that we can determine the optimal treatment, which may include a differentiated treatment strategy for different groups of cryptorchid boys. Equally, the risk of infertility and testicular cancer may not be the same in all boys with cryptorchidism.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Genome-wide assessment of the association of rare and common copy number variations to testicular germ cell cancer

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Testicular germ cell cancer (TGCC) is one of the most heritable forms of cancer. Previous genome-wide association studies have focused on single nucleotide polymorphisms, largely ignoring the influence of copy number variants (CNVs). Here we present a genome-wide study of CNV on a cohort of 212 cases and 437 controls from Denmark, which was genotyped at ~1.8 million markers, half of which were non-polymorphic copy number markers. No association of common variants were found, whereas analysis of rare variants (present in less than 1% of the samples) initially indicated a single gene with significantly higher accumulation of rare CNVs in cases as compared to controls, at the gene *PTPN1* ($P = 3.8 \times 10^{-2}$, 0.9% of cases and 0% of controls). However, the CNV could not be verified by qPCR in the affected samples. Further, the CNV calling of the array-data was validated by sequencing of the *GSTM1* gene, which showed that the CNV frequency was in complete agreement between the two platforms. This study therefore disconfirms the hypothesis that there exists a single CNV locus with a major effect size that predisposes to TGCC. Genome-wide pathway association analysis indicated a weak association of rare CNVs related to cell migration (false-discovery rate = 0.021, 1.8% of cases and 1.1% of controls). Dysregulation during migration of primordial germ cells has previously been suspected to be a part of TGCC development and this set of multiple rare variants may thereby have a minor contribution to an increased susceptibility of TGCCs.

Keywords: copy number variation, rare variants, testicular germ cell cancers

INTRODUCTION

Testicular germ cell cancer (TGCC) is the most common malignancy in young men aged 15—45 years. The incidence has increased over the last decades and is highest in the Nordic countries with 8–9 cases per 100,000, whereas the incidence in men of African and Asian ancestry is five-fold lower (Chia et al., 2010). Environmental exposure partly explains the increasing incidence and ancestral disparity (Skakkebaek et al., 2001), but there is also evidence of a substantial genetic contribution to TGCC susceptibility. The familial aggregation of TGCC is one of the highest among cancers. Brothers and sons of TGCC patients have an 8–10 times and 4–6 times higher risk to develop the disease, while the risk increases 75- and 35-fold for monozygotic and dizygotic twins, respectively (Swerdlow et al., 1997; Hemminki and Li, 2004). Despite the relatively high degree of heritability, genome-wide familial linkage analyses have not identified any loci predisposing for TGCC, and candidate studies have only found one rare deletion (2–3%) at the Y chromosome that confers a modest 2–3 fold increased risk (Nathanson et al., 2005). Recently, genome-wide association studies that search for common single nucleotide variants associated to TGCC have identified susceptibility loci at the genes *KITLG*, *SPRY4*, *BAK1*, *DMRT1*, *TERT*, and *ATF7IP*

(Kanetsky et al., 2009; Rapley et al., 2009; Turnbull et al., 2010; Dalgaard et al., 2012). The strongest association was found at *KITLG* with a greater than 2.5-fold increased risk of disease. Consistent with the relatively high familial relative risk, this is the largest effect size found for any single loci among cancers. However, a considerable portion of the heritability remains to be explained.

Here we investigate constitutional DNA copy number variations (CNVs) as another source of genetic variability that may contribute to the development of TGCC. Recent studies have described associations of common CNVs with neuroblastomas (Diskin et al., 2009), systemic autoimmunity (Fanciulli et al., 2007), psoriasis (Hollox et al., 2007), and osteoporosis (Yang et al., 2008). Rare variants, typically originating from recent and *de novo* events, constitute a significant portion of genomic variation. The thousand genomes project indicates that there are about 20,000 CNVs with allele frequencies down to 1% (1000 Genomes Project Consortium, 2010; Mills et al., 2011). The contribution of such rare, or even rarer, variants, to complex disease susceptibility is to a large extent unknown, but they seem to play an important role in psychiatric disorders (International Schizophrenia Consortium, 2008; Pinto et al., 2010) and they have been indicated to influence childhood obesity (Glessner et al., 2010). Further, identification of

de novo mutations is possible in studies of family-trios and recently three *de novo* CNVs were found in 3 out of 43 TGCC trios, a frequency higher than found in two other cancer types (Stadler et al., 2012), highlighting the paradigm of rare genetic events influencing susceptibility to TGCC. To date, case-control association studies of individual rare CNVs have insufficient power to identify disease-causing variants. To evaluate the impact of rare CNVs with respect to risk for TGCC, we therefore compared the genome-wide burden of rare CNVs and investigated whether any genes or pathways were targeted by multiple rare CNVs such that their aggregated frequency was higher in cases than in controls.

In summary, to assess the effect of CNVs on TGCC we genotyped a Danish case-control cohort (Dalgaard et al., 2012) and analyzed the resulting data with respect to the association of both common and rare germline CNVs to TGCC.
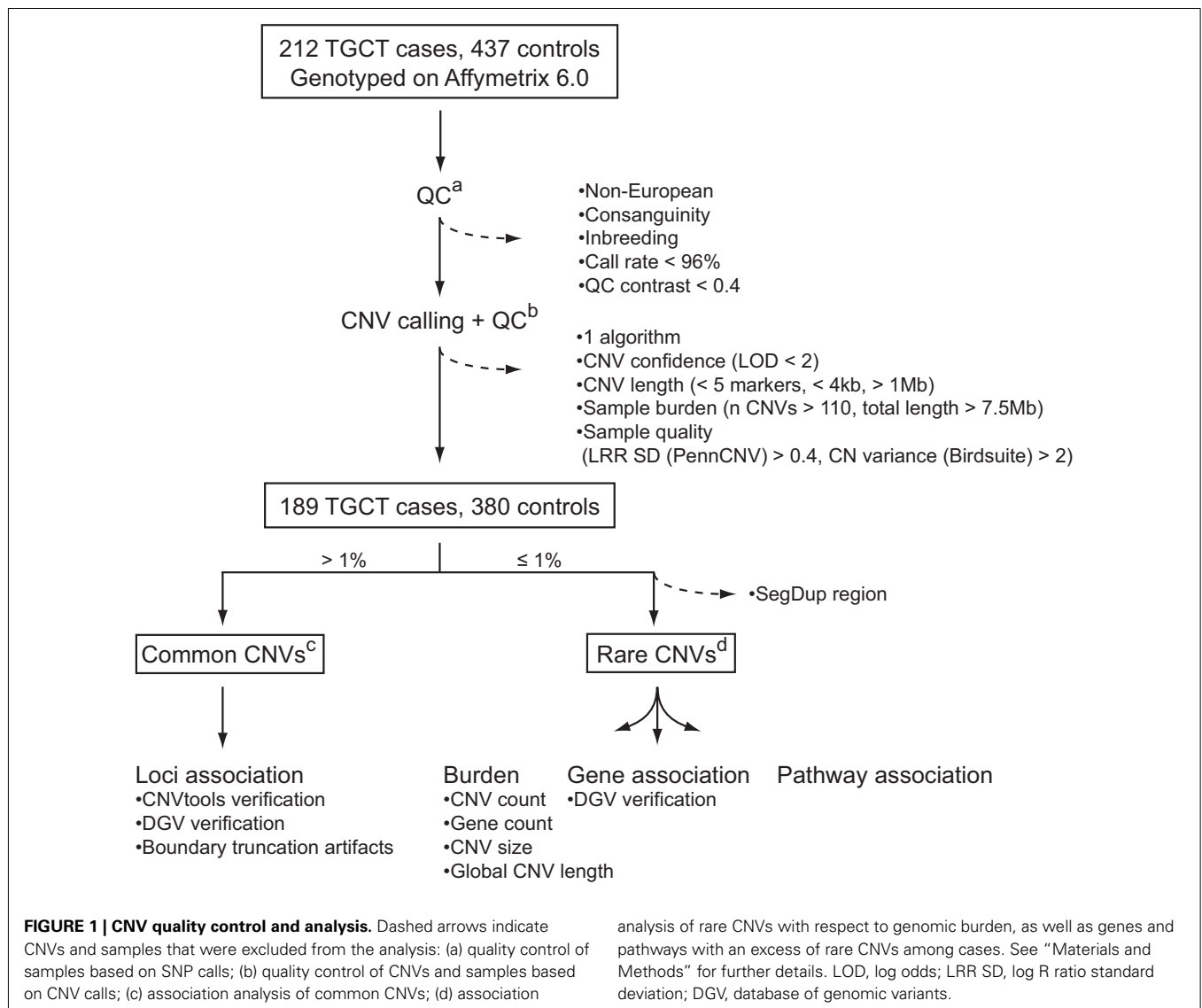
RESULTS

To identify CNVs that confer a risk to TGCC, we analyzed common and rare variants in a genome-wide dataset of approximately 1.8 million markers in a Danish cohort constituting 212 TGCC cases and 437 controls. Application of stringent quality control criteria for reliable CNV identification (**Figure 1**) resulted in a final discovery set of 189 cases and 380 controls. Common variants were defined as CNVs present in more than 1% of the study population, and rare variants as CNVs present in no more than 1% of the studied subjects. Common variants were analyzed with respect to individual locus association, and rare variants with respect to overall genetic burden, gene association, and pathway association.

LOCUS ASSOCIATION ANALYSIS

In order to identify common CNVs associated with TGCC, binary copy number state frequencies of the case and control cohorts were compared at all loci with CNV frequencies above 1%. We observed one genome-wide significant deletion at 1p13.3 covering the gene *GSTM1* ($P = 0.02$, 37.2% cases, 19.5% controls), but downstream quality control by manual inspection of the copy number intensity histogram at this locus, and application of histogram-based association analysis (Barnes et al., 2008) suggested a false positive

FIGURE 1 | CNV quality control and analysis. Dashed arrows indicate CNVs and samples that were excluded from the analysis: (a) quality control of samples based on SNP calls; (b) quality control of CNVs and samples based on CNV calls; (c) association analysis of common CNVs; (d) association analysis of rare CNVs with respect to genomic burden, as well as genes and pathways with an excess of rare CNVs among cases. See "Materials and Methods" for further details. LOD, log odds; LRR SD, log R ratio standard deviation; DGV, database of genomic variants.

finding (nominal $P = 0.26$, 51.3% cases, 58.2% controls). A histogram of the copy number signal showed three distinct clusters with 51, 40, and 8% of the cases, respectively, and 58, 37, and 5% of the controls. The three clusters were assumed to correspond to copy numbers of 1, 2, and 3. Further, the deletion allele frequency at this locus has been estimated to be ~40% in the International HapMap Phase 3 population study (Altshuler et al., 2010).

Given the varying deletion frequencies reported, and to assess the quality of the array-data CNV calling, we performed targeted sequencing of the *GSTM1* (chr1p13) gene in 62 patients. The sequencing data showed three clusters with 52, 40, and 8% of the samples, and 55 out of the 62 patients were present in the same clusters as in the array-data. The CNV frequencies of the three clusters were in perfect agreement with those from the histogram analysis of the array-data, thereby corroborating that deletion of *GSTM1* had no association to TGCC. The sequencing also revealed that the actual copy numbers were 0, 1, and 2 rather than 1, 2, and 3 since the majority of samples had no DNA present (0 sequence reads). The reason for that mis-assignment is that a copy number of 2 is generally assumed by CNV calling software to be the normal copy number state, having the major allele frequency, whereas in this case only 5–10% of the population carry two copies of the gene.

CNV BURDEN ASSOCIATION ANALYSIS

Testing whether individuals with TGCC had a greater genomic burden of rare CNVs than controls, we observed a weak indication of increased burden with respect to the number of CNVs per sample, the number of affected genes per sample, and the average length of CNVs per sample (case/control ratio: 1.08, 1.10, and 1.11 respectively), and a significant difference with respect to the total length of all CNVs per sample (case/control ratio: 1.19, $P = 0.03$; **Table 1**).

GENE ASSOCIATION ANALYSIS

Next, we explored if there were any specific genes where rare CNVs were more common in cases than controls. This analysis did not require that CNVs found in different samples overlapped each other, rather, it was sufficient that they were located within the same genic region. Two genes were found to have genome-wide significance, *PTPN1* ($P_{emp.} = 0.038$) and *KCNB2* ($P_{emp.} = 0.022$), affecting 0.9 and 1.2% of cases, respectively, whereas no occurrence in controls was observed at these loci (**Table 2**). The CNV at *PTPN1* involved five cases, all found to have a heterozygous deletion at the same intronic region (**Figure 2**). CNVs at *KCNB2* were found at three different loci: four and one deletions at two different introns and one deletion and one duplication at the promoter (**Figure 2**). Several CNVs have previously been reported in healthy individuals at *KCNB2*, but not at *PTPN1* (Database of Genomic Variants, v. 10), corroborating a true TGCC association at *PTPN1*, but weakening the possibility of an actual association to *KCNB2*.

We attempted to verify the CNV at *PTPN1* by performing qPCR on the affected samples, but all five samples with an indication of a heterozygous deletion in the array-data were observed to have two copies in the qPCR. Thus, the CNV calls from the array-data of *PTPN1* are likely to be false positives and no association between *PTPN1* and TGCC can be inferred at this stage.

PATHWAY ASSOCIATION ANALYSIS

Proteins tend to act in concert and perturbations of different components in a set of proteins that are interacting in a network may result in a dysregulation with similar outcome (Lage et al., 2007; Navlakha and Kingsford, 2010; Pers et al., 2011). We therefore conducted association analysis on the level of pathways and protein–protein interaction networks. Association was assessed in the same fashion as for genes and loci described above, that is, by comparing case and control cohorts with respect to the total frequency of rare CNV events targeting a pathway. We compiled comprehensive collections of gene sets, where a set of genes either share function or operate in the same pathway, and performed one thousand random permutations (shuffling case and control status) to estimate a local false-discovery rate (FDR) of each gene set (Efron and Tibshirani, 2002). We observed a significant differential proportion of rare CNVs between cases and controls in 14 gene sets (FDR < 5%, **Table 3**). However, 11 of these associations were driven by the gene-specific association to *PTPN1* described above.

Table 1 | Global burden of rare CNVs in cases versus controls.

Type	Burden	P	Case:control ratio	Baseline (control)	Baseline (case)
All	Rate	0.09	1.08	4.8	5.17
	Gene rate	0.19	1.10	3.3	3.7
	Mean length (kb)	0.14	1.11	76.9	85.1
	Total length (kb)*	0.03	1.19	372.5	444.3
Duplications only	Rate	0.36	1.03	1.9	1.93
	Gene rate	0.29	1.09	1.9	2.1
	Mean length (kb)*	0.01	1.33	112.3	149.0
	Total length (kb)	0.07	1.22	279.4	340.8
Deletions only	Rate	0.09	1.12	2.9	3.24
	Gene rate	0.23	1.11	1.4	1.6
	Mean length (kb)	0.49	1.00	49.9	49.9
	Total length (kb)	0.21	1.09	151.6	165.0

*Significant difference ($P < 0.05$). Genome-wide P-values were estimated by 10,000 permutations of case-control status.

The three remaining gene sets, which did not include *PTPN1*, were: "regulation of cell migration" [go:0030334, 1.8% versus 1.1%, FDR = 0.021, OR = 3.47 (1.12−11.82)], "positive regulation of catalytic activity" [go:0043085, 1.4% versus 0.5%, FDR = 0.04, OR = 5.54 (1.31 − 32.78)], and "macromolecular complex disassembly" [go:0032984, 2.3% versus 1.9%, FDR = 0.047, OR = 2.47 (1.00−6.23)]. The most significant gene set among all sets tested was "regulation of cell migration." A total of 16 individuals harbored CNVs that altogether overlapped 14 genes in this pathway, at 13 unique CNV loci (**Table 4**). Genes in this gene set that

were affected in cases but not controls included: *BCL2*, *CDH13*, *CORO1A*, *KDR*, *MUC2*, *MUC5AC*, *ONECUT2*, and *PTPRK*.

DISCUSSION

We assessed the effect of common and rare CNVs in a TGCC case-control cohort. No single locus was found to be associated to TGCC, but one potential gene network of interest with a weak significant association was identified, having an elevated frequency of rare CNVs among cases. The absence of any single locus CNV associated to TGCC is in line with the relatively few findings for other diseases, including a screening of ∼3,400 common CNVs in eight common diseases (Craddock et al., 2010). Furthermore, common CNVs are typically ancient variations, which are tightly correlated with single nucleotide polymorphisms (SNPs), and can therefore be detected by genome-wide association studies of common SNPs (Lander, 2011). However, one should not neglect the importance of common CNVs in gene-phenotype association studies, since there exists evidence that disease associated SNPs have a tendency to tag CNVs more often than random, and that such CNV-tagging SNPs are enriched for expression quantitative trait loci (eQTL; Gamazon et al., 2011). Further, rare CNVs are

Table 2 | Genes with an association of rare CNVs.

Gene	$P^1_{emp.}$	$P_{unadjusted}$	Odds ratio	Cases (%)	Controls (%)
PTPN1	3.8×10^{-2}	3.9×10^{-3}	12.31 (1.48–568.17)	0.9	0.0
KCNB2	2.2×10^{-2}	4.1×10^{-4}	16.58 (2.19–738.20)	1.2	0.0

[1]*Empirical genome-wide P-values were estimated by 1,000 permutations of case-control status using Fisher's test.*
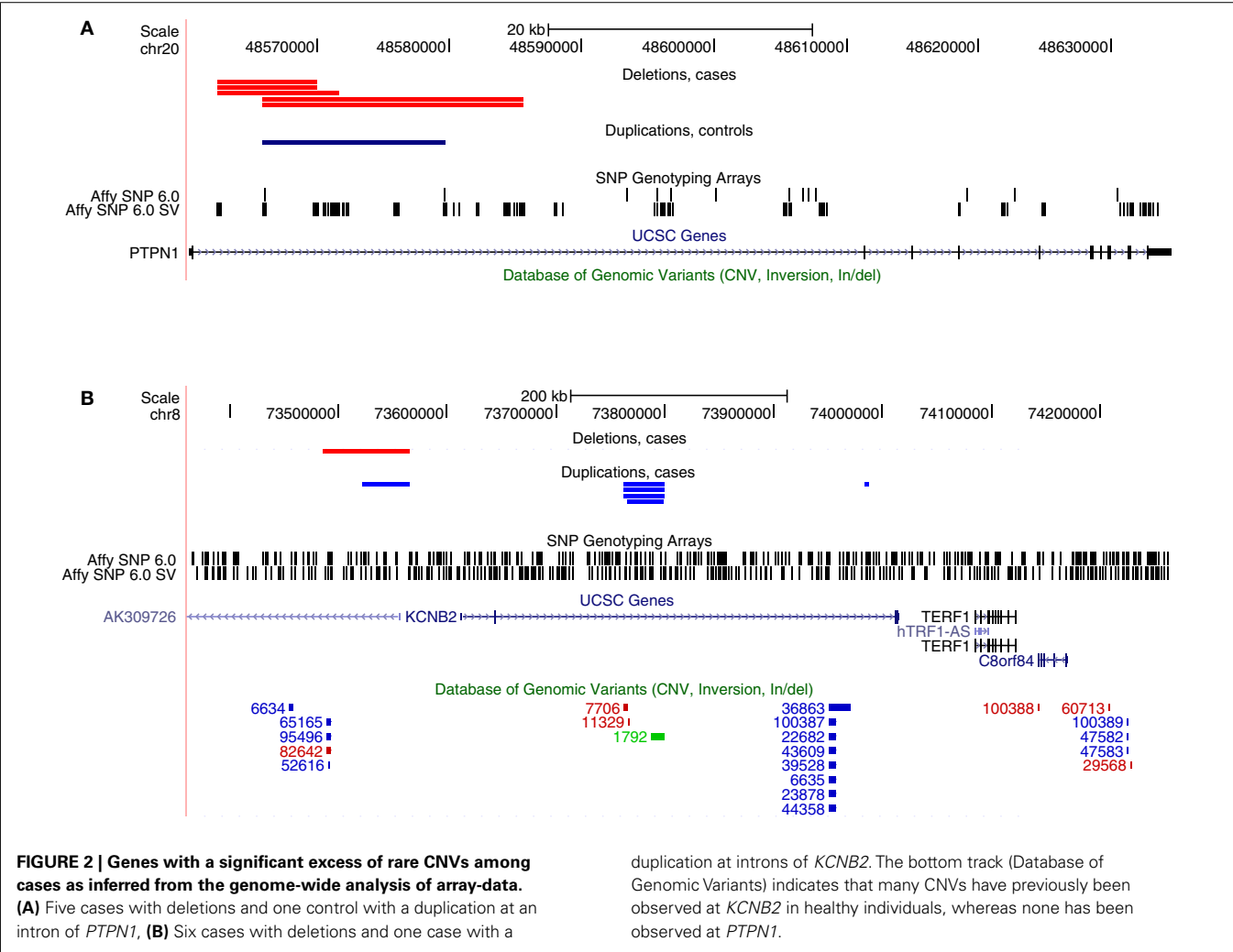

FIGURE 2 | Genes with a significant excess of rare CNVs among cases as inferred from the genome-wide analysis of array-data. **(A)** Five cases with deletions and one control with a duplication at an intron of *PTPN1*, **(B)** Six cases with deletions and one case with a duplication at introns of *KCNB2*. The bottom track (Database of Genomic Variants) indicates that many CNVs have previously been observed at *KCNB2* in healthy individuals, whereas none has been observed at *PTPN1*.

Table 3 | Gene sets with an association of rare CNVs.

Gene set type[1]	Gene set term	Posterior[2]	Local FDR	Odds ratio	Cases (%)	Controls (%)
GO BP	Regulation of cell migration	0.98	0.021	3.47 (1.1–11.8)	1.8	1.1
GO BP	Macromolecular complex disassembly	0.96	0.040	5.54 (1.3–32.8)	1.4	0.5
GO BP	Positive regulation of catalytic activity	0.95	0.047	2.47 (1.0–6.2)	2.3	1.9

[1]Many sources of gene sets were jointly analyzed but only sets of the type "gene ontology biological process" were significant, apart from gene sets that included PTPN1, which were excluded from the table.

[2]The empirical Bayes analysis of microarrays (EBAM) algorithm with 1,000 permutations was used to estimate a posterior and local false discovery rate (FDR) for every gene set.

Table 4 | CNVs targeting genes that are part of the gene set "Regulation of cell migration."

CNV	Length[1]	Copy number	Sample	Class	Genes
chr4:55607652…55616597	9	1	165855	Case	KDR
chr6:128485528…128525520	40	1	232996	Case	PTPRK
chr6:128864684…128871092	6	1	190037	Case	PTPRK
chr11:1094626…1140711	46	3	210711	Case	MUC2, MUC5AC
chr16:29474810…30099408	625	1	124873	Case	CORO1A
chr16:29488112…30085920	598	1	233682	Case	CORO1A
chr16:82119367…82175095	56	1	224567	Case	CDH13
chr16:82408574…82502970	94	1	232030	Case	CDH13
chr18:52763504…53341297	578	3	203688	Case	ONECUT2
chr18:59018003…59031365	13	1	231734	Case	BCL2
chr2:55119289…56699138	1580	3	M3088A	Control	RTN4
chr3:188880305…188936673	56	3	M1270A	Control	SST
chr12:50532205…50579767	48	3	M3576A	Control	ACVRL1
chr15:50811752…50882082	70	3	M3047A	Control	ONECUT1
chr15:97371582…97730964	359	3	M053A	Control	IGF1R
chr19:49893892…50298979	405	3	M3381A	Control	APOE

[1]Kilobases.

not tagged by common SNPs implicated by GWASs and they have been found to play a major role in neurodevelopmental disorders (Merikangas et al., 2009) and rare *de novo* CNVs were recently found in 3 out of 43 TGCC family-trios (Stadler et al., 2012). We have previously searched for CNVs associated to familial TGCTs, where the inheritance component is much higher than among sporadic cases, but failed to find a CNV that was significant across the small set of studied families. We only identified a handful of CNVs that segregated with TGCT, but these were either family-specific or relatively common variants, such as *RLN1* (Edsgård et al., 2011).

The rare association analysis presented in this study initially indicated two genes to be associated with TGCC, *PTPN1*, and *KCNB2*. *KCNB2* was considered a false positive due to the amount of previously reported CNVs in independent control cohorts at this locus, being on par with that of the frequency in the case group of this study. *PTPN1* appeared as a good candidate since it has previously been implicated with the progression of prostate cancer along with evidence that the androgen receptor is a transcriptional regulator of *PTPN1* (Lessard et al., 2010, 2012). A loss of *PTPN1* may thereby be associated with impaired responsiveness

to androgens, which would be consistent with the fact that low androgen status during development is a risk factor for TGCT (Rajpert-De Meyts and Skakkebaek, 1993; Sonne et al., 2008). Further, CNVs at the 20q13 chromosomal region have been observed in several other cancers (Nishizaki et al., 1998; Schaid, 2004; Furukawa et al., 2006). Furthermore, all affected probands in this study presented a deletion, consistent with a tumor suppressing function of oncogenic kinases, and *PTPN1* has been shown to be able to play both a pro- and anti-oncogenic role (Stuible et al., 2008). A considerable effort was made to ensure high quality of the CNV calls: by using two complementary CNV calling methods, by applying several strict QC criteria, and by manual inspection of the raw probe signals. However, despite the stringent QC the qPCR did not verify the heterozygous deletion of *PTPN1* in any of the samples. This highlights the difficulty of assessing the reliability of CNV calls from genome-wide short-nucleotide microarrays.

Our pathway analysis identified the gene set "regulation of cell migration" as having the highest difference in proportion of rare CNVs in cases compared to controls. There is a growing body of information that strongly suggests a crucial role of primordial

germ cell (PGC) biology in TGCC oncogenesis. PGCs are embryonic cells which during mid-gestation migrate from the base of the yolk sac, along the hind-gut, to the genital ridge, one of the longest migrations of all mammalian cells. Four recent SNP GWAS TGCC studies associated *KITLG* and a number of other genes related to the *KIT-KITLG* pathway (Kanetsky et al., 2009; Rapley et al., 2009; Turnbull et al., 2010; Dalgaard et al., 2012), a regulatory network which is believed to be of crucial importance in the determination of the fate of PGCs (Rapley and Nathanson, 2010). For instance, mutations of the KIT receptor, or the KIT ligand, in the mouse, blocks PGC migration, resulting in infertility (Matzuk and Lamb, 2002). In addition, a disturbance of the migration of PGCs during early fetal development may cause extragonadal germ cell cancers along the midline of the body (Oosterhuis and Looijenga, 2005). One of the afflicted genes in the "regulation of cell migration" gene set was *PTPRK*, at which two samples had a deletion. Like *PTPN1*, *PTPRK* belongs to the family of protein tyrosine phosphatases, but it is a membrane-bound receptor. *PTRPK* has been implicated in TGFβ-signaling (Xu et al., 2010), and a recent GWAS study indicated the involvement of TGFβ superfamily signaling in testicular dysgenesis (Dalgaard et al., 2012). In mouse, PGCs divide rapidly under the influence of TGFβ signaling factors, and defects in PGC development is observed in knockout models of bone morphogenetic proteins (BMPs; Lawson et al., 1999; Ying et al., 2000). In total there were 14 genes which were part of the "regulation of cell migration" pathway and that harbored CNVs. It would be of high interest to further elucidate the role of these genes by studying how the CNVs affect the expression levels of the corresponding genes. What may complicate matters is if the critical period of time is the development of the fetus, which may force one to revert to the study of animal models.

In the analysis of common CNVs we made a further assessment of the *GSTM1* CNV frequency by targeted sequencing. The main reason for this was to assess the quality of and verify the array-data CNV calls of a seemingly problematic locus. However, *GSTM1* was also partly chosen due to that the risk of developing TGCC has been shown to increase by the exposure to certain environmental factors (Skakkebaek et al., 2001) and *GSTM1* is known to be involved in the detoxification of xenobiotic compounds (Hengstler et al., 1998). Hypothetically, it is not unlikely that there exists a genetic variation that may affect the ability to metabolize environmental chemicals, which in turn affects the risk of TGCC. Apart from such gene-environment interactions it is probable that part of the unexplained heritability can be explained by gene–gene interactions, where a combination of several genetic variations cause a greater effect on the phenotype than the sum of their individual effects (epistasis). Further research is required to assess the effect of rare variants in varying genetic backgrounds.

In conclusion, this study corroborates the rejection of the hypothesis that a single CNV locus mediates a major risk for TGCC. It suggests that several rare CNVs may contribute to the oncogenesis of a subset of TGCC subjects, but the frequency after aggregation of CNVs on the implicated gene and pathways is still low, and these CNVs therefore only provide a minor contribution to the overall heritability. Larger cohorts are needed to further explore the impact of rare variants in TGCC.

MATERIALS AND METHODS

SAMPLE COLLECTION

Two hundred and twelve men with TGCCs and 439 healthy young men with semen concentrations above 60 million sperm/ml were collected at the Department of Growth and Reproduction, Rigshospitalet, Denmark. All DNA samples were obtained from men of Danish ancestry, who provided informed consent for genetic analysis. The samples were coded during the entire analysis. The project has been approved by the Regional Medical Ethics Committee (Nr H-KF-265848) and the Danish Personal Data Protection Agency (Nr 2008-41-2158). This cohort has previously been analyzed with respect to single nucleotide variation (Dalgaard et al., 2012). Sixty-two of the TGCC patients were selected for sequencing of the *GSTM1* gene.

GENOTYPING AND SNP-BASED SAMPLE QUALITY CONTROL

Germline DNA was extracted from peripheral blood using Quick-Gene DNA whole blood kits from FujiFilm Life Science according to the manufacturer's manual. Samples were genotyped using the Affymetrix Genome-wide Human SNP Array 6.0. Here we present the analysis of CNVs, SNP association analysis and details of the cohort are described elsewhere (Dalgaard et al., 2012), but an initial sample quality control was performed using SNP genotypes called by the Birdseed algorithm [Affymetrix Power Tools (APT) v. 1.10.2]. We excluded samples based on a genotyping call rate below 96%; QC contrast below 0.4 as according to the Affymetrix GCOS software; non-European ancestry by inspection of a plot of the first two principal components of the cohort and the HapMap phase III samples (Altshuler et al., 2010); high degree of relatedness based on identity-by-descent where one individual was kept among related samples; and samples with an outlying inbreeding coefficient.

CNV DETECTION AND QUALITY CONTROL

For samples that passed the SNP quality control described above we ran two CNV calling algorithms, BirdSuite (v. 1.5.5) and PennCNV (v. 2010May01). PennCNV requires a signal intensity file and a SNP genotyping file and these were generated by quantile normalization of PM-only probes with median polish probe summarization (APT v. 1.12.0) and Birdseed (v. 2 in APT 1.12.0), respectively. We excluded CNVs which failed any of the four following criteria: (1) A CNV was not called by both algorithms. A histogram of the percentage of overlap indicates that the vast majority has >90% overlap, but we set the threshold to at least 10%. (2) A CNV log odds confidence score larger than two, as recommended by BirdSuite (Korn et al., 2008). (3) CNV size was less than five markers or four kilobases, in effect excluding the 25% short-length quantile of CNVs. (4) A CNV was longer than one megabase (three outliers based on histogram). Further, samples were excluded with respect to the three following criteria: (1) extreme sample burden in terms of more than 110 CNVs (four outliers); or (2) a total length of CNVs larger than 7.5 megabases (two outliers); (3) bad sample quality in terms of high variance of copy number signal (median copy number variance larger than two, as recommended by BirdSuite, or a Log R Ratio standard deviation (LRR SD) obtained from PennCNV larger than 0.4). LRR SD was set according to PennCNV guidelines when CNV calling

are performed on Affymetrix samples. Finally, rare CNVs that had more than 50% overlap with segmental duplication regions (retrieved from UCSC hg18) were removed, since such regions have been shown to generate more false CNV calls (Pinto et al., 2010). A total of 189 TGCC cases and 380 controls remained after the completion of all quality control steps, harboring a total of 1008 and 1872 rare CNVs, respectively.

CNV ASSOCIATION ANALYSIS

Copy number variant association analysis was performed using plink (v. 1.07) and custom R (v. 2.12) scripts. Common and rare CNVs were defined as those with an allele frequency above or below 1%, respectively. The allele frequency of a CNV was determined using the locus within a CNV region with the maximum number of overlapping individual CNVs.

LOCUS ASSOCIATION

Common CNVs were evaluated by searching the whole genome for loci with a significantly higher degree of affected cases as compared to controls. Binary state frequencies were used, and a Fisher test was performed for deletion, amplification, and any type of aberration, respectively. Genome-wide significance was estimated by generating a null distribution based on one thousand case-control status permutations. For each permutation the minimal P-value was selected, thereby providing control of the family-wise error rate (FWER). Loci with significant associations were further verified by CNVtools (Barnes et al., 2008) which uses a complementary CNV calling strategy, since it employs a statistical model based on density-based clustering rather than a hidden Markov model. Furthermore, loci residing at the edges of a common CNV and associations from variation of boundary truncation were excluded.

GLOBAL BURDEN ANALYSIS

The impact of rare CNVs was assessed by three approaches: genome-burden analysis, gene association, and pathway association analysis.

The global burden of rare CNVs in cases compared to controls were assessed with respect to (i) the number of CNVs per sample, (ii) the number of affected genes per sample, (iii) the average length of CNVs per sample, and (iv) the total length of CNVs per sample.

GENE ASSOCIATION

Gene association analysis was performed using the number of case and control samples harboring a rare CNV that overlapped the gene of interest. Genes were retrieved from UCSC (hg18). CNV frequencies were compared with Fisher's test and multiple testing corrected P-values were obtained based on case-control permutation as described above for the locus association of common CNVs. Significant CNVs which were found to have a lower allele frequency in our case cohort than in the Database of Genomic Variants (DGV, v. 10), were considered false positives.

PATHWAY ASSOCIATION

Pathway association analysis was performed based on the number of case and control samples that had a rare CNV in any of the genes

of a pathway. The R package "siggenes" was used to obtain P-values corrected for multiple testing across all tested gene sets. The package provides a FDR, based on randomized case-control sampling, as well as an adjustment of the variance of an individual pathway using information from the observed variances of all pathways (Efron and Tibshirani, 2002). Gene sets were retrieved from KEGG (Kyoto Encyclopedia of Genes and Genomes; Ogata et al., 1999), Reactome (D'Eustachio, 2011), BioCarta[1], NCI-Nature curated pathways (Pathway Interaction Database; Schaefer et al., 2009), GO (Gene Ontology; Ashburner et al., 2000), COSMIC (Catalog of Somatic Mutations In Cancer; Forbes et al., 2011), Cyclebase (Gauthier et al., 2010), protein–protein interaction complexes (Lage et al., 2007), OMIM (Online Mendelian Inheritance in Man[2]), MGI (Mouse Genome Informatics[3]) and a set of candidate infertility genes from a recent review (Matzuk and Lamb, 2008). Terms annotating more than 700 or less than 5 genes were discarded, since they do not produce meaningful statistical results.

VERIFICATION OF PTPN1 COPY NUMBER

Copy number verification of PTPN1 was done using Quantitative PCR on the Mx3000P platform from Stratagene (Agilent Technologies, Santa Clara CA, USA). The protocol has been described previously (Ottesen et al., 2007). Primers for the CNV at PTPN1 were designed using Primer3 (Rozen and Skaletsky, 2000). Primer sequences for PTPN1 were: forward 5′-TTC AAC CCT AAC TAG GTA TGC A-3′ and reverse 5′-CTA AAA TGC TGG AGA CTT AGG T-3′ and primers for GAPDH: forward 5′-CTC CCC ACA CAC ATG CAC TTA-3′ and reverse 5′-TTG CCA AGT TGC CTG TCC TT-3′ (DNA Technology A/ S, Aarhus, Denmark). GAPDH was used as endogenous control gene. Mixtures of forward and reverse primers were denatured for 3 min at 95°C and incubated on ice until use. DNA concentrations were 8–17 ng, 15 µL Brilliant SYBR Green QPCR Master Mix (Stratagene), 7.0 µL primer mixture of PTPN1 (final conc.: Fw and Rev 100 nm) or GAPDH (final conc.: Fw and Rev 100 nm), and a total volume of 30 µL. Conditions for amplification were as follows: one cycle at 95°C for 10 min and 40 cycles at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min. The PTPN1:GAPDH ratio was calibrated to a normal male reference control of DNA, as previously described for other genes (Ottesen et al., 2007; Mau Kai et al., 2008). All specimens were analyzed in triplicate and the mean-ratio was used to infer integer copy number.

TARGETED SEQUENCING OF GSTM1

Design of targeted capture baits

Target capture was designed on the Agilent SureSelect capture system (Santa Clara CA, USA). Baits for capture were designed by tiling the genomic region harboring *GSTM1* with an approximately 50% overlap between consecutive baits. The bait sequences were optimized to avoid regions with extreme GC content or highly variable regions, and to avoid possible cross-hybridization or self-folding of the baits, which could decrease the hybridization

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[1]http://www.biocarta.com

[2]http://www.ncbi.nlm.nih.gov/omim

[3]http://www.informatics.jax.org

specificity. The principle of this method has been described previously (Wesolowska et al., 2011).

Library preparation, pooling, target enrichment, and sequencing

DNA shearing, library preparation, and pooling were performed using a modified protocol of the SureSelect Target Enrichment System (Agilent Technologies) to allow sample multiplexing as described elsewhere (Wesolowska et al., 2011). Three micrograms of genomic DNA was sheared by ultrasound (Covaris; Woburn, MA, USA) to yield fragments of an average size of ∼200 bp. The sheared fragments were ligated with custom made adaptors containing a unique four-base barcode, and subsequently purified and amplified by ligation-mediated PCR (LM-PCR). Amplified DNA was pooled in groups of 10 with equal amount of each, after which enrichment was performed by hybridization to custom SureSelect target capture baits (Agilent Technologies). The captured libraries were processed with Illumina Cluster Generation Station (Illumina, San Diego CA, USA) following the manufactures recommendations. We performed 100 nt single-end read sequencing on the Illumina HiSeq 2000 sequencing platform.

Data analysis

Sequence reads were trimmed and high quality reads were mapped to the human reference genome build 37 (GRCH37)

using Burrow-Wheeler Alignment algorithm (Li and Durbin, 2010). Alignments with mapping score below 30 were discarded and PCR duplicates were removed with Picard MarkDuplicates[4]. Copy numbers were estimated by calculating a sequence depth ratio from the number of reads in the targeted genomic region, normalized by size of the region and total number of reads for the sample. A histogram of the sequence depth ratio from all individuals identified three distinct clusters corresponding to two copies and heterozygous and homozygous deletions.

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[4]http://picard.sourceforge.net

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Association between polymorphisms in the aryl hydrocarbon receptor repressor gene and disseminated testicular germ cell cancer

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In the Western world, testicular germ cell cancer (TGCC) is the most common malignancy of young men. The malignant transformation of germ cells is thought to be caused by developmental and hormonal disturbances, probably related to environmental and lifestyle factors because of rapidly increasing incidence of TGCC in some countries. Additionally, there is a strong genetic component that affects susceptibility. However, genetic polymorphisms that have been identified so far only partially explain the risk of TGCC. Many of the persistent environmental pollutants act through the aryl hydrocarbon receptor (AHR). AHR signaling pathway is known to interfere with reproductive hormone signaling, which is supposed to play a role in the pathogenesis and invasive progression of TGCC. The aim of the present study was to identify whether AHR-related polymorphisms were associated with risk as well as histological and clinical features of TGCC in 367 patients and 537 controls. Haplotype-tagging single-nucleotide polymorphisms (SNPs) were genotyped in genes encoding AHR and AHR repressor (AHRR). Binary logistic regression was used to calculate the risk of TGCC, non-seminoma versus seminoma, and metastasis versus localized disease. Four SNPs in *AHRR* demonstrated a significant allele association with risk to develop metastases (rs2466287: OR = 0.43, 95% CI 0.21–0.90; rs2672725: OR = 0.49, 95% CI: 0.25–0.94; rs6879758: OR = 0.27, 95% CI: 0.08–0.92; rs6896163: OR = 0.34, 95% CI: 0.12–0.98). This finding supports the hypothesis that compounds acting through AHR may play a role in the invasive progression of TGCC, either directly or through modification of reproductive hormone action.

Keywords: aryl hydrocarbon receptor, aryl hydrocarbon receptor repressor, testicular germ cell cancers, genetic polymorphisms, genetic variation, association studies, metastasis, histology

INTRODUCTION

Testicular germ cell cancer (TGCC) represents the most-frequently diagnosed cancer in young men between 20 and 40 years of age. The rates vary considerably according to region and ethnicity, which suggests that both environmental and genetic factors contribute to TGCC. In Europe, a three- to fourfold increased incidence has been noted during the past 30–40 years, which suggests that an increased exposure to persistent environmental pollutants may play an important role.

Common persistent environmental pollutants include dioxins and polycyclic aromatic hydrocarbons, which exert their toxic and carcinogenic effects through the ligand-activated transcription factor aryl hydrocarbon receptor (AHR). In complex with its binding partner AHR nuclear translocator (ARNT), it mediates cellular responses to xenobiotic compounds. The AHR/ARNT

heterodimer stimulates the expression of AHR repressor (AHRR), which provides a negative feedback regulation by competing with ARNT for binding to AHR (Mimura et al., 1999).

Testicular germ cell cancer derives from carcinoma *in situ* (CIS) cells, also known as intratubular germ-cell neoplasia unclassified (Skakkebaek et al., 1982; Oosterhuis and Looijenga, 2005), that are believed to arise from primordial germ cells that are blocked in differentiation. The initial malignant transformation of germ cells is thought to be caused by hormonal disturbances in the microenvironment of differentiating germ cells (Skakkebaek et al., 1987; Sharpe and Skakkebaek, 1993; Rajpert-De Meyts et al., 1998). An association of TGCC with maternal estrogen and androgen levels in early pregnancy has recently been reported, supporting this hypothesis (Holl et al., 2009). In addition to altering transcription of steroidogenic genes, such as cytochrome P450 enzymes, the

AHR signaling pathway has been shown to interface with estrogen and androgen receptor signaling (Jana et al., 1999; Kizu et al., 2003; Safe and Wormke, 2003; Boverhof et al., 2006; Beischlag et al., 2008; Kollara and Brown, 2010). Additionally, AHR plays a significant role in tumor promotion and progression by deregulation of cell–cell contact and inhibition of apoptosis (Mulero-Navarro et al., 2005; Diry et al., 2006; Carvajal-Gonzalez et al., 2009; Chopra et al., 2009; Dietrich and Kaina, 2010).

The risk of environment-related carcinogenesis depends not only on the magnitude of the exposure, but also on individual susceptibility to the pollutant. Therefore, we aimed to analyze the association between polymorphic variants of genes encoding AHR and AHRR and the risk as well as histological and clinical features of TGCC.

MATERIALS AND METHODS

The cohort consisted of TGCC patients from Sweden and Denmark, two neighboring Scandinavian countries, with populations sharing largely the same genetic background as both descended from a common ancestral population.

SWEDISH TGCC PATIENTS

All TGCC patients that were referred to the Department of Oncology, Skåne University Hospital, Lund, the Department of Oncology, Radiumhemmet or Södersjukhuset, Karolinska University Hospital, Stockholm since March 1996 and November 1998 until October 2006, respectively, were asked to participate in a study focusing on reproductive function. In total 460 patients were eligible for the study, of which 75 declined to participate and 45 were excluded due to linguistic problems, bilateral testicular cancer, physical handicap, or moving to another region. Seven patients were excluded due to compromising mental conditions, 10 were excluded due to contra-lateral testicular cancer diagnosed after inclusion and 3 died of progressive disease before blood samples were obtained. Among the 320 participants in the study on reproductive function, no DNA was available for 39. Additionally, 3 patients were excluded due to extra-gonadal germ cell cancer, leaving the total at 278. All patients gave their informed written consent and the study was approved by the Ethical Boards of Lund University and Karolinska Institute.

DANISH TGCC PATIENTS

In the period between 1999 and 2008, DNA was collected from approximately 300 TGCC patients on the occasion of semen banking prior to surgery or fertility assessment after treatment. One hundred samples were random selected for this study; the criterion for selection being sufficient DNA amount. In total, 11 patients were excluded because of mistaken diagnosis ($n = 6$), purely extra-gonadal tumor ($n = 4$), and in one case because the genetic SNP analysis failed. Among the remaining 89 patients, 5 presented with CIS only and were therefore not included in the analysis of distribution of seminomas (SE) versus non-seminomas (NSE). For six patients no information on stage was available.

CONTROL SUBJECTS

Control subjects were recruited in a study of reproductive function among Swedish military conscripts aged 18–20 years in the period 2000–2001 (Richthoff et al., 2002). As part of the investigation, scrotal palpation and ultrasound was performed in order to exclude testicular tumors or microcalcifications, which are indicative of an increased risk of CIS. Furthermore, they delivered one ejaculate for semen analysis as well as a blood sample for assessment of hormone levels. Among the 305 men that were eligible, 214 men with a Swedish mother were selected as control subjects (**Table 1**).

GENOTYPING

Genomic DNA was prepared from peripheral leukocytes using QIAamp DNA Maxi Kit (Qiagen, Germany). All samples were normalized to the same DNA concentration and the genotypes were determined using the Sequenom MassArray MALDI-TOF mass spectrometry. Eleven SNPs in the gene encoding aryl hydrocarbon receptor (*AHR*) and 18 SNPs in the gene encoding aryl hydrocarbon receptor repressor (*AHRR*) with a minor allele frequency >0.05 that were identified as haplotype-tagging SNPs were selected using dbSNP (available at: http://www.ncbi.nlm.nih.gov/SNP) and SNP assays were designed using MassArray Assay Design ver. 2 software (Sequenom Inc., USA). Primers were obtained from Metabion GmbH (Germany) and all reactions were run under the same conditions, except for the primer annealing temperature of the primary PCR. PCR reactions were performed in a total volume of 6 μL containing 2.5 ng template DNA, 1.25× *Taq* PCR buffer (Hotstar, Qiagen), 0.15 U *Taq* polymerase (Hotstar, Qiagen), 3.5 nM MgCl$_2$, 0.5 mM dNTP, and 100 nM of each primer. Amplifications were performed using

Table 1 | Characteristics of controls and case patients.

	Control	TGCC-SE	TGCC-DK
	n (%)	*n* (%)	*n* (%)
n	214	278	89
Age (year)	18 ± 0	31 ± 7	31 ± 8
Histology			
NSE		153 (55.0)	34 (38.2)
SE		125 (45.0)	50 (56.2)
CIS only			5 (5.6)
Stage			
Localized		200 (71.9)	65 (73.0)
Metastasis		78 (28.1)	18 (20.2)
Unknown			6 (6.7)
Family history of TGCC			
Yes	0 (0)	9 (3.2)	7 (2.3)
No	214 (100)	250 (90.3)	77 (88.5)
Unknown		19 (6.5)	5 (9.2)
History of cryptorchidism			
Yes	7 (3.3)	23 (8.3)	13 (14.6)
No	207 (96.7)	247 (88.9)	63 (70.8)
Unknown		8 (2.9)	13 (14.6)

GeneAmp 9700 machines with dual-384 heads as follows: 95°C for 15 min, 45 cycles at 95°C for 20 s, 56°C, 60°C, or 64°C for 30 s, 72°C for 60 s, and finally 72°C for 3 min. Dephosphorylation of unincorporated dNTP was achieved using shrimp alkaline phosphatase. Concentrations of individual homogenous MassEXTEND (hME) primers were adjusted to even out peak heights in the mass spectrum. The extension reactions were carried out by mixing the adjusted primer mix (containing approximately 1 μM of each primer) with hME mix containing buffer and 50 μM of each d/ddNTP mix and 1.25 U of Thermo Sequenase (Amersham Biosciences, Uppsala, Sweden). PCR amplification of hME reactions was performed as follows: 94°C for 2 min and 99 cycles at 94°C for 5 s, 52°C for 5 s, and 72°C for 5 s. The samples were then manually desalted by using 6 mg of Clean Resin and a dimple plate and subsequently transferred to a 384-well SpectroCHIP using a nanodispenser. Randomly selected samples of each genotype were directly sequenced in order to validate the SNP assays.

STATISTICAL ANALYSIS

Single-nucleotide polymorphisms data was processed and analyzed using the web-based SNPator data analysis suite (Morcillo-Suarez et al., 2008). Agreement with Hardy–Weinberg equilibrium was tested using a χ^2 goodness-of-fit test. TGCC patients were divided in two groups depending on whether they were diagnosed with SE or NSE. Logistic regression association was used to calculate the odds ratio (OR) and 95% confident interval for developing TGCC, for developing either SE or NSE, and for developing disseminated disease [defined by stages II, III, and IV according to the Royal Marsden Hospital staging classification (Husband and Koh, 2004), with stage I disease defined as tumor confined to the testis, with no evidence of metastases]. In the latter analysis histological subtype was included as a covariant in the logistic regression. Associations were considered statistically significant at $p < 0.05$. Assuming a co-dominant additive model, we tested a linear trend of increasing effect in the different genotypes using linear-by-linear association χ^2 statistics (SPSS ver. 21). Linkage disequilibrium (LD; i.e., non-random association of alleles) was assessed for each gene. The LD between identified SNPs was determined by pairwise comparisons of correlation coefficient between SNPs (r) and the likelihood that recombination has occurred between SNPs (D'). Since we expected that the selected SNPs have a small contribution to TGCC and we regard our study to be exploratory rather than confirmatory, we have chosen to avoid correction for multiple testing. This may increase the risk of type 1 errors, but it prevents type 2 errors.

RESULTS

No significant deviations from Hardy–Weinberg equilibrium were detected for the SNPs included in the analysis.

We did not find a statistically significant association between the studied polymorphisms and the risk of TGCC.

For four SNPs in *AHRR* a significant allele association with the occurrence of disseminated disease was observed (**Table 2**). Patients with metastatic disease had significantly lower frequencies of the minor rs2466287 G, rs2672725 G, rs6879758 C, and rs6896163 G alleles compared to patients with localized disease

(5 vs 10%, 6 vs 11%, 2 vs 5%, and 2 vs 6%, respectively), which were associated with 57, 51, 73, and 66%, reduced per allele OR for developing metastatic TGCC, respectively. Heterozygous carriers of *AHRR* variants rs6879758 and rs6896163 had a 74 and 67% reduced risk of developing metastatic TGCC, respectively. Due to very low frequencies, or even absence, of homozygous carriers of the *AHRR* variants the OR could not be calculated for these groups of patients. However, trend analyses demonstrated significant associations for all four *AHRR* variants.

Polymorphisms in *AHRR* did not show a significant association with risk of TGCC or histological subtype, which was also the case for SNPs in *AHR*. Histological subtype and the occurrence of metastasic disease were not associated with SNPs in *AHR*. LD analysis demonstrated a high correlation between rs2466287 and rs2672725 as well as between rs6879758 and rs6896163 (**Table 3**).

When analyzed in the two populations individually, similar statistically significant ORs for the four SNPs were observed in the Swedish population as compared to the combined population. The associations in the Danish populations showed similar tendencies, although these did not reach statistical significance.

DISCUSSION

In this study we have analyzed associations between SNPs in genes encoding AHR and AHRR and the risk of developing TGCC, histological subtype, and the occurrence of metastasis. Whereas no associations were found with SNPs in *AHR*, four variants in *AHRR* associated significantly with the occurrence of metastatic disease.

Both AHR and its negative regulator AHRR are ligand-activated transcription factors that belong to the family of basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) proteins. They are ubiquitously expressed in almost all human tissues, with levels particularly high in the testis (Burbach et al., 1992; Tsuchiya et al., 2003). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most well-characterized exogenous ligand of AHR and it is known for its anti-estrogenic properties such as inhibition of estradiol-induced uterine weight increase and decreased levels of estrogen and progesterone receptors in uterus of rat and mice (Safe, 1995).

Several epidemiological studies have shown that TCDD promotes cancer, and experiments in AHR-deficient mice have shown that AHR is essential for the tumor-promoting effects of TCDD (Andersson et al., 2002; McMillan and Bradfield, 2007). Conversely, transgenic mice with a constitutively active AHR spontaneously develop tumors, their weights of testis and ventral prostate are decreased and the epididymal sperm reserve is reduced (Brunnberg et al., 2011). Furthermore, AHRR, the negative regulator of AHR signaling, functions as a tumor suppressor in multiple human tumors (Zudaire et al., 2008; Li et al., 2012). Several studies have shown that the AHR activation also interferes with reproductive health. An increased incidence of TGCC has been observed in workers exposed to TCDD (Kogevinas et al., 1997) and dioxins have been reported to reduce sperm number, and to decrease accessory sex gland weight and anogenital distance in rats (Mably et al., 1992; Gray et al., 1995; Sommer et al., 1996; Faqi et al., 1998; Ohsako et al., 2002).

While SNPs in either AHR or AHRR did not associate with the risk of TGCC, it is interesting that an association was observed between AHRR and dissemination of TGCC, since AHR signaling

Table 2 | Allele and genotype distributions with associated OR and trend analysis in TGCC patients stratified according to the occurrence of metastasis.

SNP (gene)	Allele and genotype	Combined				Sweden				Denmark			
		Localized n (%)	Metastasis n (%)	P-value	OR (95% CI)	Localized n (%)	Metastasis n (%)	P-value	OR (95% CI)	Localized n (%)	Metastasis n (%)	P-value	OR (95% CI)
rs2466287 (AHRR)	C	472 (90)	181 (95)		Referent	356 (90)	147 (95)		Referent	116 (89)	34 (94)		Referent
	G	54 (10)	9 (5)	0.021	0.43 (0.21–0.90)	40 (10)	7 (5)	0.036	0.42 (0.19–0.97)	14 (11)	2 (6)	0.348	0.49 (0.11–2.27)
	CC	213 (81)	86 (91)		Referent	161 (81)	70 (91)		Referent	52 (80)	16 (89)		Referent
	CG	46 (17)	9 (9)	0.061	0.49 (0.23–1.03)	34 (17)	7 (9)	0.089	0.47 (0.20–1.12)	12 (18)	2 (11)	0.026	0.098 (0.013–0.75)
	GG	4 (2)	0 (0)	–	–	3 (2)	0 (0)	–	–	1 (2)	0 (0)	–	–
	Trend			0.024				0.041				0.359	
rs2672725 (AHRR)	C	467 (89)	179 (94)		Referent	351 (89)	146 (95)		Referent	116 (89)	33 (92)		Referent
	G	59 (11)	11 (6)	0.031	0.49 (0.25–0.94)	45 (11)	8 (5)	0.028	0.43 (0.20–0.93)	14 (11)	3 (8)	0.670	0.75 (0.20–2.78)
	CC	209 (79)	84 (88)		Referent	157 (79)	69 (90)		Referent	52 (80)	15 (83)		Referent
	CG	49 (19)	11 (12)	0.104	0.56 (0.28–1.13)	37 (19)	8 (10)	0.088	0.49 (0.22–1.11)	12 (18)	3 (17)	0.014	0.078 (0.010–0.60)
	GG	5 (2)	0 (0)	–	–	4 (2)	0 (0)	–	–	1 (2)	0 (0)	–	–
	Trend			0.035				0.033				0.674	
rs6879758 (AHRR)	G	499 (95)	187 (98)		Referent	375 (94)	152 (99)		Referent	124 (95)	35 (97)		Referent
	C	29 (5)	3 (2)	0.025	0.27 (0.08–0.92)	23 (6)	2 (1)	0.023	0.21 (0.050–0.92)	6 (5)	1 (3)	0.627	0.59 (0.069–5.00)
	GG	235 (89)	92 (97)		Referent	176 (88)	75 (97)		Referent	59 (91)	17 (94)		Referent
	GC	29 (11)	3 (3)	0.032	0.26 (0.079–0.89)	23 (12)	2 (3)	0.034	0.20 (0.047–0.89)	6 (9)	1 (6)	0.147	0.32 (0.070–1.49)
	CC	0 (0)	0 (0)	–	–	0 (0)	0 (0)	–	–	0 (0)	0 (0)	–	–
	Trend			0.022				0.020				0.622	
rs6896163 (AHRR)	A	497 (94)	188 (98)		Referent	373 (94)	153 (98)		Referent	124 (95)	35 (97)		Referent
	G	31 (6)	4 (2)	0.037	0.34 (0.12–0.98)	25 (6)	3 (2)	0.035	0.29 (0.087–0.98)	6 (5)	1 (3)	0.627	0.59 (0.069–5.00)
	AA	233 (88)	92 (96)		Referent	174 (87)	75 (96)		Referent	59 (59)	17 (94)		Referent
	AG	31 (12)	4 (4)	0.040	0.33 (0.11–0.95)	25 (13)	3 (4)	0.041	0.28 (0.082–0.95)	6 (6)	1 (6)	0.180	0.41 (0.11–1.50)
	GG	0 (0)	0 (0)	–	–	0 (0)	0 (0)	–	–	0 (0)	0 (0)	–	–
	Trend			0.032				0.031				0.622	

Table 3 | Linkage disequilibrium (*D'*) and correlation coefficients (*r*) between SNPs in *AHRR* that associate with metastatic TGCC in the total population.

	rs2466287		rs2672725		rs6879758	
	D'	*r*	*D'*	*r*	*D'*	*r*
rs2466287						
rs2672725	0.978	0.909				
rs6879758	0.239	0.172	0.230	0.155		
rs6896163	0.195	0.142	0.186	0.132	0.999	0.948

is indeed known to contribute to the control of cell adhesion and migration (Carvajal-Gonzalez et al., 2009; Dietrich and Kaina, 2010). A potential role of AHR signaling in dissemination of TGCC is further supported by Koliopanos et al. (2002) who reported that AHR agonists decreased anchorage-independent pancreatic cancer cell growth.

Since testicular cancer rarely occurs before the onset of puberty, the malignant transformation of CIS cells may be associated with activation of the hypothalamic–pituitary–gonadal axis. An interaction between AHR signaling and steroid or gonadotropic hormones could therefore be an alternative mechanism underlying the association between AHRR polymorphisms and metastatic TGCC.

A strength of our study is the access to two independent TGCC populations. Although not all findings reached statistical significance in the Danish cohort when the countries were analyzed separately, most probably due to the limited number of cases in this group, the same trends with similar relative risks were found, which strengthens the relevance of our findings. We cannot exclude that a small fraction of the TGCC patients in our cohort were from non-Caucasian background. However, none of the allele frequencies of the analysed SNPs deviated from Hardy-Weinberg equilibrium, which indicates that either the non-Caucasian allele frequencies did not differ from those in the Caucasian population, or that non-Caucasians did not significantly contribute to the total cohort. The SNPs identified in this study are intronic polymorphisms, thus excluding structural changes in the proteins that these genes encode. Yet, polymorphisms in non-coding regions can still play an important role in the regulation of gene expression, e.g., by affecting mRNA stability, regulation by intronic micro RNAs, or potential splice sites. The identified SNPs could also be in LD with other genetic polymorphisms that are causally related to TGCC.

In summary, we have identified polymorphic variants in the gene encoding AHRR that associated with risk of disseminated TGCC. This supports our hypothesis that the AHR signaling pathway may affect the progression of TGCC, possibly by interfering with reproductive hormone actions.

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Cryptorchidism and testicular germ cell tumors: comprehensive meta-analysis reveals that association between these conditions diminished over time and is modified by clinical characteristics

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Introduction: Risk of testicular germ cell tumors (TGCT) is consistently associated with a history of cryptorchidism (CO) in epidemiologic studies. Factors modifying the association may provide insights regarding etiology of TGCT and suggest a basis for individualized care of CO. To identify modifiers of the CO-TGCT association, we conducted a comprehensive, quantitative evaluation of epidemiologic data.

Materials and Methods: Human studies cited in PubMed or ISI Web of Science indices through December 2011 and selected unpublished epidemiologic data were reviewed to identify 35 articles and one unpublished dataset with high-quality data on the CO-TGCT association. Association data were extracted as point and 95% confidence interval estimates of odds ratio (OR) or standardized incidence ratio (SIR), or as tabulated data. Values were recorded for each study population, and for subgroups defined by features of study design, CO and TGCT. Extracted data were used to estimate summary risk ratios (sRR) and evaluate heterogeneity of the CO-TGCT association between subgroups.

Results: The overall meta-analysis showed that history of CO is associated with four-fold increased TGCT risk [RR = 4.1(95% CI = 3.6–4.7)]. Subgroup analyses identified five determinants of stronger association: bilateral CO, unilateral CO ipsilateral to TGCT, delayed CO treatment, TGCT diagnosed before 1970, and seminoma histology.

Conclusions: Modifying factors may provide insight into TGCT etiology and suggest improved approaches to managing CO. Based on available data, CO patients and their parents or caregivers should be made aware of elevated TGCT risk following orchidopexy, regardless of age at repair, unilateral vs. bilateral non-descent, or position of undescended testes.

Keywords: testicular neoplasms, cryptorchidism, seminoma, non-seminoma, meta-analysis

INTRODUCTION

Cryptorchidism (CO), or undescended testis, affects approximately 3% of all male live births, making it one of the most common congenital disorders. Despite a high rate of spontaneous resolution during the first year of life, CO is firmly established as the primary risk factor for subsequent development of testicular germ cell tumors (TCGT) (John Radcliffe Hospital Study Group, 1992; Berkowitz et al., 1993; Thong et al., 1998; Paulozzi, 1999). TGCT are the most common form of malignancy among young men in the United States. Advances in systemic therapy have improved overall TGCT survival from 83% in 1975–1979 to 96% in 1999–2005 (Jemal et al., 2010). However, incidence of TGCT has nearly doubled during the same time period (Fast Stats), and it is now evident that significant sequelae include

subfertility, (Walsh et al., 2009) sexual dysfunction, (Magelssen et al., 2006) and elevated risk of second malignancy (Moller et al., 1993; Fossa et al., 2005; Travis et al., 2005; Van den Belt-Dusebout et al., 2007). Therefore, a clear understanding of etiologic risk factors and more comprehensive risk stratification is a priority of TGCT research.

Risk of CO and TGCT are associated with additional disorders of the male reproductive system, hypospadias, and impaired spermatogenesis. This pattern is postulated to reflect origins of all of these conditions in errors of development of the fetal testis according to the testicular dysgenesis syndrome hypothesis, which elegantly accounts for experimental research identifying genetic and early environmental factors predisposing to these phenotypes in animal models (Skakkebæk et al., 2001). Little is currently

known regarding the specific insults that may lead to elevated risk of individual and joint phenotypes in humans, or the stages of testicular development when such factors may act. In the present report we comprehensively reviewed the rich set of published observational data on co-occurrence of CO and TGCT as a first step in disentangling the complex associations among these related conditions.

Currently, little is known about the overall characterization of tumor risk in patients with a prior history of CO. Individual estimates of relative risk from the literature range from 1.35 (95% $CI = 0.73–2.48$) to 18 (95% $CI = 12–26$), (Miller and Seljelik, 1971; Mostofi, 1973; Morrison, 1976; Henderson et al., 1979; Loughlin et al., 1980; Schottenfeld et al., 1980; Wobbes et al., 1980; Fonger et al., 1981; Coldman et al., 1982; Depue et al., 1983; Mills et al., 1984; Pottern et al., 1985; Moss et al., 1986; Giwercman et al., 1987; Swerdlow et al., 1987, 1997; Gershman and Stolley, 1988; Strader et al., 1988; Thornhill et al., 1988; Haughey et al., 1989; Benson et al., 1991; Stone et al., 1991; United Kingdom Testicular Cancer Study Group (UK), 1994; Gallagher et al., 1995; Davies et al., 1996; Moller et al., 1996; Prener et al., 1996; Petridou et al., 1997; Sabroe and Olsen, 1998; Sigurdson et al., 1999; Weir et al., 2000; Stang et al., 2001; Bonner et al., 2002; Herrinton et al., 2003; Dieckmann and Pichlmeier, 2004; Kanto et al., 2004; Hardell et al., 2007; McGlynn et al., 2007; Myrup et al., 2007; Pettersson et al., 2007; Walschaerts et al., 2007; Dusek et al., 2008) suggesting significant differences in study design and/or heterogeneity of the effects of clinical characteristics which may impact risk of TGCT amongst males with CO. Potential modifying factors include anatomic location (abdominal vs. inguinal vs. ectopic) and laterality (unilateral vs. bilateral) of undescended testes, age at treatment, mode of treatment (spontaneous descent vs. hormones or orchiopexy), as well as temporal trends in TGCT risk, and tumor histology. Previous meta-analyses evaluating the CO-TGCT association have been limited to specific subsets of these factors (Castejon Casado et al., 2000; Walsh et al., 2007; Tuazon et al., 2008; Akre et al., 2009). We report a broader systematic review and meta-analysis of the overall association between CO-TGCT, and explore the possible impact of study design, temporal trends, and clinical features on this association.

MATERIALS AND METHODS

The analysis followed specifications for meta-analysis of observational studies in epidemiology (Stroup et al., 2000) and adhere to PRISMA guidelines (Moher et al., 2009). The outcome was TGCT. The exposure was CO, defined as a testicle undescended at birth that subsequently descended spontaneously, was repositioned into the scrotum by orchiopexy or hormone therapy, or remained undescended. Subgroups were defined by features of study design, CO, and TGCT.

STUDY SELECTION

We searched the MEDLINE (National Library of Medicine, Bethesda, MD, USA) Pubmed interface without language restrictions for human studies published through December 2011, using key words "CO," "undescended testicle," "undescended testis," "undescended testes," "case-control study," "cohort study"

in combination with "testicular cancer," "testicular carcinoma," "testicular neoplasia," "testis cancer," "testis carcinoma," "testis neoplasia," reviewing also reports cited in retrieved articles and review articles, and by citation indices (ISI Web of Science) for these reports. We also sought high-quality unpublished data. The Review Protocol has not been registered.

DATA EXTRACTION AND CODING

A genetic counselor and a statistician reviewed retrieved articles to determine eligibility for the meta-analysis, resolving conflicts by consensus among themselves and an epidemiologist. Data on study design, overall CO-TGCT associations, and subgroup-specific CO-TGCT associations were extracted systematically by a single reviewer and confirmed by two others. We eliminated redundant data arising from repeated publication, consulting original authors whenever possible.

We extracted published information on RRs relating CO to TGCT as follows: when provided, we recorded point estimates of the odds ratio (OR) for case-control studies and standardized incidence ratio (SIR) for cohort studies, with corresponding standard error or information from which it could be calculated (variance, CI, p-value). When only a point estimate was reported, we requested corresponding variance term from original authors. If the OR estimate was not provided, we calculated it from published tabular data.

Features of study design

We noted the following features of each study: data structure (case-control study of TGCT, cohort study of males born with CO, TGCT cases for whom frequency of CO was compared to external population), country where study was conducted, race/ethnicity of participants, and source of CO data (birth record, medical record before TGCT diagnosis, medical record at/following TGCT diagnosis, reported by participant and/or his mother). For case-control and cohort studies we noted source of reference group (population or population-based registry, hospital or neighborhood, friend). We distinguished published RR estimates extracted from those we calculated from published data; for published estimates, we tabulated covariates and matching variables in original analyses.

Features of cryptorchidism

When possible we extracted or calculated RR estimates of CO-TGCT association for subgroups defined by each of several features of CO: laterality of undescended testicle relative to TGCT (unilateral CO contralateral to tumor, unilateral CO with relation to tumor unspecified, unilateral CO ipsilateral to tumor, bilateral CO), level of maldescent (ectopic, inguinal, abdominal), whether definition of CO included spontaneous descent, means of resolving CO (spontaneous, orchiopexy or hormone therapy, remained undescended), and age at resolution. For this variable we used frequently published categories (0–9 years of age, 10–14 years, 10 years or older, 15 years or older) (United Kingdom Testicular Cancer Study Group (UK), 1994; Moller et al., 1996; Swerdlow et al., 1997) combining data from smaller intervals if provided (Swerdlow et al., 1997). Among studies with alternate cut points, one provided raw data from which

we recalculated estimates within above strata (Herrinton et al., 2003); for four others (Pottern et al., 1985; Strader et al., 1988; Myrup et al., 2007; Pettersson et al., 2007) we assigned data to closest category corresponding to above strata (**Table S3**, footnotes b–d).

Features of testicular germ cell tumors

We extracted or calculated RR estimates for subgroups defined by histology (non-seminoma, non-seminoma/mixed germ cell tumor (GCT), mixed GCT, seminoma). A separate code for mixed GCT was introduced in 1990 (ICD-0-2), and tumors of mixed histology were previously coded as non-seminoma. Therefore, for studies including diagnoses before 1990, if authors did not specify that non-seminoma excluded mixed histology, we coded reported non-seminoma as "non-seminoma/mixed GCT." As a measure of year of TGCT diagnosis, we determined midpoint of range of years of diagnosis among cases participating in each study.

Unpublished data

Original population-based case-control data (Lacson et al., 2012) were provided before publication. Briefly, 163 TGCT cases identified by the Los Angeles County Cancer Surveillance Program (CSP) and 284 age-matched neighborhood controls were enrolled. TGCT data (histologic type, laterality, age at diagnosis) were provided by the CSP, and CO data (laterality, age at
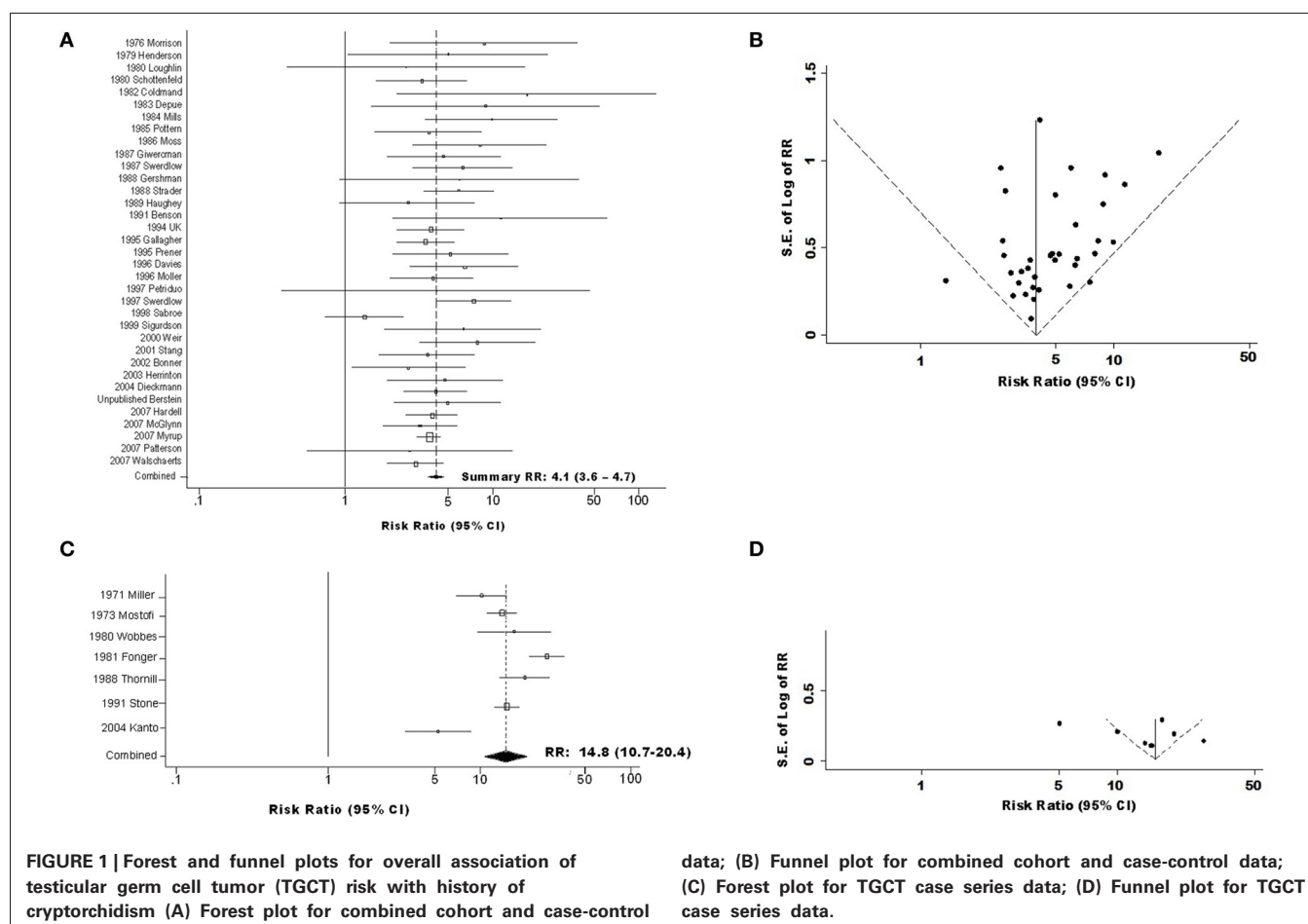
resolution, mean of resolution) were provided by participants and their mothers during in-person interviews.
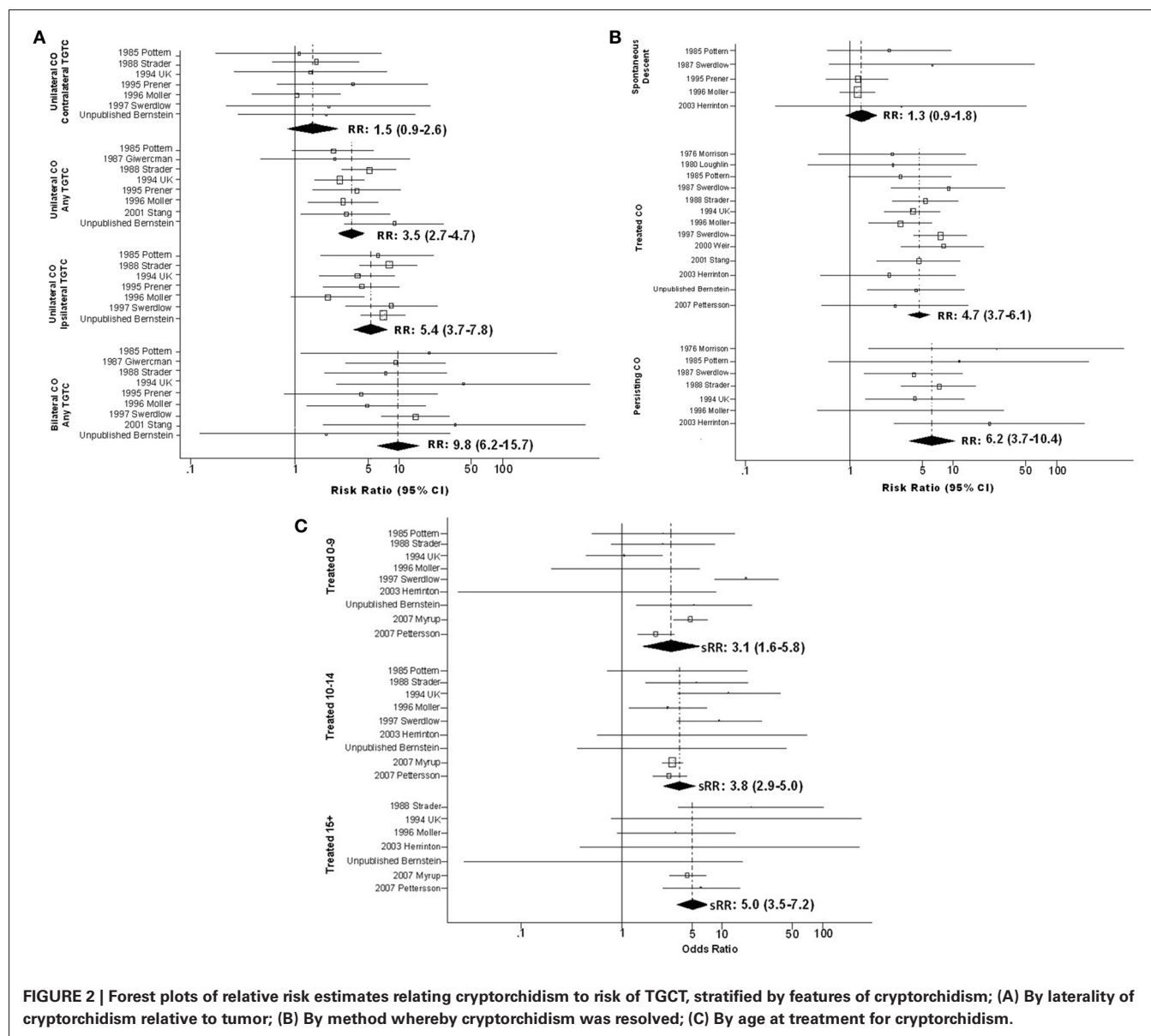
STATISTICAL ANALYSES

Estimating overall and stratum-specific summary RR of CO-TGCT association

We conducted all meta-analyses using a random effects model, which accounts for between- and within-study variance thereby incorporating the conservative assumption that individual studies estimate different effect sizes (Sutton et al., 2000). We used STATA 8.0 (Stata Corp, College Station, TX) to weight natural log of each contributing OR or SIR estimate by the reciprocal of the corresponding variance. We used this technique to estimate overall and stratum-specific summary relative risk (sRR) estimates. Forest plots were graphed displaying each study's contribution to sRRs.

Meta-analyses were performed separately for case-control (**Tables S2–S4**, columns A) and cohort (**Tables S2–S4**, columns B) studies, with summary measures subsequently pooled. Resulting sRR estimates [**Figures 1A**, **2A–C** and **3A–C**; **Tables S2–S4**, columns C (boldface)] summarize available data on CO-TGCT associations from studies with reference groups judged comparable to cases. Other articles compared CO prevalence of TGCT cases with CO prevalence from external

FIGURE 1 | Forest and funnel plots for overall association of testicular germ cell tumor (TGCT) risk with history of cryptorchidism (A) Forest plot for combined cohort and case-control data; **(B) Funnel plot for combined cohort and case-control data; (C) Forest plot for TGCT case series data; (D) Funnel plot for TGCT case series data.**

FIGURE 2 | Forest plots of relative risk estimates relating cryptorchidism to risk of TGCT, stratified by features of cryptorchidism; (A) By laterality of cryptorchidism relative to tumor; (B) By method whereby cryptorchidism was resolved; (C) By age at treatment for cryptorchidism.

populations, rather than source population of the cases; limited comparability in such studies is now recognized as a potential source of severe bias (Rothman et al., 2008). We therefore summarized these data separately (**Figure 1C**; **Tables S2–S4**, column D).
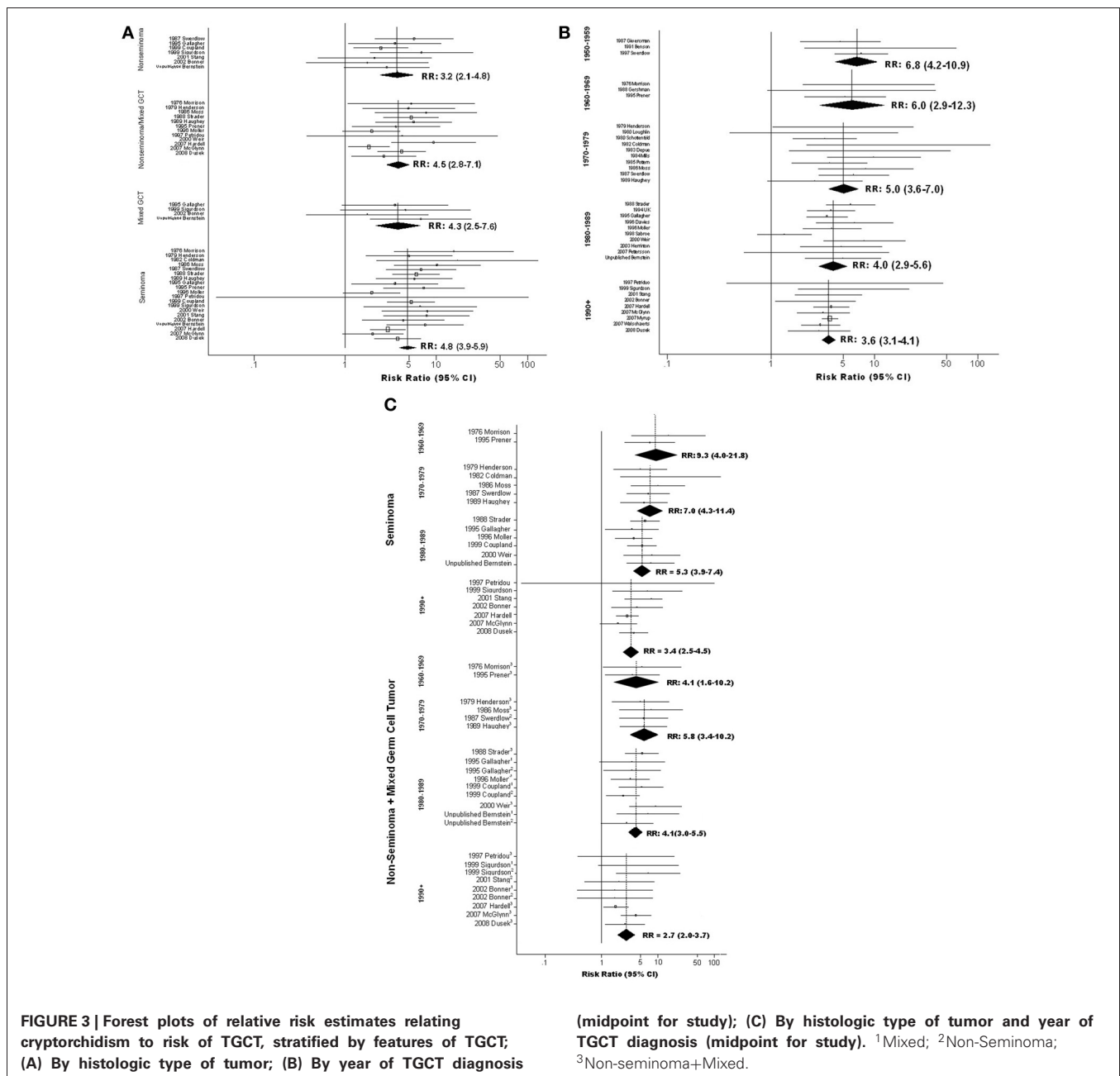
    We implemented Egger's test of publication bias, assessed heterogeneity by appropriate p-value and I^2 (Berkowitz et al., 1993) estimates, and assessed trends as described in Appendix Methods, along with procedures for imputation and sensitivity analysis.

ROLE OF THE FUNDING SOURCE

Sponsors had no role in the design, implementation, or reporting of the research. Drs. Victoria K. Cortessis and Leslie Bernstein had full access to all of the original data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

RESULTS

We identified 147 published articles reporting on both CO and TGCT in humans, eliminating those that did not provide data relating CO to TGCT risk ($N = 95$, not cited), or were duplicate reports of data included in the analysis (10 reports, **Table S1**). Data from 35 of the remaining 42 reports and raw data from one unpublished case-control study contributed to meta-analyses on which we base our inferences (**Table 1A**; **Figure S1**). We requested from authors of three of these reports (Moss et al., 1986; Strader et al., 1988; Swerdlow et al., 1987) standard error data corresponding to published histology stratum-specific point estimates, and received these for one (Strader et al., 1988). In all, data on 9542 TGCT cases contributed to the analyses. For historical interest, we separately summarized estimates from the remaining seven reports, whose authors compared CO among TGCT case-series with

FIGURE 3 | Forest plots of relative risk estimates relating cryptorchidism to risk of TGCT, stratified by features of TGCT; (A) By histologic type of tumor; (B) By year of TGCT diagnosis (midpoint for study); **(C) By histologic type of tumor and year of TGCT diagnosis (midpoint for study).** [1]Mixed; [2]Non-Seminoma; [3]Non-seminoma+Mixed.

frequencies of CO measured outside source populations of the cases (**Table 1B**).

Sensitivity analyses revealed that no single case-control or cohort study influenced either overall or stratum-specific estimates of RR sufficiently to alter interpretation (results not shown). Egger's tests revealed no evidence of publication bias among cohort data ($p = 0.68$), case-control data ($p = 0.34$), or these data types combined ($p = 0.40$); however, addition of data from the TGCT case series introduced an impression of substantial publication bias ($p = 0.01$). Visual inspection of Begg's funnel plots showed that while magnitude and standard error of RR estimates from most cohort and case-control studies (34 of 36) are

within the 95% confidence limits (**Figure 1B**), this is true of a far smaller proportion of TGCT case-series (4 of 7, **Figure 1D**).

OVERALL CO-TGCT ASSOCIATION AND EFFECTS OF STUDY DESIGN

We estimated sRR of developing TGCT following a history of CO to be 4.0(95% $CI = 3.4$–4.6) in case-control studies (**Table S2Ai**) and 4.8(95% $CI = 3.2$–7.2) in cohort studies (**Table S2Bi**). The sRR estimated by pooling these results was 4.1(95% $CI = 3.6$–4.7), with 14% of the variance (I^2) attributed to between-study heterogeneity (**Figures 1A,B**; **Table S2Ci**). By contrast, sRR estimated from studies that compared CO frequencies between TGCT case series and external populations was

Table 1 | Epidemiologic studies of cryptorchidism and testicular germ cell tumors (TGCT) included in the meta-analysis.

A. STUDIES THAT PROVIDED DATA ON TGCT CASES AND COMPARABLE REFERENCE GROUP

i. Case-control studies

First author	Year published	Country	Ethnicity[a]	Number of TGCT cases	Number of controls	Source of cases[b]	Source of controls[b]	Spontaneous descent included[c]	Number of variables matched and/or adjusted for	Source of cryptorchidism data[d]
Morrison (Morrison, 1976)	1976	USA	NS	596	602	Reg	Pop	No	0	TCMR
Henderson (Henderson et al., 1979)	1979	USA	WP	79	79	Reg	Neigh	NS	2	M
Loughlin (Loughlin et al., 1980)	1980	USA	WO	24	35	Hosp	Hosp	No	2	C + M
Schottenfeld (Schottenfeld et al., 1980)	1980	USA	WO	157	309	Hosp	Hosp + Neigh	NS	3	C + M
Coldman (Coldman et al., 1982)	1982	Canada	NS	93	79	Hosp	Hosp	Yes	2	C, TCMR
Depue (Depue et al., 1983)	1983	USA	WO	107	108	Reg	Neigh	NS	2	C
Mills (Mills et al., 1984)	1984	USA	WP	347	347	Hosp	Hosp	NS	3	TCMR
Pottern (Pottern et al., 1985)	1985	USA	WP	271	259	Hosp	Hosp	Yes	2	C + M
Moss (Moss et al., 1986)	1986	USA	WP	246	252	Reg + Hosp	Friends	NS	3	C, M
Swerdlow (Swerdlow et al., 1987)	1987	England	NS	259	489	Reg + Hosp	Hosp	Yes	2	C
Gershman (Gershman and Stolley, 1988)	1988	USA	NS	79	79	Reg	Pop	NS	2	C
Strader (Strader et al., 1988)	1988	USA	WO	326	675	Reg	Pop	No	1	C
Haughey (Haughey et al., 1989)	1989	USA	WO	247	247	Reg	Neigh	NS	2	C
UK (United Kingdom Testicular Cancer Study Group (UK), 1994)	1994	UK	WO	794	794	Reg + Hosp	Pop	No	1	C + M
Gallagher (Gallagher et al., 1995)	1995	Canada	WP	506	994	Reg	Pop	NS	1	C
Prener (Prener et al., 1996)	1996	Denmark	NS	171	366	Reg	Pop	Yes	4	PTCMR
Davies (Davies et al., 1996)	1996	England	NS	129	395	Reg	Pop + Reg	NS	6	C + M
Moller (Moller et al., 1996)	1996	Denmark	NS	514	720	Reg	Pop	Yes	1	C
Petridou (Petridou et al., 1997)	1997	Greece	NS	97	198	Hosp	Pop	NS	2	C + M
Sabroe (Sabroe and Olsen, 1998)	1998	Denmark	NS	357	704	Reg	Pop	Yes	2	BR
Sigurdson (Sigurdson et al., 1999)	1999	USA	WP	160	136	Hosp	Friends	NS	4	C
Weir (Weir et al., 2000)	2000	Canada	NS	325	490	Reg	Pop	No	5	C

(Continued)

Table 1 | Continued

First author	Year published	Country	Ethnicity[a]	Number of TGCT cases	Number of controls	Source of cases[b]	Source of controls[b]	Spontaneous descent included[c]	Number of variables matched and/or adjusted for	Source of cryptorchidism data[d]
Stang (Stang et al., 2001)	2001	Germany	NS	262	797	Reg	Pop	Yes	2	C, M
Bonner (Bonner et al., 2002)	2002	USA	WO	116	328	Hosp	Hosp	NS	1	C
Herrinton (Herrinton et al., 2003)	2003	USA	WP	183	551	Hosp	Hosp	Yes	2	PTCMR
Dieckmann (Dieckmann and Pichlmeier, 2004)	2004	Unknown	Unknown	538	551	Unknown	Unknown	Unknown	Unknown	Unknown
Hardell (Hardell et al., 2007)	2007	Sweden	NS	888	870	Reg	Pop	NS	1	C
McGlynn (McGlynn et al., 2007)	2007	USA	WP	767	928	Reg	Pop	NS	3	C
Walschaerts (Walschaerts et al., 2007)	2007	France	NS	200	585	Hosp	Hosp	NS	1	C
Dusek (Dusek et al., 2008)	2008	Czech Republic	WO	356	317	Hosp	Hosp + Neigh	NS	1	C
Lacson (Lacson et al., 2012)		USA	WP	163	284	Reg	Neigh	Yes	3	C
ii. Cohort studies of boys with Cryptorchidism										
Giwercman (Giwercman et al., 1987)	1987	Denmark	NS	6	–	Hosp	–	No	2	PTCMR
Benson (Benson et al., 1991)	1991	USA	NS	2	–	Hosp	–	Yes	1	PTCMR
Swerdlow (Swerdlow et al., 1997)	1997	England	NS	11	–	Hosp	–	No	2	PTCMR
Pettersson (Pettersson et al., 2007)	2007	Sweden	NS	56	–	Reg	–	No	2	PTCMR
Myrup (Myrup et al., 2007)	2007	Denmark	NS	110	–	Reg	–	No	2	PTCMR
B. STUDIES THAT PROVIDED DATA ON TGCT CASE SERIES, ESTIMATING RISK RATIOS USING CRYPTORCHIDISM FREQUENCY FROM EXTERNAL POPULATION										
Miller (Miller and Seljelik, 1971)	1971	Norway	NS	314	–	Reg	–	NS	–	TCMR
Mostofi (Mostofi, 1973)	1973	USA	NS	2000	–	Army Reg	–	NS	–	TCMR
Wobbes (Wobbes et al., 1980)	1980	Netherlands	NS	230	–	Hosp	–	No	–	TCMR
Fonger (Fonger et al., 1981)	1981	Canada	NS	646	–	Hosp	–	Yes	–	TCMR
Thornhill (Thornhill et al., 1988)	1988	Ireland	NS	240	–	Reg	–	NS	–	TCMR
Stone (Stone et al., 1991)	1991	Australia	NS	778	–	Hosp	–	Yes	–	TCMR
Kanto (Kanto et al., 2004)	2004	Japan	J	240	–	Hosp	–	Yes	–	TCMR

[a]NS, not specified; WO, white only; WP, white plus other ethnicities; J, Japanese.

[b]Reg, cancer registry; Hosp, hospital; Neigh, neighborhood; Pop, population.

[c]NS, not specified.

[d]C, case/control self report; M, case/control mother's report; C + M, case/control self report supplemented by mother's report; BR, birth record; PTCMR, pre-testicular cancer medical record, TCMR: testicular cancer medical record.

14.8(95% $CI = 10.7$–20.4), with 86% of variance attributed to between-study heterogeneity (**Figures 1C,D**; **Table S2Di**).

Two additional features of study design appeared to modify sRR estimated among case-control and cohort studies: time CO was recorded, and inclusion of spontaneous descent in CO definition. The RR estimate was notably greater, 9.9(95% $CI = 3.5$–28.2), in the single study (Mills et al., 1984) that determined CO history from medical record entries made around the time of TGCT diagnosis, and substantially lower, 1.3(95% $CI = 0.7$–2.5), in the single study (Sabroe and Olsen, 1998) in which CO had been recorded at birth. This latter study was likely the only one to have included in the CO group a large proportion of men whose testes would have descended in the first months of life. The same study appeared to be largely responsible for modification by whether definition of CO included spontaneous descent ($p_{\text{heterogeneity}} = 0.002$ for analysis including this study, $p_{\text{heterogeneity}} = 0.57$ for analysis excluding it, **Table S2Cvi**). Accordingly, this study was a clear outlier in the funnel plot (**Figure 1B**), while all others fell very near or within the 95% confidence limits.

MODIFICATION BY FEATURES OF CRYPTORCHIDISM

Several features of CO appeared to modify the CO-TGCT association (**Figure 2**; **Table S3**). Analyses stratified on laterality of undescended testes relative to TGCT revealed an apparent trend: sRR estimates increased steadily in progression from TGCT arising in testes contralateral to unilateral CO [sRR = 1.5(95% $CI = 0.9$–2.6)], on unspecified side relative to unilateral CO [sRR = 3.5(95% $CI = 2.7$–4.7)], ipsilateral to unilateral CO [sRR = 5.4(95% $CI = 3.7$–7.8)], and in either testis following bilateral CO [sRR = 9.8(95% $CI = 6.2$–15.7)] (**Figure 2A**; **Table S3Ci**). Data on anatomic location of undescended testes, provided for no case-control study and only one cohort study (Swerdlow et al., 1997), were insufficient to determine whether TGCT risk differs appreciably following abdominal vs. inguinal non-descent (**Table S3Cii**). Therefore, the meta-analysis did not confirm the report from a single TGCT case series (Stone et al., 1991) of far higher RR following abdominal non-descent (**Table S3Dii**).

In analyses stratified on means of resolving CO, estimates increased in the progression of testes that descended spontaneously [sRR = 1.3(95% $CI = 0.9$–1.8)], testes repositioned by surgery or hormone therapy [sRR = 4.7(95% $CI = 3.7$–6.1)], and testes remaining undescended [sRR = 6.2(95% $CI = 3.7$–10.4)] (**Figure 2B**; **Table S3Ciii**). Analyses stratified on both means of resolution and age at resolution suggested greater RR among those with older age at spontaneous descent or therapeutic resolution, although estimates for those who experienced spontaneous descent did not achieve statistical significance (**Figure 2C** and **Table S3Civ**).

MODIFICATION BY FEATURES OF TGCT

Analyses relating CO to tumor histology were conducted first in a subset of data excluding three studies that reported "no difference" in RR of seminoma vs. non-seminoma without providing stratum-specific estimates. The results hinted that RR of seminoma may exceed that of non-seminoma (**Table S4Ci**).

To minimize the possibility that systematic omission of data from studies reporting no difference had spuriously created this impression, we repeated this analysis using a fuller set of data that included values imputed for those studies; these results also suggested a stronger association of CO with seminoma (**Figure 3A**; **Table S4Cii**).

Analyses within strata defined by year of TGCT diagnosis suggest that magnitude of the CO-TGCT association diminished steadily from the 1950's forward. Highest sRR, 6.8(95% $CI = 4.2$–10.9), was estimated among studies with midpoint year of TGCT diagnosis in the 1950's, and lowest, 3.6(95% $CI = 3.1$–4.1), in studies with midpoint 1990 or later ($p_{\text{trend}} < 0.001$; **Figure 3B**; **Table S4Ciii**). Further stratification on histology, possible only for 1960's forward, revealed that this decrease results largely from a dramatic decrease in the CO-seminoma association [sRR = 9.3(95% $CI = 4.0$–21.8) for 1960–1969, sRR = 3.4(95% $CI = 2.5$–4.5) for 1990+; $p_{\text{trend}} = 0.009$), in contrast to no clear trend for other histologies ($p_{\text{trend}} = 0.320$) (**Figure 3C**; **Table S4Civ**).

TRENDS IN OCCURRENCE OF INDIVIDUAL CONDITIONS

Analyses of frequency of CO among controls participating in the case-control studies, (Morrison, 1976; Henderson et al., 1979; Loughlin et al., 1980; Schottenfeld et al., 1980; Coldman et al., 1982; Depue et al., 1983; Mills et al., 1984; Pottern et al., 1985; Moss et al., 1986; Swerdlow et al., 1987; Gershman and Stolley, 1988; Strader et al., 1988; Haughey et al., 1989; United Kingdom Testicular Cancer Study Group (UK), 1994; Gallagher et al., 1995; Davies et al., 1996; Moller et al., 1996; Prener et al., 1996; Petridou et al., 1997; Sabroe and Olsen, 1998; Weir et al., 2000; Stang et al., 2001; Bonner et al., 2002; Herrinton et al., 2003; Dieckmann and Pichlmeier, 2004; Hardell et al., 2007; McGlynn et al., 2007; Walschaerts et al., 2007; Dusek et al., 2008; Lacson et al., 2012) for which midpoint TGCT diagnosis years ranged from 1960's through 1990+, revealed no evidence that CO became more common among TGCT-free men during this period ($p_{\text{trend}} = 0.295$, data not shown).

Separate analyses of SEER 9 incidence data revealed that while annual age-standardized incidence of non-seminoma/mixed GCT increased over 28% (from 1.8 to 2.3 new diagnoses per 100,000) from 1973–1978 to 1994–1998, the increase in seminoma was far greater, 68% (from 2.2 to 3.7 per 100,000) during the same interval (**Figure S2**).

DISCUSSION

Meta-analysis of data from case-control and cohort studies suggests that young men with a history of CO experience approximately 4-fold increased risk of TGCT. Estimates from TGCT case-series whose frequency of CO was compared with separate populations were regarded as unreliable; this practice may cause severe bias (Rothman et al., 2008), which may account for the far larger sRR and between-study variation estimated from these reports.

Addressing more subtle differences in study design, we found that sRR estimates from case-control and cohort studies were notably modified by methods used to determine a man's history of CO. The stronger association estimated in the single

case-control study in which history of CO was determined from medical record notes made at TGCT diagnosis may have arisen from better recall of CO among cases than controls, since CO was a recognized TGCT risk factor when these diagnoses were made. Such recall bias may have contributed, also, to large effects reported for case series, because history of CO was similarly determined in all of these studies. Undescended testes that descended spontaneously in infancy would rarely be recorded in medical records after the neonatal period, or recalled by study participants or their mothers. We therefore anticipate that only the single study in which CO was recorded at birth (Sabroe and Olsen, 1998) would have identified a high proportion of men with this history. Weak CO-TGCT association reported in this study may indicate that boys born with CO whose testes descend spontaneously in early months experience TGCT risk approaching that of the general population. This possibility has implications for both managing CO and understanding origins of TGCT, so we hope that it will be explored further in retrospective cohorts for which there are detailed neonatal records.

The apparent modification of sRR by features of CO and TGCT may provide insight regarding TGCT etiology. Two general explanations for the CO-TGCT association have long been offered. The *common cause hypothesis* attributes the association to one or more unidentified etiologic factors shared by CO and TGCT, whereas the *position hypothesis* asserts that suprascrotal environment increases malignant potential of undescended testes. Hussman suggested two testable predictions of the position hypothesis (Husmann, 2005): (1) in unilateral CO, fully descended contralateral testes should not experience elevated TGCT risk, and (2) early orchiopexy should decrease TGCT risk. Regarding the first, we estimated a lesser sRR for contralateral testes [1.5(95% $CI = 0.9$–2.6)] than for ipsilateral testes [5.4(95% $CI = 3.7$–7.8)], suggesting a deleterious effect of suprascrotal position. Also consistent with positional effects, sRR among those with bilateral non-descent [9.8(95% $CI = 6.2$–15.7)] was, within statistical precision of the meta-analysis, indistinguishable from twice sRR of those with unilateral non-descent [$2 \times sRR_{ipsilateral} = 10.8(7.4$–15.6)]. However, published data are insufficient (24 cases in 7 studies) to rule out a small increase in risk to contralateral testes (e.g., 20%), as would be required to strictly affirm Hussman's first prediction. We note, however, that some true increase in risk to contralateral testes would not necessarily rule out the position hypothesis, because central responses to a single testis in a suprascotal position could, in theory, contribute to malignant potential of the contralateral testis. For example, in rodent models of unilateral CO created surgically, degenerative changes (Quinn, 1991; Zakaria et al., 1998) and altered gene expression (Iizuka et al., 1996) were demonstrated in contralateral, descended testes. A phenomenon observed in humans is also consistent with this possibility: among patients with unilateral TGCT who undergo biopsy of the contralateral testis, men with a history of CO are more often found to have the presumptive TGCT precursor carcinoma *in situ* testis/intra-tubular germ cell neoplasia than those without history of CO (Dieckmann and Loy, 1996). Regarding Hussman's second prediction, we observed greater TGCT risk among those who experienced later resolution. However, we cannot confidently conclude that deleterious effects of suprascrotal position are responsible. An alternate explanation,

which we cannot rule out, is that men with earlier resolution of CO experienced as a group inherently lower risk of TGCT. This might occur, for example, if this group included a higher proportion of boys destined to experience spontaneous descent if therapeutic intervention had been delayed. Because elevated risk was observed, regardless of age at orchiopexy, the clinical significance of available data is that patients undergoing orchiopexy at any age should be closely monitored; thus along with their parents or primary care givers and primary care physicians, they should be made aware of the increased risk.

Unfortunately, published data could not distinguish between risks experienced by men with histories of abdominal vs. inguinal non-descent. These distinct phenotypes may provide a means of determining relevance to human CO and TGCT of animal models of CO with high (Hsieh-Li et al., 1995; Rijli et al., 1995; Good et al., 1997; Nef and Parada, 1999; Overbeek et al., 2001) or low (Hutson, 1986; Lahoud et al., 2001) non-descent resulting from disruption of specific genes. If found to be relevant to human CO and TGCT, these models may become valuable tools in TGCT research, which has long suffered from absence of animal models of common forms of TGCT (Oosterhuis and Looijenga, 2005). Therefore, documenting position of undescended testes before orchiopexy may be useful for future research and in follow-up of men with a history of CO, particularly in the era of electronic medical records.

Modification by features of TGCT may suggest etiologic heterogeneity of these tumors.

Morrison (1976) apparently first suggested that CO is more strongly associated with seminoma than non-seminoma, although subsequent reports were inconsistent. The meta-analysis suggested greater RR for seminoma than for non-seminoma, and an intermediate value for mixed GCT, by addressing a far larger set of data than any single study and treating mixed GCT as a distinct histologic type. Although mechanisms underlying this pattern remain unknown, this finding suggests that tumors of distinct histologic types may have separate etiologies and/or result from events at different developmental stages. This possibility accords with the far more dramatic decreases in the CO-seminoma association observed over time. This trend, together with the observation that incidence of seminoma has risen more rapidly than that of non-seminoma/mixed GCT (**Figure S2**), raises the intriguing possibility that increasing occurrence of seminoma may involve increasing exposure to unidentified environmental factors through processes unrelated to CO.

In conclusion, this meta-analysis provides a detailed quantitative summary of available high-quality observational data on the association between CO and TGCT, including observations that can no longer be made due to trends toward younger age at CO repair and increasing TGCT incidence. Results of subgroup analyses indicate possible future directions in understanding both stratification of TGCT risk among boys born with undescended testes and TGCT etiology. Meta analyses addressing features of CO suggest that while bilateral CO is associated with nearly twice the TGCT risk as unilateral CO, data are inadequate to assess the role of anatomic position of the undescended testis. Early repair is associated with lower TGCT risk, but published data do not provide a basis for recommending optimal time of repair or to determine whether optimal repair can reduce risk to baseline.

Therefore, all CO patients and their families should be counseled to be aware of future risk. Additional data are needed to affirm the possibility that TGCT risk is not elevated among boys whose testes descend spontaneously after birth. Results of subgroup analyses addressing features of TGCT suggest multiple pathways to malignancy and indicate considerable heterogeneity in risk of TGCT following CO. Both possibilities warrant mechanistic examination using contemporary tools of molecular biology.

DEDICATION

The authors dedicate this report in memory of the life and work of our esteemed colleague, Dr. Brian E. Hardy.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Cancer_Endocrinology/10.3389/fendo.2012.00182/abstract

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Testicular cancer and cryptorchidism

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The failure of testicular descent or cryptorchidism is the most common defect in newborn boys. The descent of the testes during development is controlled by insulin-like 3 peptide and steroid hormones produced in testicular Leydig cells, as well as by various genetic and developmental factors. While in some cases the association with genetic abnormalities and environmental causes has been shown, the etiology of cryptorchidism remains uncertain. Cryptorchidism is an established risk factor for infertility and testicular germ cell tumors (TGCT). Experimental animal models suggest a causative role for an abnormal testicular position on the disruption of spermatogenesis however the link between cryptorchidism and TGCT is less clear. The most common type of TGCT in cryptorchid testes is seminoma, believed to be derived from pluripotent prenatal germ cells. Recent studies have shown that seminoma cells and their precursor carcinoma *in situ* cells express a number of spermatogonial stem cell (SSC) markers suggesting that TGCTs might originate from adult stem cells. We review here the data on changes in the SSC somatic cell niche observed in cryptorchid testes of mouse models and in human patients. We propose that the misregulation of growth factors' expression may alter the balance between SSC self-renewal and differentiation and shift stem cells toward neoplastic transformation.

Keywords: testis, cryptorchidism, testicular cancer, spermatogonial stem cells, somatic cell niche

INTRODUCTION

Cryptorchidism, or maldescended testes, is a common clinical diagnosis in newborn boys and one of the strongest risk factors for infertility and testicular cancer (Hutson et al., 2010). The position of the cryptorchid testicle may vary and can be located in the abdominal cavity, inguinal canal, or subcutaneous cavity, which could determine the extent of the associated phenotype. While some populations are affected at a higher frequency than others, around 2–4% of boys are globally diagnosed with either unilateral or bilateral cryptorchidism (Barthold and Gonzalez, 2003).

About 10% of all cases of testicular germ cell tumors (TGCT) occur in men with a history of cryptorchidism (Mannuel et al., 2012). Thus, a disruption of a common regulatory pathway, for example, androgen signaling, might be an underlying reason for the association of cryptorchidism and TGCTs. An alternative explanation is that the abnormal testis position itself is directly responsible for infertility and germ cell tumorigenesis. Indeed, the causative role of an abnormal testis position in infertility has been demonstrated in several animal experimental models. The elevated temperature of the undescended testis inhibits the differentiation of spermatogonia resulting in an arrest of spermatogenesis, reduced seminiferous tubule size, germ cell depletion, and fibrosis. The link between cryptorchidism and TGCT is however less clear. The most common type of TGCT in cryptorchidism is seminoma. It is commonly accepted that the precursor cancer cells are pluripotent germ cells. Whether such cells are derived from primordial germ cells (PGCs) also known as gonocytes, that continue to proliferate or undergo improper differentiation (Skakkebaek et al., 1987) or are a result of spermatogonial stem cell (SSC) transformation may be debatable. The abnormal testis position

dramatically alters the function of somatic cells providing the niche for SSC self-renewal and differentiation. We review here the epidemiologic, genomic, and experimental data that might explain the higher incidence and the causes of TGCT in cryptorchidism. Clinical aspects of this disease are beyond the scope of this review and can be found elsewhere (Isidori and Lenzi, 2008).

TESTICULAR DESCENT

During embryonic development, the gonads differentiate from the genital ridges. After completion of sex determination, both ovary and testis remain in a high pararenal position attached to the body walls by a mesenterial ligamentous complex derived from the mesonephric mesenchyme (Hutson et al., 1997; Barteczko and Jacob, 2000). At this stage, the cranial mesonephric ligament and the caudal genitoinguinal ligament (or gubernaculum) connect the gonads to the abdominal wall. The development and reorganization of these two ligaments, along with the differentiation of the epididymis, growth, and orientation of the gonads and reproductive tracts and finally the intra-abdominal pressure, direct the movement of the testis to the scrotum.

The two-stage model of testicular descent (Hutson et al., 1997), distinguishes the transabdominal phase characterized by the descent of the testis into the lower abdominal position, and the inguinoscrotal phase during which the testis moves through the inguinal canal and into the scrotum. During the transabdominal phase, the gubernacular cord and bulb are formed followed by the differentiation of muscle layers around the bulb. In humans, the first stage of testicular descent occurs between 10–15 weeks of gestation (Hutson et al., 1997). Transgenic studies in mice have identified INSL3 as the major factor in transabdominal testicular descent (Nef and Parada, 1999; Zimmermann et al.,

1999; Overbeek et al., 2001; Gorlov et al., 2002; Huang et al., 2012) INSL3 is a small peptide hormone that belongs to the relaxin/insulin-like subfamily. It is expressed in testicular Leydig cells and is first detected right before the onset of testicular descent (Adham and Agoulnik, 2004). INSL3 signals through a G protein-coupled receptor called the Relaxin Family Receptor 2 (RXFP2). Transgenic overexpression of INSL3 in female mice leads to gubernaculum differentiation and ovary descent to a low abdominal position (Adham et al., 2002). Combined with the fact that an androgen deficiency does not affect transabdominal descent, one can assume that INSL3 is the primary peptide responsible for this process. Analysis of mutant gubernaculum development and the comparisons of gene expression in mutant and wild-type tissues performed in our laboratory, indicated that the NOTCH and WNT/beta-catenin cell signaling pathways might mediate the INSL3 effects at the cellular level (Kaftanovskaya et al., 2011). The effect of INSL3 deficiency is multifold; it causes suppression of myoblast differentiation in the muscle layers of the gubernaculum, apoptosis, and reduction of androgen receptor (AR)-positive cells within the base of the gubernaculum, and a failure of processus vaginalis development (Kaftanovskaya et al., 2011).

The second inguinoscrotal stage of testicular descent is clearly androgen-dependent (Hutson et al., 2010; Kaftanovskaya et al., 2012). Suppression of androgen production or AR deficiency has been linked to cryptorchidism in humans and various other species (Bay et al., 2011). Interestingly, an increase in testosterone production in human embryos precedes inguinoscrotal testicular descent (Hutson et al., 2010). The level of serum testosterone peaks at 15–18 weeks of fetal life and declines thereafter, whereas the testis remains in the same position for 5–10 weeks after the completion of transabdominal descent. In addition, several other processes occur during this time. Starting at about 8–10 weeks the scrotum anlage is formed and the floor of the gubernaculum base inverts to become the tunica of the sac-like processus vaginalis peritonei. The muscle layers at the rim of the gubernaculum further develop to become the wall of the cremasteric sac and the testes glide along the newly formed inguinal canal and into the scrotum (Van Der Schoot, 1996). Because the majority of these processes are androgen-sensitive, it is not surprising that many abnormalities that lead to a compromised differentiation or function of stereogenic Leydig cells as well as the failure of androgen signaling result in various degrees of cryptorchidism (Hutson et al., 1997).

A non-functional hypothalamo-pituitary-gonadal (HPG) axis results in hypogonadotrophic hypogonadism. The homozygous mutant mice for gonadotropin-releasing hormone (GNRH, *hpg*), GNRH-receptor (*Gnrhr*), and the LH receptor knockout mouse (*LuRKO*) devoid of LH stimulation, all have impaired inguinoscrotal testicular descent (Klonisch et al., 2004; Pask et al., 2005; Feng et al., 2006). A number of human syndromes related to testicular feminization or genitourinary dysplasia have the hallmarks of androgen deficiency as well (Klonisch et al., 2004). Estrogen-like and anti-androgen compounds have been shown to affect testicular descent through the suppression of the functional activity of Leydig cells and decreased testosterone and INSL3 production (Klonisch et al., 2004). Little

is known, however, about local cell signaling pathways activated by androgen signaling in the developing gubernaculum, scrotum, cranial ligament, and epididymis. The link between inguinoscrotal cryptorchidism detected in the transgenic mouse and in some cases human mutants for several transcriptional factors, such as homeobox genes HOXA10 or HOXA11; Wilms tumor 1 (WT1); ARID domain-containing protein 5B (ARID5B), and androgen signaling in gubernaculum development still need to be determined (Klonisch et al., 2004; Kaftanovskaya et al., 2013).

EPIDEMIOLOGY OF CRYPTORCHIDISM

The prevalence of cryptorchidism varies somewhat from country to country with the highest incidence reported in Denmark (9.0%) compared to that of the lowest, found in Finland (2.4%) (Boisen et al., 2004). Global cryptorchidism rates of around 2–4% are generally accepted (Barthold and Gonzalez, 2003). Several studies reported an increase in incidence during the 1970s and 1980s including Lithuania and the USA (Paulozzi, 1999; Preiksa et al., 2005), however in England rates have been declining since the 1990s (Jones et al., 1998).

Variations in rates of cryptorchidism over the years and also between countries such as the difference between Finland and Denmark, may reflect the impact of many contributing environmental factors. Man-made environmentally used chemicals such as pesticides, phthalates, bisphenol A (BPA), and polychlorinated biphenyls (PCBs) are known endocrine disrupters (Acerini and Hughes, 2006). Pregnant women treated with DES gave birth to boys with a higher incidence of cryptorchidism than non-treated women (Stillman, 1982).

Consumption of liver or smoked products, which have been found to contain a higher concentration of PCB has been implicated in higher rates of cryptorchidism (Giordano et al., 2008). Likewise, maternal consumption of more than five alcoholic drinks a week has been linked to an increase in the risk of cryptorchidism in one study (Damgaard et al., 2007), however disputed by others (Moller and Skakkebaek, 1997; Biggs et al., 2002; Kurahashi et al., 2005).

Similar disputes are also a factor in the contribution of maternal smoking to cryptorchidism. One study found that smoking more than 10 cigarettes a day during pregnancy (Jensen et al., 2007), increased the risk, whereas other studies have found no viable link (Mongraw-Chaffin et al., 2008). The discrepancies in the results of studies looking at maternal dietary and lifestyle factors may be a result of inconsistent data collection methods or inaccuracies from maternal questionnaires.

Given that the inguinal stage of testicular descent is between weeks 26 and 35, premature birth and low birth weight often associated with prematurity are contributing factors to an increased risk of cryptorchidism (Boisen et al., 2004; Jensen et al., 2007). However spontaneous correction is seen more frequently in boys who reach average weight within 1 year after birth, than in those who remain smaller (Preiksa et al., 2005; Jensen et al., 2007). Lifestyle factors that contribute to reproductive birth defects such as cryptorchidism may therefore have an indirect contribution to the formation of testicular germ cells in affected individuals.

GERM CELL TUMORS IN CRYPTORCHIDISM

Testicular cancer afflicts 1% of the male population and is the most common solid tumor to affect young men between the ages of 15–34. The association between cryptorchidism and TCGT has been well documented since the 1940s. Cryptorchidism is an accepted risk factor with a relative risk of 3.7–7.5 times higher than the scrotal testis population (Thorup et al., 2010). Conversely, it has been shown that 5–10% of men who develop testicular cancer, were or are cryptorchid (Thorup et al., 2010). There is an increased cancer risk in bilateral as opposed to unilateral cryptorchidism. Some studies have indicated that there is a direct correlation between how long the testis was subjected to a cryptorchid position and TGCT incidence. This can be seen from the data on surgical correction of cryptorchidism and the reduction of the risk of testicular cancer. One particular study found 13 out of 14 uncorrected cryptorchid patients between 1934 and 1975 developed TGCT in their abdominal testes (Batata et al., 1980). A Swedish group studied almost 17,000 men treated for cryptorchidism between 1964 and 1999 with the average age of surgery being 8.6 years. In this group, 56 individuals developed testicular cancer. Individuals who had corrective surgery before the age of 13 had an incidence rate of 2.23%, whereas those who were treated after 13 had an incidence rate of 5.4% (Pettersson et al., 2007). Based on such data in recent years, the recommended age of surgical correction was reduced and now is usually performed before the age of 2 (Pettersson et al., 2007). It should be mentioned however that some other reports did not find correlation between the time of surgery and risk of TGCT (Hack et al., 2007). In any case, even after early surgical correction the risk of TGCT is somewhat higher in patients with cryptorchidism.

The other factor that appears to play a role in TGCT incidence is the relative position of the cryptorchid testes, and hence the degree of environment insults on the gonads, such as heat. It was shown that an abdominal testis presents a greater risk for TGCT than an inguinal testis (Cortes et al., 2001).

Although corrective surgery has been found to reduce the risk from fivefold to twofold, in some cases the formerly cryptorchid testis becomes cancerous, indicative of permanent epigenetic changes in the cryptorchid testes (Hutson et al., 2010). Indeed, differences in promoter methylations and corresponding gene expression of several genes have been reported in TGCT. Apart from cell transformation such changes might be a result of environmental insults in cryptorchid testis. The other aspect that was extensively studied is the risk of TGCT in normally descended contralateral testes in men with unilateral cryptorchidism. Recent meta-analysis of such data indicated that the TGCT risk factor is much higher in affected testes than in scrotal one (6.33 vs. 1.74) (Akre et al., 2009).

It should be noted, that in many epidemiologic association studies the relative position of testes, age of surgical or spontaneous correction, presence of additional developmental abnormalities, or even variable definitions of cryptorchidism were not always taken into account. However a large majority of data indicates that age of surgery and the relative position of the cryptorchid testis are contributing factors to a greater risk of TGCT.

GENETIC FACTORS IN TGCT

The genetic factors and mutations involved in the potential predisposition to TGCT in the cryptorchid testis are not entirely clear as most studies do not differentiate between individuals with a history of cryptorchidism and those without. Genome-wide studies have identified six susceptibility loci, *KITLG* and *ATF7IP* on chromosome 12, *SPRY4* on chromosome 5, *BAK1* on chromosome 6, *TERT-CLPTM1l* on chromosome 5, and *DMRT1* on chromosome 9 (Rapley et al., 2009; Turnbull and Rahman, 2011). The KITLG-KIT pathway has been implicated in PGC development and is consistent with a role in contributing to TGCT (Kanetsky et al., 2009). SPRY4 a downstream target of the KITLG-KIT pathway and an inhibitor of the protein kinase pathway was linked to TGCT. Similarly, *BAK1* was also associated with TGCT in the same GWAS study. BAK1 is repressed by the KITLG pathway and acts as a germ cell apoptosis promoting factor by binding to the apoptosis repressor BCL2 (Turnbull and Rahman, 2011). Significantly, mice with mutations in *Kitl* genes require a specific genetic background to develop testicular cancer. Mice harboring a mutation of the *Steel* locus, which deletes *Kitl*, bred on a 129 background have a higher incidence of testicular cancer than wild-type controls. These mice also exhibit PGC defects in proliferation, migration, and survival demonstrating a possible link between pluripotent cell differentiation and TGCT (Heaney et al., 2008).

The telomerase encoding TERT locus and its transcription factor regulator ATF7IP are often overexpressed in cancers. Both alleles were associated with a predisposition for TGCT in a UK based whole genome association study (Turnbull et al., 2010). A similar study conducted in the USA, identified two SNPs within the DMRT1 allele – a zinc finger-like DNA-binding motif, significantly linked to TGCT. DMRT1 is expressed in the male gonad during Sertoli cell maturation and the deletion of this gene is associated with gonadoblastoma (Kanetsky et al., 2011).

Other genetic abnormalities were found to be associated with TGCT, however the causative nature of such mutations is not clear. Among these, the alleles of AR gene with short GGN repeats were linked to an increased risk of metastatic disease (Vastermark et al., 2011).

SOMATIC MUTATIONS AND GENE MISEXPRESSION

The role of somatic mutations in p53, PTEN, and other classical tumor suppressor genes in TGCT remains contradictory, however TGCT response to cisplatin-based chemotherapy indicates cancer cell sensitivity to p53 activation (Gutekunst et al., 2011). In some studies, a downstream target of p53, DAPK-1 was hypermethylated in seminomas compared to normal testes and was found to be clinically useful for testicular germ cell tumor stage diagnosis (Christoph et al., 2007). In contrast, a study that looked at 31 primary germ cell tumors found no mutations in p53 however 9 of 14 tumors tested positive with a p53 antibody (Lothe et al., 1995).

Platelet derived growth factors (PDGFs) and their receptors expressed in the pre- and postnatal testis, have also been implicated in testicular tumor formation (Basciani et al., 2010). An aberrant 1.5 kb transcript of the PDGF α-receptor was detected in TGCT and testis parenchyma with carcinoma *in situ* and was completely absent in normal testicular tissue. Moreover, this same aberrant

transcript was detected in biopsies taken from cryptorchid testes containing CIS or germ cell tumor prior to corrective surgery. The expression of this transcript in TGCTs positively correlated with expression of the embryonic transcription factor OCT4/POU5F1. The chromosomal imbalances affecting the region containing *OCT3/4* and *KIT* genes involved in SSC maintenance, were also found in TGCT (Goddard et al., 2007; Gilbert et al., 2011).

Finally, another mouse model of TGCT is related to the overexpression of the growth factor glial cell line-derived neurotrophic factor (GDNF). GDNF is produced by Sertoli cells and targets GFRα1/RET co-receptors expressed by undifferentiated spermatogonia. It is well-established that this factor is crucial for self-renewal of SSCs. A mouse model expressing a full-length human GDNF transgene specifically in spermatogonia, began to develop tumors from 1 year of age (Meng et al., 2001). These tumors expressed the transgene, were derived from early germ cells, were alkaline phosphatase positive, and most closely resembled classical human seminomas.

Thus, several genes involved in SSC self-renewal, differentiation, and apoptosis were linked to TGCT. The association of these factors with cryptorchidism associated TGCT is less clear. It is important to note that none of the mouse mutants with an ablation of these genes exhibited cryptorchidism.

CONCEPT OF TESTICULAR DYSGENESIS SYNDROME

First proposed back in 2001 (Skakkebaek et al., 2001), Testicular Dysgenesis Syndrome (TDS) suggests an existence of a developmental disorder resulting from a disruption of embryonic programing and gonadal development during fetal life. By definition it can be manifested as one or any combination of any of the four of the following developmental abnormalities: cryptorchidism, hypospadias, testicular cancer, and reduced semen quality. The current understanding of TDS includes both the hypothesis of a common environmental cause (Sharpe and Skakkebaek, 1993; Sharpe, 2003), as well as the existence of a common genetic factor responsible for all four abnormalities.

Some groups have reported an increase in the occurrence of cryptorchidism, hypospadias, infertility, and testicular cancer and several epidemiological studies indeed have shown an association of these symptoms. Skakkebaek et al suggested a role for an endocrine disruptor, responsible for the decline in male reproductive health (Boisen et al., 2001). During fetal development, such disruptors may have a detrimental effect on Leydig cells which could impair INSL3 or testosterone production, and thus affect gubernaculum development, testicular descent, or cause the malformation of the external male reproductive organs. Hormonal imbalance or direct endocrine disruptor effects on the Sertoli cells may also disrupt germ cell development resulting in infertility or TGCT. Thus, according to the TDS concept its symptoms are due to a common defect that affects a cell signaling pathway involved in a multitude of developmental and differentiation events in males. This might be the case in AR signaling deficiency, which has repercussions in testicular descent, infertility, and the masculinization of the genital tract (Miyagawa et al., 2009). Indeed, both experimental and epidemiologic studies show a link between cryptorchidism and infertility as well as an epidemiologic association between cryptorchidism and testicular cancer. It should

be noted however that individuals presenting three or all four of the symptoms are extremely rare, and are usually associated with a 45X/46XY genotype or some forms of androgen insensitivity. Manifestations of one or two symptoms (such as cryptorchidism and infertility, or cryptorchidism and testicular cancer) are much more common.

In recent years, the common entity hypothesis of TDS has been the subject of debate. The critical evaluation of epidemiologic studies has brought into question the existence of widespread TDS due to the absence of non-casual associations between its different manifestations (Akre and Richiardi, 2009; Thorup et al., 2010). Most affected individuals exhibit one or two features, bringing into question whether one factor may be accountable for the occurrence of the abnormalities with such vastly diverse developmental etiology. On the other hand, the experimental data indicate that causative relationships can explain some of the epidemiologic correlation.

Since the TDS concept was proposed multiple attempts have been undertaken to identify genetic factors responsible for such a syndrome. The most recent genome-wide study investigated genetic variants affecting Danish males with at least one symptom of TDS in an attempt to attribute symptoms to a specific region of the genome. The study identified an association of subsets of these symptoms to genetic factors, in particular between cryptorchidism and testicular cancer (Dalgaard et al., 2012). The analysis highlighted a single nucleotide polymorphism in TGFBR3 mildly associated with all four symptoms of TDS. This gene encodes the TGFβ receptor type III found to be expressed in testicular Leydig cells and peritubular cells and when silenced in mouse, impedes Leydig cell function and normal cord formation (Sarraj et al., 2010). A member of the TGFβ superfamily, BMP7 was found to contain genomic variants in some patients with TDS symptoms, most notably those with cryptorchidism and testicular cancer. Likewise, mutations in the KITLG locus were mostly closely linked to an increased risk of testicular cancer and were not connected to any other TDS symptoms. Mutations in this gene have previously been associated with infertility as well as germ cell tumors (Galan et al., 2006). It should be pointed out however, that currently no mouse mutations in the genes encoding members of BMP/TGFβ signaling are known to cause isolated cryptorchidism.

The identification of a single or pairs of features of symptoms of TDS, as opposed to finding all four symptoms consistently running together, has further fueled speculation that the phenotypes of TDS are not due to a single cause. The occurrence of testicular cancer has increased over the last 40 years in Western countries along with rates of infertility, however rates of cryptorchidism remain unchanged. Hypospadias, most commonly associated with low birth weight, have shown a marginal increase in some studies since the 1950s but show wide epidemiological variation between countries. Interestingly, hypospadias are found isolated from cryptorchidism in 95% of cases and men with hypospadias alone do not suffer from reduced semen quality unless their symptom is accompanied with cryptorchidism. A much greater correlation between an undescended testis and a high risk of infertility or testicular cancer is supported by the alleviation offered by early orchiopexy. With 5% of germ cell tumors arising in previously cryptorchid testes, it

is apparent that many genetic and pathologic factors interplay in each feature associated with TDS (Thorup et al., 2010).

CRYPTORCHIDISM AS THE UNDERLYING CAUSE OF INFERTILITY AND GERM CELL APOPTOSIS

An alternative explanation to TDS, at least, with regard to cryptorchidism, is that the undescended testis itself causes an increased disposition for infertility and spermatogonial arrest, which in some cases may lead to abnormal germ cell differentiation and the formation of TGCT. This notion is strongly supported by the beneficial effects of surgical correction of cryptorchidism on future infertility and cancer risk. Experimental animal data also support this possibility. Induced cryptorchidism in animal models has been shown to lead to a depletion of germ cells, eventually resulting in infertility (Agoulnik et al., 2012), whereas orchiopexy restored spermatogenesis in several cryptorchid mouse mutants (Bogatcheva and Agoulnik, 2005).

The cause of germ cell depletion is strongly linked to the elevated environmental temperature of 37°C within the body cavity compared to the optimal temperature of 32°C for germ cells in the scrotum, although the mechanism behind this cause is not clear (Kumagai et al., 2000; Yin et al., 2002; Izu et al., 2004). Later stage haploid germ cells have been shown to be the most susceptible germ cells when exposed to abdominal temperature.

Apoptosis in the testis is essential for the establishment and maintenance of germ cell populations. In the cryptorchid testis, temperature induced apoptosis is responsible for the depletion of germ cells, however the molecular mechanisms behind this have not been fully determined. In the p53 knockout model, apoptosis is delayed by 3 days, from day 7 to 10 in the cryptorchid induced mouse, compared with the wild-type cryptorchid group. Thus it can be deduced that p53 is responsible for the initial phase of apoptosis in the germ cells (Yin et al., 1998). However, the initiation of apoptosis from day 10 is indicative of a p53-independent pathway after the initial phase of germ cell loss. In p53$^{-/-}$ and lpr/lpr double mutant mice, apoptosis is further delayed compared to the cryptorchid control group, showing that Fas is responsible for the later stage germ cell loss (Yin et al., 2002). In addition, the occurrence of testicular apoptosis despite the delay, suggests that a third pathway is activated in the cryptorchid testis.

SOMATIC CELL CHANGES IN CRYPTORCHIDISM

Spermatogonial cells are less affected by higher temperatures than all other germ cells and in the cryptorchid testis they survive the longest, however with time they are also eventually depleted. The question then arises, why do the spermatogonia cells decrease in the cryptorchid testis? Can it be linked to the changes in the somatic cell niche in seminiferous tubules?

The abnormal effects of the intra-abdominal environment in cryptorchid testes are not limited only to germ cells; somatic cells have also been shown to display dramatic changes in morphology, function, and gene expression. Sertoli cell vacuolization and abnormal cell adhesion are some of the most common consequences of cryptorchidism. Sertoli cells in an induced cryptorchid testis of the Rhesus monkey were found to have significant changes in the cytoskeleton (Zhang et al., 2004). Reactivation of cytokeratin 18 in adult Sertoli cells and increased expression of vimentin with

disorganized staining characterized the heat stressed Sertoli cells in these primates with similar findings in the Sertoli cells of experimentally induced cryptorchid testes in rats (Wang et al., 2002).

In another study, rats with induced cryptorchidism showed disrupted actin filaments in the basal junction regions of the Sertoli cells compared to the regular lattice structures seen in the control rats (Maekawa et al., 1995). The morphological changes in the somatic cells of cryptorchid testes were also accompanied by changes in multiple genes' expression of these cells.

In mice, Ribosomal Binding Protein Motif 3 (Rbm3) expression in adult Sertoli cells was reduced within 12 h of induced cryptorchidism (Danno et al., 2000). Likewise, the expression of FSH and ARs was dramatically reduced in cryptorchid lambs (Monet-Kuntz et al., 1987). While surgical correction restored their expression, up to 50% of seminiferous tubules did not recover, indicative of cryptorchidism-induced permanent damage.

Hadziselimovic et al. (2011) looked at differentially expressed transcripts in testis RNA isolated from patients with cryptorchidism with or without type A$_d$ spermatogonia (A dark spermatogonia are believed to be SSCs in human) and therefore at a low or a high risk of developing azoospermia later in life. In the latter group, a number of genes involved in A$_d$ spermatogonia self-renewal were not expressed, but were expressed in patients with a low risk and in the control groups. These factors included FGF9 which is essential for SSC self-renewal and acts as an inhibitor of meiosis through regulation of pluripotent genes, and FGFR3, expressed in prepubertal spermatogonia in the control and completely absent in the high risk group (Hadziselimovic et al., 2011).

Disruption of the blood-testis-barrier (BTB) enforced by Sertoli cells has also been reported in cryptorchid mice by breakdown of inter-Sertoli cell tight junctions which may have repercussions on the later stage germ cells. It was recently shown that cystic fibrosis transmembrane conductance regulator (CFTR) which enhances Sertoli cell tight junctions is significantly downregulated in cryptorchid testes and may contribute to the disruption of the BTB (Chen et al., 2012). This was also supported by the discovery that incubation of primary Sertoli cell cultures at 37°C, results in a decrease of CFTR expression compared to those incubated at 32°C. Breakdown of the BTB in both cases was shown by diffusion of injected tracker dye into the interstitial space (Chen et al., 2012). The physiological separation between pre and post meiotic germ cells, maintained by Sertoli cells in the testis, ensures that regulatory products are targeted to specific germ cell populations. Disruption of their cellular morphology, BTB, and expression of growth factors may lead to the misregulation of germ cells and abnormal differentiation, potentially leading to the formation of TGCTs.

ORIGIN OF TGCT

The TGCT most commonly associated with cryptorchidism is seminoma. It is generally accepted that the classical seminomas develop from a precursor lesion, intratubular germ cell neoplasia (or carcinoma in situ, CIS). It is proposed to develop in utero from PGCs or early gonocytes (Skakkebaek et al., 1987). After remaining quiescent during infancy, the CIS is thought to proliferate at puberty and later progress to an invasive disease

under the influence of factors such as gonadotrophins and/or testicular steroids (Skakkebaek et al., 1987). Contrary to the classical seminoma, the spermatocytic seminoma is believed to derive from differentiating spermatogonia. Recent data however showed that CIS and spermatocytic seminoma share a number of common markers with embryonic stem cells, for example, KIT, OCT3/4, SOX17, LIN28, NANOG, FGFR3, DMTR1, and others (Looijenga et al., 2003; Rajpert-De Meyts et al., 2003; Almstrup et al., 2004; Houldsworth et al., 2006; Gillis et al., 2011; Ryser et al., 2011). Thus, according to this hypothesis, expression of stem-related markers is a reflection of their origin from gonocytes. It should be pointed out however, that the expression of some of these genes is maintained and characteristic for adult SSCs (Waheeb and Hofmann, 2011).
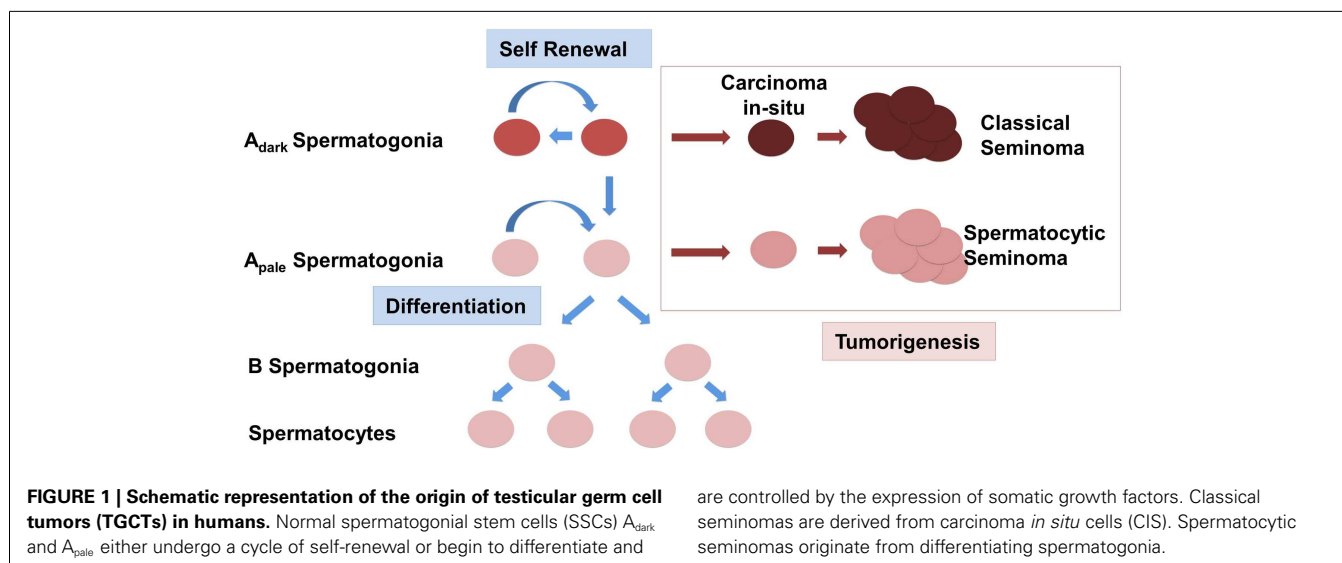
The alternative theory of CIS transformation initiated during meiosis postulates that the common duplication of chromosome 12p in TGCT occurs due to abnormal recombination during meiosis (Chaganti and Houldsworth, 1998). The amplification of genes in this genomic region including *NANOG*, *DPPA3*, *GDF3* may provide a selective proliferation advantage and subsequent reactivation of a stem-like phenotype (Houldsworth et al., 2006).

It is difficult to test experimentally which model is correct. One indirect approach is to evaluate the incidence of CIS as a precursor TGCT in cryptorchid boys. If CIS is derived from embryonic gonocytes then the CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid patients with intra-abdominal testes, abnormal genitalia, and/or abnormal karyotype, but no cases were found in any of the 1281 cryptorchid patients without these additional characteristics. The absence of neoplasm detection in patients with isolated cryptorchidism might therefore indicate that CIS and TGCT are derived from adult germ cells.

The SSC niche is tightly regulated between proliferation and differentiation by factors supplied by testicular somatic cells. GDNF acts upon the spermatogonial receptors RET and GFRα1. GDNF is critical for self-renewal; overexpression of GDNF in mouse spermatogonial cells leads to the formation of germ cell tumors (Meng et al., 2001), whereas a GDNF knockout model results in an absence of germ cells (Naughton et al., 2006). Activation of GDNF and RET/GFRα1 complex leads to the activation of two signaling pathways, which in turn up-regulate the transcription factors MYCN (Braydich-Stolle et al., 2007) and FOS (He et al., 2008). GDNF has also been linked to the upregulation of FGF2 and it was demonstrated that the addition of FGF2, along with GDNF and GFRα1 to a serum-free culture of SSCs, provided the optimal conditions for SSC colony number (Kubota et al., 2004). Long-term survival and proliferation of SSCs in culture have been tested with different concentrations and complements of growth factors. High concentrations of leukemia inhibitory factor (LIF) and FGF2 were found to have a detrimental effect on the colonization of SSCs (Kubota et al., 2004). KITLG secreted by Sertoli cells and also found to be mutated in some seminomas, facilitates the differentiation of spermatogonial cells (Pellegrini et al., 2008). In addition, colony-stimulating factor (CSF) secreted by Leydig cells and its receptor CSF1R located on undifferentiated spermatogonia have also been implicated for the sufficient proliferation of spermatogonia (Kokkinaki et al., 2009).

Changes in the expression of somatic factors in cryptorchid testes in humans have been detected using whole genome studies as previously mentioned, and interestingly, many of these differentially expressed genes such as NY-ESO, FGFR3, UTF1, and DSG2 are aberrantly expressed in seminomas (Waheeb and Hofmann, 2011). One study found that ERK1/2 – an intermediate of the GDNF pathway that leads to activation of FOS and other target genes, was increasingly phosphorylated in more than half of 26 seminomas potentially altering expression of downstream targets of the RAS/ERK1/2 pathway including FOS and ATF (Goriely et al., 2009). Taken in conjunction with the finding that GDNF overexpression in mice leads to the formation of seminomas in advanced age, it is conceivable to suggest that changes in somatic cells in the testis can lead to the deregulation of the SSC somatic niche (Clark, 2007; Kristensen et al., 2008). Instead of self-renewal and differentiation, SSCs in this scenario will be preferentially forced into the differentiation pathway (**Figure 1**). In an abnormal

FIGURE 1 | Schematic representation of the origin of testicular germ cell tumors (TGCTs) in humans. Normal spermatogonial stem cells (SSCs) A$_{dark}$ and A$_{pale}$ either undergo a cycle of self-renewal or begin to differentiate and are controlled by the expression of somatic growth factors. Classical seminomas are derived from carcinoma *in situ* cells (CIS). Spermatocytic seminomas originate from differentiating spermatogonia.

cryptorchidism-induced somatic niche environment most of the differentiating germ cells will be eliminated due to arrest of spermatogenic differentiation, lack of proper cell signaling, abnormal cell junction, and increased apoptosis. The depletion of the SSC pool will eventually lead to the complete loss of germ cells in the cryptorchid testis, which would explain the infertility in patients with surgical correction performed later in life. Accumulation of additional mutations or chromosome rearrangements, including amplification of chromosome 12p, might provide a selective growth advantage for SSC or differentiating germ cells. Instead of the spermatogenetic pathway the cells can now escape into a pre-neoplastic state. Depending on the differentiation stage when such an event occurs, this might give rise either to CIS further progressing to a classical seminoma or the development of a spermatocytic seminoma, derived from more differentiated spermatogonial cells. This scenario does not rely on a common factor causing two seminoma phenotypes with dramatically different etiology, but rather defines cryptorchidism only as an independent causative risk factor in the development of TGCT.

CONCLUSION

Testicular cancer is accountable for 1% of all cancers in men and is the most common in men between the ages of 15 and 34. The risk of developing testicular cancer due to a cryptorchid testis is increased to 5–10 times that of the general male population. This increased risk for a cryptorchid or previously cryptorchid individual is indicative of long-term damage, despite early orchiopexy in many cases. Very little is known about mechanisms of TGCT tumorigenesis and at present there is no animal model that develops testicular cancer as a result of an undescended testis phenotype. Gene expression studies on cryptorchid patients and animal models have indicated that growth factors known to be important for the balance of self-renewal and the proliferation of germ cells are deregulated. The aberrant testicular environment also has a detrimental effect on Sertoli and Leydig cells that may lead to an inability to support the stem cell population. Accumulation of mutations in the somatic cells may lead to misexpression of important growth factors and morphological breakdown. Consequently, in the cryptorchid testis, an alternative differentiation pathway for SSCs is proposed which can result in the formation of TGCT. Identification of new markers specific for fetal germ cells and SSCs in human patients will help to delineate the origin of TGCT. The detailed analysis of changes induced in the SSC somatic cell niche by cryptorchidism and the fate of SSCs will be crucial for our understanding of the link between cryptorchidism and TGCT.

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