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INVITED REVIEW

Germ Cell Biology

Etiology and early pathogenesis of malignant testicular germ cell tumors: towards possibilities for preinvasive diagnosis

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Malignant testicular germ cell tumors (TGCT) are the most frequent cancers in Caucasian males (20–40 years) with an 70% increasing incidence the last 20 years, probably due to combined action of (epi)genetic and (micro)environmental factors. It is expected that TGCT have carcinoma *in situ* (CIS) as their common precursor, originating from an embryonic germ cell blocked in its maturation process. The overall cure rate of TGCT is more than 90%, however, men surviving TGCT can present long-term side effects of systemic cancer treatment. In contrast, men diagnosed and treated for CIS only continue to live without these long-term side effects. Therefore, early detection of CIS has great health benefits, which will require an informative screening method. This review described the etiology and early pathogenesis of TGCT, as well as the possibilities of early detection and future potential of screening men at risk for TGCT. For screening, a well-defined risk profile based on both genetic and environmental risk factors is needed. Since 2009, several genome wide association studies (GWAS) have been published, reporting on single-nucleotide polymorphisms (SNPs) with significant associations in or near the genes *KITLG*, *SPRY4*, *BAK1*, *DMRT1*, *TERT*, *ATF7IP*, *HPGDS*, *MAD1L1*, *RFWD3*, *TEX14*, and *PPM1E*, likely to be related to TGCT development. Prenatal, perinatal, and postnatal environmental factors also influence the onset of CIS. A noninvasive early detection method for CIS would be highly beneficial in a clinical setting, for which specific miRNA detection in semen seems to be very promising. Further research is needed to develop a well-defined TGCT risk profile, based on gene-environment interactions, combined with noninvasive detection method for CIS.

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INTRODUCTION TO GERM CELL TUMORS AND ITS CLASSIFICATION

Germ cell tumors (GCT) are a heterogeneous group of neoplasms that most frequently occur in the gonads, both testes and ovaries.¹ GCT may also rarely occur at specific extragonadal sites along the midline of the body, such as the pineal gland-hypothalamic region, mediastinum, retroperitoneum, and sacrum, probably as a consequences of the migration route of the primordial germ cells (PGCs), being the cells of origin of gametogenesis during early embryogenesis.^{2–4}

Historically, GCT were based on histological classification only.^{5–8} However, in 2005, Oosterhuis and Looijenga⁹ proposed a classification system based on site of presentation, age of the patient at diagnosis, histological composition, pattern of genomic imprinting, and chromosomal constitution. This classification system includes five categories of GCT, which has been adopted by specialized pathologists and the World Health Organization (WHO).^{10,11} The first group (type I) consists of benign teratomas and malignant yolk sac tumors (YSTs), predominantly diagnosed in neonates and infants, with an annual incidence of about 0.12 per 100 000. The per definition malignant

seminomas and nonseminomatous GCT (type II) are the second group, which is the most common form of GCT, with an annual incidence of about 6.0 per 100 000. The third group is spermatocytic seminoma (type III), which usually affect men \geq 40 years of age with an incidence of approximately 0.2 per 100 000 per year. The last two groups are type IV (dermoid cysts, mainly the ovary) and type V (the hydatidiform mole in fertile woman).

In this review, we will focus on type II GCT of the testis (i.e. testicular germ cell tumors [TGCT]), which have their origin in a blocked maturation of a PGC.^{10,12} As indicated, the type II TGCT can be divided into seminoma (SE) and nonseminoma (NS). About 50% of all TGCT are seminomas, with a median age of patients at diagnosis of 35 years. Nonseminomas develop earlier in life, with a median age at diagnosis of 25 years. The latter can contain different histological tumor components, that is, the stem cell component embryonal carcinoma, teratoma (somatic differentiation), choriocarcinoma (extra-embryonic differentiation), and YST.^{5,13} The nonseminomas account for 40% of cases. The remaining group consist of both seminoma and nonseminoma components and occur at an intermediate age.

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TYPE II TESTICULAR GERM CELL TUMORS: INTRODUCTION

The malignant TGCT, also known as germ cell cancers, are the most frequent malignancy in Caucasian males between 20 and 40 years of age. Overall, TGCT account for approximately 1% of all solid cancers, but in young men, it is almost 60%.^{6,9} In the last 20 years, there has been a 70% increase in the incidence of TGCT,¹⁴⁻¹⁶ probably due to the action of (micro)environmental factors in relationship with (epi)genetic constitution,^{15,17,18} to be discussed in detail later. Annually, about 12 000 new cases of TGCT are diagnosed in Europe.¹⁹

It is currently accepted that the majority, and possibly all, type II TGCT (i.e. the seminomas and all histological elements of the nonseminomas) have carcinoma *in situ* (CIS) as common precursor.²⁰ However, this has not been proven so far, which also accounts for the possible presence of CIS without progression to a full blown cancer. CIS is also referred to as intratubular germ cell neoplasia unclassified (IGCNU, WHO definition) and testicular intraepithelial neoplasia, but throughout this review, the term CIS will be used in accordance with most literature.⁹ CIS originates from an embryonic germ cell, either a PGC or a gonocyte (i.e., a PGC located in the genital ridge/undifferentiated and bipotential gonad), blocked in its process of maturation.²¹ After puberty, CIS has a high risk to progress into an invasive cancer, shown to be 70% in 7 years, and assumed to be up to 100% after 10 years.^{22,23}

The overall cure rate of TGCT at 5 years, based on surgical interventional and depending on stage, combined with irradiation and/or chemotherapy,^{24,25} is more than 90%, even in case of presence of metastatic disease. However, due to treatment resistance of the cancer in some patients, TGCT-related death is the second cause of death in men between 15 and 45 years. In addition, men surviving TGCT can present long-term side effects of systemic cancer treatment, such as chronic fatigue,^{26,27} cardiovascular disease,²⁸ metabolic syndrome,^{29,30} infertility,^{31,32} and even second cancers.³³⁻³⁶

In contrast to the survival rates of patients with TGCT, the cure rate of CIS is 100%.³⁷ Local treatment is sufficient to effectively eradicate CIS, that is, a low doses of testicular irradiation³⁸ or orchidectomy. The consequence of this local therapy can be infertility and hypogonadism, especially in case of bilateral disease or monotestis.³⁹ For these patients, semen preservation must be performed in advance and hormonal support may be indicated after this local treatment. Contrary to patients with an advanced stage of TGCT, men diagnosed and treated for CIS without an invasive component continue to live without long-term side effects of systemic treatment.

Early detection of CIS is however difficult due to lack of symptoms and specific markers for screening.³⁷ A testis biopsy is currently the only reliable method to diagnose CIS of the testis. So far, it is proven to be difficult to develop a noninvasive test for CIS in men with an increased risk of TGCT, which can be used for screening purposes and case finding.⁴⁰ Defined risk factors for TGCT are cryptorchidism,^{41,42} testis atrophy,⁴³ infertility,^{44,45} a history of unilateral TGCT,^{46,47} and familial predisposition.^{48,49} However, many of the men having one or more of these risk factors will never develop a TGCT. Furthermore, many of the patients diagnosed with TGCT lack one or more of these risk factors. Therefore, these risk factors are not found to be highly informative on an individual level and it must be concluded that these risk factors alone are not specific enough to be used for screening of TGCT. In recent literature, various environmental factors may also result in a higher risk for TGCT,^{50,51} but the role of these environmental factors is still unclear. They may play a role in the early development of CIS or in the transition of CIS into TGCT. In addition, a number of recent independent

genome wide association studies (GWAS) have been conducted, indicating an association between a selected number of single-nucleotide polymorphisms (SNPs) and presence of a TGCT.⁵²⁻⁵⁴ Interestingly, the genes likely linked to these SNPs are also known to be involved in early gonadal development and regulation of germ cell survival.

In this review, the early pathogenesis of CIS of the testis and TGCT will be discussed. A better understanding of these early pathogenetic steps, influenced by (micro) environmental and (epi)genetic factors, may help to develop an informative clinical approaches for early diagnosis of CIS, allowing local gonadal treatment and prevention of long-term side effects of systemic treatment.

PATHOGENESIS OF MALIGNANT TESTICULAR GERM CELL TUMORS (TYPE II)**Normal testicular development**

During early embryogenesis, the early germ cells undergo subsequent maturation and differentiation influenced by the micro-environment of these cells.⁵⁵ Understanding these processes is essential to create insight into the mechanisms involved in the earliest events of the pathogenesis of CIS and of the derived invasive lesions, that is, TGCT.

The initial totipotent early germ cells, named PGC, initially start to migrate from their origin in the posterior wall of the yolk sac into the hindgut along the midline of the body in the 3rd week of embryogenesis.^{2,56,57} Thereafter, they move into the mesoderm and travel to the genital ridge.³ The KIT/KITLG (stem cell factor, SCF, c-KIT ligand) pathway is crucial for this migration.^{58,59}

The PGC express a number of specific (embryonic) markers during and early after their migration to the genital ridge (**Figure 1**), which are involved in different biological mechanisms during this period of embryogenesis.^{60,61} Some of these markers, like OCT3/4 (also known as POU5F1),^{62,63} c-KIT,⁵⁸ placenta like alkaline phosphatase (PLAP),⁶⁴ NANOG, SOX2 (only for nonseminoma),⁶⁰ and SOX17,⁶⁵ have diagnostic value for TGCT as well as CIS.⁶⁶⁻⁶⁸

During the 5th week of development, still the pluripotent PGC enters the genital ridge and are now called gonocytes. In the beginning of week sixth of the embryogenesis in the male genomic constitution (XY) Sertoli cells are formed, due to the expression of the transcription factor Sex-determining Region on the Y-chromosome (SRY) and subsequently SOX9 (SRY related HMG box 9).⁶⁹⁻⁷¹ If no functional SRY and the subsequent downstream pathway is activated, no Sertoli cells will be generated, but the precursors will follow the female pathway and become granulosa cells.

The primitive seminiferous cords are formed in the 7th week of embryogenesis, in which the germ cells and Sertoli cells are not yet organized. Subsequently, the germ cells migrate toward the basal lamina of the seminiferous tubules and start to lose the expression of some of the forementioned embryonic markers (i.e. PLAP, OCT3/4, and c-KIT), happening during the 13th week of embryogenesis.^{68,72,73} In fact, OCT3/4 and PLAP disappear completely, while c-KIT can still be detected at a relatively low level.^{61,67} This is in contrast to the markers VASA⁷⁴ and SOX17,⁶⁵ they continue to be expressed after birth and remain even positive throughout life.

At the 13th week of development, the male gonocytes also begin to express Testis Specific Protein on the Y chromosome,⁷⁵ which regulates the normal proliferation of spermatogonia and remain positive when the spermatogonia enter meiotic division.⁷⁶ For a good regulation of the proliferation of the gonocytes and the protection of the cells against apoptosis, the contact between the Sertoli cells and now referred to prespermatogonia is necessary. The expression pattern of proteins on the gonocytes and prespermatogonia changes during embryogenesis

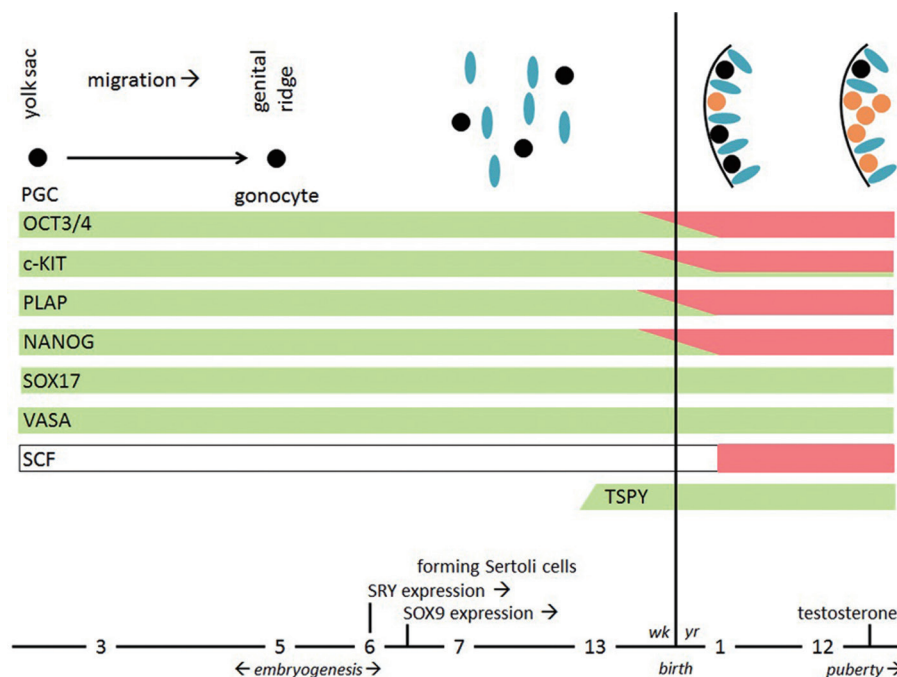


Figure 1: Marker expression in normal and impaired testicular development. Marker expression from early embryogenesis till puberty during the normal and impaired testicular development leading to carcinoma *in situ* (CIS). See text for further explanation. Black sphere: germ cell (not specified for different stages of maturation). Blue sphere: Sertoli cell. Orange sphere: CIS cell. Green bar: normal testicular development (physiological). Red bar: impaired testicular development (pathological).

substantially: the specific embryonic proteins decrease gradually in number and most of them eventually disappear.^{64,65,67,68}

Impaired testicular development and the onset of carcinoma *in situ*

The origin of TGCT probably starts at early embryogenesis, and is hypothesized to be part of the Testicular Dysgenesis Syndrome (TDS).⁷⁷ This syndrome was first described by Skakkebaek *et al.*⁷⁷ and assumes that cryptorchidism, hypospadias, impaired spermatogenesis, and TGCT may all be manifestations of impaired testicular development during early embryogenesis.

This impaired testicular development means that some early PGC/gonocytes are blocked in their process of differentiation, and as such, these germ cells retain their early (embryonic) marker profile.^{66,67,78}

The reason for this development block is not yet entirely clear. It is probably a combination of (micro)environmental factors and (epi)genetic defects.¹⁰ For example, it is believed that xeno-estrogens and anti-testosterones negatively affect the development of Sertoli cells and Leydig cells,⁷⁹ causing a suboptimal environment for germ cell differentiation and leading to development of CIS cells,⁸⁰ which are, as mentioned above, blocked embryonic germ cells. Furthermore, it is assumed that the KIT/KITLG pathway, which regulates the survival, migration and proliferation of PGC/gonocytes, is central to this development.⁸¹⁻⁸³

As indicated, these CIS cells resemble PGC/gonocytes on different aspects.⁷⁸ Both have for example expression of embryonal markers (**Figure 1:** OCT3/4, PLAP, alkaline phosphatase (AP-2γ), c-KIT, *etc.*).^{61,72} Normally, in the time window of about 6–12 months after birth all proteins related to the embryonal stage are disappeared, while VASA⁸⁴ and TSPY remain present.⁷⁵ However, there is on ongoing expression of these embryonal markers in CIS cells after birth,^{68,85} wherein the expression of SCF (KITLG) is likely more selective for the niche of the CIS cells.

In the testis, these CIS cells remain, that is, resistant to apoptosis and maturation, and eventually progress after puberty, likely due to a rise in serum testosterone, into an invasive tumor.^{22,23} The current hypothesis is that all patients with this abnormality will develop an invasive TGCT,⁹ due to the fact that in the male Caucasian population, the incidence of CIS similar is to the lifetime risk of developing a TGCT. However, the exact cause for progression of CIS to TGCT is currently unknown. It has been suggested that loss of PTEN exposure to certain and environmental factors among others play a role.⁸⁶ For the onset of CIS and the development of CIS into an invasive tumor, there is likely an interplay between genetic, epigenetic, and (micro)environmental factors also referred as the environment.^{87,88}

ETIOLOGY OF TESTICULAR GERM CELL TUMORS AND THE ENVIRONMENTAL HYPOTHESIS

Genetic risk factors

Ethnic differences in the incidence and geographical clustering of TGCT suggest a genetic component in the etiology of this disease in addition to possible environmental influences.⁸⁹ African and Asian men have a very low risk of developing TGCT.⁹⁰ Even if these men migrate to a western country they maintain their low risk, which is observed in African-Americans living in the USA for many generations.⁹¹ This shows that the supposed absence of genetic risk factors in African and Asian men, have greater influence than exposure to environmental risk factors on the incidence of TGCT.⁹² This is in contrast to selective Caucasian men migrating to another country with a higher incidence of TGCT. The second generation of these men has the same risk of TGCT as the local men.⁸⁹

Another important argument for a genetic component in the etiology of TGCT is that family history is a strong known risk factor for these malignancies.⁹³⁻⁹⁶ Several studies have shown that the risk for a brother of a TGCT patient is 8–10 fold higher as compared with

the general male population.⁹⁷⁻¹⁰⁴ The risk for a son of a father with a history of TGCT is 4–6 fold higher.^{97-99,101,103} Also twin studies, both mono- and dizygotic twins, have confirmed a strong genetic component to TGCT.¹⁰⁵⁻¹⁰⁸ It is estimated that the genetic effects accounts for 25% of TGCT, which is a high rate in comparison to other cancers and even the third among all cancer types.⁹⁴ Nevertheless most men with TGCT have no family member who also has the disease.

However, familial linkage studies and candidate gene approaches have not been very successful so far in defining genes predisposing to TGCT. An association study of the gr/gr deletion on the Y chromosome,¹⁰⁹ which carries a number of testis- and germ cell-specific genes, demonstrated that this deletion provides an approximately two-fold risk of TGCT. It is however, not clear whether it is an effect specially related to this gene mutation or to the associated infertility, also a known risk factor for TGCT.

Since 2009 there are new genetic insights starting from two TGCT GWAS, one from the UK⁵⁴ and one from the US.⁸¹ Subsequently, several additional TGCT GWAS have been done⁸³ and recently a meta-analysis is performed.⁵² SNPs with significant associations were identified in or near the genes *KITLG* (KIT ligand), *SPRY4* (sprouty 4: sprout-related, EVH1 domain containing 2), *BAK1* (BCL2-antagonist/killer 1), *DMRT1* (doublesex and mab-3-related transcription factor 1), *TERT* (telomerase reverse transcriptase), *ATF7IP* (activating transcription factor 7 interacting protein), *HPGDS* (hematopoietic prostaglandin D synthase), *MAD1L1* (mitotic arrest deficient-like 1), *RFWD3* (ring finger WD domain 3), *TEX14* (testis expressed 14) and *PPM1E* (protein phosphatase, Mg²⁺/Mn²⁺ dependent, 1E). **Table 1** highlights these genes, wherein also their chromosomal localization as well as relevant SNPs are listed.

Single-nucleotide polymorphisms at 12q22 likely related to *KITLG* have the strongest association with the development of TGCT, with a risk greater than 2.5-fold of TGCT per major allele.^{54,81} *KITLG* has been shown to be required for multiple aspects of PGC/gonocyte

development.¹¹² This gene is involved in the KITLG–KIT pathway, which system regulates the survival, proliferation, and migration of embryonic germ cells.¹¹³ Delayed differentiation of PGC/gonocytes and subsequent development of TGCT can be the result of retained activation of this pathway.¹¹⁴

In addition, for TGCT somatic alterations in KIT have been described.^{72,115,116} In 21% of seminomas and 9% of nonseminomas, an increased copy number of the *KIT* gene has been observed.^{117,118} Probably, somatic KIT mutations can occur very early in embryogenesis, because there are known cases of bilateral disease with the same mutation in both testes.^{82,119}

It is suggested that there is a relationship with these genes and infertility.^{2,114,120} The link between cancer and infertility can, therefore, further be investigated in studies of KITLG variation in men with a history of infertility with and without TGCT.

SPRY4, on chromosome 5, is an inhibitor of the mitogen-activated protein kinase pathway, which is activated by the KITLG–KIT pathway.¹²¹ An increased expression of SPRY4 can induce downregulation of the KIT–KITLG pathway.¹²² A risk of 1.5-fold of TGCT per major allele on chromosome 5 loci is described.^{54,81} BAK1, on chromosome 6, binds and then provide apoptosis by antagonizing the apoptosis suppressor activity of BCL2 and other anti-apoptotic proteins.¹²³ In testicular germ cells, expression of BAK1 is repressed by the KIT–KITLG pathway and interaction of BAK1 with anti-apoptotic proteins provides apoptosis in germ cell. Therefore, TGCT susceptibility might also come through BAK1 and its response to signaling through the KIT–KITLG pathway. A 1.5-fold increased risk was identified per major allele for the chromosome 5 and 6 loci.⁵⁴ It is suggested that a positive interaction between the loci on chromosomes 5, 6, and 12 gives a combined risk greater than the sum of the individual risks.

DMRT1 is a transcription factor with a highly conserved DNA-binding motif, which is similar to many vertebrate sex-determination pathways.^{114,124} There is much evidence that this gene is related to testicular determination, differentiation and tumorigenesis.^{114,110} Higher expression levels of DMRT1 are required for testicular differentiation and lower expression levels result in ovarian differentiation.^{111,125,126} DMRT1 deficiency in mice is associated with a high rate of teratomas, so type I TGCT.¹²⁷ In humans seems a lower expression level of DMRT1 more strongly associated with nonseminomas type II TGCT, of which teratoma is one type, than with seminomas.¹²⁶

Telomerase reverse transcriptase encodes the catalytic subunit of the telomerase ribonucleoprotein complex. Telomerase counterbalances loss of chromosome ends, which is normal in actively dividing cells, by extending the TTAGGG telomeric nucleotide repeats.¹¹⁴ Telomere shortening is associated with increased genome instability and neoplasia.¹²⁸⁻¹³¹ TERT is endogenously expressed during embryogenesis, but testicular germ cells are the only normal adult tissue in which telomerase continues to be expressed.^{132,133} Seminomas show high TERT expression, high telomerase activity and long telomeres, as seen in normal testicular germ cells.¹¹⁰ Minimal TERT expression and no telomerase activity are seen at nonseminomas, such as teratomas.^{132,133} This is also confirmed by the fact that SNP identified at this locus were more strongly associated with seminomas than nonseminomas.¹¹⁰ ATF7IP activates the expression of TERT and its associated RNA component, TERC. Knock down of ATF7IP results in a significant decrease in TERT and TERC expression and also in telomerase activity.¹³⁴ In short, both TERT and ATF7IP maintain telomere length and are reactivated in a range of tumor types.

Hematopoietic prostaglandin D synthase is expressed in the early embryonic male gonad in mice and seems to control nuclear localization

Table 1: The TGCT risk-SNPs and their likely related genes

Gene	Chromosome	SNP	References
<i>UCK2</i>	1	rs4657482	Rapley <i>et al.</i> 2009 ⁵⁴
<i>HPGDS</i>	4	rs17021463	Chung <i>et al.</i> 2013 ⁵²
<i>CENPE</i>	4	rs4699052	Rapley <i>et al.</i> 2009 ⁵⁴
<i>TERT</i>	5	rs2736100	Turnbull <i>et al.</i> 2010 ¹¹⁰
<i>TERT/CLPTM1L</i>	5	rs4635969	Turnbull <i>et al.</i> 2010 ¹¹⁰
<i>SPRY4</i>	5	rs4624820	Rapley <i>et al.</i> 2009 ⁵⁴
		rs4324715	Kanetsky <i>et al.</i> 2009 ⁸¹
		rs6897876	Kanetsky <i>et al.</i> 2009 ⁸¹
<i>BAK-1</i>	6	rs210138	Rapley <i>et al.</i> 2009 ⁵⁴
<i>MAD1L1</i>	7	rs12699477	Chung <i>et al.</i> 2013 ⁵²
<i>DMRT1</i>	9	rs755383	Turnbull <i>et al.</i> 2010 ¹¹⁰
			Kanetsky <i>et al.</i> 2011 ¹¹¹
		rs7040024	Kanetsky <i>et al.</i> 2011 ¹¹¹
<i>AFT7IP</i>	12	rs2900333	Turnbull <i>et al.</i> 2010 ¹¹⁰
<i>KITLG</i>	12	rs995030	Rapley <i>et al.</i> 2009 ⁵⁴
		rs1508595	Rapley <i>et al.</i> 2009 ⁵⁴
		rs3782179	Kanetsky <i>et al.</i> 2009 ⁸¹
		rs4474514	Kanetsky <i>et al.</i> 2009 ⁸¹
<i>RFWD3</i>	16	rs4888262	Chung <i>et al.</i> 2013 ⁵²
<i>TEX14</i>	17	rs9905704	Chung <i>et al.</i> 2013 ⁵²
<i>PPM1E</i>	17	rs7221274	Chung <i>et al.</i> 2013 ⁵²

The Single-Nucleotide Polymorphisms (SNPs) with their chromosomal localization that appear to be associated with the presence of TGCT, which is demonstrated in recent independent Genome Wide Association Studies (GWAS). The genes likely linked to these SNPs are also known to be involved in early gonadal development and regulation of germ cell survival. See text for further explanation

of the SOX9 protein.^{52,135} Disruption of HPGDS leads to modification of the phenotype of *apc^{Min/+}* mice.¹³⁶ *MAD1L1* gene encodes MAD1, prevents aneuploidy and maintains genomic stability.⁵² MAD1 is in fact a spindle assembly checkpoint protein that delays the onset of anaphase in the mitotic cell cycle until all sister chromatids achieve proper alignment and microtubule attachment.¹³⁷ Remarkably, the risk allele (C) at the most significant SNP without study heterogeneity, rs12699477, is more prevalent in population of European (29%) than those of African ancestry (8%) in 1000 Genomes.¹³⁸ This might indeed be one of the possible explanations for the intrinsic incidence of TGCT among Caucasians compared to Africans, as well as Asians, as mentioned above. This also indicates that the genomic constitution of these SNPs is dominant over the exposure to environmental compounds. RFW3 is an E3 ubiquitin ligase that controls p53 stability by forming a RFW3-MDM2-p53 complex, thereby protecting p53 from degradation by MDM2 polyubiquitination.^{139,140} The role of p53 in the pathogenesis is of interest in this context as well.

In conclusion, there are identified a number of SNPs for TGCT predisposition within fourteen genes that are biologically plausible candidates for disease susceptibility. This increased understanding of the genetic etiology of TGCT will lead to further improvement in the clinical management of this disease.

Environmental and other risk factors

In recent years, various potential risk factors for TGCT have been investigated, both prenatal, perinatal as well as postnatal. These include a variety of risk factors, such as related to environmental exposures, lifestyle factors, and prenatal characteristics. The knowledge on the impact of these particular risk factors is based on epidemiological research only,¹⁴¹ because there are so far no informative animal models available for TGCT.⁵⁰ Some studies have focused on the etiology of TGCT in utero and early life, because CIS is supposed to develop in that period (as discussed above), and other studies have examined potential risk factors during childhood and adolescence. Overall, the results of these studies are inconsistent,¹⁴² partly because of their small sample sizes, multiple testing, self-report data, and recall bias. Recent meta-analyses have provided insight into the influence of these factors on the development of TGCT.^{50,51,143} In this review only the most important environmental factors in the context of development of TGCT will be highlighted.

The following prenatal risk factors for the development of TGCT have been investigated; maternal exposure to estrogens and nausea during pregnancy (being related to the first), diethylstilbestrol (synthetic estrogen DES), maternal hypertension, preeclampsia, maternal bleeding during pregnancy, maternal smoking, and maternal age.^{17,18,51} No association is found for nausea,¹⁴³ and excess of endogenous hormones (likely estrogen) that can cause nausea, during pregnancy,¹⁴⁴ similarly as for maternal hypertension, preeclampsia, and maternal age. DES, a nonsteroidal estrogen, has been used from the late 1940s to the early 1970s to prevent abortions and pregnancy complications,¹⁴⁵ has been suggested that it can cause TGCT as well, but it remains uncertain so far.¹⁴⁵⁻¹⁴⁷ Maternal bleeding during pregnancy is a risk factor for TGCT with an odds ratio (OR) of 1.33 (95% confidence interval (CI) 1.02-1.73) in a recent meta-analysis.⁵¹ The association between maternal smoking and TGCT is inconsistent.¹⁴⁸⁻¹⁵⁴

Many perinatal factors have been investigated, including cryptorchidism, birth weight, gestational age, inguinal hernia, twinning, and hypospadias. Cryptorchidism is the most defined factor associated with TGCT, with a population attributable risk between 4.3% and 10%.^{143,155-159} Low birth weight was associated with an increased

risk TGCT (OR 1.19-1.34)^{18,143} and gestational age was inversely related to the risk of TGCT.¹⁴³ A meta-analysis of two other perinatal factors, inguinal hernia and twinning, shows OR of, respectively, 1.63 (95% CI 1.37-1.94) and 1.22 (95% CI 1.03-1.44).¹⁴³ Due to the small number of patients who participated in only a few studies no meta-analysis of hypospadias as a risk factor for TGCT could be performed.^{43,160,161} The variables birth length, breast feeding, and neonatal jaundice were not associated in a meta-analysis with TGCT.¹⁴³ Some perinatal factors of the mother were examined and birth order and sibship size were inversely associated with the risk of TGCT.⁵¹ For breech delivery and cesarean, no association with TGCT was found.⁵¹

Various postnatal factors influence the development of TGCT, which are subdivided in direct case-related factors, lifestyle of men, and environmental influences. Most studied case-related factors are age at puberty, body mass index (BMI), height, infertility, history of TGCT, and socioeconomic status (SES). Late age at puberty is associated with a large reduction in the risk of TGCT.¹⁶²⁻¹⁶⁵ The results of studies of BMI are often inconsistent¹⁶⁶⁻¹⁷² as well as height as TGCT risk factor.^{164,167,169,170,172} Infertility is associated with a higher risk for TGCT.^{44,164,173,174} A history of TGCT has a strong relationship with the risk of TGCT.^{46,47,175} SES has been studied in various ways, as well as social class as education, however, the association with TGCT is uncertain.^{163,176-179} The influence of some lifestyle factors on risk of TGCT has also been studied. There is no association with smoking and TGCT risk.¹⁷ The effect of physical activity on the risk of TGCT is unclear.^{160,179-181}

The environmental influences during life on the risk of TGCT developing have been studied extensively, but there are no clear associations between TGCT and environmental exposure so far. Various industrial exposures have been investigated, like working in the paper industry¹⁸²⁻¹⁹³ plastic-related industries^{190,192} and metal industries,^{188,194-197} with different results and no clear association with TGCT. Studies on construction and related occupations,^{187,188,192,193,196,198-200} like construction workers, electrical workers, painters, wood workers, and lumber-jacks, find some with inconsistent results and others with no association. Other jobs that have been studied, like firemen,²⁰¹⁻²⁰⁵ policemen,^{185,196,206,207} and military and related occupations^{187,196,208-215} shows divergent results. In general, no significant results were found in all studies who investigated TGCT risk with pesticides exposures among agricultural workers, pesticides applicators or in occupations associated.^{187,188,193,196,199,216-224}

Other environmental influences that have been studied are magnetic and electric field exposure, organochlorines exposure and living in rural areas. Magnetic and electric field exposure have been investigated in five studies^{193,199,225-227} without a clear association with TGCT. Environmental exposure to organochlorines was studied by using blood samples^{220,223,228-233} or questionnaires,^{220,223} and overall no relation was found. Living in rural areas has been suggested as a surrogate for environmental exposure to pesticides,^{192,193,223,234-236} but the several studies are inconsistent in their results. In conclusion, there are a number of well-defined prenatal and perinatal risk factors for the development of TGCT. The only prenatal factor that indicates a risk for TGCT is maternal bleeding during pregnancy. Perinatal risk factors of the son are cryptorchidism, a lower gestational age, inguinal hernia, and twinning. Birth order and sibship size are the two perinatal factors of the mother who are inversely associated with the risk of TGCT. There is a lack of association of most postnatal factors who could be influence the development of TGCT, which is in line with current hypotheses of the early embryogenic origin of TGCT. Only infertility and a late age at puberty are well-established postnatal factors.

Environmental hypothesis

Current evidence for the etiology of TGCT included genetic and environmental factors as mentioned above, but at this time, these factors separately are insufficient to make a risk assessment for TGCT on an individual level. Probably the combined action of (epi)genetic factors and (micro)environmental factors will lead to the development of TGCT,²²⁹ named as the environmental hypothesis (Figure 2).^{87,88}

EARLY DETECTION OF TESTICULAR GERM CELL TUMORS

Current methods

To date, an open testicular biopsy is still the gold standard for the diagnosis of CIS.²³⁷ A single biopsy of at least 3 mm is usually sufficient to detect CIS,^{238,239} since sensitivity for CIS detection is above 99% provided that at least 10% of tubules contain CIS cells. Contralateral biopsy is not routinely performed in most countries.²⁴⁰ It may be considered in selected cases, such as in men with cryptorchidism, atrophic testes and ultrasonographic abnormalities.

Guidelines on the use of fixatives for testicular biopsies are contradictory on the use of Stieve's or Bouin's solution or formalin.^{241,242} Formalin is commonly used for fixation, despite some shrinkage artefacts, along with at least one solid immunohistochemical marker such as OCT3/4,^{63,243} PLAP,²⁴⁴ AP-2 γ ^{61,245} or c-KIT.²⁴⁶ With the use of these robust CIS markers only in less than 0.5% false-negative biopsies occur,²⁴⁷ as a consequence of a heterogeneous distribution of CIS within the testis²⁴⁸⁻²⁵⁰ or surgical damage of the tissue.

An open testicular biopsy is, however, an invasive procedure with potential complications, although infrequent: overall, 2.8% complications were noted,²⁵¹ like superficial wound infections, intra-testicular hematoma and decline in semen quality secondary to the biopsy. A diagnostic testicular biopsy is nowadays only considered in men at high risk of TGCT. These include men with risk factors for TGCT, like infertility,^{174,252} cryptorchidism,^{43,253} atrophic testes⁴³ and a history of TGCT,²⁵⁴ together with ultrasonographic abnormalities.^{255,256} The most significant abnormalities found on the ultrasound are testicular microlithiasis (TM, Figure 3a), an inhomogeneous

parenchyma (Figure 3b) and solid testicular lesions (Figure 3c).²⁵⁷ TM is defined as all hyperechogenic foci smaller than 3 mm without shadowing irrespective of their number.²⁵⁸ An inhomogeneous parenchyma of the testis is described as a heterogeneous parenchyma with hypo- and hyper-echoic areas and often very small cysts.²⁵⁷ Testicular lesions are presented as focal solid hypo-echoic or hyper-echoic structures inside the parenchyma.²⁵⁷

Use of markers in semen of infertile males and testicular germ cell tumors patients

Efforts have been made to develop a noninvasive method for the detection of CIS cells in semen in the last decades, since these CIS cells are exfoliated from the seminiferous tubules into seminal fluid.²⁵⁹ The first studies were based on cytological examination by plain microscopy²⁶⁰⁻²⁶³ or after immunohistochemistry (IHC) using PLAP²⁶⁴ and IHC with magnetic beads and the M2A antibody,^{265,266} which all proved to be unsuccessful or too laborious. Another approach was to use the aneuploid DNA content in CIS cells as a marker.²⁶⁷⁻²⁷⁰ However, normal semen contains cells with differences in numbers of chromosomal copies, and possible CIS cells could be proved only in a very small window.

After the discovery of novel stem cell-related markers of CIS, including AP-2 γ ,^{271,272} NANOG,²⁷² and OCT3/4,^{272,273} an immunocytochemical assay for the detection of CIS cells in semen was tested again (Figure 4). In contrast to previous studies, which used cytoplasmic markers of CIS, these proteins are localized in the nucleus, and thereby better protected from degradation in semen or during processing of the sample. In these studies, the identification of CIS cells in semen has a high specificity; nevertheless, the sensitivity remained relatively low. Even the double staining method with of immunocytological staining for AP-2 γ or OCT3/4 and rapid cytochemical AP reaction has its limitations, like unspecific cross-reaction and still some false-positive results due to a weak staining of AP-2 γ and OCT3/4 in the epithelial cells of the epididymis, prostate gland, and seminal vesicles.^{274,275}

Most recently, the two cancer-testis antigens MAGE-A4²⁷⁶ and NY-ESO-1, which are expressed by TGCT, were tested in semen by

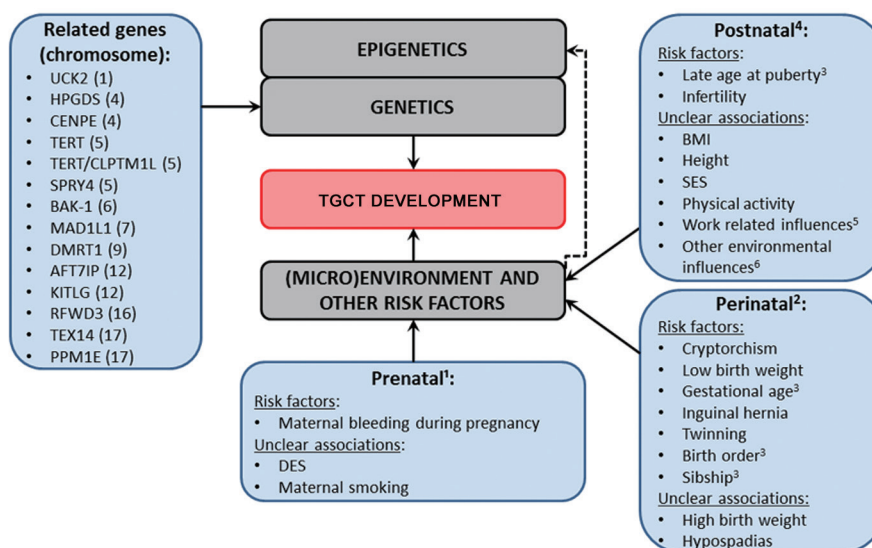


Figure 2: Environmental risk model. The environmental risk model shows the different etiological factors which have an influence on the development of testicular germ cell tumors. ¹No association is found for nausea during pregnancy, maternal hypertension, preeclampsia and maternal age. ²No association is found for birth length, breastfeeding and neonatal jaundice. ³Inversely related. ⁴No association is found for smoking and organochlorines exposure. ⁵Working on the paper industry, construction and related occupations, fireman, policemen and military and related occupations. ⁶Pesticides exposures, magnetic and electric field exposure and living in rural areas.

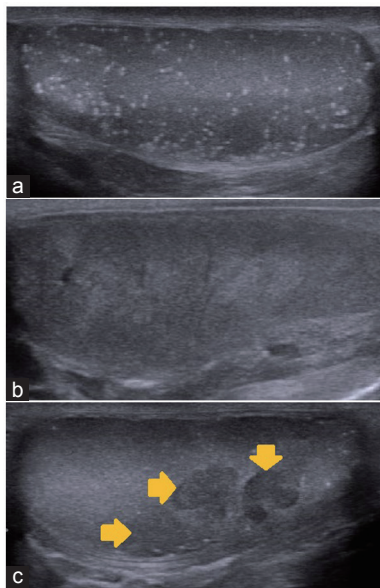


Figure 3: Ultrasonographic abnormalities that may be present in men at risk for testicular germ cell tumors. (a) Testicular microlithiasis in the parenchyma of the testis. (b) Inhomogeneous parenchyma of the testis. (c) Hypo-echoic lesions in the parenchyma of the testis.

immunological staining in TGCT patients and healthy semen donors.²⁷⁷ Although this may seem promising results, there are too much false-positive cells. Moreover, there is only assumed that these markers also are expressed in CIS cells, but this has never been demonstrated in semen of men with CIS.

Despite some progress, none of the above-mentioned semen-based methods for CIS detection are currently used in standard clinical practice. This is due to different causes, like for example the low numbers of CIS cells in seminal samples and degradation of the CIS cells or impaired shedding from the seminiferous tubules. In addition to this, the relatively high number of false-positive results in the most promising immunocytochemical techniques is a problem too. False-positive cells were observed in some patients, probably due to a weak expression of some of the used markers, that is, OCT3/4, AP-2 γ , NANOG, in the epithelial cells of epididymis, prostate and seminal vesicles.²⁷⁸ The limits of these investigated markers are an important reason not to apply these techniques in standard clinical practice. Thus, this field still awaits a noninvasive method for CIS detection, ultra-sensitive and fail-safe, which could be applied to routine screening of population at risk for TGCT.

Detection of microRNA in blood and semen of testicular germ cell tumors patients and infertile males

microRNAs (miRNAs) are small noncoding RNA molecules, with approximately 22 nucleotides in length.^{279,280} In the early 1990s, the first miRNAs were characterized in *C. elegans*,²⁸¹ but their role in biological processes is better understood since the beginning of this age. These relatively small sequences interact with messenger RNA (mRNA) in the mammalian system, and they are involved in the fine-tuning of the translation process from mRNA to protein.²⁸² It has been found that these miRNAs are crucial for normal development, of which some in stem cell formation.^{283,284}

Over 500 miRNAs exist within the human genome²⁸⁵ and different sets of expressed miRNAs are found in various cell types and tissues.²⁸³ It is found that aberrations in regulation of expression of miRNAs can be involved in the development of cancers,^{286–291} so they can act as both

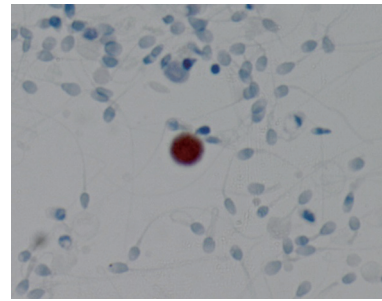


Figure 4: Carcinoma *in situ* (CIS)-cell in semen with OCT3/4 staining. A positive CIS-cell in semen, detected with the immunocytochemical marker OCT3/4.

oncogenes and tumor suppressor genes.^{290,292,293} There is increasing interest in their potential use as biomarkers in various cancer types and other disorders due to their secretion into body liquids, in which they have proved to be very stable.^{294–296}

Recently, it is demonstrated that miRNAs of the miR-371~373 (miR-371-372-373)^{297,298} and miR-302/367 (miR302a, b,c,d/367) clusters²⁹⁹ are overexpressed in all TGCT,³⁰⁰ regardless of patient age, histologic subtype (except teratoma), or anatomic site of the tumor. All main members of the miR-371~373 and miR-302/367 clusters were elevated in the serum and returned to normal after treatment of the disease within only a few days or even less.^{301–303} The hypothesis reported that these miRNAs are not only expressed in all TGCT, but also in CIS.³⁰⁴ Remarkable is that these miRNAs are not up-regulated in other tumor types or disorders.

Clinical management of TGCT is greatly based on serum biomarker monitoring.³⁰⁵ At the moment the markers alfa-fetoprotein, b-human chorionic gonadotropin, and lactate dehydrogenase are used, but only 60% of all TGCT patients have elevations of these markers.³⁰⁶ For this reason, the above-mentioned miRNA are promising candidate biomarkers for disease monitoring and potentially also in the diagnosis of TGCT.^{303,304,307} Possibly, these miRNAs may also be used as screening method of semen in men at risk for CIS/TGCT. To date, no studies have been performed of these TGCT miRNA in semen and further research is required.

However, specific miRNA for infertility in semen has been tested in several studies in infertile population.^{308–313} Gene expression is active during spermatogenesis and miRNAs are differentially expressed during this differentiation period.^{314,315} To understand the role of miRNAs in the different forms of spermatogenic failure, miRNA expression profiles have been studied in normal and infertile testicular tissues in mice.³¹⁶ With this knowledge, several clinical case-control studies were conducted in men and five of these studies have mapped the well-defined expression profiles of miRNAs in semen between normal and infertile men (**Table 2**).^{308–311,317} Each study has made its own choice which miRNAs are tested in the semen and they all made a different case-selection. Therefore, the results of the studies are difficult to compare with each other. Yet, because of these studies, there is sufficient evidence that it is possible to detect in semen specific miRNAs related to infertility.

It can be concluded that specific seminal plasma miRNAs have been explored as potential biomarkers for the diagnosis and classification of male factor infertility. Therefore miRNAs, both for male factor infertility and CIS/TGCT, need to be further investigated in different patient population and follow-up studies.

CONCLUSIONS AND REMARKS

This review focused on the etiology and early pathogenesis of TGCT, its

Table 2: Case-control studies of detection specific miRNAs for infertility in semen

	Cases	Controls ^a	A ^b /C	AO ^c /C	O ^d /C	OA ^e /C	IF ^f /C	NOA ^g /C	OA ^h /A ²
Wang <i>et al.</i> 2011 ³⁰⁸	n=181 ^b	n=68	↓ miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, miR-513a-5p	↓ miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, miR-513a-5p	-	-	-	-	-
Liu <i>et al.</i> 2012 ³⁰⁹	n=86 ⁱ	n=86	-	-	-	-	↑ miR-122, miR-185, miR-193b, miR-297, miR-373, miR-574-5p, miR-1275 ↓ miR-16, miR-19b, miR-23b, miR-26a, miR-100, miR-512-3p	-	-
Wu <i>et al.</i> 2012 ³¹⁰	n=192 ^j	n=96	-	-	Not sign	-	-	↑ miR-19b, let-7b	-
Wu <i>et al.</i> 2013 ³¹¹	n=100 ^k	n=100	-	-	-	-	-	↑ miR-7-1-3p, miR-141, miR-429	-
Abu-Halima <i>et al.</i> 2013 ³¹⁷	n=18 ^l	n=9	↑ miR-141, miR-200a ↓ miR-34b, miR-122	-	-	↑ miR-141, miR-200a ↓ miR-16, miR-34b, miR-34c-5p, miR-122	-	-	Not sign

To date, in 5 case-control studies of infertile men miRNA in semen is investigated. The studies examined different miRNA in semen and also have made a different case-selection. ^aControls (n): fertile controls; ^bA: azoospermia; ^cAO: asthenozoospermia; ^dO: oligozoospermia; ^eOA: obstructive azoospermia; ^fIF: infertile males; ^gNOA: nonobstructive azoospermia; ^hN: 181 – A: 73, AO: 79, O: 34; ⁱN: 86 – IF: 86; ^jN: 192 – O: 96, NOA: 96; ^kN: 100 – NOA: 100; ^lN: 18 – A: 9, OA: 9. ↑: significant upregulated miRNA; ↓: significant downregulated miRNA. miRNA used in different studies: miR-34c-5p, miR-122, miR-16, miR-19b, let-7b, miR-141. Used miRNA per study – Wang *et al.* 2011.³⁰⁸, n=7: miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, miR-513a-5p. Liu *et al.* 2012.³⁰⁹, n=21: miR-574-5p, let-7b, miR-297, miR-122 (1, 2, 5), miR-1275, miR-1281, miR-373, miR-185, miR-193b, miR-145, miR-214, miR-31, miR-455-3p, miR-100, miR-517a, miR-512-3p, miR-16, miR-523, miR-19b, miR-23b, miR-26a. Wu *et al.* 2012.³¹⁰, n=2: miR-19b, let-7b. Wu *et al.* 2013.³¹¹, n=3: miR-141, miR-429, miR-7-1-3p. Abu-Halima *et al.* 2013.³¹⁷, n=6: miR-141, miR-200a, miR-122, miR-34c-5p, miR-34b, miR-16. miRNAs: microRNAs

relation to testicular development and the current and future possibilities for noninvasive early detection methods of TGCT. Currently, the early detection methods for TGCT by the detection of CIS are not well-suited for clinical use, while there are considerable health benefits if CIS could be detected in a noninvasive way. All men diagnosed and treated for CIS only instead of a TGCT continue to live without long-term side effects of systemic treatment, such as chronic fatigue, cardiovascular disease, metabolic syndrome, infertility, and even second cancers. An open testicular biopsy is currently the only reliable method to diagnose CIS of the testis. Therefore, this biopsy is only considered for a specific group of patients, such as in men with cryptorchidism, atrophic testes, and ultrasonographic abnormalities. However, for screening purposes, it is required to develop a sensitive and specific noninvasive early detection method and to compose a well-defined TGCT risk profile.

Key aspects of the etiology of TGCT were discovered in the past few years. Since 2009, a several GWAS have found SNPs with significant associations in or near the genes *KITLG*, *SPRY4*, *BAK1*, *DMRT1*, *TERT*, *ATF7IP*, *HPGDS*, *MAD1L1*, *RFWD3*, *TEX14*, and *PPM1E*. Many of these genes are involved in the early gonadal development, which explains their involvement in the pathogenesis of CIS. Prenatal, perinatal, and postnatal risk factors also influence the onset of CIS. These genetic and environmental factors play an essential role in the pathogenesis of TGCT but are individually insufficient to identify men at high risk for TGCT. Additional national and international collaborative studies, to obtain enough power, into the combined effects of these factors are required to develop a well-defined TGCT risk profile for screening purposes.

Research has been done on a noninvasive method for the detection of CIS cells in semen for many years. Diagnostic methods like cytological examination and IHC with cytoplasmic and nucleus markers, like OCT3/4, AP-2c, NANOG, *etc.*, have been assessed. None of these semen-based methods for CIS cell detection proved to

be sufficiently valid, due to the high number of false positive results. A promising noninvasive method for CIS screening seems to be the detection of specific TGCT miRNAs in semen, because there was demonstrated that miRNAs of the miR-371~373 and miR-302/367 clusters are highly overexpressed in serum in all TGCT and specific miRNA associated to infertility has already been found in semen.

In summary, a screening method for population at increased risk for TGCT is needed to diagnose men at the CIS-stage, such that they do not have to undergo systemic treatment and suffer from the related long-term effects. Further research is needed to develop a well-defined TGCT risk profile, based on environmental interactions, and a noninvasive detection method, in which the miRNA detection in semen seems to be very promising.

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