Charles University

Faculty of Science

Study programme: Special Chemical and Biological Program

Branch of study: Molecular Biology and Biochemistry of organisms

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Testicular germ cell tumor related sperm and testis germ cell pathologies: experimental and clinical angle

Patologie spermií a zárodečných buněk varlat související s germinálním nádorem varlat: experimentální a klinický úhel pohledu

Bachelor thesis

Supervisor: Mgr. Jana Svobodová, Ph.D.

Prague, 2021

Poděkování

Děkuji za odborné vedení práce a za cenné rady své školitelce Mgr. Janě Svobodové, Ph.D. Dále děkuji RNDr. Kateřině Komrskové, Ph.D. za příležitost zpracovávat bakalářskou práci v Laboratoři reprodukční biologie.

Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 1.5.2021

Podpis

Abstract

This bachelor thesis focuses on testicular germ cell tumors (TGCT) and associated sperm and tissue pathologies in the context of different clinical and experimental perspectives. Testicular cancer is the most common malignancy among young men in the reproductive age and the worldwide incidence of testicular cancer is on the rise. This thesis emphasizes the importance of better cooperation between scientific findings from primary research and their clinical implementations in cancer management. The main aim of this thesis is not only to summarize current knowledge of healthy and tumor affected sperm and testes tissue, but primarily define clinical and especially experimental approaches of how to reveal, study and treat TGCT. In order to gain a deeper understanding and insight into TGCT, the data discussed in this thesis comes from the genetic, epigenetic, proteomic, endocrinologic and metabolic fields. More research on the underlying mechanisms of testicular cancer will further improve the quality of life of many young people faced with this diagnose.

Keywords: sperm parametrs, testicular germ cells, testicular tumors, tumor treatment, diagnostic strategies

Abstrakt

Tato bakalářská práce se zaměřuje na patologii spermií a testikulární tkáně asociovanou s germinálním nádorem varlat. Je zahrnut jak klinický, tak experimentální pohled. Germinální nádor varlat je nejčastější rakovinou postihující mladé muže v reprodukčním věku a incidence této rakoviny nekompromisně stoupá. Důraz je v této práci kladen na propojení vědeckých poznatků z primárního výzkumu s klinickou praxí a na přetavení těchto poznatků v nové a efektivní způsoby léčby. Cílem této práce je nejen shrnutí soudobých poznatků o zdravé a postižené tkáni a spermiích, ale hlavně analýza klinických a experimentálních přístupů, které rakovinu studují a odhalují nové možnosti léčby a diagnostiky. Použitá data pochází z několika disciplín, jejichž poznatky práce dává do kontextu a snaží se je propojit. Tyto disciplíny zahrnují genetické a epigenetické studie, studie endokrinologických hodnot a nádorového metabolismu. Další výzkum mechanismů nádorové transformace germinálních buněk je potřeba pro zlepšení kvality života mnoha mladých mužů postižených tímto typem rakoviny.

Klíčová slova: parametry spermií, zárodečné buňky varlat, nádory varlat, léčba nádorů, diagnostické strategie pro hodnocení spermií a tkáně varlat

List of abbreviations

AFP Alpha-feto protein

AOT Acridine orange test

AR Acrosomal reaction

ART Artificial reproductive technologies

ATP Adenosine triphosphate

BPA Bisphenol A

CD Cluster of differentiation

CIS Carcinoma *in situ*

CT Computed tomography

ER Estrogen receptors

FSH Follicle-stimulating hormone

GADH Glyceraldehyde dehydrogenase

GAPDS Glyceraldehyde-3-phosphate dehydrogenase-S

SCSA Sperm chromatin structure assay

STIs Sexually transmitted diseases

TDS Testicular dysgenesis syndrome

TGCT Testicular germ cell tumor

WHO World Health Organisation

GCNIS Germ cell neoplasia *in situ*

GPER G-protein coupled estrogen receptor

hCG Human chorionic gonadotropin

IVF *In vitro* fertilisation

LDH Lactate dehydrogenase

LH Luteinizing hormone

LOH Loss of heterozygosity

MMP Mitochondrial membrane potential

MRI Magnetic resonance imaging

OXPHOS Oxidative phosphorylation

PGC Primordial germ cell

RLGS Restriction landmark genomic scanning

Table of content

1 Introduction

Infertility is a rising problem troubling many couples. According to the National Institute for Health and Care Excellence (NICE) 80% of couples in the UK general population conceive within 1 year, if the female is under 40 years of age and has regular intercourse (2013). Which leaves us with 20% of couples questioning their fertility and some seeking help in fertility centers. The male factor is the cause for infertility in at least 30% of couples undergoing *in vitro* fertilisation in the UK (Karavolos *et al.*, 2013). Male semen and sperm decrease in quality is a growing issue in the western world. There has been a very significant decline in sperm count reported in the last decades (Carlsen *et al.*, 1992; Geoffroy-Siraudin *et al.*, 2012; Levine *et al.*, 2017; Sengupta, Dutta and Krajewska-Kulak, 2017). According to Sengupta the difference in sperm count is almost 60% (2017). But not only the western world is battling this problem, the African continent showed an astonishing drop in the last 35 years as well. In Nigeria the male factor alone makes up to 45% (Ikechebelu *et al.*, 2003).

Infertility is a very complex problem and finding a single cause is nearly impossible. The most common reported causes of sperm damage, inferior semen parameters or sexual dysfunction are: environmental (Carlsen *et al.*, 1995; Jurewicz *et al.*, 2009; Sharpe, 2010), genetic (Ferlin, Arredi and Foresta, 2006; O'Brien, Varghese and Agarwal, 2010), due to an infectious agent (Schuppe *et al.*, 2017) or medication-related (Fusco *et al.*, 2014). Although our understanding of male infertility grew rapidly in the past, the cause of infertility remains idiopathic in 30% of all cases (Cavallini, 2006).

An increase in testicular germ cell cancer (TGCT) has also been documented (Carlsen *et al.*, 1995; Bray *et al.*, 2006; Chia *et al.*, 2010). This may be connected with increasing male infertility and may have a similar cause. Men in infertile couples are 1,6 times more likely to develop testicular germ cell cancer than the general population of men (Jacobsen *et al.*, 2000). When the male factor of infertility is confirmed, men are almost three times more likely to develop TGCT than men in infertile couples without such a confirmation (Walsh *et al.*, 2009). Thus, infertility can be a risk factor although probably not helpful for diagnostics because most men are diagnosed between 15 and 35 years of age, prior to starting a family. Other risk factors linked to both TGCT and infertility are cryptorchism, hypospadia or pathospermia (Carlsen *et al.*, 1995).

Testicular germ cell tumors have incidence of up to 10 in 100 000 men in Denmark (Danckert *et al.*, 2019). Denmark is a region with the highest incidence. In the US the rate of new cases is 5,9 per 100 000 men with white men four times more likely to develop this type of cancer than black men (National Cancer Institute, 2020). 97% of all types are cured (Nur *et al.*, 2008). Germ cell tumors in general make up only 1-2% of all malignancies but it is the most common solid tumor in men between 15-34 years of age (Motzer *et al.*, 2009). Testicular tumors can be categorized as testicular germ cell

tumors (95% of all testicular tumors) and stromal tumors (Barrisford *et al.*, 2015). We further subdivide testicular germ cell tumors into seminomas and non-seminomas based on their histology. Seminomas comprise solely of germ cells. Whereas non-seminomas can be mixtures of various types of cells: embryonic (embryonal carcinoma), extraembryonic (choriocarcinoma, yolk sac tumor), somatic (teratoma), and germ cells as well (Som, Wen and Tu, 2013; Liu *et al.*, 2019). Clinically it is important to know which type of tumor it is, since seminomas are highly sensitive to radiotherapy, while non-seminomas and mixed tumors need a multimodal approach (Liu *et al.*, 2019).

This thesis further explores testicular germ cell cancer, its effect on sperm and testicular tissue, various treatments and the ability to father a child after undergoing these treatments. Additionally, it investigates several experimental methods that detect sperm and testes abnormalities that are currently used. Further studies on this topic are necessary because a broader spectrum of diagnostic markers and better understanding of molecular mechanisms of male infertility is needed for clinical applications. Hopefully, the future will bring new knowledge and methods to improve successful conception for couples battling infertility and for men fighting testicular germ cell tumors.

2 Sperm parameters and testicular tissue

2.1 Sperm parameters

The World Health Organization (WHO) has been publishing laboratory manuals for the examination of human semen since 1980 when a growing need for standardization of procedures could not be overlooked anymore. Since then, it has been updated three times. The last edition (2010) has provided more detail and is more specific in methods so that results from laboratories all over the world can be truly comparable. When semen is collected, there is a number of routinely checked parameters. The macroscopic examination includes: the speed of liquefaction, viscosity, appearance of the ejaculate, semen volume and pH. Microscopic investigation reveals: the presence of other cell types, agglutination and aggregation of spermatic cells and sperm motility. Other assessed categories are sperm vitality, sperm numbers, sperm morphology and a few others. A man with sperm considered to be normal should

Table 1 - Lower reference limit – the 5th centile and its 95% confidence interval.

Parameter	Lower reference limit
Semen volume (ml)	1.5 (1.4–1.7)
Total sperm number (10^6 per ejaculate)	39 (33–46)
Sperm concentration (10^6 per ml)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)

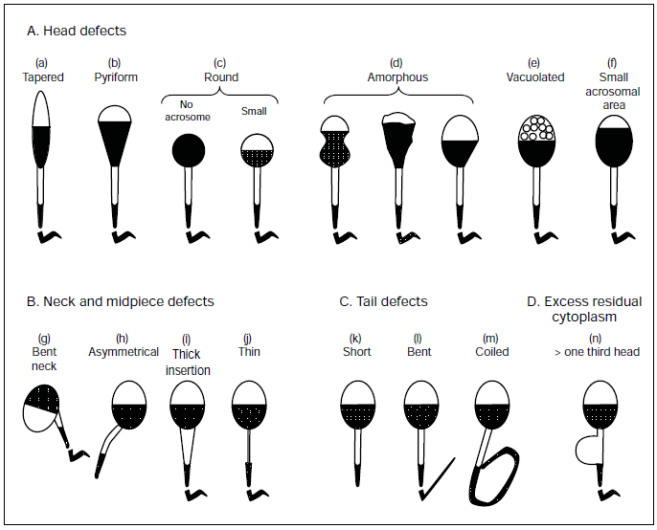
fit into reference limits shown in table 1 (World Health Organization, 2010, p. 224). These procedures are standard, there are many more optional and research procedures. These will be discussed in a different chapter.

2.1.1 Sperm morphology

According to the WHO manual (2010) a normal sperm consists of a head, a midpiece and of tail components. Under examination with a light microscope, spermatozoa should have a visible head and tail including the midpiece and principal piece. All parts of a spermatozoon in a sample that has been stained and processed according to WHO recommendations must be morphologically normal for the spermatozoon to be considered normal. All spermatozoa with slight alternation are called borderline and classified as abnormal.

The head should be oval, and its length should fall within the range of 4.0-5.0 µm. Its width should be 2.5-3.5 µm. The length-to-width ratio must be 1.50-1.75. The acrosomal region should comprise 40-70% of the head area. The midpiece should be thin and about 1 µm wide and 1.5 times longer than the head, attached axially. Cytoplasmic droplets are allowed but should not be greater in size than half a regular head piece. The tail should be approx. 45 µm long. It should be thinner than the midpiece, straight and uncoiled. There must not be any sharp angle that indicates a flagellar break (World Health Organization, 1999). See Figure 1 for a schematic illustration (adapted from World Health Organization, 2010, p. 70). In the last edition of the WHO manual (2010), there are no exact measures given. The head should be smooth, regularly contoured and oval. No vacuoles should be visible in the post-acrosomal region and only up to two small vacuoles are allowed in the acrosomal region. The midpiece can contain residual cytoplasm but not in excess, meaning it exceeds one third of

Figure 1- schematic sperm defects. White part of the head is the acrosome, black is the remaining cytoplasm and nucleus. Neck and midpiece are shown as the white part in between the head and tail. Adapted from World Health Organization, 2010, p.70.



the sperm head size. The tail is assessed the same. Although these are indicative markers for infertility, threshold values for sperm concentration, motility and morphology cannot be used as diagnostic markers (Guzick *et al.*, 2001), therefore there is a need for other more conclusive tools.

2.1.2 Sperm motility, mitochondria, energetic metabolism

Sperm motility is key for male fertility and it is an indicator of sperm fitness. For motility, sperm critically depends on ATP production. To this day, it is not clear whether sperm produces ATP mainly by glycolysis in the sperm head cytoplasm or by oxidative phosphorylation (OXPHOS) in the mitochondrion (Barbagallo *et al.*, 2020). It seems it is a combination of both, and the most successful spermatozoa are able to supply the demand according to its current needs. Glycolysis in the cytoplasm is quicker but inferior in the efficacy of conversion of glucose to ATP (Nascimento *et al.*, 2008). Sperm mitochondria have a different anatomy than mitochondria in somatic cells. They are localized exclusively in the midpiece where they surround the outer dense fibers that are paired with the microtubule doublets of the axoneme. Mitochondria are wrapped around the flagellum helically and are tightened by numerous disulphide bridges. Where the midpiece ends, there is a fibrous sheath that encircles the flagellum further. This part is called the principal piece (Lindemann and Lesich, 2016). The sperm mitochondrion also has certain unique isoforms of proteins involved in electron transport chain or other mitochondrial metabolic pathways (Barbagallo *et al.*, 2020).

There are many studies that link mitochondrial function and sperm motility (Evenson, Darzynkiewicz and Melamed, 1982; Ruiz-Pesini *et al.*, 2000; Marchetti *et al.*, 2002; Wang *et al.*, 2003; Gallon *et al.*, 2006; Paoli *et al.*, 2011). It seems that some specific mitochondrial dysfunctions could be the underlying cause of idiopathic asthenozoospermia (spermatozoa with reduced or no motility) (Ruiz-Pesini *et al.*, 1998). In these studies, mitochondrial function was measured by mitochondrial membrane potential (MMP), which is critical for chemiosmotic gradient required for OXPHOS. Fertilization ability has been associated with sperm MMP (Kasai *et al.*, 2002; Sousa *et al.*, 2011; Marchetti *et al.*, 2012; Vončina *et al.*, 2016). Mitochondria are believed to be very important for sperm motility as many studies show, but cytosolic glycolysis seems to be very important as well. Miki *et al*. (2004) conducted a study on mice where they determined that a central enzyme of glycolysis is necessary for sperm motility and male fertility. In mammalian sperm, there is a unique isoform of glyceraldehyde dehydrogenase (GADH) called glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS). This glycolytic protein is located on the fibrous sheath of the flagellum. Mice with disrupted *Gapds* expression were infertile and motility was decreased, there was no forward progression in the sperm.

More research on sperm energetic metabolism needs to be done. There are many studies having conflicting results regarding this OXPHOS versus glycolysis dispute.

2.1.3 DNA damage and chromatin integrity

Series of mitotic and meiotic changes precedes the formation of a mature spermatozoon. One of these changes is the replacement of somatic histones with transition proteins and the addition of protamines. This structure keeps chromatin in a much more condensed state than in somatic cells, which allows the sperm nucleus to be small and compact. This DNA organization also protects the DNA and provides access to it for the embryo once it starts decondensing. A chromatin structure of poor quality is prone to DNA denaturation and can predict the failure to conceive, sperm DNA integrity is essential for transmission of genetic information. The diagnostic value of sperm DNA damage parameters has been established in evaluating male infertility (Agarwal and Said, 2003).

DNA fragmentation can be caused by many various conditions such as cryptorchidism, cancer, varicocele, age, leucocytospermia and others (Evenson and Wixon, 2006). The environmental factor has a contribution as well. Radiation, medicine (Fusco *et al.*, 2014), air pollution (Rubes *et al.*, 2005), smoking (Sharpe, 2010) or pesticides (Whorton *et al.*, 1977; Recio *et al.*, 2001) and other chemicals can damage sperm DNA. There also is a threat from inside the sperm itself – reactive oxygen species (ROS) – produced by the very active mitochondria. ROS are highly reactive substances such as hydrogen peroxide or superoxide or free radicals that damage the phosphodiester bonds of DNA's backbone. ROS can cause both single- and double- strand breaks and they can alter bases. DNA is protected by its tight packaging and by antioxidants (Agarwal and Said, 2003). There are four major tests for sperm DNA fragmentation: Connet assay, Tunel test, sperm chromatin structure assay (SCSA) and the acridine orange test (AOT) (Evenson and Wixon, 2006). The sperm chromatin structure assay (SCSA) was used in multiple studies and has proved to be a good predictor of infertility having the most stable clinical results. A man is considered subfertile if the number of DNA damaged (measured as DFI – DNA fragmentation index) spermatic cells exceeds 30% (Evenson *et al.*, 1999).

2.2 Testicular tissue

Testis is a paired organ found in the scrotum outside of the body. The scrotum is halved by a septum in two more or less symmetrical chambers in which the testes lay. The scrotum has a lot of smooth muscle that provides thermoregulation, the deepest layer of the scrotum is made of fascia. It is vascularized and innerved (Fietz and Bergmann, 2017). Testes produce not only mature spermatozoa, but it also generates steroid sex hormones (mainly testosterone). A fibrous sheath called *tunica albuginea* surrounds the testis parenchyma. From this sheath there are *septula testes* expanding towards the interior. In the interior these septulas meet and make a proper septum called *mediastinum testis*. The septula meeting in the middle subdivide the testis in approximately 250-350 lobules. Every lobule contains 1-4 seminiferous tubules, where spermatogenesis takes place. These tubules, which are very

condensed and twisted, straighten, and meet with other tubules to form a bigger tubule (*tubuli seminiferi recti*) which then becomes *rete testis*. From the *rete testis* sperm goes into the epididymis (Fietz and Bergmann, 2017). In between these cells, there is the interstitial tissue. This comprises of loose connective tissue, blood and lymph vessels, nerve fibers and some other cells such as Leydig cells. The Leydig cells produce testosterone, small amounts of estrogen and insulin-like factor 3, a marker for Leydig cell differentiation and onset of puberty. They surround blood vessels and are usually found in groups. Other than Leydig cell, the interstitial compartment can contain macrophages, mast cells or lymphocytes, although the testis is an immune privileged organ (Fietz and Bergmann, 2017).

With testicular cancer or other testicular diseases, this anatomy can be disrupted, most often manifesting in a unilateral testicular scrotal mass (Laguna *et al.*, 2019). For the diagnosis of testicular cancer and testicular diseases in general, ultrasound of the scrotum is often used. By ultrasound, location and characteristics of intratesticular lesions can be determined. Tumors appear as lesions of the testicular parenchyma. According to a prospective study by Schwerk *et al*. (1987) ultrasound does not generate any false negative results and therefore is a great tool. Cancers are in approximately 95% of cases hypoechoic in comparison to the surrounding parenchyma (Schwerk, Schwerk and Rodeck, 1987). Although there are some differences between seminomas and nonseminomas on ultrasound, the type of tumor tissue cannot be determined solely by its ultrasonographic appearance. Ultrasonography is also not very accurate in predicting tumor size, neither can it differentiate accurately between benign and malignant lesions (Shtricker *et al.*, 2015). For staging, more imaging methods are commonly used. Magnetic resonance imaging (MRI) and computed tomography (CT) are dominant in the field. MRI is currently used for clinical decisions, preoperative determining of benignity or malignancy or local staging (Liu *et al.*, 2019). CT of the abdomen shows retroperitoneal lymph nodes, where doctors search for metastasis (Thomas *et al.*, 2020).

2.2.1 Spermatogenesis

Understanding spermatogenesis is key in the context of TGCTs. TGCTs are believed to arise from defective gonocyte maturation. During spermatogenesis, a primordial germ cell differentiates into a functional haploid cell (Batool *et al.*, 2019). Spermatogenesis is a very complex process happening in the seminiferous tubules. Many structures, cells and molecules are involved such as: hormones, paracrine factors, genes, epigenetic regulators and many types of cells (Neto *et al.*, 2016). All the seminiferous tubules are situated in a wall of collagen fibers called *lamina propria*. Germ cells and all the stages of their differentiation as well as somatic Sertoli cells can be found in the seminiferous epithelium (Fietz and Bergmann, 2017). Undifferentiated germ cells – spermatogonia – undergo many changes. It starts with the proliferation of spermatogonia, then these differentiate into spermatocytes and divide meiotically to create spermatids. Unmatured spermatids are round and after specialization

become spermatozoa that further go into the testicular tubule. A key type of cells in spermatogenesis are Sertoli cells. These cells support germ cells on their way to the lumen of the tubule. Their shape is irregular and polarized, they are sitting on the basal membrane and their apex is turned towards the lumen. Germ cells are immunogenic and can potentially trigger an immune response and they also need a regulated environment. For these reasons, there is a blood testis barrier. During spermatogenesis there are changes in DNA packaging. 85% of histones are replaced by protamines (Neto *et al.*, 2016).

3 Testicular cancer

Testicular cancer is a rare disease with the highest incidence of 10 in 100000 (Danckert *et al.*, 2019). Incidence of GCTs has been increasing in the last 40 years. Although it is so rare overall, it is the most common type of cancer in men in the age group of 15-34 years (Motzer *et al.*, 2009). This cancer has a great cure rate of up to 97% in the UK (Nur *et al.*, 2008). A testicular germ cell tumor often manifests in a suspicious painless testicular mass in the testes. When this is found, a diagnostic process starts: physical examination, ultrasonography (100% sensitivity), measurement of serum tumor markers (Batool *et al.*, 2019). For lymph nodes metastasis abdominopelvic and chest computed tomography or magnetic resonance imaging is usually performed. Typical serum tumor markers are: human chorionic gonadotropin (hCG), alpha-fetoprotein (AFP) and lactate dehydrogenase (LDH) (Laguna *et al.*, 2019). Other symptoms may be gynaecomastia (abnormal enlargement of men's breasts, appears in 7% of cases), scrotal pain (27% of cases, usually the first symptom). Back pain can be present due to metastasis (11% of cases). Orchiectomy must be performed in all malignant cases of GCTs. In uncertain cases, testicular biopsy is done (Laguna *et al.*, 2019).

3.1 Classification

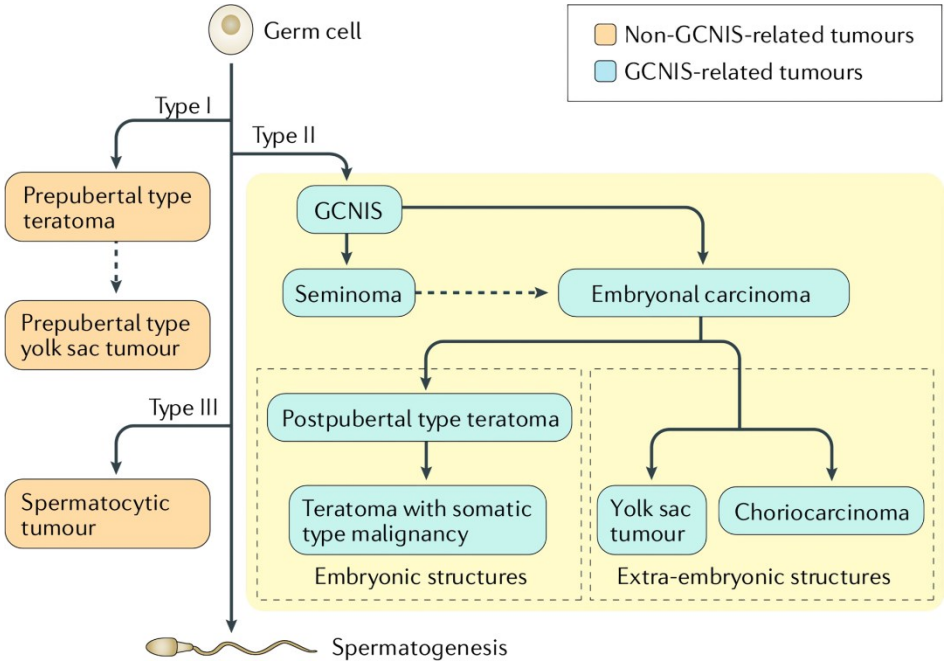


Figure 2 – schematic overview of testicular cancer classification. Author: Cheng et al. 2018.

There are many different types of testicular tumors. This thesis focuses on germ cell tumors, which make up to 95% of testicular malignancies (Barrisford *et al.*, 2015). TGCTs can be further divided in germ cell tumors derived from germ cell neoplasia *in situ* (GCNIS) and germ cell tumors unrelated to GCNIS. GCNIS unrelated tumors occur mostly in prepubertal patients. Germ cell neoplasia *in situ* is the new name for previously used "intratubular germ cell neoplasia" or "carcinoma *in situ*". It has been chosen because it is more precise while combining the older terms at the same time (Williamson *et al.*, 2017).

Germ cell neoplasia *in situ* is the lesion that is considered to be the precursor of adult malignant TGCTs. Based on histology, GCNIS are divided into seminomas and nonseminomas. The lesion is composed of seminoma-like cells with altered nuclei or multiple histological types of cells. These cells are uniformly positive for marker OCT3/4 and both express NANOG (Williamson *et al.*, 2017) which will be talked about later. Seminomas tend to occur later in life, in the fourth or fifth decade. It is composed of a homogenous population of neoplastic gonocytes. Nonseminomas affect a younger age group. They appear in young men in their twenties/ thirties and are typically more aggressive. They are a heterogenous mass of different histological types. Typical cell populations are: teratomas, yolk sac tumors and choriocarcinomas. Nonseminomas can also contain pluripotent cells which make a so-called embryonal carcinoma. Seminomas typically express SOX17 and are negative for SOX2. Embryonal carcinomas on the other hand express SOX2 and not SOX17 (Buljubašić *et al.*, 2018).

3.2 Testicular dysgenesis syndrome

There has been a rise in incidence of testicular cancers, cryptorchidism, hypospadias and subfertility during the last 50 years. It has been proposed that all of these reproductive disorders may be symptoms of one underlying disease, testicular dysgenesis syndrome. Men with undescended testicles have an increased risk of developing testicular cancer (Batata *et al.*, 1982; Giwercman *et al.*, 1987). There are also associations between cryptorchidism and hypospadias and reduced male fertility. The clinical finding in men with TDS vary and are treated by an endocrinologist, andrologist, urologist, oncologist or another clinician. That seems to be the problem in connecting the dots – all the problems are treated separately. For the clinical treatment of the individual diseases, it is important for the clinicians to bear in mind that their patient may have an increased risk of having more than one of the TDS symptoms. Needless to say, not all cases of subfertility, undescended testis and hypospadias are caused by TDS. But most cases of testicular cancer are probably a result of TDS (Olesen *et al.*, 2007).

A lot of cases of male infertility have a genetic background such as: rare complex syndromes, deletions of the Y-chromosome and other genetic disorders affecting the function of the testes. Although this is true, the rise in male infertility cannot be explained by genetics alone (Olesen *et al.*, 2007). TDS may play an important role and the true question with TDS is: could testicular dysgenesis syndrome be an environmental disease? TDS might result from genetic aspects, but recent studies indicate the association between the environment and lifestyle. That is particularly alarming for highly industrialized countries. Xing and Bai (2018) concluded in their review based on previous studies, that TDS is indeed predominantly triggered by environmental exposure and genetics. Lifestyle on the other hand is not so dominant. The exposure to endocrine disrupting chemicals such as phthalates, BPA, synthetic estrogen and others, has also a role in developing symptoms.

3.3 Sperm

Sperm parameters in TGCT patients do not differ significantly from the general population although subfertile men have increased risk of developing testicular cancer (Hanson *et al.*, 2016). Testicular cancer patients have higher DNA damage compared to sperm donors. TGCT patients with abnormal sperm parameters are more prone to DNA strand breaks (Kumar *et al.*, 2018). Patients with a seminomatous tumor have semen of better quality than men with a nonseminoma (Fraietta *et al.*, 2010). Cancer treatment often alters spermatogenesis and sperm parameters; therefore, cryopreservation is recommended before initiating treatment.

3.4 Tissue

Testicular tissue is firstly examined by ultrasound. The clinician should be looking for any suspicious mass, hypoechoic places or testicular microlithiasis. Microlithiasis appears as small and bright echoes. These might be carcinoma *in situ* cells, histological calcium microliths or unusual Sertoli cells with thickened membranes. The clinical decision then depends on circumstances. If the man is otherwise healthy and has no other testicular cancer risk factors, it probably will lead to only annual screenings. If there is a history of testicular cancers in the family or other risk factors, this finding might lead to tissue biopsy (Hoei-Hansen *et al.*, 2007).

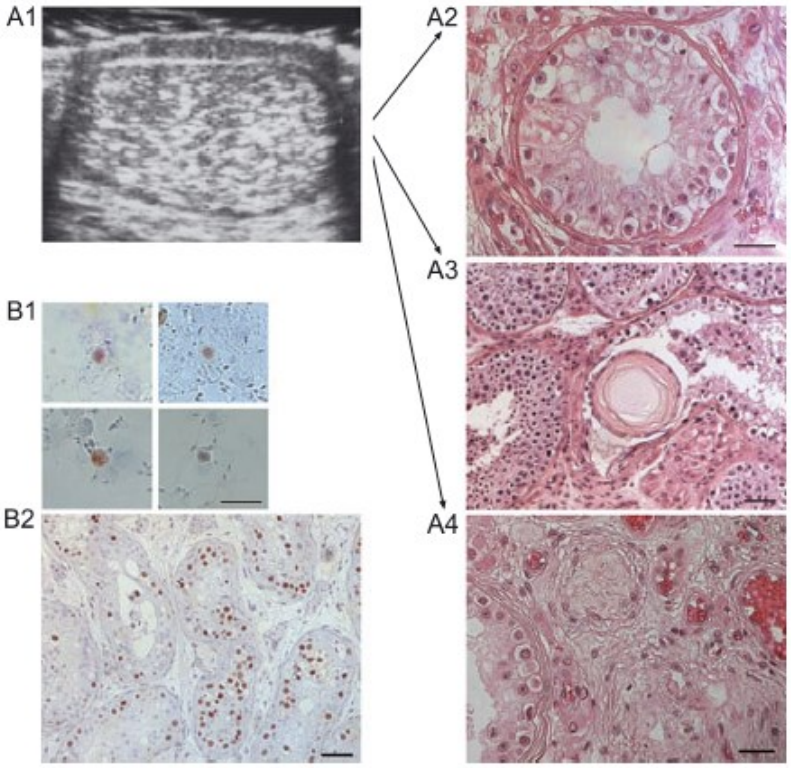


Figure 3 – adapted from Hoei-Hansen et al., 2007. A1) shows an example of an ultrasonic examination. Testicular mirolithiasis is clearly visible – small bright spots. In patients with these findings, other histological features may be found in tissue biopsy. A2) are carcinoma n situ cells – big cells with distinct nucleoles and thickened basement membranes. These appear often in undersized seminiferous tubules. A3) staining shows microliths clearly. Microliths are eosinophilic and therefore appear blueish. A4) shows hyalinized seminiferous tubules without spermatogenesis. B1) is a picture of immunostained CIS cells. Anti-AP-2γ were used. B2) the same staining, clear AP-2γ expression in CIS cells.

Modified Stieve's fixative, haematoxylin and eosin fixation; (B1–2) formalin fixation, AP-2γ staining. Scale bars: A2, A4, B1 = 25 µm; A3, B2 = 50 µm.

The presence of CIS (carcinoma *in situ*, since 2016 this has been abandoned and the term germ cell neoplasia *in situ* is used now) is usually confirmed by an open surgical biopsy. Extracted tissue should be fixed in Stieve's or Bouin's agent and stained with hematoxylin and eosin. Although CIS cells have a characteristic appearance, for the correct recognition of CIS cells, immunohistochemical staining is used – markers OCT3/4 or AP-2γ, which are mentioned later (Hoei-Hansen *et al.*, 2007).

4 Clinical methods of treatment

Testicular cancer is a very rare disease, it makes only 1% of all cancers in men. But in young adults aged 15-34 it is the most common malignancy. The cure rate is very good and still improving

especially with stage I or stage II TGCT (Howlader *et al.*, 2019). Although testicular cancer has a high cure rate, 10-20% of patients with metastatic TGCT ultimately lose their lives (Singla *et al.*, 2019).

Standard treatment of TGCTs leads to removal of one or both testicles – orchiectomy. For patients with stage II and III seminomas there is a combination of the surgical removal and chemotherapy or radiotherapy, while stage I patients can be managed with orchiectomy only, if scanned regularly afterwards. Non-seminomas are a bit harder to manage and all stages are usually treated with both orchiectomy and chemotherapy. Dissection of retroperitoneal lymph nodes is needed in some stages (Jostes, Nettersheim and Schorle, 2019). The "go to" therapy for advanced TGCT is the platinum-based chemotherapy. According to Batool (2019), small interfering RNA therapy, microRNA therapy and immunotherapy are potential strategies for TGCT managing. Developing noncytotoxic therapies has been overall largely unsuccessful. There is a clear need for better understanding of molecular mechanisms lying behind testicular cancer to find new effective approaches for clinical treatment (Singla *et al.*, 2019). Experimental methods, and primary research in general, are very important for clinical applications.

4.1 The effect of cancer treatment on sperm and spermatogenesis

As mentioned, TGCTs have a high cure rate, which is an outstanding achievement of modern medicine. However, the treatment methods can disrupt spermatogenesis which causes temporary or permanent azoospermia. Bearing in mind the young age of the affected men, it is essential to give them the opportunity to cryopreserve sperm. New noncytotoxic treatments which do not affect the fertilizing ability are a glimpse of the future.

Both men who undergo chemo- and radiotherapy show a significant decrease in sperm parameters. Interestingly, after chemotherapy, the biggest drop is seen three months after the treatment whereas with radiotherapy the most relevant decrease is seen 6 months after. In a study with 166 patients divided in two groups depending on the treatment, only 3% of chemotherapy patients and 6% of radiotherapy patients remained azoospermic two years after therapy (Gandini *et al.*, 2006). The same study contradicts the premise, that the recovery of spermatogenesis is a function of pre-therapy sperm parameters. Another link was studied – the connection between therapy dose and the recovery of spermatogenesis. For chemotherapeutic dose, there is no significant difference between two, three or four chemotherapy cycles. For radiotherapy on the other hand, the reduction in total sperm count is associated with the total applied dose. This reduction is usually not permanent but the dose is a predictive factor of the time necessary to recover (Gandini *et al.*, 2006).

With this said, it is currently not possible to predict which patients will remain azoospermic and which will recover fully. We must also bear in mind that the number and morphological parameters of

sperm cells do not necessarily define a fertile man. Cancer treatment can also affect chromatin condensation and DNA integrity in sperm and therefore the sperm might not be able to fertilize an egg.

5 Experimental approaches related to TGCTs

Sperm and testis abnormalities go hand in hand with testicular cancer. Although many other diseases may present with such abnormalities including sexually transmitted diseases (STIs) and other urogenital infections. Experimental methods are key for understanding cell-cell contacts, protein-protein interactions, underlying molecular mechanisms and connected pathologies. The result of this primary research should help with early on diagnosis and better or even personal treatment.

5.1 Proteomics

Proteomics is a very dynamic technology-driven new field. Proteomics has been used in the exploration of human reproduction and fertility and it has given us valuable insight in the physiology of human reproduction and fertility. To this day, more than three decades of using assisted reproductive technologies, the success rate of these technologies is still quite poor. Proteomics promises new diagnostic tools for sperm, egg and embryo selection (Kosteria *et al.*, 2017). Not only artificial reproductive technologies (ART) should be profiting from this knowledge – many groups are trying to find new and more precise biomarkers for TGCTs, which might help in clinics. For the treatment of TGCT patients it is crucial to distinguish between seminomas and nonseminomas. Biomarkers currently used for the diagnosis and differentiation of TGCTs are suboptimal (Milardi *et al.*, 2019).

Currently used serum biomarkers for the diagnosis of cancer include AFP, βhCG and LDH. These biomarkers are insufficient and there is a desperate need for new and more precise tools. AFP and hCG have specificity of 90% but both are relatively low in sensitivity. 40% of men with cancer reoccurrence have normal levels of these markers (Milardi *et al.*, 2019). New approaches are focusing on biomarkers in serum but also other body fluids and components – biomarkers in semen and testicular tissue. To this day, biopsy is needed for the detection of carcinoma *in situ*. On this tissue immunohistochemical staining must be performed. In the light of new research on stem cell-related markers of carcinoma *in situ*, that are located in the nucleus and are protected from degradation in semen and during sample processing, a possibility of semen screening arose. This non-invasive method could be a game-changer. The candidate protein markers are AP-2γ, NANOG and OCT3/4. These are found on neoplastic CIS cells. NANOG seems unsuitable, whereas both AP-2γ and OCT3/4 had specificity at approx. 90%. However, the sensitivity was only around 50% in this arrangement. The downside of this method is difficult detection of the CIS cells, which are present in low numbers in semen (Hoei-Hansen *et al.*, 2007).

5.1.1 Surface and fusion proteins

Many laboratories all over the world are trying to detect the most important surface fusion proteins on gametes. Finding these crucial proteins is important for better understanding of gamete attachment and fusion process and IVF technologies. With artificial insemination there is a need to select sperm. For this selection we need much more information about how to spot "the best" sperm cell possible. Studying sperm-egg interaction on a protein level can help greatly. Tetraspanins were found on the oocyte as well as on spermatozoa and are now under extensive research in the context of gamete contact and fusion ability. Cluster of differentiation (CD) proteins in somatic cells have many functions: cell movement, growth, aggregation, lipid raft formation, and they were found on both the egg and sperm, where their function is debated. They all have four transmembrane domains and together with integrins they have an ability of forming large webs called the tetraspanin webs.

CD9 is known to be present on the whole surface of the egg, while on the sperm it has only recently been studied. This protein forms a complex with two CD81 oligomers, the position of both is in the acrosomal cap in humans. After the acrosomal reaction (AR), CD9 is relocated to the equatorial segment while CD81 disappears from the acrosome and is detectable only in the post-acrosomal region. The placement of CD9 after AR supports the idea of CD9´s importance in sperm-egg fusion (Frolikova *et al.*, 2018). Cholesterol seems to play a crucial role, since it is exiting the membrane during capacitation and the acrosomal reaction heavily and it has been found that the conformation of these two proteins relies on cholesterol. The generated fluidity then enables the formation of a positive curvature to resemble and adapt to the oocyte membrane once the sperm and egg start fusing (Frolikova *et al.*, 2018). Another tetraspanin that might have an important role in gamete fusion as well is CD151. This protein is located onto the inner acrosomal membrane and has the ability to interact with integrins. After the acrosomal reaction, CD151 gets exposed (Jankovicova *et al.*, 2020). The ability of tetraspanins to produce such webs together with integrins that we know from somatic cells and the fact that they are found in the sperm (and the egg as well) makes them the perfect candidates for key fusion components and may be great targets for studying new possible markers for TGCTs.

The protein CD46 is a sperm membrane protein that also might have a significant role in sperm-egg interaction. CD46 is a ubiquitously expressed protein on cell surfaces that protects the cells from autoimmune complement attack. On sperm there is a specific isoform. A connection has been found between the lack of expression of CD46 sperm isoform and male idiopathic infertility (Nomura *et al.*, 2001). By mapping the dynamic of CD46 and β1 integrin subunit location and performing proximity ligation assay an association has been found between the binding pair CD46 and β1 with actin cytoskeleton. CD46 plays an important role in fertilization and maintaining acrosome integrity and stability (Frolikova *et al.*, 2016).

5.2 Genetics

Understanding the genetic background of testicular tumors might improve patient outcomes – especially for patients with poorer prognosis or chemoresistant disease. Testicular tumors are very heterogenous, which makes them hard to study and it is difficult to find correlations in genetics and TGCT development. Inheritance is very clearly visible in TGCTs. Family history is considered to be the biggest risk factor, the increase in this case is up to 10-fold (Singla *et al.*, 2019). No single high penetrance gene has been found in TGCTs. Inheritance is undoubtable, so a variable polygenic model is proposed. TGCTs show very little somatic mutations but very frequent chromosomal anomalies. Chromosome 12p gains are the most common in TGCTs. Some genes on chromosome 12 might play a significant role in the development and pluripotency of TGCTs. Some candidates are: *CCND2, KRAS, TNFRSF1A, GLUT3, REA, NANOG, DPPA3* or *GDF3* (Batool *et al.*, 2019). Some single nucleotide polymorphisms have been associated with familial TGCT risk. Candidate genes are mainly linked to germ cell differentiation pathways including receptor tyrosine kinase gene *KIT/KITLG*, phosphodiesterase *PDE11A* and some others. KIT/KITLG tyrosine kinase system regulates the proliferation and migration of germ cells (Rapley *et al.*, 2009). Although there are candidate genes that may cause TGCT, studies suggest a polygenic model of TGCT development (Rapley *et al.*, 2009; Litchfield *et al.*, 2015). A study by Taylor-Weiner *et al*. (2016) suggests that sensitivity to platinum-based chemotherapy relies on intact *TP53*, reciprocal LOH (loss of heterozygosity) and high mitochondrial priming. Chemoresistant tumors on the other hand accumulate additional reciprocal LOH and lose expression of pluripotency markers and apoptosis regulators. This might be a significant finding for clinical sets.

5.3 Epigenetics

Primordial germ cells (PGCs) arise from the epiblast or from gonocytes that have already settled in the genital ridge. PGCs and gonocytes are undifferentiated embryonic cells which have gone through "reprogramming". This reprogramming erases and reestablishes DNA methylation and an exchange in histone modifications happens in the gestation week six. The demethylation is necessary for the development of correct sex of the child. Due to this methylation erasure, it seems that an incorrect combination of epigenetically activated and inactivated genes in gonocytes may transform these cells into GCNIS. Normal spermatogonia undergo remethylation of genes, whereas GCNIS genome remains unmethylated in adults. GCNIS resemble fetal germ cells as they express many similar transcription factors: OCT3/4, NANOG, KIT above mentioned and some more. All these transcription factors are associated with pluripotency of embryonic stem cells. OCT3/4 is a protein that is critical for the regeneration of undifferentiated embryonic stem cells (Buljubašić *et al.*, 2018). It was shown that transgenerational epigenetic inheritance requires an epigenetic alteration in the germline that include

methylation of cytosines, histone changes and the third regulatory option is the involvement of RNAs. The inability to maintain the hereditary epigenetic pattern can result in diseases such as TGCTs through inactivation of tumor suppressors or activation of oncogenes.

Despite the fact that DNA methyltransferase1 is active in GCNIS cells, these cells have very low methylation levels. Studies have found that GCNIS cells produce proteins that facilitate the demethylation of DNA – AID/APOBECI or BER. In normal male germ cells, after birth DNA gets hypermethylated. Smiraglia et al. (2002) suggested a model where seminomas arise from GCNIS that were globally demethylated whereas nonseminomas result from GCNIS cells with de novo methylation. Using Restriction landmark genomic scanning (RLGS) Smiraglia et al. found that CpG island methylation in nonseminomas is in a normal range seen in other cancers as well (mean 1,11%). But the methylation of CpG islands in seminomas is strikingly low with a mean of 0,08%! The same scientists used two more methods to investigate their hypothesis. Both the Southern blot data and data from the cytosine extension assay support the deficient in methylation in seminomas (Smiraglia *et al.*, 2002). Histone modifications also lead to different results, thus balance in the activity of histone deacetylase and histone acetyltransferase must be established correctly by epigenetics. In many cancers, histone deacetylase is overexpressed which leads to silencing of tumor suppressors. Cells can then proliferate freely and are insensitive to cell apoptosis signals. Epigenetic studies promise a bright future in TGCT managing. Histone deacetylase inhibition is one of the newest approaches in combination with cytoskeleton-interfering molecules or other anticancer compounds. Merging two drugs in one molecule is the ultimate goal of many studies which would help greatly in drug resistance, dosage and drug interference management (Münster *et al.*, 2007; Steinemann *et al.*, 2019). The last mechanism involved in epigenetic regulation of male gamete differentiation is RNA interference. This interference aims to destroy specific mRNAs after transcription, and it is executed by non-coding RNAs (such as microRNAs and siRNAs). It seems that epigenetic regulations are more important for TGCT development than genetic.

5.4 Steroidogenesis

Hormonal imbalance in testicular cancer patients causes secondary endocrine symptoms such as premature puberty or gynaecomastia. In a healthy individual, the gonadotropin releasing hormone (GnRH) is secreted by the hypothalamus and it regulates the release and secretion of gonadotropins – luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Gonadotropins are released from the anterior pituitary gland and these hormones regulate testicular function. Feedback loops for all these components establish homeostasis. Sertoli cells have FSH receptors, Leydig cells have LH receptors and both regulate the synthesis of healthy testosterone levels, which maintain normal spermatogenesis and other testes function (Darbandi *et al.*, 2018). Tumors produce testosterone – either by the

overstimulation of normal Leydig cells by elevated hCG levels in TGCTs or by the hyperproduction of testosterone in defective Leydig cells in Leydig cell tumors. Another factor is the aromatization of testosterone by the enzyme aromatase which converts testosterone to estrogens. The excess of estrogens causes the impairment of spermatogenesis and gynaecomastia. This is the reason why some cancer patients have unusual sperm parameters prior to the diagnose (Morrish *et al.*, 1990; Rajpert-De Meyts, Skakkebaek and Toppari, 2000). Aromatase has been proved to be in cancerous tissue. Estrogen receptors (ER) and G-protein coupled ER (GPER) are studied extensively in testicular cancer. *In vitro* ERβ and GPER that are expressed in testicular germ cell cancers are able to regulate seminoma cell proliferation when exposed to endocrine disruptors. The suppressive ERβ is downregulated in seminomas and the overexpressed GPER is a promoting receptor. Estrogens activate these receptors and therefore TGCC is considered to be estrogen dependent (Fénichel and Chevalier, 2019). Exposure to estrogens in adulthood has not been proved to be a significant risk factor, however, fetal exposure has been strongly associated with the development impairment of spermatogenesis and testicular cancer (Xing and Bai, 2018).

Many cancers including TGCTs show an interesting metabolic switch. The cancerous cells start producing ATP mainly with aerobic glycolysis. This phenomenon is called the Warburg effect. The phenotype involves the overexpression of certain metabolism related proteins and clinically these cancers are much more aggressive. In TGCTs we usually see this in higher stages of non-seminomas (Bonatelli *et al.*, 2019). Therefore, the investigation of the molecular mechanisms resulting in this phenotype and their contributions to cancer initiation and development is of major importance for clinical treatment.

Furthermore, the mitochondrion in steroidogenic cells is a key organelle for the biosynthesis of steroid hormones (Miller, 2013). The mitochondrion is also the producer of reactive oxygen species (ROS) which are generated from the oxidative phosphorylation, although cancer cells prefer glycolysis as their source of ATP. Mitochondria are very susceptible to oxidative damage. Chemical substances from the environment can cause oxidative stress in the mitochondria which can lead to several pathologies related to the testis and its steroidogenesis. If mitochondria are damaged, it can lead to apoptosis and autophagy (Diemer *et al.*, 2003). Some chemical substances interrupting metabolic pathways might be of clinical significance. For example, shikonin (a natural alkannin) has been showed to stop the proliferation of testicular cancer cells and even induce apoptosis and increase autophagy in these cells by downregulating glycolysis-related proteins and upregulating autophagy-related proteins (Yao *et al.*, 2020). Zearalenone, a mycotoxin disrupting the steroidogenesis pathway found as a contaminant in cereal crops, causes testicular germ cell apoptosis in rats (Kim *et al.*, 2003), affects mouse fertility (Yang *et al.*, 2007) and is of harm to male health when exposed neonatally (Koraïchi *et*

al., 2013). Zearalenone is a non-steroidal estrogen, but its toxicity might not be linked only to its estrogenic activity. It induces cell apoptosis by affecting mitochondrial function and by altering ROS production. This oxidative stress is caused by the increase in energetic metabolism through the rise of fatty acid uptake and β-oxidation. Another result of zearalenone exposure is the inhibited steroidogenesis which leads to hormonal imbalance (Li *et al.*, 2014).

6 Conclusion

Testicular cancer is a rising issue troubling more and more men every year worldwide, although the western industrialized world is much more affected. From the 1970s the cure rate has risen significantly, which is an amazing achievement of modern medicine. This type of cancer is a very peculiar one – it burdens young men, which makes the need for prevention and screening programs even bigger. Primary research is very important for the further development of diagnosis and treatment. Open surgery to obtain tissue for biopsy is still needed to properly recognize the cancer. Thus, noninvasive diagnostic tools are of high importance. Ejaculate screening for AP-2γ and OCT3/4 is one of the suggested models instead of serum marker (hCG, AFP, LDH) usage for the diagnosis of TGCT. From the experimental angle, the focus is on protein analysis, genetic or epigenetic studies along with the endocrine research in TGCT patients. Genetic screening in cancer patients might be a way to personalize treatment to avoid drug resistance. It is known that cancer has long-term consequences for male offspring fertility, inducing alterations in sperm epigenome (e.g., global DNA methylation, specific histone modifications and miRNA expression). Epigenetic studies brought histone deacetylase inhibition-based drugs that are under clinical trial. Proteomic research offers a better understanding of sperm surface proteins (e.g., CD46) and their mutual interactions which might be soon implemented in improved sperm quality parameter analysis and utilized in artificial reproductive technology (ART).

To this day little is known about the mechanisms underlying TGCT pathospermia. So, sperm banking should be recommended routinely by clinicians to ensure that patients have the option of fathering a child in the future if fertility is compromised by treatment or the cancer itself. Again, primary research is of great significance when it comes to cryopreserving and especially for artificial reproductive technologies used in reproductive health centers. Knowing and recognizing crucial proteins on sperm and egg surfaces can enable us to deliver much more optimistic results. IVF success rate is alarmingly insufficient with first cycle success only a bit over 25% in the youngest age group of women under 35 years, in women over 42 years it is only a little above 1% (Society For Assisted Reproductive Technology, 2018). This statistic is merciless and quite troubling in the 21st century with sperm count and overall fertility rapidly declining. From the environmental perspective, endocrine disruptors seem to be the biggest hazard for men's reproductive health – prenatal, postnatal and adulthood exposure

show to have negative impacts. The environment in the western world endangers men's fertility and reproductive health. More caution is needed when it comes to chemical pollutants in the water we drink or crops we eat or even the air we breathe. Some researchers call this increase in TGCT incidence and the decrease in both quality and quantity of sperm a male reproductive health crisis.

7 Literature

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