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Comprehensive analyses of genetic and clinical factors in patients affected by Testicular Germ Cell Tumor

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Apának, Anyának és Gábornak,

“Nella vita non bisogna mai rassegnarsi, arrendersi alla mediocrità, bensì uscire da quella "zona grigia" in cui tutto è abitudine e rassegnazione passiva, bisogna coltivare il coraggio di ribellarsi."

"In life you never need to resign, surrender to mediocrity, but get out of that "gray area" where everything is a habit and passive resignation, you need to cultivate the courage to rebel."

Prof. Rita Levi-Montalcini

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I. ABSTRACT

Introduction: Testicular Germ Cell Tumor (TGCT) is a multifactorial, polygenic, and complex disease. It is the most common malignancy of men in their reproductive ages. This neoplasm has one of the highest heritability (37–48,9%) based mainly on epidemiological and Genome-Wide Association Study data. Epidemiological studies support that there is an increased familial cancer risk among TGCT patients' family members. However, the studies are heterogeneous and sometimes controversial. Despite of the growing body of evidence regarding the involvement of genetic factors in TGCT susceptibility, our knowledge about its genetic basis remains scarce. In the latest study, aiming at the evaluation of 48 established DNA Repair (DR) genes in the etiopathogenesis of TGCT, *CHEK2* has been identified as a new susceptibility gene with moderate penetrance. In addition, in a recent case report, TGCT has been linked to Lynch syndrome.

Objectives: In order to identify new clinical and genetic risk factors of TGCT, and to explore whether TGCT may be part of a more generalized cancer predisposition, we performed two projects: i) an epidemiological study, where we aimed to estimate the familial cancer risk among TGCT patients' relatives (first-degree and grandparents), and ii) Whole Exome Sequencing (WES) in TGCT patients with positive family history of cancers, to identify monogenic causes of the malignancy and to determine the role of DR genes in the etiopathogenesis of TGCT.

Materials and Methods: For both projects included in this thesis, the patients were recruited at the Andrology Unit, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Centre of Excellence DeNothe, University of Florence, Florence, Italy and at the Andrology Department, Fundació Puigvert, Universitat Autònoma de Barcelona, Instituto de Investigaciones Biomédicas Sant Pau (IIB-Sant Pau), Barcelona, Spain.

In the epidemiological study, which was a multicentric, retrospective, case-control study, a total of 1407 subjects were enrolled. Among them, 592 were affected by TGCT, 352 had oncohematological (OH) malignancy, and 463 were fertile cancer-free controls. Statistical analyses were performed using SPSS Software.

In the second project, WES was carried out for 32 TGCT and one Leydig tumor patients, who had two or more family members affected by any type of malignant tumors. DNA was extracted form peripheral blood lymphocytes and WES was performed by NovaSeq 6000 System (*Illumina*) using the SureSelect Human All Exon V6 (*Agilent Technologies*) kit. After filtering for rare (Minor Allele Frequency < 0,01), presumed as deleterious, non-synonymous and splicing variants, we performed a first cross with 653 DR genes, and a second cross with 1731 Mendelian autosomal dominant genes from OMIM and COSMIC databases. For further classification of the variants' pathogenicity, we

used the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) criteria. Finally, a series of bioinformatic analyses were carried out.

Results: In the first, epidemiological study we found that TGCT patients' relatives have significantly more cancers (*p value* = 0,0001) than controls' relatives. As comparing the TGCT cohort with another type of malignancy, such as OH, which affects young males, we did not observe significant differences. This implies that OH patients also have an increased familial cancer risk (*p value* = 0,0045). Furthermore, we report some site-specific associations, other than TGCT aggregation in case of familial TGCT, and OH malignancy aggregation in case of familial OH. The novelty of our study is that we defined the semen phenotype for all subjects of the different cohorts, thus we were able to assess not only the impact of tumors *versus* non-tumors on familial incidence of neoplasms but also to compare whether non-normozoospermic subjects have more tumors among family members. We report a 1,57-fold higher risk (*p value* = 0,0048) for tumor development among family members if the patient had severe spermatogenic disturbances (azoospermia and severe oligozoospermia with TSC < 5 million). Another interesting finding of our study was that we observed significantly less siblings among TGCT (mean number of siblings: 1,16) and OH (1,09) cases in respect to controls (2,07).

In the WES study, we identified rare, predicted as damaging, germline variants of DR genes in 5 out of 32 (15,6 %) TGCT patients. We report seven variants in seven genes in the five subjects. Five out of 7 variants were loss-of-function, whereas two were missense. Three variants were classified as pathogenic, two as likely pathogenic, and two as "hot" VUS, according to ACMG criteria classification. Our main findings are related to Lynch syndrome (LS). We found mutations in three established LS genes (*MSH6, MLH3, MLH1*) in three patients with typical LS-associated tumor types among their relatives. The other mutated genes are involved in homologous recombination (*FANCD2, XRCC3*), nucleotide excision repair (*ERCC3*), and oxidative DNA damage repair (*MUTYH*). Further important finding of our study was the identification of two patients, both carrying two variants in different DR genes and presenting typical tumor types for both genes among their family members.

Conclusions and wider implications: Our results show association between TGCT, increased familial cancer incidence, and sub/infertility. Therefore, there might be a common link between spermatogenic defects and a systemic problem leading to higher morbidity including cancer development. The biological explanation for such a relationship could be an overall genomic instability/DNA repair defects in the family, which is reflected in the occurrence of multiple cancers and subfertility.

WES is a powerful tool for searching monogenic causes of TGCT in highly selected patients as it has been in our study on selected TGCT patients with family aggregation of cancers. DR genes might

have a role in the etiopathogenesis of TGCT, which could explain the increased frequency of cancers among TGCT patients' relatives. We therefore suggest to perform sequencing of DR genes in selected TGCT cases with clear signs of familial predisposition to cancer. We also propose that TGCT may be part of the Lynch syndrome associated urological malignancies. Therefore, an onco-andrological screening is suggested for the male members of Lynch syndrome families. On the other hand, in case of pathogenic variants in DR genes, other types of malignancies could occur later in life, hence a careful follow-up of these patients is strongly advised.

II. INTRODUCTION

2. Testicular Germ Cell Tumor

2.1 Epidemiology

Testicular cancer (TC) is a multifactorial and complex disease of the young adulthood. It is the most common neoplasm among men between 15-45 years of European ancestry (1,2). Of the 71,000 new cases estimated worldwide in 2018 (3), over one-third occur in Europe, with the highest age standardized incidence rates observed in Denmark (12/100,000) and Norway (11.8/100,000) (4). The lowest rates are in India (0.5/100,000) and Thailand (0.4/100,000). The annual incidence of TC has doubled over the past half century with an increasing trend over time, particularly in Caucasian males (5). Interestingly, the recently observed attenuation of incidence rates in Danish and Swiss cohorts are in contrasts with the rapidly increasing rates seen in lower-risk populations, such as Finland, Slovenia, and Croatia (6–9). A recent study by Znaor and his collaborators (10) confirms a past peak and a predicted decline in incidence rates in the four high-risk countries of Denmark, Norway, Switzerland, and Austria, whereas a robust increase in particular in historically lower risk countries (11). In fact, the predicted increase in the number of TC patients by 2035 will be the most substantial in Eastern Europe, where cancer survival rates are currently among the lowest (10).

2.2 Etiopathogenesis

In the vast majority of cases (95%) germ cell tumors are diagnosed in the testis. Testicular germ cell tumor (TGCT) is a peculiar malignancy differing in many aspects from other tumors derived from somatic cells, which accumulate typical somatic mutations for the given cancer during ageing.

The histology and pathogenesis of TGCT depend on the developmental stage of the germ cell of origin. TGCTs are divided into two major groups: those that are derived from a precursor lesion, termed as germ cell neoplasia *in situ* (GCNIS) and those that are not (see Table 1.). GCNIS was previously called with three different names such as carcinoma *in situ* (CIS), testicular intraepithelial neoplasia and intratubular germ cell neoplasia unclassified (IGCNU). TGCTs are characterized by extreme histological heterogeneity due to largely retained pluripotency and aberrant somatic differentiation.

Table 1. – Histological classification of testicular tumors according to the WHO (12).

Tumors	Usual age at onset	Frequency	Derived from GCNIS	Genotype	Behaviour
Teratoma (prepubertal) **Yolk sac tumor (prepubertal)** **Dermoid cyst** **Epidermoid cyst**	< 6 years	1-2 %	**NO**	Diploid or aneuploid No i12p gains	Very good, Mostly benign
Seminoma **Embryonal carcinoma** **Choriocarcinoma** **Teratoma** **Yolk sac tumor**	Post-pubertal 20-35 years	95 %	**YES**	Aneuploid, Frequent gains and losses, Overexpression of isochrome 12p	Malignant, Good response to therapy
Spermatocytic Tumor	> 50 years	< 1 %	**NO**	Aneuploid, No i12p gains	Excellent
Leydig cell tumor **Sertoli cell tumor**	Various age	1-3 % < 1 %	**NO**	–	Usually Benign*
Rhabdomyosarcoma	Children < 15 years	**Rare** (17% of all malignant intrascrotal tumors in children)	**NO**	–	Malignant
Liposarcoma **Primary lymphoma**	Older men (> 60 years)	**Extremly rare,** **< 5 %**	**NO**	–	Malignant

Human primordial germ cells (PGCs) first appear in the yolk sac wall during the 3–4 weeks after conception. From week 4 to 5, they migrate under control of SCF/c-KIT signaling system (13) in the hind gut epithelium and then they colonize the genital ridges, the precursors of both gonads, the ovary, and the testis. At the 6th gestational week, the expression of *SRY* gene leads to the determination of the testes from the bipotent gonad (14). This process induces the expression of SOX9, a transcription factor that initiates the differentiation of supportive cells into Sertoli cells (15). At the 7th week, primitive seminiferous cords, a particular structure in which germ cells and Sertoli cells are not yet organized, are formed. Later on, PGCs migrate toward the basal lamina of the seminiferous cords and Sertoli cells organize the microenvironmental niche regulating their differentiation into spermatogonia. PGCs differentiation passes through three stages which three different types of germ cells: gonocytes, intermediate cells, and spermatogonia, present concurrently in the fetal testis and distinguishable by morphologic and immunohistochemical traits (16). At the 10th gestational week, gonocytes are the more abundant type of germ cells located centrally within the seminiferous cords and separated from the basal lamina by Sertoli cells. Then, gonocytes become intermediate cells, with similar morphology but located peripherally within the seminiferous cords and in contact with the basal lamina. By the 15th gestational week, many intermediate cells are present together with gonocytes. It has been hypothesized that when these cells reach the basal lamina, they lose their pluripotency and start to differentiate into spermatogonia. From the 18th week onward, spermatogonia represent the most common germ cell population and are located peripherally to the basal lamina. They undergo mitosis until the first month post-partum, when the mitotic arrest takes place (17).

Through the characterization of GCNIS cells for pluripotency markers, such as c-KIT, NANOG, and OCT3/4, it has been proposed that GCNIS derives from fetal gonocytes that fail to differentiate due to inadequate early gonadal development (18). The malignant transformation of GCNIS into TGCT involves secondary genomic aberrations during adaptation to the adult testicular cellular niche (19,20). In fact, fetal gonocytes whose development into spermatogonia is blocked may undergo abnormal cell division. Their further invasive growth is mediated by postnatal and pubertal gonadotropin stimulation. GCNIS cells adjacent to cancerous zone usually exhibit a gain of the short arm of chromosome 12, leading to increased 12p copy number, i.e. one or more copies of isochromosome i(12p) or tandem duplications of 12p, but also to the overexpression of the cyclin D2 gene (*CCND2*) located at chromosome band 12p13. This event suggests that gain of 12p could play a key role for TGCTs to acquire invasive ability. In fact, the chromosomal region corresponding to 12p contains genes that could be associated to TGCT development. Amplification of *CCND2* activates CDK4/6, allowing the cell to progress through the G1-S checkpoint, resulting in the re-initiation of the cell cycle and genomic instability. Other genes, such as *NANOG* and *STELLAR* are associated with the maintenance of pluripotency in stem cells (21). Another characteristic chromosomal aberration associated with GCNIS is polyploidization (22).

It is also supposed that these early PGCs/gonocytes blocked in differentiation are tightly regulated by epigenetic modification in terms of microRNA expression and DNA methylation, retaining their early marker profile (23).

2.3 Histopathology

The most frequent TGCTs are those arising from GCNIS in post-pubertal adolescents and in young adults (24). The clinically most important histotypes are reported in Table 1. These tumors are divided into morphologically homogeneous, pure seminomas (around 60% of cases, peak incidence at 35 years) and heterogeneous non-seminomas (peak incidence at 25 years). Non-seminomatous tumors can be composed from one or more of the following components: embryonal carcinoma, teratocarcinoma, yolk sac tumor, choriocarcinoma, and teratoma. TGCTs comprising both seminomatous and non-seminomatous elements are clinically classified as non-seminomas.

Other cancer types of the testis are much less commonly observed. Pre-pubertal teratomas or yolk sac tumors, dermoid or epidermoid cysts are probably derived from PGCs, but not associated with GCNIS or (i)12p. They have a benign behavior and do not recur or cause metastasis.

Spermatocytic tumors (SpT), previously termed as spermatocytic seminomas, are still germ cell tumors but they derive from post-natal precursors and despite its name, it is now thought to derive

from spermatogonial cell populations. It tends to occur in older patients (mean age at diagnosis is 54 years), represent < 1% of cases.

Similarly to pre-pubertal TGCTs, SpT does not show evidence to be derived from GCNIS e.g. it does not possess chromosome (chr) 12p abnormality, it is negative for OCT3/4, and has no extragonadal counterpart (25–27). It is suggested that SpTs arise through neoplastic transformation of pre-meiotic germ cells, probably at a transition stage between spermatogonia and spermatocytes (28). Moreover, immunohistochemistry studies propose the existence of distinct sub-classes of SpTs, implying that SpTs are not a single entity but represent a heterogeneous tumor type with multiple cellular and/or developmental origins. So far, the etiopathogenesis leading to SpT formation have not been fully elucidated, although it is proposed that some of these types of tumors represent the extreme and rare outcome of a universal process termed as "selfish spermatogonial selection" that takes place in the testis of all men as they get older (29). In a subset of cases (around 20%) oncogenic gain-of-function mutations in genes involved in pathways that increase spermatogonial survival and proliferation, e.g. *FGFR3* and *HRAS* (30). These "selfish" mutations lead to clonal expansion of this mutant spermatogonial cells in the testis over time. *FGFR3* and *HRAS* are expressed in subpopulations of cells in normal seminiferous tubules. The encoded proteins are physiologically connected, and the activation of these receptors can lead to the activation of RAS-MAPK and PI3K-AKT pathway, among others. SpT is characterized also by the gain of chr 9, with a frequent amplification of a locus on the short arm involving *DMRT1*, a gene involved in testis development and spermatogenesis (31). Other authors found that these tumors exhibit extensive aneuploidy (50±99 autosomes/tumor) involving whole-chromosomes, with recurrent gains of chr 9 and chr 20 and loss of chr 7, suggesting that aneuploidy itself represents the initiating oncogenic event i.e. gene imbalance mediated *via* whole-chromosome aneuploidy.

This tumor is mostly benign but occasionally, progression or dedifferentiation of SpT into different types of sarcoma (mainly rhabdomyosarcoma) have been observed, in which the tumor behavior is markedly more aggressive (12).

In older men, primary lymphomas of the testis can be identified also, with 71 years, as a median age at onset, based on a Danish non-Hodgkin's lymphoma registry (3). Other type of tumors, arising from endocrine structures, such as Leydig cell tumors or Sertoli cell tumors, can occur at various ages but are uncommon and generally benign.

Rarely, tumors arising from paratesticular structures, such as rhabdomyosarcomas in children, and liposarcomas in older men can be identified (12).

2.4 Diagnosis

The diagnosis is based on clinical symptoms, scrotal ultrasound, and serum markers. A common clinical manifestation of TGCT is a painless lump in the body of the testis. Possible symptom could be episodic pain, due to hemorrhage, but also could mimic epididymo-orchitis, or cause hydrocele. Rarely, TC may present symptoms related to metastases, such as backache from enlarging abdominal lymph nodes or chest symptoms from lung metastases such as cough, pain, or hemoptysis. β subunit of human chorionic gonadotrophin (β-hCG) production by non-seminomas (choriocarcinoma) can cause nipple tenderness and gynecomastia. A small amount of germ cell tumors (less than 5%) arises from an extragonadal primary site, such as the pineal gland, retroperitoneum, or mediastinum (along the journey of the PGCs). These type of germ cell tumors are much more common among patients with Klinefelter syndrome (47,XXY).

Suspected tumors can be confirmed by scrotal ultrasound, which is the gold standard modality for evaluating scrotal diseases and has a sensitivity to detect intratesticular lesions of almost 100%. To further confirm the diagnosis and to distinguish between tumor types, preoperative serum tumor markers, including β-hCG, α-fetoprotein (AFP), and lactate dehydrogenase (LDH), should be measured. β-hCG is produced mainly by choriocarcinoma, and concentrations greater than 500 IU/L are usually associated with non-seminomas containing a choriocarcinoma component but occasionally it has been reported at a much lower level also in seminoma (likely containing the same component). AFP is secreted by yolk sac tumors and embryonal carcinomas with some yolk-sac differentiation and is never secreted by seminomas. Early detection based on circulating microRNAs (targeted miRNA-based blood tests for miR-371-3 and miR-367 clusters) had a great promise as universal markers for diagnosing germ cell tumors (23,32,33).

Percutaneous needle biopsy is contraindicated, and standard initial management is orchidectomy in continuity with the spermatic cord, performed with an inguinal approach (orchifunicolectomy).

Early diagnosis would be preferred before the appearance of an overt clinical picture. Although, personalized screening models based on known clinical risk factors may offer enhanced TGCT risk discrimination, presently a population-level testing is not available yet.

2.5 Treatment and survivorship

Over the last several decades the mortality rates of TGCT have declined. The 5-year survival rate is above 95%, (around 80% in cases, when metastasis has already occurred) thanks to the combined treatment of orchifunicolectomy and either radiotherapy or platinum-based chemotherapy (carboplatin/cisplatin, bleomycin, etoposide). Although highly efficient to cure TGCT, cytotoxic therapy may induce long-term morbidity, including cardiovascular disease, metabolic syndrome,

infertility, and secondary malignancies. On the other hand, there are still some patients unresponsive to chemotherapy, and especially those presenting disseminated non-seminomas, unfortunately die of the disease. These facts show the relevance to further improve our knowledge i) on the underlying pathogenic basis of this disease and ii) on the mechanisms involved in resistance to cytotoxic therapies. In fact, improved disease monitoring is another clinical priority. Conventional tumor markers have been used effectively for early risk stratification and detection of relapse in non-seminomas but have limited sensitivity and specificity for patients with seminoma. Circulating microRNAs have been proposed as markers not only for diagnosis but also for disease monitoring (23,32,33).

2.6.1. Clinical Risk Factors of TGCT

Besides young age, a number of medical conditions have been associated with the increased incidence of TGCT. These conditions include for example cryptorchidism, contralateral testicle tumor, testicular microlithiasis, testicular atrophy. In 2001, Skakkebaek and his collaborators (34) proposed the existence of a new clinical entity, termed as Testicular Dysgenesis Syndrome (TDS), that combines four pathological conditions: i) TGCT; ii) impaired spermatogenesis; iii) cryptorchidism; iv) hypospadias; as they seem to have a shared etiopathogenesis (35). Recently, short anogenital distance (AGD) has also been linked both to TDS and TGCT (36).

Perinatal factors including inguinal hernia, twinning, maternal bleeding, birth order and sib-ship size have also been reported as conferring an increased risk of TC in some meta-analyses (37,38). These factors are likely to be indirectly linked to TC development due to shared intrauterine environmental exposures.

2.6.1.1. Testicular Dysgenesis Syndrome-related risk factors

All five TDS components are considered as manifestations of disturbed embryonic programming and gonadal development during fetal life. It has been proposed that the etiology of TDS is due to unfavorable environmental factors, including hormone disruptors, acting on a susceptible genetic background (for instance genetic polymorphisms in genes involved in hormonal regulation of male genital tract development). This results in: i) **Sertoli and peritubular cell dysfunction** leading to impaired germ cell differentiation, therefore, to disturbed spermatogenesis and GCNIS formation; and ii) **decreased Leydig cell function** leading to androgen deficiency, thus, to decreased testosterone production, cryptorchidism (along with insulin-like hormone 3 deficiency), hypospadias, and short AGD (see Figure 1.).

During the past couple of decades and still nowadays, the incidence of TDS-related conditions is undoubtedly growing in the developed Western countries. It is suspected that environmental exposures arising from modern lifestyle, rather than genetics, are the most important factors to explain the observed trend (39). In fact, the production of the so-called endocrine disruptors (EDs) has also been increased over the last 50 years. The term of EDs describes a highly heterogeneous group of substances including both manufactured chemicals and natural compounds. Most of them are structurally similar to endogenous hormones and can disrupt their action. The group involve industrial solvents and their by-products (e.g. polychlorinated biphenyls), plastics and plasticizers (e.g. bisphenol A and phthalates respectively), pesticides, dioxins, pharmaceutical agents (e.g. diethylstilbestrol), fungicides, phytoestrogens, mycotoxins and heavy metals (e.g. arsenic or mercury). EDs are detected almost everywhere: in the air, soil, body fluids, drinking water, food, cosmetics, household products, electronic devices, and textiles. They could not only be found in the site of production but are also transferred to long distances through water and wind and could be resistant to degradation.

ED exposure during fetal and neonatal life can occur through placenta and breast feeding and might act either directly or *via* epigenetic mechanisms. In the latter case, the effects of adverse environmental exposures might have an impact for several generations post-exposure (transgenerational effect).

TDS-related conditions such as cryptorchidism, hypospadias, and short AGD are clearly related to impaired androgen action taking place in a critical phase of development (34), termed as masculinization programming window (MPW) (40).

Animal studies showed that the exposure to certain EDs with anti-androgen activity during this phase might induce abnormal male reproductive development (40–42). In fact, ED exposure negatively affects reproductive hormone concentrations, including testosterone and LH, as well as testicular volume and sperm concentration and quality (43–48). It is worth noting, that TGCT has not been replicated in rodent models following the exposure to anti-androgens, probably due to species specificity.

The existence of a fetal MPW has been proposed also in human during the 8-14th gestational weeks (40). The exposure to environmental factors with potential anti-androgenic, or estrogenic effect may lead to the disruption of the hormonal balance during MPW with consequent adverse effects on male reproductive health (49). In accordance with the link between EDs and TDS, in 1982 Stillman and colleagues (50) observed that boys whose mothers were treated with DES during pregnancy were more likely to suffer from hypospadias, or cryptorchidism or decreased sperm count.

Cryptorchidism, or undescended testis, is a common genital birth defect affecting 2-9% of male newborns (51). The process of testicular descent can be divided in two phases: firstly, the transabdominal phase, which depends on the insulin-like hormone 3 (INSL-3) produced by interstitial Leydig cells, and secondly, the inguinoscrotal phase in which testosterone is essential (52). Testicular maldescent can be due to transient hormone deficiencies in fetal life and may occur on one or both sides. The testicle(s) could stuck anywhere along the "path of descent," such as: i) high in the retroperitoneal abdomen; ii) in the inguinal canal; iii) high scrotal; or ectopic from the path of descent. Cryptorchidism has been shown to increase testicular cancer risk by four-fold (38). The incidence of both defects shows different prevalence in different geographic regions, and in several countries increasing trends have been reported (53–56). In Denmark, cryptorchidism rate was significantly elevated as compared to that observed in the 1960s (55). Danish cryptorchidism rate at birth was approximately two-fold higher in respect to the rates observed in other countries in the 1980s and 1990s (54,57–59). A prospective study performed in 1997–2001 showed an even higher difference in cryptorchidism rate at birth between Denmark (9.0%) and Finland (2.4%) (54). The observed differences could be ascribed to the variation of environmental or lifestyle related factors, including exposure to ED or other toxicants, which are likely to be at higher levels in Denmark, but they may be also related to genetically determined factors.

Hypospadias is a rare malformation of the urethral opening, affecting 0.2-1% of male newborns. Its incidence shows geographic differences, and similarly to cryptorchidism and TGCT, the highest rates are observed in Denmark (464/10,000 births) (60). In physiological conditions during the first trimester of pregnancy, the urethral folds fuse in a proximal to distal direction under the influence of androgens produced by the fetal testis (61). The failure of this fusion results in a ventral positioning of the urethral opening.

AGD, i.e. the distance between the anus and the genitals, is a sexually dimorphic measure of genital development since males have anogenital lengths longer than females (62). Given its dependence on androgen action, it is considered as a well-established and sensitive biomarker for endocrine disruption within MPW in animal and human. In fact, decreased distance has been associated with testicular dysfunction and may be shorter in infant males with genital anomalies, such as hypospadias and cryptorchidism (41,63–65). Eisenberg and his collaborators (66) found that AGD may predict normal male reproductive potential, since infertile men possessed significantly shorter mean AGD and penile length compared to the proven fertile controls (AGD: 31.8 vs 44.6 mm, PL: 107.1 vs 119.5 mm, p = 0,0.01). (24). A recent study by Moreno-Mendoza and colleagues (36) including 156 Spanish TGCT cases and 110 tumor-free normozoospermic controls demonstrated a significantly shorter AGD in the TGCT group in respect to controls ($p < 0.001$), independently from sperm count and

TGCT histology. Subjects with decreased ano-penile and ano-scrotal distance showed a significantly increased risk for TGCT (OR= 4.97, 95% CI= 2.01-12.33, p = 0.001 and OR= 4.11, 95% CI= 1.89-8.92, p ≤ 0.001, respectively). Another novel study by Priskorn et al. (67) also found that TGCT survivors had a shorter ano-scrotal distance (-0.84 cm, 95% CI: -1.31; -0.37) compared to men from the general population.

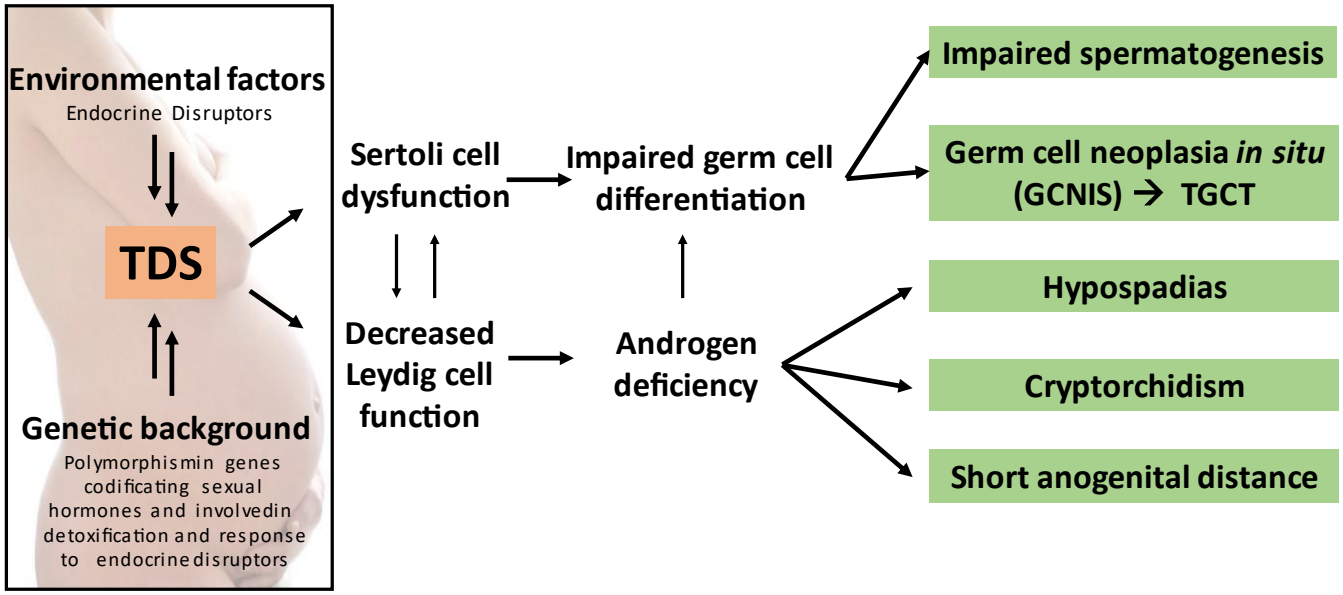
The observations concerning AGD underline the importance of its evaluation in male patients since it is a significant risk factor for TGCT.

Impaired spermatogenesis: After the meta-analysis by Carlsen et al. (68), which suggested a robust reduction in the mean sperm count occurring between 1938-1990, a number of papers with comparable, or with contradictory results were published, generating a major debate (69–72). Numerous original studies published since 2000 in various countries suggest an overall decreasing trend in sperm concentration and/or total sperm count of young men (73–75), male partners in infertile couples (76–81), fertile men (82,83) and semen donors (84–86) or semen donor candidates (87–90). An overall decline in sperm count among unselected men from several populations (−0.70 million/ml/year) was reported by Levine and colleagues (91) between 1973 and 2011, which is consistent with, but not as steep as previously reported for an earlier period (Carlsen et al. (68) between 1938-1990, −0.93 million/ml/year; and Swan et al. (69) between 1934-1996, −0.94 million/ml/year). The annual percentage change in sperm count reported in the study of Levine et al. (91) was −0.75% million/ml, comparable to −0.83% found by Carlsen et al. (1992) (68). However, studies reporting no significant decrease or, in contrast, founding a slightly increasing trend, have also been published (92–98). As a conclusion to this longstanding debate, in 2015, a systematic review and meta-analysis (99) of 124 articles has summarized these heterogenic findings and evaluated the changes in sperm concentration over a 75 years' period (1938– 2013), observing a significant decline in sperm concentration (p < 0.001). In parallel with the other elements of TDS, variability in mean sperm count between geographical regions have been described. Cross-sectional studies have shown differences in sperm production among men living in northern countries. For instance, total sperm counts were significantly higher in Finland as compared to Denmark in fertile males (aged between 20-45 years, with a currently pregnant female partner from natural conception) (100). Levine et al. (91) confirms the geographical differences in a wider perspective i.e. a significant decline in sperm count between 1973 and 2011 in unselected men from Western populations, such as North America, Europe, Australia and New Zealand (−1.38; −2.02 to −0.74; *p value* < 0.001), while no significant trends in South America, Asia and Africa. The significant decline of sperm production during the past decade, indeed, has major consequences. In fact, nowadays, male infertility affects approximately 7-12% (101) of the general population, causing a widespread problem in the developed

Western countries. In the era of Assisted Reproductive Techniques (ART), up to 6% of children (6.1% in Denmark, 4.9% in Slovenia, 4.6% in Belgium, 4.2% in Israel, 3.3% in Australia, 1.6% in the United States, 1.5% in Japan, and 1.7%–2.2% in the largest European countries), are now born after *in vitro* fertilization (IVF), IntraCytoplasmic Sperm Injection (ICSI), donor or homologous insemination (102–108).

The different elements of TDS can be found often in combination within the same individual. However, as in other clinical syndromes, not all patients suffering from TDS present all signs. The mildest manifestation of TDS may be "only" impaired spermatogenesis without any other symptoms, or with slightly reduced testosterone levels (109,110), whereas more severe forms are characterized by more than one TDS components. The increasing number of components confers a proportionally higher risk for TGCT development.

Figure 1. – **The etiology of Testicular Dysgenesis Syndrome (TDS) from Skakkebaek (34).**



2.6.1.2. Testicular Microlithiasis

Testicular microlithiasis (TM) is an incidental finding of the scrotal ultrasound examination. It is a relatively rare condition of unknown etiology that results in the formation of intratubular calcifications. The phenomenon was first described by Priebe and Garrett in 1970 (111), after seeing bilateral diffuse testicular calcifications on a pelvic X-ray of a 4-year-old boy. The first sonographic identification of TM was reported by Doherty in 1987 (112), as described five or more hyperechoic

focus measuring between 1-3 mm in the testicular parenchyma without posterior shadow cone. History of cryptorchidism is considered as a risk factor for TM (113). Moreover, bilateral TM is associated with moderately decreased testicular volume, sperm concentration, and total sperm count (114). Indeed, the prevalence of TM seems higher among men with testicular dysfunction, and it may be a risk factor for GCNIS in men with additional risk elements. Most men with TM from the general population will not develop TGCT, calculating from the incidence of TM and the low frequency of TGCT (around 0.006%), whereas in infertile men this risk is significantly higher (around 1%). Furthermore, de Gouveia Brazao and colleagues (115) demonstrated that GCNIS is present in 20% of infertile men with bilateral TM. However, regarding the heterogeneity of the literature data, it is still a controversial issue. A meta-analysis from 2018 (114), including 40 articles on this topic, reported that cryptorchidism associated with TM does not seem to be a risk factor for TGCT. In contrast, infertility associated with TM confers a higher tumor risk.

Current European guidelines (116) do not recommend any follow-up in cases of only TM with no other specific risk factors for TGCT, such as personal/familial history of TGCT, testicular atrophy, infertility, or cryptorchidism.

2.6.1.3. Contralateral Testicular Tumor

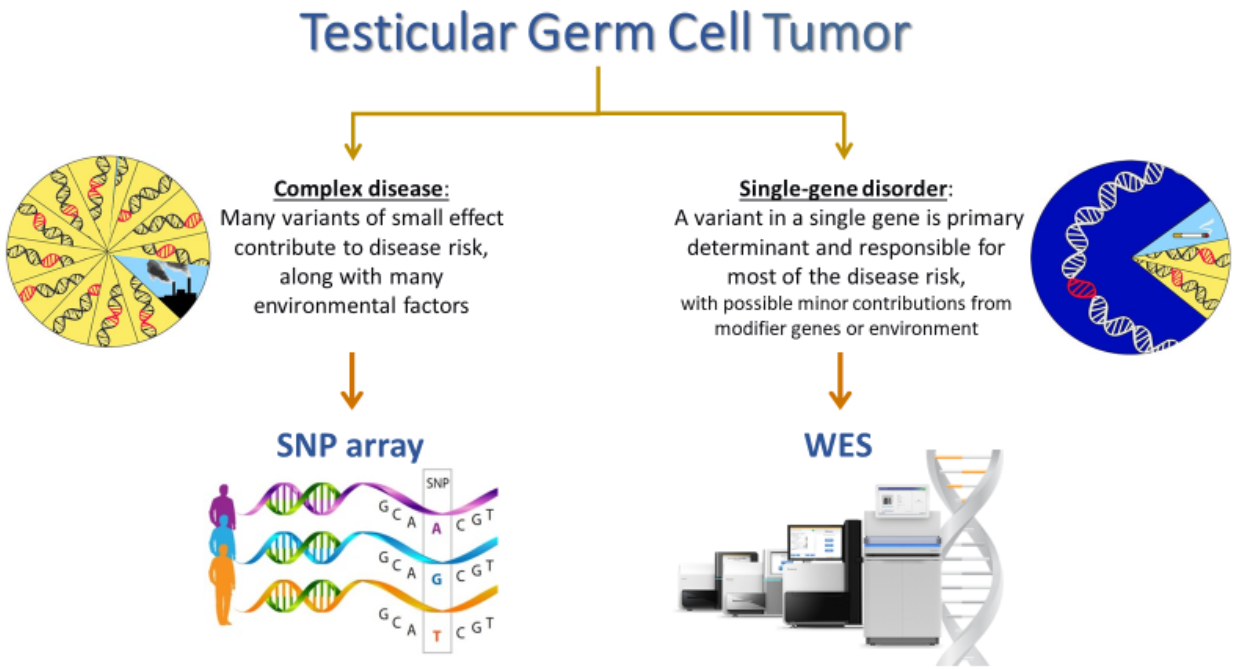
Due to the rising incidence of TGCT and the high survival rates, increasing number of unilateral TGCT patients are at risk of developing a subsequent (i.e., metachronous) contralateral testicular cancer (MCTC) (117). Caucasian population with unilateral TGCT has a 12- to 38-times elevated risk of developing another testicular cancer compared with males from the general population (118–121). Studies demonstrated that the 15-year cumulative risk of developing a MCTC was 1.9%, whereas 20–25 years after the first cancer diagnosis this risk rises up to 2.4% - 5.2% (119,120,122–126). Some studies (119,123,127) have also investigated the influence of age at diagnosis of initial TGCT, histology, extent of disease, and treatment on the development of MCTC. Diagnosing the first cancer before the age of 30 years has been observed as a relevant risk factor (119). Furthermore, patients with unilateral seminoma seems to have a higher risk of developing a MCTC compared to patients with a unilateral non-seminoma (119).

2.6.2. Genetic Risk Factors of TGCT

Robust evidence supports that besides environmental factors, genetic factors may also be involved in TGCT development. In fact, family, twin, and migration studies indicate a strong inherited genetic basis of TGCT susceptibility. Familial studies defined that the risk of developing TGCT is 8- to 10-

fold elevated for brothers of affected males, and 4- to 6-fold elevated for fathers/sons of cases, compared with the general male population (128–131). Litchfield and his collaborators (132) estimated the level of heritability of TGCT by performing both an analysis of the Swedish population registry, comprising 15.7 million individuals, and a genome-wide complex trait analysis using Genome-Wide Association Study (G-WAS) dataset of 6,000 individuals. Based on this population-wide assessment, the research group estimated the heritability of TGCT to be 37–48.9%, applying the genomic and population data, respectively. These values point out that nearly half of all TGCT risk is determined by inherited genetic factors, which is substantially higher than in other common tumor types, such as breast or prostate cancer (128). Epidemiological studies showed that the incidence of TGCT also varies considerably between ethnic groups. It is well defined that the white Caucasian population have five-times higher risk of developing TGCT, compared with the Asian or African populations. In addition, Asian or African descent maintains their low risk, even after several generations since migration, although living in a geographic area of high risk (130,133). These studies clearly show the importance of genetic components in the etiology of TGCT. The advent of genome-wide array (Single Nucleotide Polymorphisms, SNP) and Next-Generation Sequencing (NGS) technologies helped the fast and cost-effective genetic analyses in large populations, which sought to reveal the genomic architecture of TGCT (see Figure 2.).

Figure 2. – Differences between the research methods in case of complex diseases and single gene disorders. Testicular Germ Cell Tumor (TGCT) may belong to both models.



2.6.2.1. Y chromosome-linked gr/gr deletion

Given the association between impaired fertility and TGCT, genetic alterations associated with infertility have been investigated as potential risk factors of TGCT.

The human Y chromosome harbors essential genes for testis development and function. The *SRY* gene on the short arm of the Y chromosome (Yp), is the "master gene" of male sex determination. The absence/malfunction of this gene is associated with the 46,XY complete gonadal dysgenesis (Swyer syndrome), which leads to female phenotype and non-functional, fibrotic, streak gonads.

The long arm of the Y chromosome (Yq) contains a large set of testis expressed genes, most of them are located in a specific region called the AZoospermia Factor region (AZF), which were discovered more than 40 years ago by Tiepolo and Zuffardi (134). Further research shed light to the existence of three subregions, in proximal, middle, and distal Yq11, designated as AZFa, AZFb, and AZFc (135). AZF microdeletions are well-established genetic causes of severe spermatogenic failure (5–10% of azoospermic and 2–5% of severe oligozoospermic men) (136). Partial deletion, which removes half of the gene content (1.6 Mb) of the AZFc subregion, called as "gr/gr deletion", represents a population-dependent, significant risk factor for oligozoospermia (137). Population-dependent means that the risk for a gr/gr deletion carrier to be affected by oligozoospermia varies between different ethnicities and shows the highest values in the Mediterranean area (OR=4.2, CI 2.0–8.8) (138). Three studies analyzed the role of complete AZF deletions in TGCT, but the link with the malignancy has not been proved (139–141). Regarding the Y haplogroups and increased risk of TGCT, no association has been observed neither (142–144). The role of gr/gr deletion in TGCT has been evaluated in four studies (144–147), with slightly contradictory results. In fact, two published studies observed an increased risk, whereas the other two studies not. The first, large (1842 TGCT cases) multicenter study by Nathanson and collaborators (145), confirmed a 2-, and 3-fold elevated risk for sporadic and familiar TGCT in subjects carrying the gr/gr deletion, respectively. The two subsequent studies based on relatively small cohorts from England (263 TGCT cases) and Italy (118 TGCT cases) have not found the relationship between gr/gr deletions and TGCT (144,146). In 2019, Moreno-Mendoza and colleagues (147) conducted the largest European case–control study to date, analyzing 497 TGCT patients and 2030 controls from two Mediterranean populations. The authors observed that gr/gr deletion is an independent predisposing factor of TGCT. Interestingly, the highest risk was observed in the normozoospermic group: while only 0.9% of controls were gr/gr deletion carrier, this percentage was significantly higher in normozoospermic TGCT patients (3.4%) and even higher in normozoospermic seminoma patients (4.1%). Thus, gr/gr deletion in normozoospermic subjects confers almost 4-fold increased risk for TGCT susceptibility.

As a conclusion, we can state that since the two largest studies have found the association between gr/gr deletion and TGCT, diagnosing this partial microdeletion in affected patients has a potential clinical impact. This implies that the male relatives, especially the brothers and sons, of gr/gr deletion carriers (both TGCT and infertile patients), should undergo preventive measures such as regular autopalpation or ultrasound scan of the testis in long-term. In some countries, gr/gr deletion screening is already part of the routine genetic diagnostic work-up of oligozoospermic men (148).
However, the absolute frequency of the gr/gr deletion is relatively low (observed in ~2% of cases) in TGCT, accounting only for approximately 0.5% of the total genetic risk of TGCT development (132).

2.6.2.2. Genome-Wide Association Studies and TGCT

Advances in genomic technologies have enabled considerable progress in understanding the genetic landscape of TGCT. G-WAS are based on specific arrays analyzing SNPs. SNPs are the most frequent genetic differences among individuals, accounting for 4-5 million SNPs/person's genome. With SNP-arrays over millions SNPs/person could be analyzed. Since G-WAS allows the definition of Linkage Disequilibrium (LD) blocks, which are nonrandom associations of alleles at different loci, the final number of SNPs for which information can be obtained is much higher than the number of hybridized SNPs in the array. Although G-WAS contributed to the understanding of TGCT oncogenesis, SNP arrays are not part of the clinical practice.
Immediately after the first G-WAS in the early 2000s it became clear that a single high-penetrance risk locus is unlikely to exist (149,150). The authors suggested that multiple susceptibility loci with low effect size might contribute to the disease development. The number of SNPs has increased progressively in the past 20 years. The most recent study of Pluta et al. along with The Testicular Cancer Consortium (151) has shed light to novel susceptibility loci in association with TGCT. This meta-analysis brings the TGCT risk SNPs to a total of 78, accounting for 44% of disease heritability. In this study, besides discovering 22 novel SNPs, the authors replicated 44 of the 56 already known SNPs in a cohort of 10,156 cases and 179,683 controls. Some of these sentinel variants occur on introns or in very close proximity of genes that could be associated with TGCT with high plausibility. The vast majority of these SNP-related genes are associated with seven biological pathways: i) KIT-KITLG signaling, ii) male germ cell development, iii) male sex determination and differentiation, iv) genomic integrity, involving DNA damage repair, telomerase function, centrosome cycle and actin, cytoskeleton and microtubule assembly, v) apoptosis, vi) mRNA translation, and vii) enzymatic functions. Pathways, such as DNA repair or telomerase function have already been linked to oncogenesis of other cancers, while other pathways, such as sex determination or germ cell development are specific to germ cell tumors (152).

i) KIT-KITLG signaling and its related pathways

In 2009, two independent research groups (153,154) have discovered the strongest risk locus so far, at 12q21, containing the gene *KITLG*. This individual SNP (rs995030, in the 3'untranslated region of exon 10) carrying per-allele odds ratio (OR) >2,6, is among the highest reported in G-WAS of any other disease phenotype. *KITLG* encodes the ligand for the receptor-type protein-tyrosine kinase (KIT), which regulates the survival, proliferation, and migration of germ cells (155,156). Functional studies in human cell lines have elucidated that the signal of association at this locus is mediated through an allele-specific p53 binding effect and subsequent upregulation of KITLG expression, which may increase KIT signaling and germ cell proliferation. The SNP's Minor Allele Frequency (MAF) is different between ethnicities. In the non-Finnish European population is 0.816, whereas in the African population is much lower, MAF = 0.339, which might explain the different incidence of the disease between these two ethnicities (157). To date, four additional KITLG-related SNPs (rs1508595, rs3782179, rs4474514, rs3782181) have been identified in association with TGCT (153,154,158).

The importance of the **KIT-KITLG signaling** in TGCT is further supported by the discovery of three other risk loci: i) 6p21 comprises the gene *BCL2* Antagonist/Killer 1 (*BAK1*); ii) 11q14.1 comprises the gene *GAB2*, from the GRB2-associated binding protein (GAB) family; iii) 5q31 contains the gene sprouty homologue 4 (*SPRY4*). The *BAK1* plays a role in the mitochondrial apoptotic process and its' expression is regulated by *KIT*. *GAB2* encodes an adapter protein which acts downstream of several membrane receptors including cytokine, antigen, hormone, cell matrix and growth factor receptors to regulate multiple signaling pathways, such as KIT–KITLG signaling cascade (159). *SPRY4* inhibits the mitogen-activated protein kinase (MAPK3–MAPK1) pathway which in turn is activated by KIT signaling. Regarding the OR of these SNPs in the replication studies is between 1.50 and 1.17 (160).

ii) Male germ cell development pathway

Other pathways associated with **male germ cell development** have also been recurrently observed in TGCT G-WAS, including genes *DAZL* at 3p24.2 and *PRDM14* at 8q13.3. *DAZL* encodes an RNA-binding protein that has been shown to have a crucial role in the early differentiation of human primordial germ cells. Expression studies showed that *Dazl−/− knock out* mice are infertile, with differentiation of the germ cells arrested at the type A spermatogonia phase (161). Furthermore, knockout of *Dazl* causes spontaneous gonadal teratomas, likely due to prolonged expression of pluripotency genes (162,163). *PRDM14* encodes a transcriptional regulator and modulates primordial germ cell specification due to controlling key pluripotency genes' expression, such as *POU5F1* (alias *OCT4*), *NANOG* and *SOX2* (164).

iii) Male sex determination and differentiation pathway

Another risk locus at 9p24 is containing a single gene *DMRT1*. This gene encodes a transcription factor which function is in **male sex determination and differentiation** by controlling testis development and male germ cell proliferation. It inhibits meiosis in undifferentiated spermatogonia and promotes mitosis, leading to spermatogonial development and allowing abundant and continuous sperm production. It also plays a key role in postnatal sex maintenance by balancing between male and female sex determination pathways, with the activation of male-specific genes and the repression of transcription of female promoting genes. It is associated with testicular cancer, since studies showed that 90% of *Dmrt1*−/− 129Sv *knock out* mice developed teratoma (165). *DMRT1* may act as a tumor suppressor.

iv) Genomic integrity pathway

Other biological pathways associated with TGCT relate to **genomic integrity**, such as telomerase function, DNA damage repair, and centrosome cycle and microtubule assembly. Three risk loci have been identified containing genes in relation to **telomerase function**: 5p15 (*TERT/CLPTM1L*), 12p13 (*ATF7IP*) and 5q31.1 (*PITX1/CATSPER3*). The locus at 5p15 is recognized as a so-called 'cancer hub' because it has been frequently reported in association with multiple other cancer types (e.g., melanoma and other skin cancers, melanoma, glioma, lung, pancreas, breast, ovaries, cervix, urinary bladder, prostate cancers) (166,167). The identified SNP (rs2736100) lies on intron 2 of *TERT*. The enzyme encoded by *TERT* consists of a protein component with reverse transcriptase activity and an RNA component which serves as a template for the telomere repeat (TTAGGG). Telomerase expression plays a role in cellular senescence, resulting in progressive shortening of telomeres. Dysregulation of telomerase expression in somatic cells is involved in oncogenesis. *Knock out* mouse studies showed that telomerase also participates in chromosomal repair, since *de novo* synthesis of telomere repeats may occur at double-stranded breaks. *TERT* was firstly identified by Turnbull and colleagues in 2010 (166) in association with the development of TGCT, with an OR of 1.48 (1.32-1.66) in seminomas and 1.26 (1.12-1.42) in non-seminomas. Other genes related to telomerase function are *PITX1* and *ATF7IP*.

TGCT-associated locus identified at 16q22.3 contains SNP rs4888262, which lies in exon 8 of *RFWD3*, whereas within the LD block locus at 17q22 the gene *RAD51C* is embraced. Both genes, *RAD51C* and *RFWD3*, are involved in **DNA Repair** mechanism**, which** has a dominant role in cancer predisposition. *RAD51C* is involved in the homologous recombination (HR) repair pathway of double-stranded DNA breaks arising during DNA replication or induced by DNA-damaging agents. It has an early function in DNA repair in facilitating phosphorylation of the checkpoint kinase (*CHEK2*) and thereby transduction of the damage signal, leading to cell cycle arrest and HR activation. It also plays a role in the regulation of mitochondrial DNA (mtDNA) copy number under

conditions of oxidative stress together with *XRCC3*. RAD51C/XRCC3 is an additional component of the mitochondrial nucleoid having nucleus-independent roles in mtDNA maintenance. RAD51C/XRCC3 localizes to the mtDNA regulatory regions in the D-loop along with the mitochondrial polymerase *POLG*, and this recruitment is dependent upon Twinkle helicase. In case of replication stress, *RAD51C* and *XRCC3* are further enriched at the mtDNA mutation hot spot region D310. Particularly, the absence of RAD51C/XRCC3 affects the stability of *POLG* on mtDNA. As a consequence, RAD51C/XRCC3-deficient cells exhibit reduced mtDNA synthesis and increased lesions in the mitochondrial genome, leading to overall unhealthy mitochondria. Moreover, *RAD51C* contributes to DNA cross-link resistance, sister chromatid cohesion and genomic stability, but it is involved also in the maintenance of centrosome number in mitotic cells.

Further TGCT-related gene set include those SNPs which are mapping to loci containing genes involved in the **centrosome cycle and microtubule assembly** such as: *CENPE, TEX14, PMF1* and *MAD1L1* (168,169). The *TEX14* (testis expressed 14) encoded protein is a kinase over-expressed in the human male germ cell, and *TEX14 knock out* male rodents are shown to be infertile, whereas female *knockouts* are not (170,171). In vitro data show that *TEX14* is a regulator of kinetochore-microtubule assembly and spindle assembly checkpoint in testicular germ cells, a process which requires recruitment of a number of proteins including *CENPE* (Centromere-associated protein E), which is encoded within the TGCT-associated locus at 4q24 (171,172).

v) Apoptotic pathway

The risk loci at 16p13.13 (rs4561483) observed by Litchfield et al. is in LD block with the gene *GSPT1*. It is a proto-oncogene, associated with the process of programmed cell death, the **apoptosis**. The dysfunction of this biological mechanism (defective or excessive) is a negative factor in many human conditions, including several types of tumors as well. *GSPT1* has been shown to be upregulated in gastric, prostate and breast cancers (173–175), and in TGCT (160,176).

Another novel risk factor of TGCT, rs351418 at 1p11.1, identified by Pluta et al. (151) is associated to the gene *BCL2L11*, which interacts with *BAK1* since both of them are members of the BCL-2 family. These genes together tightly regulate the mitochondrial apoptotic response to either facilitate or prevent cell death depending upon intercellular stimuli. BCL-2 is a large protein family, and all members contain at least one of four Bcl-2 homology domains. Certain members (Bcl-2, Bcl-XL and Mcl-1) are anti-apoptotic, whilst others (Bax, Bak, Bok) are pro-apoptotic proteins. Bax and Bak, two functionally similar proteins of the family, are known as the gateway to apoptosis because of their essential role as effectors of mitochondrial outer membrane permeabilization (MOMP), a major step during mitochondria-dependent apoptosis. *BCL2L11* (Bim) is a pro-apoptotic BH3-only protein that can activate Bak, but preferentially activates pro-apoptotic effector Bax. It is worth noting, that in

rodent models, Bim and Bik cooperate to initiate early germ cell apoptosis in a biological pathway that appears to require Bax, but not Bak (177). Bax also controls apoptosis of fetal germ cells during their migration, and in Bax *knock out* mice ectopic germ cells with retained primitive markers are observed (178,179). Further studies suggest that down-regulation of *BCL2L11*, implies improper survival of arrested germ cells and their transformation to pre-GCNIS (151).

vi) mRNA translation

The latest pathway analysis showed that several moderately and highly ranking target genes encode proteins that interact in **mRNA translation**, including one of the ribosomal proteins (*RPL4*), translation termination protein eRF3A (*GSTP1*) and translocon-associated protein subunit gamma (*TRAP*-gamma, encoded by *SSR3*), which is the general ribosomal interactor participating in the co-translational translocation of proteins into the endoplasmic reticulum. Furthermore, multiple DNA-binding transcription factors are implicated in TGCT predisposition, such as *HNF1B, PITX1, PKNOX2, PRDM14, SP1, TFCP2L1, ZFPM1, ZNF64*, and *ZNF217*. Several are zinc finger proteins (ZNF) critical for proper germ cell development, like male primordial germ cells specification and epigenetic reprogramming (180).

vii) enzymatic functions

Genes, such as *MPV17L*, *TKTL1* and *UCK2* were found in association with TGCT risk loci. *MPV17L* at 16p13.11 participates in reactive oxygen species metabolism by up- or down-regulation of the genes of antioxidant enzymes. The gene *TKTL1* on the X chromosome, encodes a transketolase that acts as a homodimer and catalyzes the conversion of sedoheptulose 7-phosphate and D-glyceraldehyde 3-phosphate to D-ribose 5-phosphate and D-xylulose 5-phosphate. This reaction links the pentose phosphate pathway with the glycolytic pathway. The gene *UCK2* encodes a pyrimidine ribonucleoside kinase. The encoded protein catalyzes phosphorylation of uridine and cytidine to uridine monophosphate and cytidine monophosphate, respectively; hence, its related pathway among others is nucleotide metabolism.

Although many of these genes and pathways implicated in TGCT have extensive evidence to support their role in the formation of this malignancy, a G-WAS signal points only to a genomic block of linkage disequilibrium. Unfortunately, the putative biological mechanism of many identified TGCT SNPs is still unclear.

In fact, these 78 SNPs do not have a cause-effect relationship with TGCT, they only contribute to predispose to the tumor. The various combinations of disease-associated SNPs allow distinguishing distinct haplotypes, each of them may lead to a different predisposition entity. The so-called **Polygenic Risk Score** (PRS) is a valid tool for the calculation of overall risk for a given disease. This

approach is defined as a single value estimate, which shows an individual's genetic liability to a phenotype, calculated as a sum of their genome-wide genotypes, weighted by corresponding genotype effect size estimates. PRS combined with lifestyle and clinical factors is useful to obtain an even more precise risk estimate. Concerning TGCT, Litchfield and his colleagues (181) calculated the combined effect of 18 TGCT risk loci. To accomplish this, PRSs were calculated based on the sampling of two million randomly generated genotypic combinations and combining these with OR per SNP, per individual. Results show that men within the top 10% of genetic risk have a 4.2-fold increased relative risk of TGCT, whereas men within the top 1% have an 8.7-fold elevated relative risk of TGCT compared with the median population risk. This value is much higher than that calculated in other cancers such as breast cancer and prostate cancer. Subjects in the top 1% for the combined effect of 71 risk loci for breast cancer, and 65 for prostate cancer, showed only 3- and 5-fold elevated risk, respectively (182,183). Since the absolute lifetime risk of TGCT is low (0.5% in Caucasian males), even for men in the top 1% with an almost nine-fold elevated relative risk, their absolute lifetime risk is shifted to a modest 4.4%. In a subsequent study by adding 4 more, newly identified risk loci, the genetic risks raised to 10.4-fold relative and 5.2% lifetime risk of TGCT among men in the top 1% (160). In 2018, Loveday and his colleagues (184) calculated the PRS for 37 TGCT susceptibility SNPs in 236 familial and 3931 sporadic TGCT cases versus 12.368 controls. They observed a significant enrichment of risk alleles in familial cases compared with sporadic cases ($p = 0.0001$) underpinning familial clustering of TGCT. The latest study (151), reported a calculation of PRS for men in the top 5% (95th percentile) based on 78 SNPs, revealing a 6.8-fold increased risk, with 3.4% lifetime risk. Unfortunately, these authors did not report the risk estimate for the top 1%.

2.6.2.3. Whole Exome Sequencing and TGCT

Thanks to the widespread diffusion of Next Generation Sequencing (NGS), a rapid and cost-effective sequencing of the whole-genome (WGS) or the whole-exome (WES) became feasible. It is also known as high-throughput sequencing (or massive parallel sequencing), implying the parallel sequencing of millions of small DNA fragments. Depending on the target of interest, WGS will provide data on all the three billion bases in the human genome whereas WES will give information on the 22.000 protein coding genes. This modern technique has revolutionized the medical field facilitating the identification of novel genetic factors of various Mendelian diseases.

As described above, there is strong evidence of heritability, around 37-49% in TGCT (132). Since no major gene has been identified with SNP-arrays so far, much expectation was given to NGS based approach for the identification of hidden genetic factors.

Four WES studies (185–188) have been performed in the past few years, which sought to identify monogenic and polygenic causes of TGCT. Litchfield and his collaborators conducted two large studies on familial and sporadic cases of TGCT, but both of the studies have failed in the detection of a recurrent, high-penetrance genetic alteration. In the first study (185) they searched for genes that are recurrently affected by rare variants (MAF < 1%) with presumptive damaging effects (nonsense, splice acceptor/donor and indel frameshift) and with a low burden of comparable variants in controls. In the second study (186), they analyzed a larger study population, and extended the analysis also to the missense, and low-frequency (MAF 1–5%) variants. Based on the segregation of heterozygous mutations with TGCT among family members, the two studies identified three candidate genes and gene families: *DNAAF1* and other cilia-microtubule genes (CMGs), *DNAH7* and *BOLL*.

Regarding the first study (185), *DNAAF1* and the CMGs have been identified in a discovery level analysis of 153 independent families (n = 328 cases) with TGCT and 1644 UK males with no history of malignancy. Variants in *DNAAF1* (*alias LRRC50*) (p.Arg636Ter and p.Gly434ProfsTer4) and in its paralogue genes i.e., *LRCC6* (p.Ser27ValfsTer13), and *CNTRL* (p.Arg1038Ter and p.Glu724LysfsTer6) have been found in 9 cases in 5 families, among them 4 in complete segregation with the disease. Incomplete segregation have been detected in case of *CNTRL* (p.Glu724LysfsTer6), where the affected uncle was *wild type* for the mutation. By performing a gene set enrichment analysis, the top ranked set of genes belonged to the CMG gene family with 8 members, including also *DNAAF1*. Almost 9% of TGCT families carry rare disruptive mutations in the CMG genes as compared with 0.5% of controls (*p value* = 2.1×10^{-8}). Functional studies in zebrafish have supported the role of *DNAAF1* in testis cancer predisposition (94% of *dnaaf1*^{hu255h} (+/-) zebrafish model developed TGCT versus 14% in case of wild-type genotype), as well as immunohistochemistry staining of *DNAAF1* showed its complete absence in 3/3 tumors available from mutation carriers. In the replication part of the study, the mutation frequencies of the CMG set, and the rare variants found in *DNAAF1* and paralogue genes, *MAP4, DRC1, CEP290* and *DYNC2H1* were compared in 634 sporadic TGCT cases from the UK and 21.173 Non-Finnish European, cancer-free controls from the Exome Aggregation Consortium (ExAC Browser) database. The obtained WES data revealed additional mutations within these genes. However, none of the genes were significant according to segregation analysis alone after correcting for exome-wide analysis in 27,173 ExAC controls, in 1,644 UK controls and in 4,300 European controls. In addition, missense *DNAAF1* variants were not considered in this article because even if they were predicted as pathogenic, their frequency was equivalent between TGCT cases and control series (189). Therefore, these genes are not marked as "major" TGCT predisposition genes.

The oncogenic role of CMG's inactivation remains to be established yet; however, ciliation and the cell cycle are mutually exclusive with both processes competing for the centrosome. Thus, cilia inactivation may bias towards cell cycle progression and proliferative growth. Worth to note that the loss of cilia function is emerging more broadly as an important pathway in oncogenesis in multiple cancer types (e.g. clear cell renal cell carcinoma, breast cancer, etc.) (190).

Regarding the results of the second study (186), two missense variants have been found in *DNAH7* (c.1895C>G; p.Ser632Cys and c.6340A>G; p.Thr2114Ala), which were fully segregated in 2 three-case and 8 two-case pedigrees. The total MAF of the first variant is 0.0436, whereas for the latter is 0.0303 and according to the authors the variants were predicted as *damaging*. Since the publication of this paper novel data became available and we have reassessed the pathogenicity of these variants; according to the VarSome database both of them are predicted as *benign*. On the other hand, one variant in *BOLL* (c.62C>A; p.Ser21Tyr, population MAF= 0.001) has been identified also in complete segregation in the a four-case pedigree. However, no evidence of association has been found when they genotyped the three variants in the full case-control study population (3999 unselected TGCT cases *versus* 4011 controls). The MAF of the *BOLL* variant was 0.03 in patients *versus* MAF = 0.04 in controls, p value > 0.5). Following re-evaluation of the mutation, the MAF dropped to 0.000912, and it is classified as VUS by VarSome. Besides the above-described investigation of familial TGCT cases, Litchfield et al. carried out other analyses in the frame of a case-control study (919 affected patients *versus* 1609 healthy controls) such as the gene burden testing for i) 114 established high- or moderate-penetrance cancer susceptibility genes; ii) genes associated with TGCT loci. No gene was significant at the exome-wide level with a Bonferroni-corrected threshold of p value $< 8 \times 10^{-7}$. The authors suggest that to significantly improve the discovery of rare variants for TGCT, studies at least ten-fold larger in scale are required. Since such studies are not available in the literature, the lack of high-penetrance recurrent gene mutations favors the previously proposed model of polygenic susceptibility, in which much of the heritable risk of TGCT is associated with common genetic variants.

Another WES study has been performed in 2018 by Paumard-Hernandèz (187) without notable results. Firstly, they aimed to identify genetic alterations in 19 familial cases of TGCT and examined the obtained WES variants under a monogenic and polygenic model. The variants were filtered by their type (non-synonymous, essential splice site, indels or gain/loss of stops) and by MAF < 5%. Their potential damaging effect was evaluated by *in silico* prediction tools. A total of 171 variants have been identified in the frame of monogenic (n = 125) and polygenic (n = 46) model. All of the variants have been confirmed by Sanger sequencing, and have been prioritized, which resulted in 120

variants to further evaluate in a case-control association study. Due to quality control, further 25 variants have been filtered out, but the remaining 95 variants were genotyped (*OpenArray system by Applied Biosystems*) in 391 sporadic Spanish TGCT cases and 1170 healthy Spanish men. In this case-control study population five variants resulted statistically significant. The variants were mapped to genes *GRP* (rs149962068), *PLEC* (rs138924815), *DNAH7* (rs62623377), *EXO5* (rs150018949), *GPRC6A* (rs: not available, p.Tyr775delinsTer) with a MAF between 0.01-0.03. However, only three of them were replicated in a second cohort of 101 Caucasian TGCT cases from The Cancer Genome Atlas database (TCGA) and 27.000 Non-Finnish European, cancer-free controls from the ExAC Browser database. All three variants were predicted as highly pathogenic by the prediction tools used in the study (PredictProtein, CADD, Polyphen, SIFT and Condel) The variant in *PLEC* (MAF = 0.012) with complete segregation in 2 families has been validated as a high susceptibility risk allele with an OR=6.28 and *p value* = 6.42×10^{-23}. It was found in 25/365 cases and 27/1129 controls of the Spanish population. The gene encodes Plectin, which is a prominent member of an important family of structurally, and in part functionally related proteins, termed plakins or cytolinkers, that are capable of interlinking different elements of the cytoskeleton. It is expressed in Sertoli cells at the nuclear surface and at sites of attachment to elongated spermatids. Although at the time of publication the variant on *PLEC* was predicted as pathogenic based on *in silico* prediction tools, it is currently classified as benign by VarSome and ClinVar. The other two mutations, one in *EXO5* (MAF = 0.013) and one in *DNAH7* (MAF = 0.018), both showing incomplete penetrance in single independent families, were found as moderate (OR=3.37) and low (OR=1.64) susceptibility risk alleles, respectively. *EXO5* encodes a single-stranded DNA-specific bidirectional exonuclease involved in DNA repair, whereas *DNAH7* encodes a protein found among others in sperm flagella, and has dynein ATPase activity, hence essential in spermatogenesis. The reassessment of these variants shows that the variant in *EXO5* is classified as likely benign by VarSome, whereas *DNAH7* as VUS, with a much lower MAF = 0.00000403.

Similarly to the previous WES TGCT studies, the above data indicate that although familial TGCT has a strong genetic component, the genetic basis of this tumor is likely to be determined by the co-inheritance of multiple risk variants. In order to demonstrate such a model much larger study populations are needed.

The last study by AlDubayan and his colleagues from 2019 (188) evaluated the role of 48 DNA repair genes in TGCT with established cancer risk, from the curated COSMIC (Catalogue of Somatic Mutations in Cancer) germline cancer census gene set (v86; http://cancer.sanger.ac.uk/census). They based their hypothesis on a study by Taylor-Weiner (2016) (191), which tried to explore the origins

of TGCT by conducting an integrative analysis of tumor genomic and transcriptomic data. Taylor-Weiner et al. showed that TGCTs were exceptionally enriched for arm-level and chromosome-level gains of one parental allele with simultaneous loss of the other parental allele, leading to reciprocal loss of heterozygosity. This DNA double-strand break-enriched genomic signature suggested that increased DNA damage with impaired repair might be important in the development and progression of TGCTs. This finding is further supported by previous G-WAS that highlighted several validated TGCT-risk SNPs located in or near DNA-damage sensing and repair genes, such as *RAD51C* and *RFWD3* (168). However, since the role of inherited DNA repair defects in TGCT pathogenesis is still uncharacterized, the study of AlDubayan aimed at the systematic evaluation of germline pathogenic variants in 48 Mendelian cancer predisposition DRGs in unselected 205 men with TGCT compared with 27,173 cancer-free non-Finnish European individuals from the ExAC cohort. Significant findings from the discovery phase were replicated both in independent cohorts of 448 unselected men with TGCT and 442 population-matched controls, and in 231 high-risk TGCT subjects (those with two or more individuals affected by TGCT in the family, or those with bilateral TGCTs) and 3090 cancer-free men. Germline variants in cases and controls from all cohorts were independently assessed for pathogenicity by three clinical geneticists, using the American College of Medical Genetics and Genomics guidelines (ACMG) (192,193). Only pathogenic and likely pathogenic variants were taken into consideration in the study.

The authors observed a significantly higher than expected rate of inherited *CHEK2* pathogenic variants in men with TGCT then in controls. They identified a low-penetrance, common founder variant in *CHEK2* (c.470T>C; p.Ile157Thr) in 28/884 TGCT cases and 25/3532 controls. The mutation carriers were significantly more likely to develop TGCT compared to controls.

Furthermore, the authors found that germline, rare (MAF < 1%) LOF variants in *CHEK2* (such as c.1100delC p.Thr367Metfs; complete deletion of exon 9 and 10; c.1361G>A p.W411*; c.444+1G>A; c.85C>T p.Q29*, etc.), in all unselected and high-risk cohorts (n = 18/884), appear to confer a significantly increased risk of TGCT compared with *wild type* allele carriers. *CHEK2* encodes a cell cycle checkpoint regulator and putative tumor suppressor. This protein is phosphorylated by the DNA damage-sensing protein ATM (ataxia-telangiectasia mutated) to subsequently regulate more than 20 downstream effector proteins, such as BRCA1, BRCA2, p53, and Rb, which are crucial in the mechanisms of DNA double-strand break repair, cell cycle regulation, and cellular apoptosis.

So far, *CHEK2* is considered as the only moderate-penetrance gene. The clinical importance of this finding is that germline, rare LOF alterations in *CHEK2* confer a moderate increase in the risk of TGCTs in around 4% of affected men. Beyond the potential for TGCT risk stratification, the identification of pathogenic germline *CHEK2* variants in TGCT subjects may also be informative for

the clinical management of these individuals. First, germline *CHEK2* LOF-variant-carrier TGCT patients develop the tumor significantly earlier than those TGCT subjects with *wild type CHEK2* alleles. In addition, individuals with *CHEK2*-mutated TGCTs are more likely to develop other cancers, such as prostate and colorectal cancers, for which specific cancer screening strategies already exist. Furthermore, as several studies exploring the efficacy of targeted therapeutic interventions (such as CHEK1 and CHEK2 inhibitors) in DNA–repair–proficient and DNA–repair–deficient tumors are in progress, the authors identified potentially targetable DNA-repair defects that might be exploited in chemotherapy-resistant TGCT.

As a conclusion, all the four WES studies failed to identify rare, recurrent, high-penetrance, disease causing gene variants, leaving the conclusion that such variants must be very rare in unselected patients (see Table 2.). It remains to be answered, whether high-penetrance genetic defects may underlie the development of TGCT in highly selected TGCT cases, for instance those who present several tumors among first-, second-degree family members.

Table 2. – Conclusions of the four Whole Exome Sequencing (WES) studies.

Abbreviations: an. – analysis, CMGs – cilia-microtubule genes, ctrl – controls, Fm – familial, IHC – immunohistochemistry, Sp – sporadic, TGCT – Testicular Germ Cell Tumor, UK – United Kingdom, ZFM – zebrafish model.

Authors	Year	Subjects (case/ctrl)	Cohorts and methods	Main Results	Conclusions
Litchfield et al.	2016	328/1,644 634/27,173	Familial Discovery Gene burden analysis Gene set enrichment an. Sporadic Replication (UK/ExAC) Somatic alterations (IHC) Functional study (ZFM)	**CMGs** ***DNAAF1*** ***dnaaf1 (+/-)***	**CMGs as cancer susceptibility genes**
Litchfield et al.	2018	919/1,609 (613/306 Sp/Fm) 3,999/4,011	Discovery Gene burden analysis Replication	***BOLL*** ***DNAH7***	**No «major» high-penetrance gene**
Paumard Hernàndez et al.	2018	41/30 391/1,170 101/27,000	Familial Discovery Sporadic Discovery Replication (TCGA/ExAC)	***PLEC*** ***EXO5*** ***DNAH7***	**Favour of a polygenic model of inheritance**
AlDubayan et al.	2019	205/27,173 448/442 231/3090	Unselected Discovery Unselected Croatian Replication High risk Replication	**loss-of-function *CHEK2* variants ,** **low-penetrance *CHEK2* variant**	***CHEK2* as a novel moderate-penetrance TGCT susceptibility gene**

2.7. TGCT and familial cancer risk

Since 1996, the question whether the family members of TGCT patients are at an increased risk for overall cancer development became a topic of debate. The elevated incidence of familial TGCT is well established, but regarding the risk for the development of any kind of other cancers, is still controversial.

Westergaard et al. (194), observed only the aggregation of TGCT, but no excess of other cancers while investigating the cancer risk of fathers and brothers of TGCT patients. Similarly, the early studies of Kroman et al. (195) and Heimdal et al. (196) from 1996 did not confirm that families of TGCT patients are prone to cancer. Kroman and his collaborators calculated the relative risk (RR) for having an estrogen dependent cancer among TGCT patients' mothers. They observed that the RR for developing breast cancer was 0.8 (95% CI 0.6 – 1.1), for endometrial cancer 0.6 (95% CI 0.3 –1.0) and for ovarian cancer 1.0 (95% CI 0.6 – 1.6). Hence, the authors concluded that mothers of TGCT patients are not at an increased risk for developing estrogen-related cancers. Heimdal and his collaborators found that the standardized incidence ratio (SIR) for overall cancer development is 0.89 (95% CI 0.78 -1.00), but it was probably due to underreporting. Further studies by Spermon et al. (2001) (197) and McMaster et al. (2015) (198) confirmed the above-mentioned results, i.e. first-degree relatives of TGCT patients do not have an increased risk for cancers overall. However, they observed specific associations, such as TGCT aggregation, or respiratory and other genital tract cancers, leukemia, and soft tissue sarcoma excess.

On the other hand, six papers support that there is an overall increased risk of cancer among family members of TGCT patients (126,199–203). Bajdik et al. (2001) (199) and Bromen et al. (2004) (200) demonstrated an increased risk of other cancers among first- and second-degree family members; for instance, a higher risk of breast cancer, and cancers in the female genital organs of mothers or sisters of TGCT subjects. Kaijser et al. 2004 (204) reported that mothers affected by lung cancer had more frequently son with TGCT. Hungarian authors (201) examined the cancer susceptibility in the first-degree relatives of TGCT patients and found that malignancies occurred with higher probability in brothers and children of TGCT patients than in those of controls. Comparing the number of cancers among family members of controls and TGCT patients, no cancer was found among family members in 76.8% and in 63.8%, respectively (OR: 0.533; 95% CI 0.393-0.724), while one cancer occurred in 18.9% among relatives of controls, and this proportion nearly doubled (33.4%) among relatives of TGCT patients (OR: 2.151; 95% CI: 1.564-2.958). However, there was no excess in the prevalence of other cancers than testicular tumors in the brothers, and six rare childhood cancers in the offspring, such as bilateral Wilms' tumor, neuroblastoma, medulloblastoma, acute lymphoblastic leukemia,

histiocytosis-X, and testicular tumor. Dong and colleagues (126), besides confirming familial TGCT clustering (fathers SIR: 3.8, 95% CI 2.0–6.8, brothers SIR: 8.3, 95% CI 5.7–12.2, sons SIR: 3.9, 95% CI 2.0–6.7) and increased overall cancer development risk (SIR: 1.2 95% CI 1.1–1.3), evaluated the different histological subgroups separately. The authors found that only seminoma was associated with a significant increased risk for other cancers, such as pancreas and nervous system cancers. In mothers of TGCT patients, the SIR was increased for lung, non-endometrium uteri and connective tissue cancers and for melanoma, but no significant differences were found among the histological subgroups. Other study by Nordsborg and his collaborators (2011) (205) also asserted the higher incidence of cancers, furthermore, shed light to specific associations with non-Hodgkin lymphoma and esophageal cancer among first-degree relatives. The only study which analyzed the maternal and paternal lineage differences was conducted by Chia et al. (2009) (202) and identified that maternal relatives had a statistically significant increased risk of all cancer (RR=1.16, 95% CI, 1.04−1.30), digestive tract (RR=1.52, 95% CI, 1.15−2.00), and male genital organ cancer (RR=1.70, 95% CI, 1.15−2.51), but these observations remained without biological explanation.

Based on the above observations i.e. elevated familial cancer incidence in TGCT patients, and the latest WES study finding reporting the *CHEK2* mutation, it was not surprising that Lobo et al. (2020) (206) observed a mutation in a DNA repair gene in TGCT patient whom uncle was diagnosed with Lynch syndrome.

Lynch syndrome (LS) is an autosomal dominant disease, due to germline mutations in the DNA mismatch repair (MMR) gene family, including genes such as *MSH2, MSH6, MLH1, MLH3, PMS2, EPCAM.* The function of proteins encoded by MMR genes is to maintain genomic integrity with the detection and correction of DNA mismatches that occur during DNA synthesis. MMR function requires the coordination of multiple MMR gene products, thus mutation in any of the MMR genes results in the dysfunction of the overall repair system, leading to cancer predisposition. Disrupted DNA MMR system is due to the inactivation of both alleles in any of the MMR genes. The first allele inactivation originates from the inherited germline mutation, resulting in loss of function of the encoded protein, whereas the second allele is inactivated by another mechanism, such as ‘second hit’ somatic mutation, loss of heterozygosity, or epigenetic silencing. With the inactivation of the second allele, downstream genetic mutations accumulate. This generally occurs in the microsatellite regions of DNA, causing alterations in the tandem repeats’ length, referred to as microsatellite instability (MSI).

From a clinical point of view, the cancer spectrum of LS is wide. Most frequently associated malignancies are nonpolyposis colorectal and endometrial cancers, hepatobiliary tract (mainly pancreas), ovary and urinary tract, such as upper tract urothelial cancer or prostate cancer.

The case reported by Lobo et al. (206) is a TGCT patient, who had a pathogenic germline *MSH2* mutation (c.2152C>T, p.Gln718Ter) in heterozygosity. The patient's uncle, who was diagnosed with LS, carried the same germline variant. Immunohistochemistry for MMR-associated proteins was performed in the testicular seminoma, disclosing loss of *MSH2* and *MSH6* expression. Besides the presence of the germline *MSH2* mutation, tumor tissue sequencing revealed the presence of the same mutation along with loss of heterozygosity (LOH) at the *MSH2* locus. Additionally, the tumor was confirmed to harbor high microsatellite instability (MSI-H).

Thus, the authors suggest for the first time in the literature, that TGCT may be part of the Lynch syndrome associated urological malignancies. Since there is an association between MMR-deficiency, MSI and cisplatin resistance, it may be worthwhile testing for the MMR status of recurrent and/or resistant TGCT cases (207).

This is the first and so far, the only study reporting a genetic link between TGCT and familial cancer risk.

III. OBJECTIVES

3.1 Primary objectives

In order to identify new clinical and genetic risk factors of TGCT, and to explore whether TGCT may be part of a more generalized cancer predisposition, we carried out two projects: i) an epidemiological study, where we aimed to estimate the familial cancer risk among TGCT patients' relatives (first-degree and grandparents), and ii) Whole Exome Sequencing (WES) in selected TGCT patients with positive family history of cancers, to identify monogenic causes of the malignancy and to determine the role of DNA Repair genes in the etiopathogenesis of TGCT.

3.2 Secondary objectives

First project – Epidemiological study:

- To identify the type of neoplasms, which are most frequently observed among TGCT patients' family members.
- To determine the differences between seminoma and non-seminoma in regard of familial cancer risk.

- To compare whether non-normozoospermic subjects have more tumors among family members.
- To define the number of siblings in the different cohorts (TGCT, oncohematological patients, control group)

Second project – Whole Exome Sequencing in TGCT patients with multiple cancers among family members:

- To find specific genotype-phenotype correlations.

IV. MATERIALS AND METHODS

4.1 Description of the study population

For both projects included in this thesis, the patients were recruited at the Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Centre of Excellence DeNothe, University of Florence, Florence, Italy (head: Prof. Csilla Krausz, collaborator: Dr. Matteo Vannucci) and at the Andrology Department, Fundació Puigvert, Universitat Autònoma de Barcelona, Instituto de Investigaciones Biomédicas Sant Pau (IIB-Sant Pau), Barcelona, Spain (head: Prof. Eduard Ruiz Castañé, collaborator: Dr. Daniel Moreno-Mendoza, Dr. Antoni Riera-Escamilla).

First project: Epidemiological study

Study design: Multicentric, retrospective, case-control study.

Inclusion criteria:

i) Patients with confirmed TGCT and known family history. Included histopathological categories: seminoma, non-seminoma (Teratoma, Choriocarcinoma, Embryonal carcinoma, Yolk Sac tumor) and Mixed Germ Cell Tumors (seminoma and non-seminoma components together, considered as non-seminoma).

ii) Patients with confirmed hematopoietic malignancies and known family history. Included histopathological categories: Hodgkin lymphoma, Non-Hodgkin lymphoma, all types of leukemia.

Exclusion criteria:

- Unknown histology of testicular cancer/hematopoietic malignancy
- Benign testicular cancer (Leydigoma, Spermatocytic tumor, etc.)

- Unknown family history

Controls were enrolled at the Andrology Department, Fundació Puigvert, Universitat Autònoma de Barcelona, Instituto de Investigaciones Biomédicas Sant Pau, (IIB-Sant Pau). They arrived at our Clinic at Barcelona to undergo a minimal invasive andrological intervention, vasectomy.

In the first project, a total of 1407 subjects were recruited in the study, among them 592 were affected by TGCT, 352 had oncohematological malignancy, and 463 were fertile cancer-free controls. In the TGCT group 322 had seminoma, and 270 had non-seminoma (NS) as cancer histology. The oncohematological group involved three subtypes, 206 patients with Hodgkin's lymphoma, 123 patients with Non-Hodgkin lymphoma (NHL), and 23 patients with leukemia. In the control group, a total of 463 men were enrolled.

Second project: Whole exome sequencing in TGCT patients with multiple cancers among family members

Inclusion criteria: Patients with confirmed testicular cancer: i) available DNA form peripheral blood lymphocytes, ii) two or more family members affected by any type of malignant tumors.

Exclusion criteria:

- not available DNA from the proband
- less than two malignant tumors among family members

In the second project, WES was carried out for 32 TGCT and one Leydig tumor patients. Among the TGCT subjects, 20 had seminoma and 12 had non-seminoma.

4.2 Andrological visit

Patients were recruited in the frame of an onco-andrological visit at the two centers. During the visit the patients completed a detailed questionnaire with the andrologist. The questions covered the main queries regarding the patients' family history, fertility status, past medical history, known andrological alterations. They had to respond to the following questions among others, such as: i) Does anyone in your family have/had any oncological diseases? What kind of tumors they have had, and who had the tumor? Which lineage? ii) Do you have/had undescended testicles at birth? Do you have/had hypospadias?

After the questionnaire, the patients underwent a physical examination (height, weight, blood pressure, heart rate, scrotal evaluation, size, and consistence of the testicles, etc.) followed by a blood withdrawal for hormonal dosage (LH, FSH, Testosterone, Sex Hormone Binding Globulin), and for

DNA extraction. After that, a sperm-analysis was carried out for cryopreservation, according to the 5th manual of semen analysis by the WHO 2010 (208).

4.3 Statistical analyses

A comprehensive database (*Microsoft Excel Software*) was created to process all of the obtained data from the patients. The statistical analyses were performed with the SPSS Software (*version 25.0 Chicago, IL, USA*). We tested the significance of the observed differences regarding the familial tumor incidence between TGCT patients, oncohematological patients, and the control group, using Fisher´s exact test. Our null hypothesis was that the incidence is the same in the TGCT patients, oncohematological patients, and in the control group. For the comparison of the medians between the different groups, the non-parametric Mann–Whitney U-test for independent samples was applied. Correlations were assessed using Pearson's method for normally distributed data. A p-value ≤ 0.05 was considered statistically significant for each test.

In the first project, to compare the tumors among relatives, data were matched for type of relationship: TGCT patients' mothers versus oncohematological patients' mothers or controls' mothers; fathers versus fathers, grandparents versus grandparents, siblings versus siblings.

4.4 DNA extraction from peripheral blood lymphocytes

The genomic DNA was extracted from peripheral blood lymphocytes, with the presence of anticoagulant ethylenediaminetetraacetic acid (EDTA), and then frozen at -20 °C for at least 12 hours. For DNA extraction we used the standard "*Salting out*" method, based on the solubility of proteins depending on the salt concentration or ionic strength. Therefore, adding salt reduces the solubility of the sample proteins which, through the interaction of their hydrophobic side chains, will tend to aggregate and therefore precipitate. For the extraction, 5-10 mL of blood were used.

4.5 Quantitative and qualitative evaluation of the extracted DNA

The extracted DNA was analyzed using Nanodrop ND-100 (*Thermo scientific, Wilmington USA*), a spectrophotometer that provides DNA concentration and purity values, measuring the absorbance of the sample at different wavelengths. The spectrophotometer measures the absorbance of DNA at 260nm, which corresponds to the wavelength at which nucleic acids absorb. The concentration is automatically calculated by the Lambert-Beer law which correlates the quantity of light absorbed by the material (A), with the concentration of the material itself (C), the thickness of the medium passed through (b) and a coefficient of proportionality called molar absorptivity (ε):

$$A = C \cdot b \cdot \varepsilon$$

The quality of the analyzed DNA derives from the relationship between: i) the absorbance of the sample at 260nm with the absorbance of the same at 280nm, the wavelength at which the protein absorbance peak is found (A260/A280); ii) the absorbance of the sample at 260nm with the absorbance of the sample at 230nm, the wavelength at where the maximum absorption of phenols, carbohydrates, aromatic compounds, such as ethanol is (A260/A230). The sample is considered to have adequate purity when the A260/A280 ratio is approximately 1.8 and the A260/A230 ratio is greater than 2. A deviation from the ideal values of the ratios indicates the presence of contamination, such as an excess of phenols, which could cause Taq polymerase inhibition during the PCR reaction. The DNA "mother" solution was frozen at -20 °C, whereas the aliquot with around 100 ng/µL concentration is stored at + 4 °C for subsequent analyzes.

4.6 Whole Exome Sequencing (WES)

For all of the 33 patients WES was carried out. WES's protocol involved the preparation and acquisition of DNA libraries using the SureSelect Human All Exon V6 (*Agilent Technologies*) kit, following the protocol provided by the company. The enriched libraries were quantified with Qubit (*Life Technologies*) and Bioanalyzer 2100 (*Agilent Technologies*). Exome sequencing, performed with the NovaSeq 6000 System (*Illumina*), made it possible to generate 2x120 nucleotide long sequences, with a coverage of 20X for all exonic regions in the genome. Subsequently, the quality of the readings was verified through FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reading fragments, called "reads", were analyzed using the Illumina guidelines: Burrows-Wheeler Aligner (*BWA*) software was used for the alignment with the reference genome GRCh37 (hg19), for the Broad Institute (GATK - Genome Analysis Tool Kit) genotyping (https://gatk.broadinstitute.org/hc/en-us); and the ANNOVAR (ANNOtate VARiation) bioinformatics tool (http://www.openbioinformatics.org/annovar/) for annotating the variants. Raw data, including FastQC, BAM, and variant call format (vcf) files, were provided for further analyses.

4.7 Bioinformatic analyses

The results obtained from the WES analysis were filtered from the vcf files of each patient. The bioinformatic workflow is reported in Figure 3.

i) Firstly, variants with a total coverage below 12X were excluded because of the insufficient reads, which might be due to a machine error (artefact). Similarly, variants found in more than two carriers were discarded, to avoid considering false positive results due to a high variant frequency or machine

read errors. In order to further eliminate possible artifacts, we excluded the variants with a mutated/*wild type* ratio below 25%.

ii) In the subsequent step, a filter was applied for the variant frequency value in general, and in different populations, for example European non-Finnish population, obtained from the GnomAD database (https://gnomad.broadinstitute.org/): Minor Allele Frequency (MAF) < 0,01 or not available (n.a.). This allowed us to exclude polymorphisms (MAF > 5%), or common genomic alterations (MAF > 1%), which highly likely will not be the cause of the disease *per se*, due to their high frequency.

iii) Then the functional effect of the variants was taken into account, excluding the synonymous ones and variants from the untranslated regions (UTR5', UTR3'), since they do not produce any amino acid change. The non-synonymous variants, such as missense, insertions, deletions, splice-site, frameshift, stop-gain, and stop-loss, were kept.

iv) Combined Annotation Dependent Depletion (CADD, https://cadd.gs.washington.edu/) score was concerned for pathogenicity evaluation. Variants with CADD Phred score higher or equal of 20 were included in the analysis only.

v) The obtained variants were crossed with a gene list of 653 genes associated with DNA repair by Gene Ontology (GO Term Enrichment, http://geneontology.org/), thus applying a **hypothesis-driven approach**.

vi) Afterwards, an "*in house"* pathogenic index (IP) was calculated from a numerical score (from 0 to 1) based on the verdict of five "*in silico"* bioinformatics prediction tools: *SIFT* (Sorting Intolerant from Tolerant, https://sift.bii.a-star.edu.sg/), two types of *PolyPhen-2 (HDIV* and *HVAR*, http://genetics.bwh.harvard.edu/pph2/), *MutationTaster* (http://www.mutationtaster.org/) and *MutationAssessor* (http://mutationassessor.org/r3/). For each variant, the scores obtained from the five prediction tools, were summed up and divided by the number of tools which had a verdict for that specific variant. With this algorithm final IP value was computed. Regarding the "*in house"* cut-off values: **a)** in case of the point variants (SNVs, Single Nucleotide Variants) IP higher or equal of 0.7 or not calculable were retained, whereas **b)** in case of the insertions and/or deletions (indels), all of the variants were included in the analysis, independently of the IP, since IP calculation was not possible for this type of mutations.

vii) The remained, predicted as pathogenic, DNA repair gene variants were crossed with a list containing already reported Mendelian genes (n = 1731) by the OMIM database (https://www.omim.org/) with confirmed dominant inheritance, and further genes with established cancer risk, by the curated COSMIC (Catalogue of Somatic Mutations in Cancer) germline cancer census gene set (v86; http://cancer.sanger.ac.uk/census).

viii) SNVs with CADD and IP scores above the threshold values, and all indels were further analyzed manually by VarSome (https://varsome.com), Franklin by Genoox (https://franklin.genoox.com/clinical-db/home), and InterVar by WGLAB (https://wintervar.wglab.org/), which all use the American College of Medical Genetics and Genomics (ACMG, https://www.acmg.net/) and Association for Molecular Pathology (AMP, https://www.amp.org/) classifications (192,193). They are "*in silico"* prediction tools, capable to generate a verdict on the putative pathogenic effect of the single variant ("Pathogenic" – P, "Likely Pathogenic" – LP, "Uncertain Significance" – VUS, "Likely Benign" – LB, and " Benign" – B) according to the above-mentioned updated guidelines for the clinical interpretation of sequence variants with respect to human diseases. Therefore, a more precise estimation of the variants' pathogenicity was carried out, excluding those predicted as "Benign" or "Likely Benign". If the verdicts are discordant, or only one tool predict the variant "Likely Benign" while the other two tools predict it "Pathogenic", "Likely Pathogenic" or "VUS", the variant was re-evaluated manually. A variant is classified as "hot" VUS, when no benign supporting ACMG criteria is known.

ix) After applying all of the previously described criteria on the WES variants, the candidate ones were further assessed, considering different aspects:

- Evaluation of the variant whether it has already been reported in the medical literature in association with any kind of tumors, or found in affected patients, using different browsers such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), LOVD (https://www.lovd.nl/) and HGMD (http://www.hgmd.cf.ac.uk/ac/index.php);
- Evaluation of the variant whether it has already been identified in any type of cancers, through two cancer databases such as COSMIC (https://cancer.sanger.ac.uk/cosmic) and ICGC (https://icgc.org/);
- General information about the gene with the given variant, through the GeneCards database (https://www.genecards.org/), concerning its function and pathways.

- Evaluation of the phenotype in *"knock out"* mouse using the bioinformatics tool MGI (Mouse Genome Informatics: http://www.informatics.jax.org/), whether functional analyses have already been carried out to study the gene's role in oncogenesis.
- Tissue expression of the gene in adults and in the fetus (The Human Protein Atlas (https://www.proteinatlas.org/) and Human Proteome Map (http://www.humanproteomemap.org/) databases, respectively);
- The gene with the given variant is already reported in the medical literature in relation to TGCT or to other type of tumors, using the PubMed Browser (https://www.ncbi.nlm.nih.gov/pubmed);
- Evaluation of the interaction of the obtained DNA repair genes by HuRI (The Human Reference Protein Interactome Mapping Project, http://www.interactome-atlas.org/) , and the expressed proteins encoded by the candidate genes using STRING (https://string-db.org/).
- Evaluation of the protein in case of amino acid changes by the predictions of protein structure in 1D and 2D (secondary structure, solvent accessibility, transmembrane segments, disordered regions, protein flexibility, and disulfide bridges), and protein function (functional effects of sequence variation or point mutations, Gene Ontology GO terms, subcellular localization, and protein-, RNA-, and DNA binding). For this assessment, Predict Protein by ROSTLab (https://predictprotein.org/) was used. Moreover, the conservation of the amino acid changes in different species was analyzed by PRALINE (https://www.ibi.vu.nl/programs/pralinewww/), which is a fully customizable multiple sequence alignment program to obtain more information about the homology-extended alignment, predicted secondary structure and/or transmembrane structure data and iteration capabilities.

Finally, a complex prioritization of the selected variants was performed, considering both the characteristics of the variant and the characteristics of the gene.

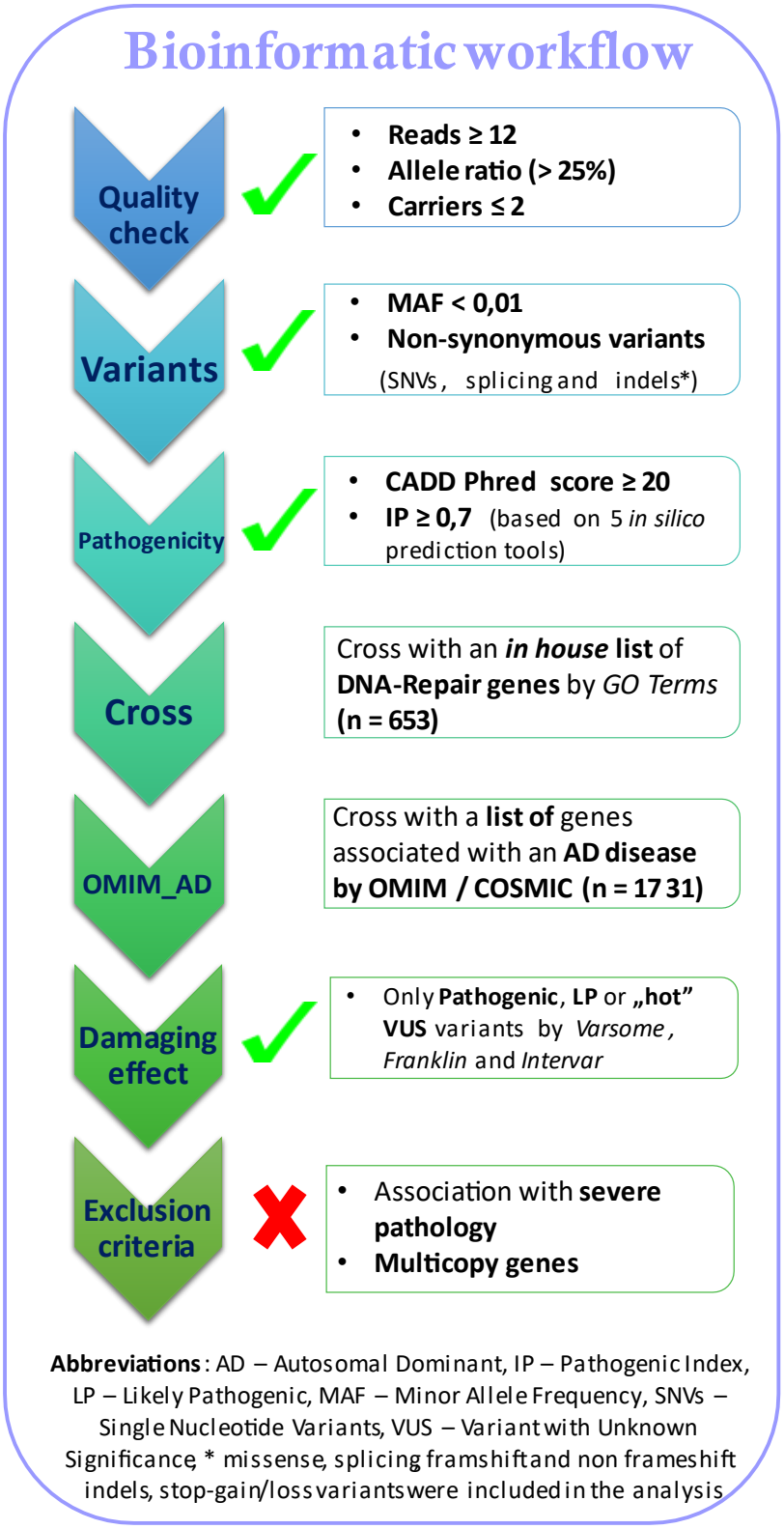


Figure 3. – Bioinformatic workflow "step by step" used for the analysis of Whole Exome Sequencing (WES) data.

4.8 Validation of the selected variants

The validation of the selected variants was performed by Sanger sequencing, a technique consisting of several phases: 1) amplification of the DNA sequence by PCR, containing the selected variant; 2) purification of the PCR product; 3) labeling reaction; 4) purification of the labeled product; 5) reading the sequences.

General overview of the PCR technique

PCR (Polymerase Chain Reaction) allows an exponential amplification of a target sequence of genomic DNA, provided that the two ends that flank are known. This amplification is carried out with Taq polymerase, a thermostable DNA polymerase, deriving from a thermophilic bacterium (*Thermus acquaticus*), which is capable to maintain its function even at high temperatures without denaturation. It is able to synthesize a new DNA strand complementary to a single-stranded template. For this process, a primer, i.e. a complementary single-stranded oligonucleotide is needed. More precisely, two primers are used (Forward 5'-3' and Reverse 3'-5'), each of which matches at the 3' end of the region of interest on one of the two complementary filaments. Thanks to these primers, the DNA polymerase adds complementary deoxynucleotides to the template (Taq polymerase does not have proofreading activity like the other DNA polymerases, hence it is not able to correct incorrectly incorporated bases). PCR is based on cyclically repeated three steps which allow to obtain a high quantity of amplified genomic DNA: 1) *denaturation*, 2) *annealing*, 3) *extension*

To avoid contamination, a negative control is always inserted, which should show any type of amplification since no DNA is added.

The amplificate obtained from the PCR reaction is analyzed with gel electrophoresis method, using agarose gel marked with ethidium bromide. This molecule intercalates the DNA double helix, which allows the visualization of the amplificate by UV transilluminator. The amplified target DNA sequence is visible as a band formation. For a correct interpretation of the observed bands on the gel, it is necessary to apply a molecular weight marker (a set of fragments of known increasing molecular weight) to the same electrophoretic run, in order to trace the length of the amplified fragment.

General overview of Sanger sequencing

Sanger sequencing, i.e. automated sequencing with chain terminator method, was developed in 1977 by Frederick Sanger and his collaborators. This technique applies modified nucleotides (triphosphate dideoxynucleotides, ddNTPs), which differ from the classic ones due to the lack of hydroxyl group, essential for the formation of the phosphodiester bond with the next nucleotide. When ddNTPs are incorporated into the nascent filament, they block its elongation. Since Taq polymerase is not able to

distinguish between ddNTPs and dNTPs, at the end of the labeling reaction (described below), the obtained DNA strands will differ with one nucleotide in length, which is due to the incorporated dideoxynucleotide. The DNA sequence of interest could be reconstructed by analyzing the terminator nucleotide beginning from the first incorporated one, up to the longest strand. In automated sequencing, the nucleotide sequence determination occurs through a single electrophoresis test within a sequencer, which is possible due to four ddNTPs marked with different fluorochromes. When the fluorochrome is intercepted by the laser, it emits a fluorescence signal precepted by the instrument. These fluorochrome emitted signal sets are converted into a graph called electropherogram: series of peaks appear in four different colors, each corresponding to a nucleotide (Adenine: green, Thymine: red, Cytosine: blue, Guanine: black); and the consecutive reading of the nucleotides corresponds to the sequence of the fragment of interest. The obtained electropherogram should be compared with the reference sequence derived from UCSC Genome Browser (http://genome.ucsc.edu/index.html).

PCR reaction for selected variants

In order to validate two candidate variants detected by WES and to perform segregation analysis, a specific PCR reaction was performed for each of them. Specific primers have been designed to amplify the genomic region containing each variant.

Gene and variant	***Primer Forward***	***Primer Reverse***	***Amplified region***
MSH6 NM_000179.2; c.2906_2907delAT; p.Tyr969LeufsTer5	CGATGGGATAC AGCCTTTGAC	TACATCCCTC CGTTCTTCAG C	351 bp
MLH3 NM_001040108.2 c.3232_3237delTGTACT; p.Cys1078_Thr1079del	GGATCTTACTC CTTGTCCAGCA	AATCCGGTA GAAGATGCC ACA	331 bp

The reaction protocol applied for the amplification of the variant in *MSH6* involved the following reagents (reaction mix for a single sample): 5µL of PCR Master Mix 2x (*Promega, Madison, WI, USA*); 1µL of 10µM Forward+Reverse Primer; 3µL of Nuclease-Free Water; 1µL of 100ng/µL DNA, for a final volume of 10µL. The PCR reaction was performed using the Applied Biosystem Veriti Thermal Cycler (*Thermo Fisher*), the program settings are described in the Table 3. below.

Step	Temperature	Time	N°cycles
Initial denaturation	94°C	5 min	
Denaturation	94°C	45 sec	x 35 cycles
Annealing	60°C	45 sec	
Extension	72°C	45 sec	
Final extension	72°C	10 min	
HOLD	4°C		

Table 3. – PCR program settings used to amplify genomic sequences containing selected variants in *MSH6* and *MLH3* genes.

At the end of each amplification reaction, an electrophoretic run of the PCR products was performed on 2% agarose gels in 0.5X TAE (*Tris-Acetate-EDTA*) buffer, then analyzed by UV transilluminator to visualize the bands. This is performed to verify the actual amplification of the specific fragment.

Labeling reaction and sequence reading for the selected variants

After the gel electrophoresis, each PCR product was subjected to an enzymatic purification using the ExoSAP-IT kit (*Affymetrix, Santa Clara, CA, USA*) in order to remove excess primers and nucleotides. After an initial incubation step at 37°C for 15 minutes, the amplificate underwent a second incubation at 80°C for another 15 minutes, according to the protocol provided by the company. Then the labeling reaction was carried out using "The BigDye® Terminator v3.1 Cycle Sequencing Kit" (*Lifetechnologies, Foster City, CA, USA*) in a final volume of 10µL for each sample: 4µL of BigDye™ Terminator 3.1 Ready Reaction Mix, 2.5X; 1µL of Forward Primer 3.2 µM; 1µL of Reverse Primer 3.2µM; 4µL of Nuclease-Free Water; 1µL of purified PCR product. The program settings of the thermal cycler were the following (Table 4.):

Step	Temperature	Time	N°cycles
Activation	96°C	1 min	
Denaturation	96°C	10 sec	x 25 cycles
Annealing	50°C	5 sec	
Extension	60°C	4 min	
HOLD	4°C		

Table 4. – Labeling program settings used for variant analysis in *MSH6* and *MLH3* genes.

At the end of the labeling reaction, further purification was performed on the product using the NucleoSEQ Columns kit (*Macherey-Nagel*), based on the protocol provided by the company. With this purified product, a mix was made for each sample, consisting of 8µL of Formamide and 7µL of purified Marked Product. Afterwards, this mix was analyzed using the 3130 Genetic Analyzer automated four-capillary sequencer (*Applied Biosystems, Carlsbad, CA, USA*).

4.9 DNA extraction from buccal swab

In order to verify the way of transmission, QIAamp DNA Mini Kit was used for DNA purification from buccal swabs (*C.E.P., cotton Omni Swabs, Whatman Bioscience*) in case of A2301 patient' parents, due to the long distance of their residency.

To collect samples, the swab was firmly scraped against the inside of each cheek at least 6 times. After collection, the swab was air-dried for at least 2 hours. DNA extraction was performed according to the protocol provided by the manufacturer.

DNA was extracted from peripheral blood lymphocytes for all available family members (mother and father) in case of patient 18-1040.

V. RESULTS

5.1. EVALUATION OF FAMILIAL CANCER RISK AMONG TGCT PATIENTS

5.1.1 Clinical characteristics of the study population

A total of 1407 subjects were recruited in the study, among them 592 were affected by TGCT (Seminoma, Non-Seminoma), 352 had oncohematological (OH) malignancy –Hodgkin's lymphoma (HL), Non-Hodgkin lymphoma (NHL), leukemia– and 463 were fertile cancer-free controls. Clinical data on the whole study population is reported in the "Materials and Methods" section and in Table 5.

***Mean age at diagnosis**:* the mean age at the disease onset among patients was not significantly different. The earliest onset of the disease was among the HL group (27,14 ± 7,48 years ± SD), followed by non-seminoma (28,71 ± 6,70 years ± SD), whilst the latest disease onset was for NHL (34,68 ± 9,35 years ± SD). The control group had a mean age of 39,97 ± 4,99 years ± SD, which was significantly higher in respect to TGCT or OH cohorts.

Anthropometric parameters: The lowest BMI was observed in patients affected by HL (23,70 ± 4,21 SD), probably due to critical systemic illness. Whereas the highest BMI was observed among the controls (26,54 ± 3,78 SD).

Semen phenotype: Significantly more non-normozoospermic patients were in the TGCT group as compared with the oncohematological group (51,35% *versus* 34,66%, *p value* < 0.0001), with an almost two times higher risk for TGCT patients to be non-normozoospermic (OR: 1.99, 95% CI: 1.52 – 2.62). The highest number of oligozoospermic patients (total sperm count < 39 million) were among the non-seminoma group (47,41%), whereas the highest number of normozoospermic patients were observed among the NHL patients' group (73,98%). These findings were confirmed when the total sperm count (TSC) and the total motile sperm count (TMSC) were investigated as well: statistically significant differences were observed when TGCT and OH cases were compared to the control group (*p value* < 0.0001) and when TGCT versus OH patients were compared (*p value* < 0.0001).

The frequency of ***cryptorchidism and hypospadias*** was also evaluated in the study, given the association between TGCT and these conditions in the context of TDS. As expected, cryptorchidism was much more frequently observed among TGCT cases as compared to OH cases, or to controls (14,19% *vs* 4,83%, or *vs* 1,94%, *p value* < 0.0001). The risk of having cryptorchidism was increased from 3.26 (95% CI 1.90 – 5.59) to 8.34 (95% CI 4.15 – 16.78) in TGCT patients *versus* OH and controls, respectively. This marked difference was not observed concerning hypospadias (1,18% *vs* 0,57%, *p value* = 0.36, or 1,18% *vs* 0,22%, *p value* = 0.11, respectively).

Table 5. - Main clinical data related to the study cohorts.

	TGCT	*Seminoma*	*Non-Seminoma*	***OH***	*HL*	*NHL*	*Leukemia*	***CTRL***	*p value*[1]	*p value*[2]	*p value*[3]
Total number of cases	**592**	322	270	**352**	206	123	23	**463**	-	-	-
Mean age (y) ± SD	**31,40 ± 6,87**	33,31 ± 6,28	28,71 ± 6,70	**30,00 ± 9,00**	27,14 ± 7,48	34,68 ± 9,35	30,61 ± 9,26	**39,97 ± 4,99**	< 0,001	< 0,001	0,12
BMI ± SD	**24,66 ± 3,57**	24,72 ± 3,40	24,59 ± 3,77	**24,28 ± 4,26**	23,70 ± 4,21	25,16 ± 4,26	24,93 ± 4,00	**26,54 ± 3,78**	0,001	0,001	0,007
Total sperm count (x million) ± SD	**86,03 ± 136,01**	90,40 ± 154,42	80,80 ± 110,03	**152,92 ± 188,96**	131,51 ± 174,88	183,90 ± 201,49	181,50 ± 223,35	**234,97 ± 220,16**	< 0,0001	< 0,0001	< 0,001
Total motile sperm count (x million) ± SD	**34,71 ± 64,39**	37,54 ± 75,34	33,00 ± 50,46	**63,55 ± 109,11**	57,49 ± 101,39	72,61 ± 115,37	76,55 ± 132,33	**125,78 ± 131,75**	< 0,0001	< 0,001	< 0,001
Non-normozoospermia	**304 (51,35%)**	164 (50,93%)	140 (51,85%)	**122 (34,66%)**	82 (39,81%)	32 (26,02%)	8 (34,78%)	**48**	< 0,0001	< 0,0001	< 0,0001
Oligozoospermia	**265 (44,76%)**	137 (42,55%)	128 (47,41%)	**94 (26,70%)**	66 (32,04%)	24 (19,51%)	4 (17,39%)	**48**	< 0,0001	< 0,0001	< 0,0001
Azoospermia	**39 (6,59%)**	27 (8,38%)	12 (4,44%)	**28 (7,95%)**	16 (7,77%)	8 (6,50%)	4 (17,39%)	**0**	0	0	0,43
Cryptorchidism	**84 (14,19%)**	47 (14,60%)	37 (13,70%)	**17 (4,83%)**	12 (5,82%)	5 (4,06%)	0 (0%)	**9 (1,94%)**	< 0,0001	0,02	< 0,0001
Hypospadias	**7 (1,18%)**	5 (1,55%)	2 (0,74%)	**2 (0,57%)**	0 (0%)	2 (1,62%)	0 (0%)	**1 (0,22%)**	0,11	0,43	0,36

[From left to right: p value[1]: TGCT *versus* CTRL, p value[2]: OH *versus* CTRL, p value[3]: TGCT *versus* OH. **Abbreviations:** BMI – body mass index, CTRL – controls, HL– Hodgkin's lymphoma, NHL – Non-Hodgkin lymphoma, OH – oncohematological patients, SD – standard deviation, TGCT – Testicular Germ Cell Tumor patients, y – years]

5.1.2 Aggregation of cancer cases among the first-degree relatives and grandparents of TGCT and OH patients, and cancer-free controls: comparison of the frequency of cancers in the different cohorts of the study population

The main objective of this study was to evaluate whether the relatives of TGCT patients have an increased risk for tumors, compared to two cohorts: i) subjects affected by OH malignancies, ii) cancer-free controls. We analyzed the presence or absence of neoplasms among the family members of the three different cohorts (see Table 6.). No differences were observed between the TGCT and OH cohorts: 241 out of 592 TGCT patients have positive family history for tumors (40,5%) whereas 136 out of 352 (38,6%) in OH patients [*p value* = 0.235]. When the same parameter was compared between TGCT and the cancer-free control group, a statistically significant increase of tumor incidence among family members of TGCT patients was observed: 241/592 (40,5%) *vs* 135/463 (29,16%) [*p value* = 0,0001, OR 1,668; 95% CI 1,29 – 2,16]. We found that also OH patients presented a significantly higher incidence of positive family history for tumors as compared to controls [*p value* = 0,0045 OR 1,530; 95% CI 1,14 – 2,05].

In case of positive family history, the mean number of tumors among relatives of cases and controls was compared, using *Mann-Whitney U* test for independent samples. Among TGCT family members a mean number (± SD) of 1.51 ± 0,71 tumors were identified, among OH family members it was 1.41 ± 0,74 tumors, whereas among controls' relatives it was only 1.21 ± 0,46. These observed differences were statistically significant both between TGCT *versus* controls, and OH *versus* controls (*p value* < 0.001 and 0.016, respectively), but not between TGCT *versus* OH patients (*p value* = 0.102).

Table 6. – Comparison of the incidence of positive family history for tumors and mean number of tumors among relatives of TGCT cases, OH cases, and controls.

	TGCT (n = 592)	OH (n= 352)	CTRL (n= 463)	p value[1]	p value[2]	p value[3]
Positive fam. history for tumors	**241 (40,5%)**	**136 (38,6%)**	**135 (29,16%)**	0,0001	0,0045	0,235
Mean number of tumors in case of positive fam. history ± SD	**1,5062 ± 0,714**	**1,4118 ± 0,745**	**1,2148 ± 0,463**	< 0,001	0,016	0,102

[From left to right: *p value*[1]: TGCT patients *versus* CTRL; *p value*[2]: OH *versus* CTRL; *p value*[3]: TGCT *versus* OH. **Abbreviations:** CTRL – controls, fam. – family, n – number, OH – oncohematological patients, SD – standard deviation, TGCT – Testicular Germ Cell Tumor patients]

5.1.3 Comparison of the frequency of the positive family history for tumors in the different subgroups in function of tumor' histotype and sperm phenotype

To further elaborate the above observations, we analyzed the frequency of positive family history for tumors in the different subgroups of the cases (see Table 7.). The two TGCT histotypes show very similar frequency of positive family history for tumors: 41,3% (133/322) in case of seminoma *versus* 40% (108/270) in case of non-seminoma. Regarding the OH patients, we confronted HL with other types of hematological malignancies, i.e. NHL and leukemia, and no significant differences were observed.

When we evaluated the sperm phenotype, we observed a higher frequency of positive family history for tumors among non-normozoospermic, i.e. oligozoospermic, cryptozoospermic, azoospermic patients compared to normozoospermic patients (see Table 7.). In case of TGCT, 43,75% non-normozoospermic patients (133/304) had tumor(s) among their relatives, whereas this percentage was 37,5% (108/288) among normozoospermic TGCT patients. In case of OH patients, 39,34% (48/122) with positive family history was non-normozoospermic and 38,26% (88/230) were normozoospermic. Thus, modest, but not significant differences were identified between groups.

Comparing the various subgroups with the control group, statistically significant differences were observed in almost all instances, except leukemia patients. This is in contrast with the fact that leukemia patients have the second highest positive family history for tumors (43,48%), but this was the smallest subgroup, and it is likely the cause for not reaching statistical significance.

Table 7. – Comparison of the incidence of positive family history distributed in different subgroups, in function of histotype and sperm phenotype.

	Patients with tumors in their family	*p value[1] OR (CI 95%)*	*p value[2] OR (CI 95%)*
Seminoma (n = 322/592)	133 (41,3%)	0,75 1,06 (0,76 – 1,47)	**0,0004** **1,71 (1,27 – 2,31)**
Non-Seminoma (n = 270/592)	108 (40,0%)		**0,0027** **1,62 (1,18 – 2,22)**
TGCT N (n = 288/592)	108 (37,5%)	0,12 0,77 (0,56 – 1,07)	**0,018** **1,46 (1,07 – 1,99)**
TGCT NN (n = 304/592)	133 (43,75%)		**< 0,0001** **1,89 (1,40 – 2,56)**
HL (n = 206/352)	77 (37,38%)	0,56 0,88 (0,57 – 1,36)	**0,035** **1,45 (1,03 – 2,05)**
NHL (n = 123/352)	49 (39,84%)		**0,024** **1,61 (1,06 – 2,43)**
Leukemia (n = 23/352)	10 (43,48%)		0,15 1,87 (0,80 – 4,37)
Oncohemat N (n= 230/352)	88 (38,26%)	0,84 0,96 (0,61 – 1,50)	**0,016** **1,51 (1,08 – 2,10)**
Oncohemat NN (n= 122/352)	48 (39,34%)		**0,032** **1,58 (1,04 – 2,39)**
CTRL (n = 463)	135 (29,16%)	-	-

[From left to right: *p value*[1]: comparing the two subgroups from a given category, i.e. Seminoma *vs* Non-Seminoma; TGCT N *vs* TGCT NN; LH *vs* NHL and Leukemia; OH N vs OH NN; *p value*[2]: comparing the given subgroup *vs* CTRLs. **Abbreviations:** CTRL – controls, n – number; N – normozoospermic patients; NN – non-normozoospermic patients, OH – oncohematological patients, TGCT – Testicular Germ Cell Tumor patients]

5.1.4 The "Top10" tumor types among relatives belonging to the three cohorts

We collected data on the malignant tumor types among the first-degree relatives and grandparents. As a second control group, we extrapolated the Italian and Spanish general population data from the ECIS - European Cancer Information System (https://ecis.jrc.ec.europa.eu/). **The "Top10" cancer types among relatives belonging to the three cohorts** are shown in Table 8. In Table 8. we reported all tumors present among the family members, according to their frequency. Seven out of the "Top10" cancers are in all four cohorts (colorectal, anal, and other gastro-intestinal tract cancers, breast, lung, prostate, urinary tract, and hematopoietic cancers).

Some differences have been observed among the frequency of the above-mentioned malignancies, such as prostate cancer is the most recurrent in the general population, whereas in our cohorts is only in the 4th- 5th position. Concerning hematopoietic malignancies, it is the second most recurrent cancer type among OH patients' relatives, while in other cohorts are at the 7th- 9th position.

We confirm the data reported in the literature concerning that TGCT patients' relatives have a higher risk for testicular and extra-testicular germ cell cancer development. Another interesting finding is that pancreas cancer is in the "Top10" tumors among TGCT relatives and in the Italian and Spanish general population, but not in the other two cohorts, i.e. among OH patients' and controls' relatives.

Table 8. – Identified tumor types among the family members ordered by their frequency (from highest to lowest). The "Top10" most recurrent tumor types are highlighted in red. The number in the list remained the same, if the observed number of affected relatives were equal for more tumor types.

TGCT	Oncohemat	CTRL	Gen.Pop. ESP-ITA
1 CRC, ANAL	1 BREAST	1 CRC, ANAL	1 PROSTATE (14.4)
2 BREAST	2 HEMATOPOIETIC	2 BREAST	2 BREAST (12.9)
3 LUNG	3 CRC, ANAL	3 LUNG	3 CRC, ANAL (8.7)
3 OTHER GIT	4 LUNG	4 PROSTATE	4 LUNG (6.6)
4 URINARY TRACT	5 PROSTATE	5 OTHER GIT	5 URINARY TRACT (3.3)
5 PROSTATE	6 OTHER GIT	6 URINARY TRACT	6 UTERUS (2.7)
6 PANCREAS	7 URINARY TRACT	7 HEMATOPOIETIC	7 PANCREAS (2.3)
7 TESTICULAR	8 UTERUS	8 CNS	8 OTHER GIT (2.0)
7 HEMATOPOIETIC	8 CNS	9 MELANOMA, SKIN	9 HEMATOPOIETIC (1.8)
8 CNS	9 MELANOMA, SKIN	10 OVARIAN	10 MELANOMA, SKIN (1.5)
8 OTHER CANCER (ET-GCTs, OSTEOSARCOMA, THROAT, NECK, VOCAL CORDS)	10 LARYNGEAL	11 OTHER CANCER (VOCAL CORD, NECK, THROAT)	10 OVARIAN (1.5)
9 MELANOMA, SKIN	10 OTHER CANCER (SPLEEN, CERVIX, OSTEOSARCOMA,)	11 TESTICULAR	11 THYROID (1.3)
9 THYROID	11 OVARIAN	12 UTERUS	12 CNS (0.9)
10 UTERUS	11 PANCREAS	12 THYROID	13 LARYNGEAL (0.6)
11 OVARIAN	12 TESTICULAR	12 PANCREAS	14 TESTICULAR (0.5)
12 ESOPHAGEAL	12 ESOPHAGEAL	13 ESOPHAGEAL	15 ESOPHAGEAL (0.4)
13 LARYNGEAL	12 THYROID	-	16 OTHER CANCER

[**Abbreviations:** CNS – cranio-nerv system malignancies, CRC – colorectal cancers, ET-GCT – extra-testicular germ cell cancer, ESP – Spanish, GIT – gastro-intestinal tract cancers, ITA – Italian]

5.1.5 Familial cancer risk estimation for each cancer types observed in the three cohorts

We performed a risk estimation for the occurrence of the different type of cancers depending on the relationship of the relatives to the index case. Although the number of affected individuals in the subgroups are small, for some cancer types, significant differences were observed between subgroups. Results of this set of analyses is reported in Table 9.

Taken all relatives together, we observed that TGCT patients' relatives show a significantly higher frequency for six concordant malignant tumor types (breast, colorectal, anal, and other gastro-intestinal tract cancers, pancreas, urinary tract, and other cancers, such as extra-testicular germ cell tumor) and for testicular cancer.

In case of OH patients' relatives, we confirm the increased risk for hematopoietic malignancy development (*p value* < 0.001; OR: 4.14; 95% CI: 1.84 – 9.32). Furthermore, a statistically increased risk was identified for breast cancer also among OH patients' family members (*p value* = 0.02; OR: 2.37; 95% CI: 1.37 – 4.11).

Regarding the type of kinship, no significantly higher risk was found for any type of cancer development in mothers and fathers comparing the three different cohorts. A significantly lower frequency of colorectal (CRC) and anal cancer was observed in OH patients' fathers versus controls' fathers. It is probably due to the relatively younger age of the OH patients' fathers, given the later onset of CRC and anal cancers. When cancer risk was assessed for siblings, a significantly higher risk for TGCT development in brothers of TGCT patients (*p value* = 0,03 OR: 9,94; 95% CI: 1,22 – 81,18) was identified, thereby confirming data in the literature.

The most pronounced differences were observed among the grandparents. In case of TGCT patients' grandparents *versus* controls, the frequency was significantly higher for five tumor types, such as lung cancer (*p value* = 0,02), breast cancer (*p value* = 0,0004), colorectal and anal cancer (*p value* = < 0,001), other gastro-intestinal tract cancers (*p value* = 0,001), and urinary tract cancers (*p value* = 0,009). In case of OH patients' grandparents, a significantly higher risk was identified for the development of hematopoietic malignancies (*p value* = 0,004), breast cancer (*p value* = 0,006), colorectal and anal cancer (*p value* = 0,001), and other gastro-intestinal tract cancers (*p value* = 0,02).

Table 9. - Risk estimate for individual cancers in first-degree relatives and in grandparents of patients and controls

[The statistically significant differences are highlighted in red. *p^1 value:* TGCT patients' relatives *versus* CTRLs' relatives; *p^2 value:* OH patients' relatives *versus* CTRLs' relatives.

* Sex specific cancers were calculated only for the given sex, i.e. prostate and testis only for males, and ovarian and uterine cancer for females.

** Other cancers among the **i) TGCT group:** extra-testicular germ cell tumors, osteosarcoma, head, and neck cancers, etc**; ii) OH group:** spleen, cervical cancer, osteosarcoma, etc; **iii) control group:** head and neck cancers, such as esophagus, vocal cord, neck, and throat cancers.

Abbreviations: CNS – cranio-nerv system malignancies, CRC – colorectal cancers, ET-GCT – extra-testicular germ cell cancer, GIT – gastro-intestinal tract cancers]

Cancer site	Mothers					Fathers					Grandparents				
	TGCT (n=592)	OH (n=352)	CTRL (n=463)	p[1] OR (CI 95%)	p[2] OR (CI 95%)	TGCT (n=592)	OH (n=352)	CTRL (n=463)	p[1] OR (CI 95%)	p[2] OR (CI 95%)	TGCT (n=2368)	OH (n=1408)	CTRL (n= 1852)	p[1] OR (CI 95%)	p[2] OR (CI 95%)
Lung	2	2	0	-	-	8	7	11	0,22 0,56 (0,22 – 1,41)	0,71 0,83 (0,32 – 2,17)	27	13	8	**0,02** **2,66 (1,20 – 5,87)**	0,09 2,15 (0,89 – 5,20)
Breast	23	19	16	0,71 1,13 (0,59 – 2,16)	0,18 1,59 (0,81 - 3,15)	0	0	0	-	-	32	13	3	**0,0004** **8,44 (2,58 – 27,62)**	**0,006** **5,74 (1,63 – 20,19)**
CRC, Anal	5	3	7	0,32 0,55 (0,18 – 1,76)	0,40 0,56 (0,14 – 2,18)	9	1	13	0,15 0,53 (0,23 – 1,26)	**0,03** **0,10 (0,02 – 0,76)**	44	19	5	**< 0,001** **7,00 (2,77 – 17,67)**	**0,001** **5,05** **(1,89 to 13,76)**
Other GIT	3	2	2	0,86 1,17 (0,20 – 7,06)	0,78 1,32 (0,19 – 9,40)	5	2	6	0,48 0,65 (0,20 – 2,14)	0,31 0,43 (0,09 – 2,17)	27	10	3	**0,001** **7,12 (2,15 – 23,47)**	**0,02** **4,41** **(1,21 to 16,54)**
Urinary T,	0	1	2	-	0,73 0,66 (0,06 – 7,27)	8	3	4	0,46 1,57 (0,47 – 5,25)	0,99 0,99 (0,22- 4,44)	18	5	2	**0,009** **7,09 (1,64 – 30,57)**	0,15 3,30 (0,64 – 17,02)
*Prostate**	-	-	-	-	-	7	9	11	0,15 0,49 (0,19 – 1,28)	0,87 1,08 (0,44 – 2,63)	14	12	6	0,22 1,83 (0,70 – 4,79)	0,05 2,66 (0,99 - 7,12)
*Testis**	-	-	-	-	-	3	1	2	0,86 1,17 (0,20 – 7,10)	0,73 0,66 (0,06 – 7,27)	**6**	0	0	-	-
Pancreas	1	0	0	-	-	2	0	2	0,81 0,78 (0,11 – 5,57)	-	**16**	2	0	-	-
Hematop	5	4	1	0,21 3,94 (0,46 – 33,80)	0,14 5,31 (0,59 – 47,72)	3	4	4	0,48 0,58 (0,13 – 2,62)	0,70 1,32 (0,33 – 5,31)	5	14	3	0,72 1,30 (0,31 – 5,46)	**0,004** **6,19 (1,78 – 21,58)**
CNS	3	0	0	-	-	0	2	4	-	0,63 0,66 (0,12 – 3,60)	8	3	2	0,15 3,14 (0,67 – 14,78)	0,46 1,98 (0,33 – 11,84)
Mel, Skin	2	1	1	0,71 1,57 (0,14 – 17,33)	0,85 1,32 (0,08 – 21,12)	5	1	0	-	-	4	1	2	0,61 1,57 (0,29 – 8,55)	0,73 0,66 (0,06 – 7,26)
*Ovarian**	3	2	3	0,76 0,78 (0,16 – 3,89)	0,89 0,88 (0,15 – 5,27)	-	-	-	-	-	3	0	0	-	-
*Uterus**	6	2	2	0,29 2,36 (0,47 – 11,75)	0,78 1,32 (0,19 – 9,40)	-	-	-	-	-	4	4	0	-	-
Thyroid	0	0	1	-	-	0	0	0	-	-	0	0	0	-	-
*Other cancers**	0	1	0	-	-	4	1	4	0,73 0,78 (0,19 – 3,14)	0,32 0,33 (0,04 – 2,94)	6	5	0	-	-

Cancer site	Brothers					Sisters					All relatives				
	TGCT (n=352)	OH (n=210)	CTRL (n= 491)	p[1] OR (CI 95%)	p[2] OR (CI 95%)	TGCT (n=335)	OH (n=173)	CTRL (n= 465)	p[1] OR (CI 95%)	p[2] OR (CI 95%)	TGCT (n=4239)	OH (n=2495)	CTRL (n= 3734)	p[1] OR (CI 95%)	p[2] OR (CI 95%)
Lung	0	0	0	-	-	0	0	0	-	-	37	22	19	0,06 1,72 (0,99 – 3,00)	0,08 1,74 (0,94 – 3,22)
Breast	0	0	0	-	-	1	1	2	0,77 0,69 (0,06 – 7,68)	0,81 1,35 (0,12 – 14,94)	56	33	21	**< 0,001 2,37 (1,43 – 3,92)**	**0,02 2,37 (1,37 – 4,11)**
CRC, Anal	0	0	1	-	-	0	0	1	-	-	58	23	27	**0,006 1,91 (1,20 – 3,01)**	0,39 1,28 (0,73 – 2,23)
Other GIT	2	0	0	-	-	0	0	1	-	-	37	14	12	**0,003 2,73 (1,42 – 5,25)**	0,16 1,75 (0,81 – 3,79)
Urinary T,	0	0	0	-	-	0	0	1	-	-	26	9	9	**0,02 2,55 (1,20 – 5,46)**	0,39 1,50 (0,59 – 3,78)
*Prostate**	0	0	0	-	-	-	-	-	-	-	21	21	17	0,79 1,09 (0,57 – 2,10)	0,06 1,85 (0,97 – 3,52)
*Testis**	7	0	1	**0,03 9,94 (1,22 – 81,18)**	-	-	-	-	-	-	16	1	3	**0,01 4,74 (1,38 – 16,29)**	0,54 0,50 (0,05 – 4,76)
Pancreas	0	0	0	-	-	0	0	0	-	-	19	2	2	**0,04 8,40 (1,96 – 36,10)**	0,69 1,50 (0,21 – 10,63)
Hematop	1	1	0	-	-	2	3	0	-	-	16	22	8	0,19 1,76 (0,75 – 4,13)	**< 0,001 4,14 (1,84 – 9,32)**
CNS	1	0	1	0,81 1,40 (0,09 – 22,40)	-	0	0	0	-	-	12	6	7	0,39 1,51 (0,59 – 3,84)	0,65 1,28 (0,43 – 3,82)
Mel, Skin	0	1	2	-	0,90 1,17 (0,11 – 12,97)	0	0	0	-	-	11	4	5	0,22 1,94 (0,67 – 5,59)	0,79 1,20 (0,32 – 4,46)
*Ovarian**	-	-	-	-	-	1	0	1	0,82 1,39 (0,09 – 22,29)	-	7	2	4	0,49 1,54 (0,45 – 5,26)	0,74 0,75 (0,14 – 4,12)
*Uterus**	-	-	-	-	-	0	0	0	-	-	10	6	2	0,06 4,41 (0,96 – 20,14)	0,06 4,54 (0,91 – 22,54)
Thyroid	0	0	1	-	-	1	1	0	-	-	1	1	2	0,50 0,44 (0,04 – 4,86)	0,81 0,75 (0,07 – 8,26)
*Other cancers**	4	0	0	-	-	1	0	0	-	-	15	7	4	**0,03 3,31 (1,10 – 9,99)**	0,12 2,62 (0,77 – 8,97)

5.1.6 Impaired spermatogenesis in the index cases is a significant risk factor for malignancies in their family members

In the entire study population (a total of 1407 subjects), we evaluated whether decreased sperm count is associated with an increased cancer risk among family members. 192 out of 1407 had impairment of spermatogenesis: 87 out of 192 (45,31%) subjects had a positive family history for tumors, compared with 322 out of 933 (34,51%) normozoospermic subjects. A significantly higher risk (*p value* = 0.0048, OR 1.57; 95% CI 1.15 – 2.15) was identified for tumor development among family members if the patient had severe spermatogenic disturbances.

Table 10. – Comparing the frequency of positive family history for tumors in all subjects with azoospermia and severe oligozoospermia (total sperm count < 5 million), *versus* normozoospermic (total sperm count > 39 million) subjects.

	Number of AZOO AND SEVERE OLIGO	*Number of positive fam. history for tumors (%)*	***Number of NORMOZOO***	*Number of positive fam. history for tumors (%)*	*p value OR (CI 95%)*
TOTAL	**192**	**87 (45,31%)**	**933**	**322 (34,51%)**	**0,0048 1,57 (1,15 – 2,15)**

5.1.7 Observed differences regarding the number of sibling(s) and the mean total sperm count of the index cases in the three cohorts

We were interested to define whether decreased sperm count of the index cases is associated with subfertility among the subjects' parents. We observed significantly lower number of siblings among TGCT and OH patients in respect to controls (*p value* < 0,0001). Furthermore, mean sperm count was significantly lower among TGCT subjects with no siblings versus those with siblings (*p value* < 0,001). Also joining the three cohorts this difference remained significant (*p value* < 0,001).

Tables 11. and 12. – The upper/left table reports the mean number of siblings in the three cohorts, and the mean total sperm count of the index cases with and without siblings. From left to right: *p value*[1] was calculated for TGCT patients *versus* CTRLs, *p value*[2] for OH patients *versus* CTRLs, *p value*[3] for TGCT *versus* OH, and *p value*[4] for mean TSC of TGCT patients without siblings *versus* with siblings. The lower/right table shows the mean total sperm count of the index cases with and without siblings after joining the three cohorts.

	TGCT	OH	CTRL	p value1	p value2	p value3	p value4
Mean nr. of siblings	1,160	1,094	2,065	< 0,0001	< 0,0001	> 0,05	-
Nr. of subjects without siblings (%)	122 (20,6%)	92 (26,1%)	24 (5,2%)	< 0,0001	< 0,0001	0,0502	-
Mean TSC of subjects without siblings ± SD	60,38 ± 93,57	161,4 ± 188,76	229,01 ± 191,32	< 0,001	> 0,05	< 0,01	0,029
Mean TSC of subjects with ≥1 siblings ± SD	90,53 ± 135,65	149,97 ± 189,32	235,29 ± 221,82	< 0,0001	< 0,001	< 0,001	

	No siblings	MEAN TSC	With siblings	MEAN TSC	p value
TOTAL	238 (16,9%)	116,10 ± 159,02	1169 (83,1%)	159,89 ± **195,54**	**< 0,001**

[**Abbreviations:** CTRL – controls, nr. – number, OH – oncohematological patients, SD – standard deviation, TGCT – Testicular Germ Cell Tumor patients, TSC – total sperm count]

5.2 WHOLE EXOME SEQUENCING IN TGCT PATIENTS WITH MULTIPLE CANCERS AMONG FAMILY MEMBERS

5.2.1 Clinical characteristics of the examined TGCT patients

Given the high frequency of affected family members with any type of cancers of TGCT patients in respect to controls, we aimed to perform an analysis in order to shed light on the potential genetic basis for this phenomenon. DNA-Repair genes are involved in oncogenesis of many different cancer types; hence, we carried out a targeted Next-Generation Sequencing-based analysis of these genes. We performed WES on 32 independent TGCT and one Leydig tumor cases of two Mediterranean populations i.e. Spanish and Italian to identify rare germline variants involved in DNA-Repair genes. The mean age at diagnosis (± SD) was 32,64 ± 6,52. Regarding the tumor histology, among the 33 TGCT patients, 20 had seminoma, 12 had non-seminoma, and one patient had Leydigoma. Fifteen patients were normozoospermic, whereas 18 were non-normozoospermic, ranging from oligozoospermia to azoospermia. The mean number (± SD) of affected family members with any type of cancers was 2,82 ± 0,917, among them 15 had two affected relatives, 11 had three, 5 had four, and 2 had five affected relatives. A total of 100 neoplasms were present among the family members. The "Top 5" tumors were breast, colon, gastric, lung and prostate cancer (see Table 13A, 13B). These five neoplasms are also among the "Top10" list (see Table 8.) from our first, epidemiological study (chapter 5.1).

Table 13.A	TGCT + one Leydig tumor cases with positive family history
Number of cases	**33**
Mean age (y) ± SD	**32,64 ± 6,52**
Histology	
• **Seminoma**	**20**
• **Non-Seminoma**	**12**
• **Leydigoma**	**1**
Semen phenotype	
• **Normozoospermia**	**15**
• **Non-Normozoospermia**	**18**
Mean nr. of tumors among family members ± SD	**2,82 ± 0,917**
	15
	11
• **2 tumors**	**5**
• **3 tumors**	
• **4 tumors**	
• **5 tumors**	**2**

Table 13.A – Main clinical features of the TGCT study cohort.

Table 13.B **Type of neoplasms**	**Number of neoplasms in TGCT family members**
• **Breast cancer**	**17**
• **Colon cancer**	**13**
• **Gastric cancer**	**11**
• **Lung cancer**	**8**
• **Prostate cancer**	**6**
• **Pancreas cancer**	**5**
• **Liver cancer**	**4**
• **Leukemia**	**4**
• **Uterus cancer**	**4**
• **Ovarian cancer**	**4**
• **Skin cancer**	**3**
• **Bladder cancer**	**3**
• **Renal cancer**	**3**
• **Laryngeal cancer**	**3**
• **Unspecified**	**2**
• **Testis cancer**	**2**
• **Lymphoma**	**1**
• **Dysgerminoma**	**3**
• **Other cancers (Osteosarcoma, Thyroid, Mesothelioma)**	
Total number of neoplasms	**100**

Table 13.B – Number and type of tumors among TGCT relatives.

5.2.2 Description of the variants obtained during the various steps of filtering process

A total of 7.037.422 germline variants were identified in 32 TGCT and one Leydig tumor patients. The majority of the variants were Single Nucleotide Variants, such as missense and splicing variants (n = 5.922.775), whereas the minority were indel (frameshift and non-frameshift insertions and deletions) and stop-loss / stop-gain variants (n = 1.114.647). A mean of 213.255 ± 7989 variants/person was identified. In order to eliminate possible artifacts, quality check was performed, and the following variants were excluded: i) under 12 reads, ii) a mutated/*wild type* ratio below 25%, and iii) more than 2 carriers. After this step, 326.357 variants were obtained, 275.623 SNV and splicing, and 50.734 indel variants, which correspond to a mean of 9890 ± 728 variants/person. This was followed by filtering for MAF ≤ 0,01 (or not available), obtaining 252.359 variants (209.019 SNV and splicing, and 43.340 indel variants), and after filtering for coding and splicing variants, we obtained 21.582 variants (19.659 SNV and splicing, and 1923 indel variants, 654 ± 61,5 variants/person). Then, a filter for CADD Phred score (≥ 20) was applied, only for the SNV and splicing variants (n = 8790 variants) since this score is not available for the indel variants. The total of 8790 SNVs + 1923 indel variants were crossed with the DNA-Repair gene (DRG) list (n = 653), obtaining 331 variants (10 ± 4,4 variants/person). In order to further evaluate the variants' pathogenicity, an *in house* pathogenic index (IP) was applied using five *in silico* prediction tools. Variants with an IP under 0,7 were excluded (for details see chapter Materials and Methods). A second cross was carried out on the remaining variants (n = 165) with the OMIM AD/COSMIC gene list (n= 1731), obtaining 34 variants.

Two out of these 34 variants belonged to *FLNB* gene (NM_001164318 c.4391G>C and c.6464C>T) were excluded for the discrepancy between the expected and observed phenotype. Based on OMIM database, pathogenic mutations mapping within this gene cause severe disease phenotypes with an autosomal dominant way of inheritance, such as Atelosteogenesis type I and III (OMIM number: #108720, #108721), Boomerang Dysplasia (#112310) and Larsen syndrome (#150250). These disorders affect the development of bones throughout the body, i.e. affected individuals present with several congenital abnormalities (broad forehead, hypertelorism, clubfeet, camptodactyly, syndactyly, etc.) and underdeveloped or absent bones in the spine, rib cage, pelvis, and limbs, which lead to very short arms and legs (dwarfism). Due to the orthopedic abnormalities, they also have delayed development of motor skills, and severe respiratory problems, such as respiratory failure. Hence, these conditions are usually result in stillborn or neonatal death. In rare cases, the affected individuals survive longer, but with intensive medical support. Our patients did not show the above features.

The 32 variants were classified according to the ACMG criteria by using three additional bioinformatic tools (VarSome, Franklin, InterVar). Only 7 variants classified as pathogenic, likely pathogenic or "hot" VUS variants were considered of clinical interest. Conventionally, the variants classified as VUS, likely benign, or benign were discarded.
Further details about ACMG criteria classification are in section "Materials and Methods".

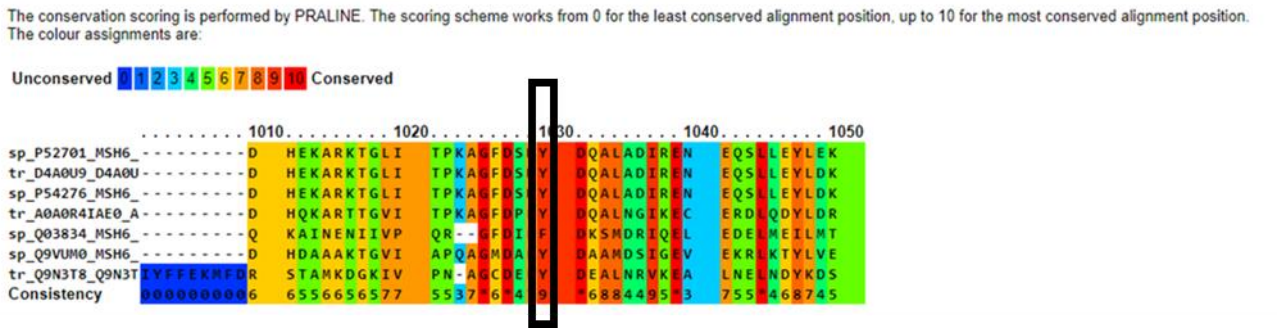
The 7 candidate variants mapping to 7 genes belonged to 5/32 TGCT patients (15,62%). For the 7 variants the minimum reads were 118 (gene *MLH1*) and the maximum were 260 (gene *MLH3*).

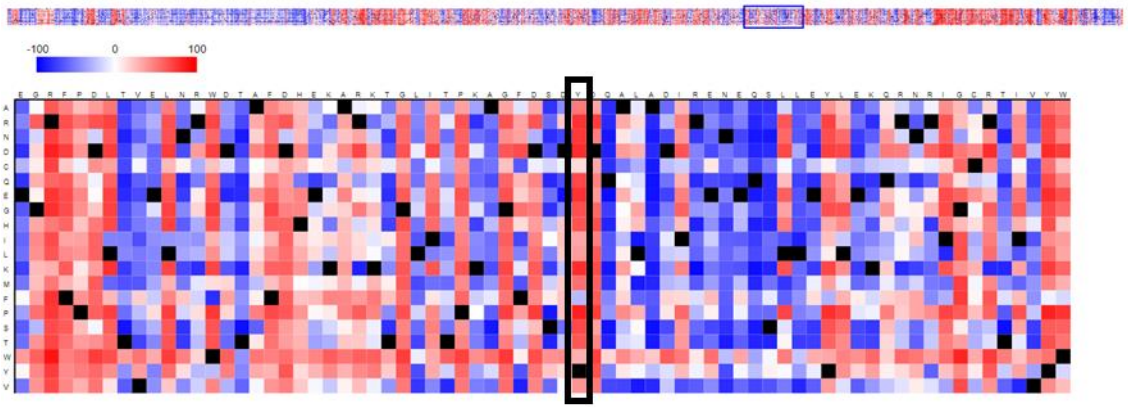
The variants mapping to 7 genes belong to four different biological pathways.

i) Variants mapping on genes involved in mismatch repair pathway

An already known pathogenic deletion of two nucleotides in exon 4 of the *MSH6* gene was found, (NM_001281494 c.2906_2907delAT), causing a translational frameshift with a predicted alternate stop codon. This alteration is expected to result in loss of function due to premature protein truncation or nonsense-mediated mRNA decay. As such, this alteration is interpreted as a disease-causing mutation. For the variant position PRALINE indicates high conservation in various species (*Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio, Xenopus laevis, Drosophila melanogaster*) and Predict Protein indicates high level of intolerance of the amino acid change in human (see Figure 4.). This mutation has already been reported in Lynch syndrome (LS) cases. For instance, it was identified in a cohort of 1260 individuals undergoing panel testing for Lynch syndrome because of a diagnosis of a Lynch-associated cancer and/or polyps (209).

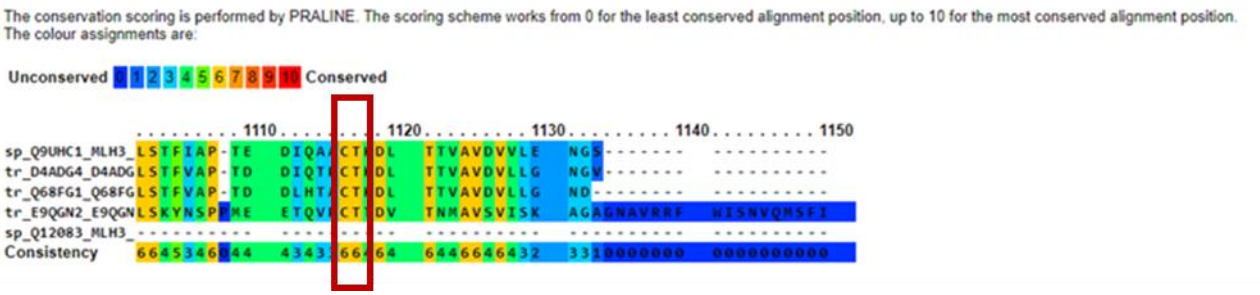
Figure 4. – PRALINE (upper) and Predict Protein (lower) outputs for pathogenic variant mapping on *MSH6* 48028026 position. In case of Predict Protein, the darker red squares indicate the greater intolerance, whereas the lighter red, rose color, and blue squares indicate the medium intolerance, neutrality, and the tolerance of amino acid changes, respectively.

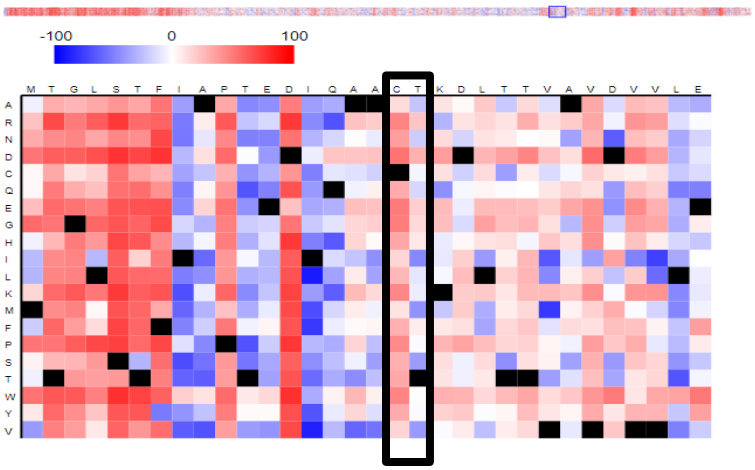




We identified also a novel non-frameshift deletion variant on *MLH3* (NM_001040108 c.3232_3237delTGTACT), classified as pathogenic according to ACMG criteria. The variant interests the 2nd exon, which is one of the main exons along with the 13th. This gene takes part of the LS causing genes among others, but the variant has not been reported in LS patients yet. For the variant position PRALINE indicates medium conservation in some species (*Mus musculus*, *Rattus norvegicus*, *Danio rerio*, and *Saccharomyces cerevisiae*) and Predict Protein indicates medium level of intolerance of the amino acid change in human (see Figure 5.).

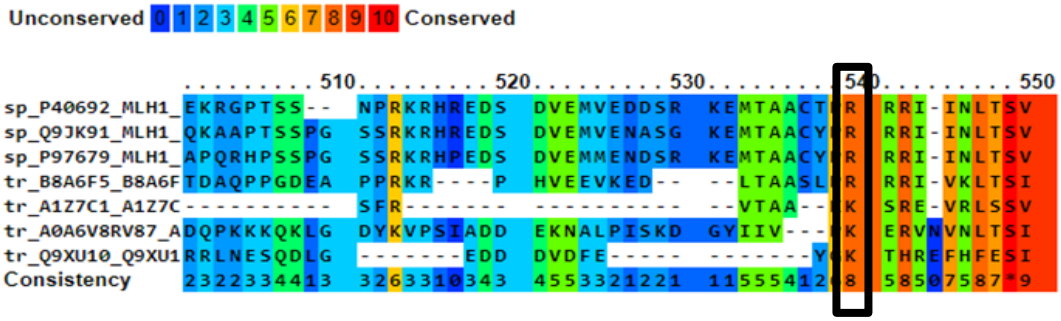
Figure 5. – PRALINE (upper) and Predict Protein (lower) outputs for pathogenic variant mapping on *MLH3* 75513121 position. In case of Predict Protein, the darker red squares indicate the greater intolerance, whereas the lighter red, rose color, and blue squares indicate the medium intolerance, neutrality, and the tolerance of amino acid changes, respectively.

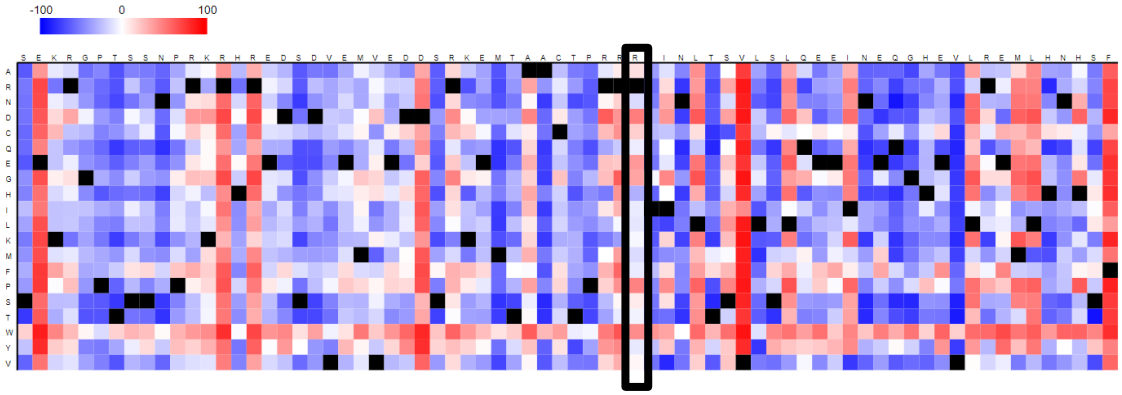




A novel missense variant in exon 13 of *MLH1* (NM_001167617 c.1203G>T) was identified. It was classified as likely pathogenic according to ACMG criteria. The amino acid of interest is at position 499 of 757, which belongs to the DNA mismatch repair protein Mlh1 domain. For the variant position PRALINE indicates relatively high conservation in various species (*Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio, Xenopus laevis, Drosophila melanogaster, Canis lupus familiaris*) and Predict Protein indicates neutral level of intolerance of the amino acid change in human (see Figure 6.).

Figure 6. – PRALINE (upper) and Predict Protein (lower) outputs for pathogenic variant mapping on *MLH1* 37070362 position. In case of Predict Protein, the darker red squares indicate the greater intolerance, whereas the lighter red, rose color, and blue squares indicate the medium intolerance, neutrality, and the tolerance of amino acid changes, respectively





ii) Variants mapping on genes involved in homologous recombination pathway

A missense variant on exon 25 of *FANCD2* (NM_033084 c.2252T>C), and a frameshift duplication on exon 8 of *XRCC3* (NM_001100119 c.978dupC) has been found. Both variants are classified as "hot" VUS according to ACMG criteria. The *FANCD2* variant is in position 758 of 1472, which might disrupt the proteins' main domain. The variant's, maximum MAF belongs to the Latino population, and it is 0,00327. According to gnomAD database, the variant of *XRCC3* has been identified only once in heterozygosis, in a Non-Finnish European male, so the variants' maximum MAF is 0,00000955. The identified variant interests one of the main functional domains, i.e. the DNA recombination and repair protein Rad51, C-terminal domain.

iii) Variant mapping on genes involved in nucleotide excision repair pathway

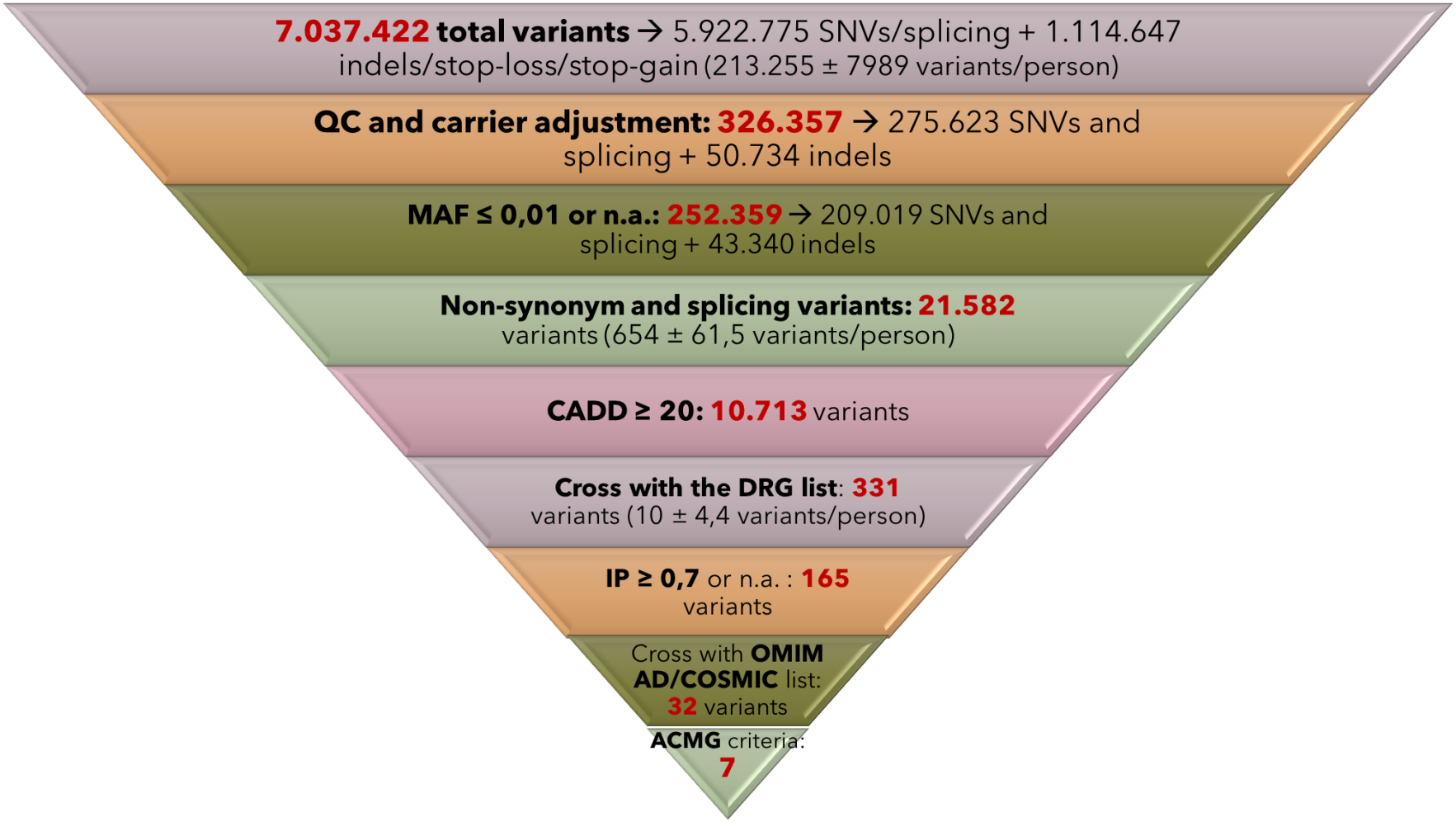
We found a novel null variant in gene *ERCC3* (NM_000122 c.2065-2A>C), for which loss-of-function is a known mechanism of disease, thus classified as pathogenic according to ACMG criteria. The variant is at intron 13 of 14, position 1778, with -2 of splice site.

iv) Variant mapping on genes involved in oxidative DNA damage repair pathway

A frameshift variant (NM_001048172 c.1598delC) on *MUTYH* gene has been identified, situated only 3 amino acids from the end of the protein, which loss-of-function is a known mechanism of disease. Hence, this variant is classified as likely pathogenic, according to ACMG criteria. Despite its terminal position, the variant might have an effect on three different functional domains, such as the MutY, C-terminal, the NUDIX hydrolase domain, and the NUDIX hydrolase domain-like domain. The variant has been identified twice, in a Non-Finnish European and in a Finnish European person, thus the maximum MAF is 0,0000462.

Figure 7. reports the flow-chart with the results. All data on the candidate variants are reported in Table 14.

Figure 7. – Variants defined as pathogenic / likely pathogenic / "hot" VUS according to American College of Medical Genetics and Genomics (ACMG) criteria evaluation including three bioinformatic databases (VarSome, Franklin, InterVar). DNA Repair Gene (DRG) list contains 653 genes. Further details about Pathogenic Index (IP) ≥ 0,7 see in chapter Material and Methods. OMIM AD/COSMIC list contains 1731 genes.



Patient	*Chr.*	*Gene*	*Variant*	*Variant type*	*IP*	*Max. MAF*	*ACMG classification*	*Pathway*	*Associated diseases/cancers in the literature*
18-1040	*2*	*MSH6*	c.2906_2907delAT p.Tyr969fs	frameshift deletion	n.a.	n.a.	PATHOGENIC	MMR	LS
A2301	*14*	*MLH3*	c.3232_3237delTGTACT p.Cys1078_Thr1079del	non-frameshift deletion	n.a.	n.a.	PATHOGENIC	MMR	LS
18-048	*3*	*MLH1*	c.1203G>T p.R401S	missense	0,7	n.a.	LIKELY PATHOGENIC	MMR	LS
	3	*FANCD2*	c.2252T>C p.I751T	missense	0,9	0,00327	HOT VUS	DNA cross-link repair/ chr.stability	Fanconi anemia (AR), HBOS, OH
A2315	*14*	*XRCC3*	c.978dupC p.Ser327fs	frameshift insertion	n.a.	0,00000955	HOT VUS	homologous recombination	Breast, prostate melanoma
A2049	*2*	*ERCC3*	c.2065-2A>C	splicing	1	n.a.	PATHOGENIC	nucleotide excision repair	HBOCS
	1	*MUTYH*	c.1598delC p.Ala533fs	frameshift deletion	n.a.	0,0000462	LIKELY PATHOGENIC	oxidative DNA damage repair	colorectal cancer

Table 14. – Description of the candidate heterozygote variants. Pathogenic Index (IP) was calculated based on five *in silico* prediction tools (SIFT, MutationTaster, MutationAssessor, PolyPhen2 HVAR, PolyPhen2 HDIV). **Abbreviations:** HBOCS – Hereditary Breast and Ovarian cancer syndrome, n.a. – not available, LS – Lynch syndrome, MMR – mismatch repair, OH – oncohematological malignancy

5.2.3 Main features of the 7 candidate genes, and their role in oncogenesis

To obtain information on the 7 genes' function and role in oncogenesis, we used the GeneCards and OMIM databases. Further description on the applied bioinformatic tools can be seen in section "Materials and Methods".

i) Genes involved in mismatch repair pathway

We identified mutation in *MSH6, MLH3* and *MLH1* genes which are known members of the mismatch repair (MMR) gene family. Somatic mutations within these genes frequently occur in tumors exhibiting microsatellite instability, and germline mutations have been linked to Lynch syndrome (hereditary nonpolyposis colorectal cancer syndrome). *MSH6* gene encodes a member of the DNA MMR MutS family. The MutS protein helps in the recognition of mismatched nucleotides prior to their repair. A highly conserved region of approximately 150 amino acids, called the Walker-A adenine nucleotide binding motif, exists in MutS homologs. The encoded protein heterodimerizes with MSH2 to form a mismatch recognition complex that functions as a bidirectional molecular switch that exchanges ADP and ATP as DNA mismatches are bound and dissociated. *MLH3* and *MLH1* are also known LS genes, which encode a protein that can heterodimerize with mismatch repair endonuclease PMS2 to form MutL alpha, part of the DNA MMR system. *MLH* genes are implicated in maintaining genomic integrity during DNA replication and after meiotic recombination.

ii) Genes involved in homologous recombination pathway

Among our 7 clinically relevant genes, *FANCD2* and *XRCC3* are involved in homologous recombination (HR), which is a well-established DNA repair pathway associated with several human cancer types (e.g. breast, ovarian, OH, prostate cancer).

FANCD2 is required for the maintenance of chromosomal stability. Promotes accurate and efficient pairing of homologs during meiosis. Involved in the repair of DNA double-strand breaks, both by HR and single-strand annealing. May participate in S phase and G2 phase checkpoint activation upon DNA damage. Plays a role in preventing breakage and loss of missegregating chromatin at the end of cell division, particularly after replication stress.

The gene *XRCC3* is also involved in the HR repair pathway of double-stranded DNA, thus the encoded protein repairs chromosomal fragmentations, translocations, and deletions. XRCC3 is part of a PALB2-scaffolded HR complex containing BRCA2 and RAD51C and which is thought to play a role in DNA repair by HR. It also plays a role in regulating mitochondrial DNA copy number under conditions of oxidative stress in the presence of RAD51 and RAD51C.

iii) Genes involved in nucleotide excision repair pathway

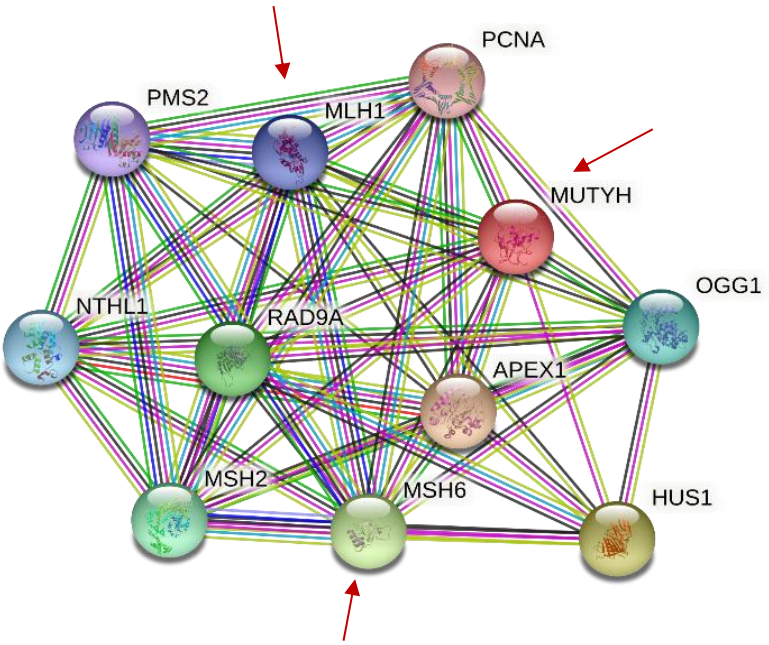
ERCC3 is an ATP-dependent 3'-5' DNA helicase, component of the general transcription and DNA repair factor IIH (TFIIH) core complex. Associated malignancy is the Hereditary Breast and Ovarian cancer syndrome in case of gene malfunction. This complex is involved in general and transcription-coupled nucleotide excision repair (NER) of damaged DNA, and in RNA transcription by RNA polymerase II. In NER, TFIIH acts by opening DNA around the lesion to allow the excision of the damaged oligonucleotide and its replacement by a new DNA fragment. The ATPase activity of XPB/ERCC3, but not its helicase activity, is required for DNA opening.

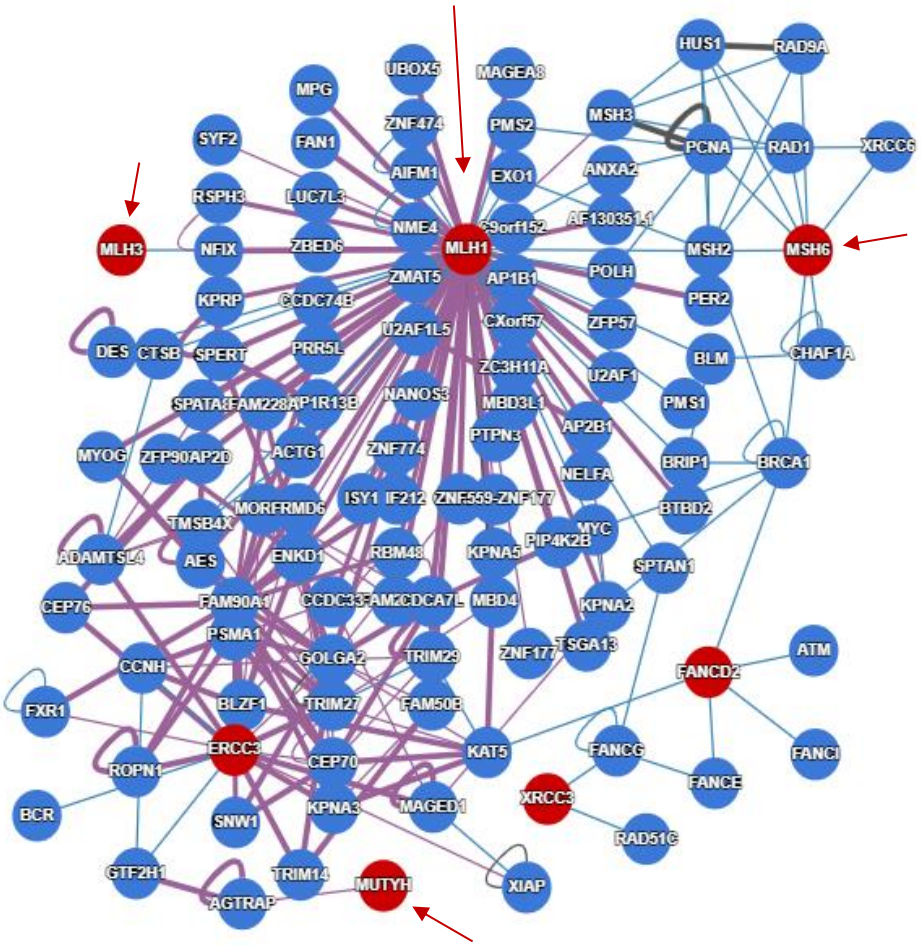
iv) Genes involved in oxidative DNA damage repair pathway

The gene *MUTYH* encodes a DNA glycosylase involved in oxidative DNA damage repair. Mutations within this gene are associated with colorectal and gastric cancer among others. The enzyme excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired with guanine, cytosine, or 8-oxo-7,8-dihydroguanine. The protein is localized to the nucleus and mitochondria. This gene product is thought to play role in signaling apoptosis by the introduction of single-strand breaks following oxidative damage.

The 7 genes belonging to four different pathways involved in oncogenesis. By performing STING and Interactome analyses only 4 out of 7 genes, the Lynch syndrome genes and *MUTYH*, are interacting (see Figure 8.).

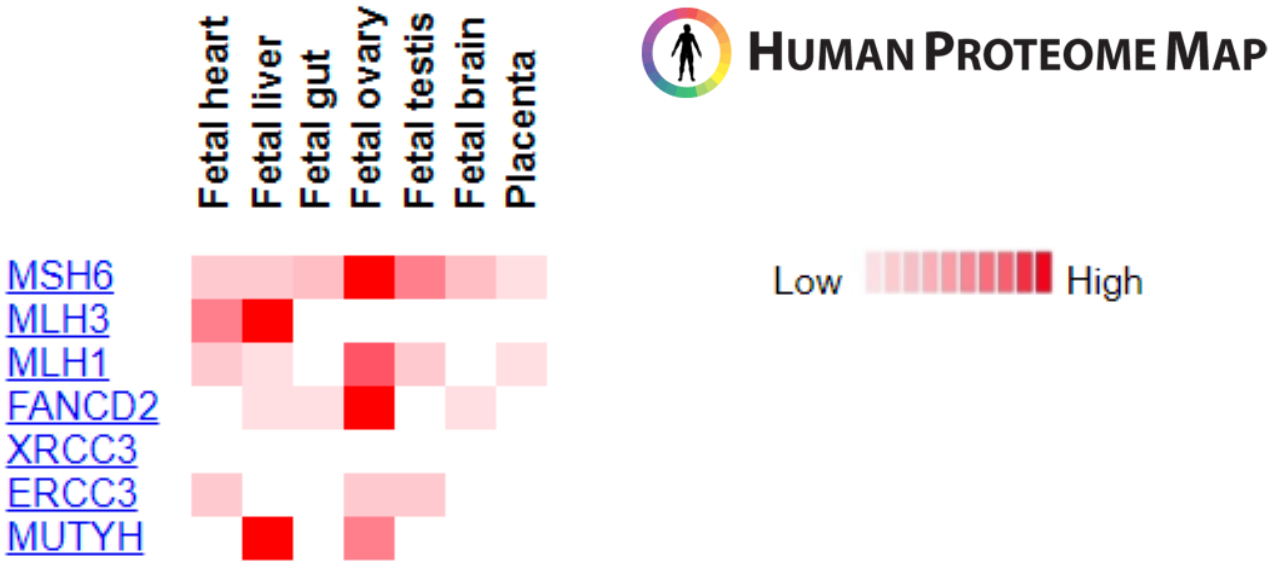
Figure 8. – STRING (upper), Interactome (lower). The 7 candidate genes are in red circles in the Interactome output. The arrows indicate the interacting genes.





Further analyses revealed that all encoded proteins by the candidate genes are expressed in all adult human tissue types and have low tissue specificity. In case of *FANCD2,* the protein is highly expressed in the lymphoid tissue and in the testis, whereas in case of *XRCC3,* the protein is enhanced in the human skin. On the other hand, fetal tissue expression is not detected in all cases. For example, *XRCC3* is not expressed in fetal tissues, whereas *MSH6* or *MLH1* are highly expressed in several fetal tissue types, such as the ovary or the testis (see Figure 9.).

Figure 9. – Gene expression in different human fetal tissue types, such as heart, liver, gut, ovary, testis, brain, and the placenta. Light colors indicate low protein expression, whereas darker colors high protein expression, encoded by the given gene.



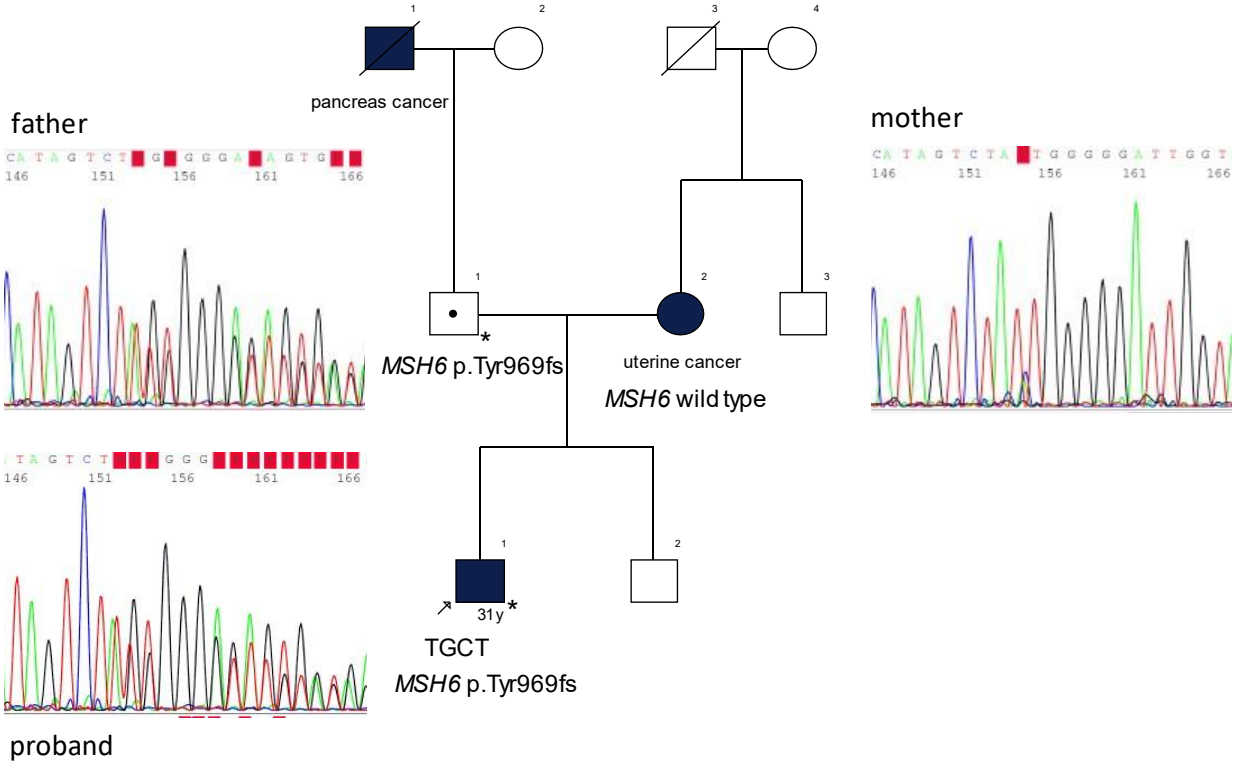
Using Mouse Genome Informatics (MGI) database, neoplastic phenotype in *knock out* mice was found in 3 out of 7 genes (*MSH6, MLH1, FANCD2*). The observed cancers were concordant with the identified cancer types in human. For the remaining four candidate genes, neoplastic phenotype is observed only in human, but not in *knock out* mouse.

5.2.4 Genotype-phenotype associations in the TGCT cohort carrying mutations in the seven, clinically relevant genes

The mean age of the five candidate patients was 32,4 ± 2,58 years (± SD). Regarding the cancer histology, three had seminoma and two had non-seminoma. As sperm phenotype, we did not identify specific associations. Four patients were normozoospermic, whereas one had azoospermia. Segregation analysis was performed only in two families (including parents of patient 18-1040 and patient A2301). Data on the genotype-phenotype correlations is reported in Table 15. Regarding the affected family members, the mean number was 3 ± 0,632 (± SD) among the five patients with the 7 candidate mutations, which is slightly but not significantly above the whole study cohort (2,82 ± 0,917).

Patient 18-1040: carried a pathogenic variant in *MSH6* gene (NM_001281494 c.2906_2907delAT). The patient's mother was affected by uterine cancer, whereas his paternal grandfather had pancreas cancer (see Figure 10.). Both cancer types are involved in the LS associated cancer spectrum. Interestingly, the mutation was inherited from the paternal lineage, which could be congruent with the data in the literature, that variants of *MSH6* may be in incomplete penetrance, i.e. can skip generations, and associated with later disease onset.

Figure 10. – Pedigree of patient 18-1040.

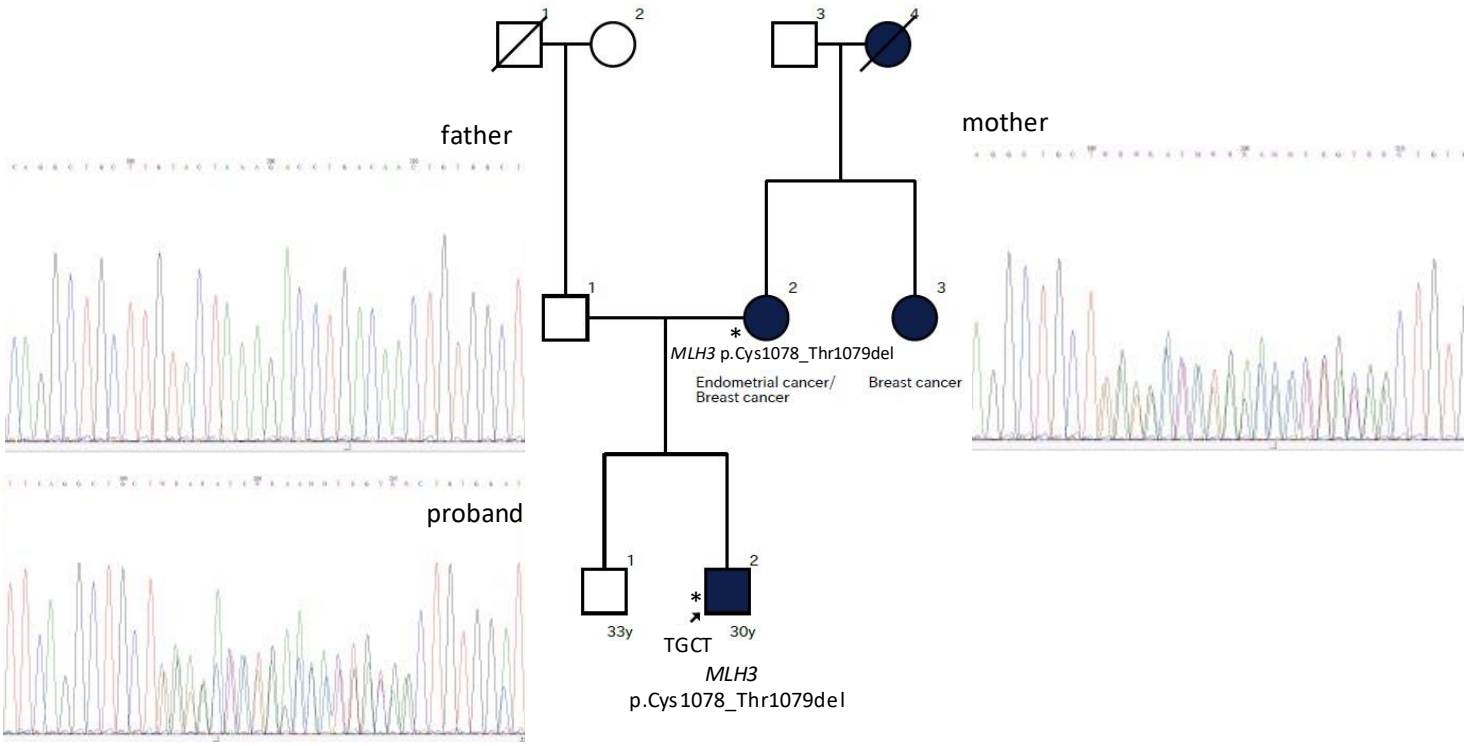


[Filled symbols indicate affected patients. Proband is marked by an arrow, carrier status was studied in available relatives, and those carrying the variant are shown with the variant symbol (*).
Sequence of the *MSH6* frameshift deletion (c.2906_2907delAT) mutation in the patient 18-1040, in his carrier father, and his *wild type* mother, obtained with the forward primer.]

Patient A2301: carried a pathogenic variant on *MLH3* (NM_001040108 c.3232_3237delTGTACT) The patient's mother had endometrial and breast cancer, his maternal aunt had breast cancer, and his

maternal grandfather had pancreas cancer, which are typical LS associated cancers (see Figure 11). The mutation was inherited from the mother, whereas the father was *wild type*, confirmed by Sanger sequencing. Interestingly, the mother and the brother of the patient carry a *BRCA1* mutation as well whereas our patient is *wild type*.

Figure 11. – Pedigree of patient A2301.



[Filled symbols indicate affected patients. Proband is marked by an arrow, carrier status was studied in available relatives, and those carrying the variant are shown with the variant symbol (*). Sequence of the *MLH3* non-frameshift deletion (c.3232_3237delTGTACT) mutation in patient A2301, his mother and father, obtained with the reverse primer.]

In **patient 18-048** two predicted as pathogenic variants have been identified: i) a missense variant of *MLH1* (NM_001167617 c.1203G>T), and ii) another missense variant in *FANCD2* (NM_033084 c.2252T>C). *MLH1* is a known LS associated gene, whereas *FANCD2* is associated with Fanconi Anemia, if it is inherited in a homozygous way (autosomal recessive disease). On the other hand, in case of dominant transmission of pathogenic mutations in *FANCD2*, Hereditary Breast and Ovarian Cancer syndrome (HBOC) and some type of lymphomas can occur. Typical tumors associated to both

genes are present in the patient's family, since the patient's mother had ovarian cancer, and the maternal grandmother had lymphoma. The paternal uncle had colon cancer, which is typical for LS.

Patient A2315: a frameshift duplication (NM_001100119 c.978dupC) in *XRCC3* has been found. Pathogenic variants within this gene are mainly associated with breast cancer and melanoma, but polymorphisms within the gene could significantly increase the risk for the development of prostate cancer and acute or chronic myeloid leukemia. However, in case of leukemia, the association is controversial, and might be found only in selected populations (ref: Association of *XRCC3* Thr241Met polymorphism and leukemia risk: evidence from a meta-analysis). On the paternal lineage the patient's family members are affected by prostate cancer (grandfather and great-grandfather), whereas the maternal grandmother is affected by leukemia.

Patient A2049: two candidate variants were identified: i) a splicing mutation in gene *ERCC3* (NM_000122 c.2065-2A>C), and ii) a frameshift deletion in *MUTYH* (NM_001048172 c.1598delC) gene. *ERCC3* is associated with HBOC, an inherited disorder with an increased risk for breast and ovarian cancers. Whereas mutations in gene *MUTYH* result in heritable predisposition to colorectal cancer, termed MUTYH-associated polyposis (MAP). Tumors observed among the proband's family are uterine and breast cancer in the maternal aunt, breast cancer in maternal cousin, colon cancer in the maternal grandfather, and gastric cancer in the paternal grandfather.

Table 15. – Genotype-phenotype correlations regarding the 7 candidate heterozygote variants in 5 patients. The different colors indicate the genotype-phenotype correlation, i.e. what kind of tumors are associated with the given gene. Color legend: pink – Lynch syndrome-associated tumor types; blue – Hereditary Breast and Ovarian cancer syndrome-associated tumor types, orange – *XRCC3*-associated tumor types, green – *MUTYH*-associated tumor types.

[**Abbreviations:** Azoo – azoospermia; Cryptozoo – cryptozoospermia; n.a. – not available; Normozoo – normozoospermia; NS – Non-Seminoma; OAT – oligoasthenoteratozoospermia; Sem – Seminoma; HBOCS – Hereditary Breast and Ovarian cancer syndrome, LS – Lynch syndrome (e.g. colorectal, endometrial, ovarian, breast, pancreas, and prostate cancer), MMR – mismatch repair, OH – oncohematological malignancy]

Patient	*Gene*	*Variant type*	*Variant*	*Tumor histology*	*Sperm phenotype*	*Associated diseases/cancers in the literature*	*Tumor types present in the family*
18-1040	*MSH6*	frameshift deletion	c.2906_2907delAT p.Tyr969fs	Sem	Normozoo	LS	UTERUS (mother) PANCREAS (paternal grandfather)
A2301	*MLH3*	non-frameshift deletion	c.3232_3237delTGTACT p.Cys1078_Thr1079del	NS	Normozoo	LS	UTERUS/BREAST (mother) PANCREAS (maternal grandfather) BREAST (maternal aunt)
18-048	*MLH1*	missense	c.1203G>T p.R401S	Sem	Normozoo	LS	OVARY (mother) HL (maternal grandmother) COLON (paternal uncle)
	FANCD2	missense	c.2252T>C p.I751T			Fanconi anemia (AR), HBOS, OH	
A2315	*XRCC3*	frameshift insertion	c.978dupC p.Ser327fs	Sem	Azoo	Breast, prostate melanoma, OH	PROSTATE (paternal grandfather) PROSTATE (paternal great-grandfather) LEUKEMIA (maternal grandmother)
A2049	*ERCC3*	splicing	c.2065-2A>C	NS	Normozoo	HBOCS	UTERUS/BREAST (maternal aunt); BREAST (maternal cousin) COLON (maternal grandfather) GASTRIC (paternal grandfather)
	MUTYH	frameshift deletion	c.1598delC p.Ala533fs			colorectal cancer	

VI. DISCUSSION

TGCT is a multifactorial, polygenic, and complex disease. It is the most common malignancy of men between 15-40 years. TGCT originates from primordial germ cells (PGCs), which undergo aberrant differentiation arrest during fetal life (210). This results in the formation of a pre-malignant lesion, first observed by Skakkebaek in 1972 and termed as carcinoma *in situ* (CIS) of the testis (24). According to the latest WHO nomenclature, now it is called as germ cell neoplasia *in situ*, GCNIS (12). The aberrant gonocytes are arrested in differentiation, acquire abnormal characteristics, such as increased size of the nucleus, increased DNA content and appearance of chromatin aggregates, and express pluripotency markers (210). They are in a dormant state until puberty, however an accumulation of chromosomal aberrations and mutations in genes involved in cell proliferation and differentiation occurs in these cells. At the time of puberty, the production of androgens rises physiologically, which stimulates the process of spermatogenesis. In the presence of aberrant PGCs, these hormones stimulate their malignant growth as well, leading to the development of TGCT (19,23).

This neoplasm has one of the highest heritability (37–48,9%) (132), based mainly on epidemiological and population-wide assessment data. The hypothesis that genetic mechanisms are involved in TGCT development is supported by several lines of evidence. Epidemiological studies confirm a higher incidence of TGCT among non-Hispanic Caucasian population as compared to the African or Asian population, suggesting a link between ethnicity and TGCT (211). Ethnic variation in its incidence and lack of migration effect have been interpreted as supporting the role of genetic factors. On the other hand, strong evidence is provided also by the analysis of familial cases demonstrating a 4- to 6-fold increased risk of TGCT occurrence in children of an affected individual, and an 8- to 10-fold increased risk in siblings (129). In monozygotic and dizygotic twins, this risk rises to 75- and 35-fold, respectively (128). These increased risks for family members are among the highest compared to any other tumor types, where barely exceeds 4 times (129). Early age of disease onset and a higher than by chance expected proportion of bilateral cases also suggest the involvement of genetic factors.

Data in the literature supports that for a subgroup of subjects, the etiopathogenesis of TGCT is due to an imbalance in the action of sex hormones during fetal life. It has been hypothesized that genetic background (common polymorphisms) in combination with environmental factors (endocrine disruptors) may interfere with the canonical signaling of Sertoli and Leydig cells, during the time window of gestation in which masculinization occurs (40,210). This could lead to altered development of the male reproductive system, with the potential development of five

conditions: cryptorchidism, hypospadias, decreased ano-genital distance, impaired spermatogenesis, and TGCT. These pathologies have been proposed as entities of a single syndrome called Testicular Dysgenesis Syndrome (TDS) (34). However, TGCT may also be attributable to other genetic factors (monogenic causes) that affect genes involved in the proliferation or differentiation of PGCs. Thus, they undergo differentiation arrest and malignant transformation, leading to TGCT (19,23).

It is well known that quantitative/qualitative sperm parameters are considered as biomarkers of the general male health, and impaired spermatogenesis is associated with higher morbidity and mortality in respect to fertile men (212–218). In addition, the correlation between infertility and TGCT is also well described (100,219,220), just as well as the association of azoospermia and increased risk of cancer development in general (221). Moreover, in a study of Choy and Eisenberg in 2018 (217,222), it was clearly demonstrated that male infertility may serve not only as a biomarker for the individual man's health, but also as a marker of oncologic risk for the affected man's family members. Anderson and colleagues (223) observed that in a retrospective cohort study of 12.889 men who underwent semen analysis for couple infertility, matched with 12.889 fertile control men, the first-degree relatives of the subject who underwent semen analysis had a 52% increased risk of TGCT compared with the first-degree relatives of the fertile control males. Furthermore, first- and second-degree relatives of men with confirmed azoospermia were found to have a significantly increased risk of thyroid cancer development compared with the relatives of the control cohort (223). In a subsequent cohort study of 10.511 men who underwent semen analysis and their 63.891 siblings, and 327.753 cousins revealed that oligozoospermia was associated with a two-fold increased risk of childhood cancer in the subfertile men's siblings, as well as specifically a three-fold risk of acute lymphoblastic leukemia, compared with the siblings of fertile men (224). Although the origins of these familial associations are yet unclear, shared genetics or environmental exposures might be plausible mechanisms.

Based on the aforementioned correlations, we aimed to investigate the link between TGCT and familial cancer risk. We performed an epidemiological study of 1407 subjects, where 592 were affected by TGCT, 352 had oncohematological (OH) malignancy, and 463 were fertile cancer-free controls. We found that TGCT patients' relatives (first-degree and grandparents) have significantly more cancers (*p value* = 0,0001) than controls' relatives. The risk was 1.67-fold increased for tumors among TGCT patients' family members. As comparing the TGCT cohort with another type of malignancy which affects young males (OH), we did not observe

significant differences. This implies that OH patients also have an increased familial cancer risk, which was confirmed by our results (*p value* = 0,0045). Among TGCT family members a mean number (± SD) of tumors was 1.51 ± 0,71, and it was very similar among OH family members, which mean number was 1.41 ± 0,74 tumors. As compared to controls' relatives (1.21 ± 0,46), the observed differences were statistically significant both between TGCT *versus* controls, and OH *versus* controls (*p value* < 0.001 and 0.016, respectively). According to our knowledge, we are so far the first comparing these two tumor types, which affect males in their reproductive age, regarding familial cancer incidence. Similar studies have been published only for TGCT and for OH malignancies, separately and it is still debated whether TGCT relatives have an increased cancer risk. Studies favoring our results have found similarly increased cancer risk among first-degree relatives: Nordsborg et al. (205) found in Denmark a 1.13-(1.02–1.26) fold increased relative risk, Spermon et al. (2000) (197) and Dong et al. (2001) (126) both a 1.2-fold increased standardized incidence ratio (SIR), whereas Bodrogi et al, (2004) (201) a 1.4-fold increased risk (OR). The only study which assessed first- and second-degree relatives as well, was conducted by Chia and colleagues (2009) (202). They report an overall increased risk for cancer among first-degree relatives of cases compared to controls (RR=1.17, 95% CI 1.01−1.35). Still increased significantly but dropped slightly to RR = 1.14 (95% CI 1.05−1.24) when second-degree relatives were added.

On the contrary, Heimdal and his collaborators found in 1996 (196) that in the families of the Norwegian TGCT patients, the total number of cancers among relatives was significantly lower (SIR: 0.89, 95% CI 0.78 -1.00) than the expected number derived from national incidence rates. Still in Norway, in 2015, McMaster et al. (198) found likewise no increase in overall cancer risk (SIR = 0.8; 95% CI: 0.6–1.1), nor in the United States (SIR = 0.9; 95% CI: 0.7–1.3).

In case of OH malignancies, articles mainly report the familial aggregation of the different OH cancers only (225,226), which is similar to that observed concerning the familial aggregation of TGCT. As far as we know, there are only two studies dealing with this topic, i.e. OH malignancies and familial cancer risk (227,228). In an Italian study of Negri and her collaborators (2006) (227) found an overall increased risk for any tumors among first-degree family members of Non-Hodgkin Lymphoma (NHL) patients. Whereas, in another study by Chatterjee et al. (228) this correlation was not observed.

Regarding the different histotypes of TGCT, we did not find specific differences. This corresponds to the observations of Dong et al. (126) since they reported exactly the same SIR (1.2, 95% CI 1.1–1.4) of familial cancer incidence both for seminoma and teratoma patients. However, some studies report that seminoma patients have higher tumor incidence among

family members. Bromen et al. (2004) (200) found that the risk estimates for seminoma patients and controls are consistently higher in all analyses than in NS patients and controls. In the study of Zhang et al. (2018) (203) seminoma patients seemed to share more familial associations with discordant cancers than non-seminoma (NS) patients. It is well established that the average age at onset of disease is higher for patients with seminoma than with NS, and this is shown in our dataset as well. Thus, the lower familial cancer incidence when focusing on NS patients may be due to the earlier age at diagnosis, potentially reducing the probability for having older family members, whose are at higher risk for cancer development.

In case of OH malignancies, we found the higher tumor incidence among leukemia patients' family members, but the differences did not reach statistical significance, probably due to small sample size. Supporting data in the literature is scarce.

When we performed the site-specific analyses to define which cancer types are in excess or in deficit among TGCT and OH patients' relatives, we identified some specific associations. First of all, TGCT patients' relatives have a higher risk for testicular and extra-testicular germ cell cancer development. These cancer types are in the "Top10" among TGCT patients' relatives despite of their low frequency in the general population (absolute lifetime risk 0,5% in males, ref). We report a 9.94-fold increased risk for TGCT development in brothers of TGCT patients, and a 4.74-fold increase in risk counting for all male relatives, i.e. brothers, fathers, grandfathers. In the study of Chia et al. (202) a 4.51-fold increase in total testicular cancer risk was reported for male first-degree relatives, which is really similar to our results.

Apart from TGCT, other significant associations were found with breast, colorectal, other gastrointestinal and anal cancers, pancreas, urinary tract cancers. Data concerning these specific correlations is highly heterogenic. In the study of Dong et al. (126) only seminoma was associated with the risk for other cancers, such as pancreatic and nervous system cancers among TGCT patients' family members. The results of Bromen and colleagues (200) support the hypothesis that cancers of other genital organs occur more frequently in first-degree relatives of TGCT patients than expected. For sisters only, they found a significantly higher prevalence of breast cancer in the cases' families. As well as they observed ''clusters'' of genital and breast cancers for the most part in relatives of cases. The study of McMaster et al. (198) revealed a leukemia excess (O/E = 6.5; 95% CI: 3.0–12.3), deficit of female breast cancer (O/E = 0.0; 95% CI: 0.0–0.6) and increased risk of soft tissue sarcoma (O/E = 7.2; 95% CI: 2.0–18.4), which findings are not corresponding to our results. Our results are discordant also with the observations of Nordsborg et al., (205) as they estimated a higher incidence ratio of NHL and esophageal cancer among TGCT patients' relatives. However, these may be "chance findings"

as the author suggested. Significantly increased risks of digestive tract cancer, hematopoietic cancer, melanoma, and non-melanoma skin cancer were observed among first- and second-degree relatives in the study by Chia et al. (202). Furthermore, a significantly increased risk of prostate cancer (RR=1.39, 95% CI 1.07−1.81) was reported also for male relatives. In contrast, a decreased risk of lung cancer was detected, particularly among male family members (RR=0.78, 95% CI, 0.62−0.99).

Concerning hematopoietic malignancies, it is the second most recurrent cancer type among OH patients' relatives, while in the other three cohorts (i.e. TGCT patients' relatives, controls' relatives, and Mediterranean general population) are not that frequent. In fact, our OH patients' relatives have a 4.14-fold increased risk for hematopoietic malignancy development (*p value* < 0.001). This result confirms the literature data since there is mounting evidence for familial hematolymphoproliferative cancer aggregation (225,226). Furthermore, we identified a significantly higher risk (OR 2.37) for breast cancer also among OH patients' family members (*p value* = 0.02). This is also in line with the findings of Negri et al.,(227) where they found a 1.9- (95 % CI 1.0-3.5) fold increase for the incidence of breast cancer among first-degree relatives. These results underline the importance of the genetic basis regarding these types of malignancies, and the association of breast cancer and hematopoietic malignancy predisposing genes. Another site-specific association in the Italian study was the increased risk for liver (OR 2.3, 95% CI 1.2-4.6) and kidney (OR 4.5, 95% CI 1.0-20.2) cancer, which we did not identify. We found only by the grandparents a significantly increased risk (OR 4,41, 95% CI 1,21-16,54, *p value* = 0,02) for gastrointestinal cancers other than colorectal and anal cancers but including liver cancer. In the other study (228) regarding this issue, the analysis of various other cancers provides modest but not significant evidence for familial aggregation of NHL with melanoma of the skin, gastric, pancreatic, and prostate cancer.

The novelty of our study is that we defined the semen phenotype for all subjects of the different cohorts, thus we were able to assess not only the impact of tumors versus non-tumors on familial incidence of neoplasms but also to compare whether non-normozoospermic subjects have more tumors among family members or not. We report a 1.57-fold higher risk (*p value* = 0.0048) for tumor development among family members if the patient had severe spermatogenic disturbances (azoospermia and severe oligozoospermia with TSC < 5 million). This is in line with the observations of Choy and Eisenberg (2018) (222). Regarding the different subgroups, non-normozoospermic TGCT patients have more affected relatives than normozoospermic TGCT patients (43,75% *versus* 37,5%), but this did not reach statistical significance (*p value* = 0.12).

An interesting finding of our study was that we observed significantly less sibling among TGCT (mean number of siblings: 1,16) and OH (1,09) cases than among controls (2,07). Nowadays fertility rate is 1,34 in Italy and 1,27 in Spain (https://www.worldometers.info/demographics/), which is higher as compared to our observations among TGCT and OH patients. This difference was observed also when we calculated the fertility rate from the exact decade where our subjects were born, for the TGCT and OH cohorts with a mean age of 31 and 30 years respectively, this rate was 1,3 in Italy and 1,5 in Spain in the 1990', whereas for the Spanish control cohort with a mean age of 40 years this rate was 2,6 children per mother in Spain in the 1980'. When we investigated the mean total sperm count (TSC) of subject without siblings, we found a significantly decreased mean TSC as compared to subjects with siblings (*p value* = 0,001). The lowest rate was observed among TGCT patients without siblings (TSC 60,38 ± million spermatozoa).

The decreased number of siblings together with the observed differences in mean total sperm count might suggest a general subfertility among TGCT patients' families. These findings further support a possible common etiology between impaired spermatogenesis, subfertility, and oncogenesis. It might be due to an overall genomic instability (224) of the pedigree.

Among the main mechanisms of oncogenesis aberrant gene expression is considered a leading force causing essential changes in biological processes within cancer cells. Hanahan and Weinberg in 2000 (229) described six fundamental changes in cancers (growth factor independence, evading growth suppressors, avoiding cell death, angiogenesis, maintaining replicative potential, invasion/metastasis) that largely explain their malignant behavior. This was updated subsequently, with the addition of two emerging hallmarks (reprogramming energy metabolism, evading immune destruction) and two enabling characteristics (genomic instability, inflammation) (230).

Genomic instability relates to the state in which cancer cells lose control of the integrity of their genetic material and acquire an increasing repertoire of mutational changes that progressively alter their biology and promote the hallmarks of cancer. In the context of male infertility, some specific manifestations of genomic instability, such as increased Copy Number Variation (CNV) burden (231), microsatellite instability (MSI) (232) have been observed. In addition, mutations in mismatch repair and tumor suppressor genes, which could potentially lead to genomic instability, have been identified in some infertile men and in *knock out* animal models (233). Microsatellites are short stretches of the DNA composed of 1 to 6 nucleotide tandem repeats. MSI is characterized by expansion or contraction of these regions due to the gain or loss of one or more microsatellite repeats (234) and is an important marker for several types of

cancers arising as a result of defective DNA repair mechanisms. MSI typically results from mutations in DNA mismatch repair (MMR) genes, but in some instances, it could occur through epigenetic silencing of MMR genes (235). The fact that DNA repair gene mutations have been reported as cause of azoospermia (for review see (236)), we can speculate that an increased genomic instability can be the link between spermatogenic defects and a more systemic problem leading to higher morbidity including cancer.

Given the link between infertility and TGCT, genetic alteration associated with impaired spermatogenesis have been investigated as potential risk factors of TGCT development. Partial deletion, which removes half of the gene content of the AZFc subregion, termed as "gr/gr deletion", represents a population-dependent, significant risk factor for oligozoospermia (137,138). Thus, it has been assessed whether gr/gr deletion could be a risk factor for TGCT development. The two largest studies have found a significant association between this type of microdeletion deletion and TGCT. However, the absolute frequency of the gr/gr deletion is only ~2% in TGCT, accounting only for around 0,5% of the total genetic risk of TGCT development (176). Since the gr/gr deletion is a CNV, it can be an expression of a more generalized genomic instability.

In addition to this Y chromosome linked risk factor, other genetic risk factors have been identified through large-scale SNP array-based analysis. Genome-Wide Association Studies (G-WAS) identified many SNPs, each carrying a limited risk for TGCT, but when inherited together result in a consistent predisposition to this type of cancer. Till date, 78 risk loci have been identified (151) and most of the SNP-related genes are associated with seven biological pathways including those related to genomic integrity (telomerase function, DNA damage repair, and centrosome cycle and microtubule assembly pathways). A TGCT risk locus related to DNA damage repair has been found in association with *RAD51C*, a gene involved in the homologous recombination (HR) repair pathway. It has an early function in DNA repair in facilitating phosphorylation of the checkpoint kinase (*CHEK2*) and thereby transduction of the damage signal, leading to cell cycle arrest and HR activation. It also plays a role in the regulation of mitochondrial DNA copy number under conditions of oxidative stress together with *XRCC3*.

In recent years, the methodology based on massive parallel sequencing allowed the fast and cost-effective analysis of the entire exome, called Whole Exome Sequencing (WES). WES was implied also in the search for monogenic factors underlying TGCT. Altogether four WES studies have been performed in the past few years, which sought to identify rare, disease-causing variants of TGCT. The first three works (185–187) using this technique failed to find

evidence of a single, recurrently mutated gene with high penetrance. The last study (188),which evaluated the role of 48 DNA repair genes in the etiopathogenesis of TGCT, found *CHEK2* as a new susceptibility gene with moderate penetrance. The protein encoded by the gene Checkpoint Kinase 2 (*CHEK2*), is a cell cycle checkpoint regulator and putative tumor suppressor. It contains a forkhead-associated protein interaction domain essential for activation in response to DNA damage and is rapidly phosphorylated in response to replication blocks and DNA damage. When activated, the encoded protein is known to inhibit CDC25C phosphatase, preventing entry into mitosis, and has been shown to stabilize the tumor suppressor protein p53, leading to cell cycle arrest in G1. In addition, this protein interacts with and phosphorylates BRCA1, allowing BRCA1 to restore survival after DNA damage. Mutations within *CHEK2* have been linked with Li-Fraumeni syndrome, a highly penetrant familial cancer phenotype usually associated with inherited mutations in *TP53*. Also, mutations in this gene are thought to confer a predisposition to sarcomas, prostate, breast, and brain tumors. AlDubayan and his collaborators (188) have identified *CHEK2* loss-of-function variants in 2% of the affected subjects, whereas their frequency is only 0,4% among the control group (n = 3090) and 0,6% on the ExAc database (n = 27.173).

Very recently, in a case report by Lobo et al. (206) a pathogenic germline *MSH2* mutation was identified in a patient with seminoma and in his uncle with confirmed Lynch syndrome. *MSH2* is a known DNA repair gene and Lynch syndrome is an autosomal dominant cancer prone disease, due to germline mutations in the DNA mismatch repair (MMR) gene family, such as *MSH2* among others. This is the first evidence to date, supporting a genetic link between TGCT and familial cancer risk.

After we observed a significantly increased cancer risk among TGCT patients' relatives in our epidemiological study, we aimed at the definition of potential genetic defects, which may cause not only TGCT but may lead to a more generalized cancer predisposition. Our hypothesis is based on the followings: i) DNA Repair genes are involved in oncogenesis in general, ii) several TGCT risk loci-associated genes linked to the DNA repair pathway, iii) *CKEK2* is considered as a novel susceptibility gene of TGCT, and iv) recently TGCT has been linked to Lynch syndrome. Thus, we aimed to investigate the role of DNA repair genes in the etiopathogenesis of TGCT. For this purpose, we performed WES on 32 TGCT patients with positive family history of malignancies.

We identified rare, potentially disease-causing, germline variants of DNA repair genes in 5 out of 32 (15,6 %) TGCT patients. We report seven variants in seven genes in the five subjects. Five out of 7 variants were loss-of-function, whereas two were missense. Three variants were classified as pathogenic, two as likely pathogenic, and two as "hot" VUS, according to ACMG criteria classification. Regarding the cancer histology and semen phenotype we did not observe specific associations.

Our main findings are related to Lynch syndrome (LS). We found mutations in three established LS genes (*MSH6, MLH3, MLH1*) in three patients (18-1040, A2301, 18-048, respectively) with typical LS-associated tumor types among their relatives. The *MSH6* frameshift deletion (NM_001281494 c.2906_2907delAT) is an already known pathogenic mutation. According to ClinVar database, it has already been reported in four articles. Yurgelun et al. (209) performed germline analysis with NGS using a 25-gene containing panel for 1260 individuals who underwent clinical genetic testing for LS. All patients had a history of LS-associated cancers and/or polyps. 111 mutations (61%) have been found in known LS genes (*MLH1, MSH2, MSH6, PMS2, EPCAM*). Among them, the *MSH6* gene was mutated in 26 cases (23%). Further three subjects presented with two mutations, one in an MMR gene (*MSH2* twice and *MSH6*). Our variant has been identified in patient 1095079885, but the exact phenotype was not reported. The only information available is a general description of the 111 subjects with MMR gene mutations. The majority of them had colorectal (CRC) and endometrial cancer (EC), and few of them had ovarian, breast, or other LS-associated tumors. The two other papers reporting our variant are published by The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) consortium (237,238). One of these studies using also a multigene (*MLH1, MSH2, MSH6, PMS2,* and *EPCAM*) panel testing on 34,980 individuals (237), 618 MMR and *EPCAM* mutations were identified in 612 (1.7%) subjects. Mutations in *MSH6* were observed in 29.3%. Also in this paper, the exact clinical manifestations of the *MSH6* mutation carrier were not described. In the fourth, smallest study (239) the authors performed molecular analysis of *MSH6* on genomic DNA of 143 probands from Hereditary Non-Polyposis Colorectal Cancer (HNPCC) families and 125 patients with EC. They found that *MSH6* mutations are more common in EC patients than HNPCC families. Unfortunately, clinical information on our *MSH6* mutation was not available. The other MMR variants, the pathogenic non-frameshift deletion on *MLH3,* and the likely pathogenic missense on *MLH1* have not been reported yet in the medical literature. However, the genes are also well-known LS/ HNPCC-associated genes.

Currently, in the spectrum of LS-associated urological malignancies, only upper tract urothelial carcinoma and prostatic cancer are included (240). The association of TGCT and LS is inconclusive and limited to a single case report (240). as mentioned above, only Lobo and his collaborators (206) reported clear evidence regarding the genetic link between MMR gene *MSH2* and TGCT, where the patient had seminoma and his uncle had confirmed LS, both carrying the same mutation.

We were able to perform segregation analysis only for patient 18-1040 with the *MSH6* mutation, and for patient A2301 with the *MLH3* mutation. Surprisingly, in the former case, the mutation was inherited from the father, who is so far a healthy carrier, whereas the mother was affected by uterine cancer. It might be due to "generation skipping" or later disease onset, which is well described in relation to *MSH6* mutations. On the paternal lineage, the grandfather had pancreatic cancer, which is also a typical LS-associated tumor type. In a case report by Mannucci et al. (2020) (241) a pathogenic *MSH6* variant has been identified in a family with hereditary pancreatic cancer without diagnosed cases of colorectal adenocarcinoma. Seven family members were affected by the *MSH6* nonsense variant. Three had pancreatic adenocarcinoma at 65, 57 and 44 years; one had endometrial cancer at 36 years. None of the remaining three subjects (75, 45 and 17 years old) had developed any cancer yet. These observations underline the possible incomplete penetrance concerning pathogenic *MSH6* alterations. The pedigree analysis of patient A2301 gave a much clearer picture. The *MLH3* (NM_001040108 c.3232_3237delTGTACT) mutation was inherited from the mother who was affected by endometrial and breast cancer. Other neoplasms on the maternal lineage were breast cancer of the proband's aunt, and pancreas cancer of the proband's grandfather. It is relevant that the patient's mother has a *BRCA1* mutation as well. Since different types of neoplasms occurred in the mother's family, the two mutations carried by the mother in *MLH3* and *BRCA1* must be present (probably as single gene defect) in her affected family members as well. Important to state, that our patient did not inherit the *BRCA1* mutation. Our patient 18-048, carrying the *MLH1* variant, presented also a second gene mutation hence showing a digenic condition (see below).

Digenicity is a relatively novel finding, and it was present in two patients (18-048, A2049). Interestingly, the two variants in different DNA repair genes were associated with typical tumor types for both genes among the family members. Index case 18-048 has two missense variants, one in *MLH1* (NM_001167617 c.1203G>T), and the other one in *FANCD2* (NM_033084

c.2252T>C). *MLH1* belongs to the DNA mismatch repair gene family and pathogenic germline mutations of the gene are known causes of Lynch syndrome. *FANCD2* is part of the Fanconi Anemia gene family and in case of dominant inheritance of pathogenic mutations, Hereditary Breast and Ovarian Cancer syndrome (HBOC) and some type of OH malignancies (acute leukemia, multiple myeloma, T and B cell lymphoma) (242–244) can occur. Recent studies showed a functional interaction between *FANCJ* and the MMR complex MutLα, which is essential for establishment of DNA interstrand cross-links. *FANCD2* is required for binding between *MSH2* and *MLH1*, which are involved in the mono-ubiquitination of FANCD2, leading to recruitment of DNA checkpoint cluster ATR and then activation of *CHK1* and *TP53*. Furthermore, MutSα and MutLα complex have been shown to be required for the recruitment of ATR to DNA damage lesion. Taken together, these results support that there might be a functional overlap between the MMR and Fanconi Anemia-BRCA pathways. In the family of proband 18-048, the mother had ovarian cancer, and the maternal grandmother had lymphoma, so it seems that the *FANCD2* variant was inherited from the maternal lineage. On the paternal lineage, the patient's uncle had colon cancer, which is the most typical cancer type in LS, hence could be related to the *MLH1* gene. Which one of these two genes is responsible for TGCT in our index case remains to be established.

The other patient (A2049) possessing two candidate variants, has a pathogenic, splicing mutation in gene *ERCC3* (NM_000122 c.2065-2A>C) and a likely pathogenic, frameshift deletion in *MUTYH* (NM_001048172 c.1598delC) gene. Growing body of evidence supports the role of *ERCC3* in the development of HBOC, which is an inherited disorder with an increased risk for breast and ovarian cancers. *ERCC3*, a nucleotide excision repair gene encoding an ATPase/helicase protein involved in DNA repair, emerged as a good candidate to explore in patients with HBOC. Recurrent heterozygous *ERCC3* truncating mutation was described to increase the risk (OR 1.53, *p value* = 0.023) of breast cancer in individuals with Ashkenazi Jewish ancestry (245). Some other studies have identified similar germline truncating or nonsense *ERCC3* variants in other populations (e.g. Afro-American women) with HBOC as well (246,247). As far we know, no splicing mutations have been identified in relation with HBOC/breast cancer patients. Mutations in gene *MUTYH* result in heritable predisposition to colorectal cancer, termed MUTYH-associated polyposis (MAP), but they are linked to other human neoplastic and non-neoplastic diseases as well. In a recent paper from 2020 (248) is well described that *MUTYH* mutations can contribute to the development of sporadic gastric cancer (GC) (249). It has been found that GC patients with low *MUTYH* expression showed a poor outcome when compared to those expressing high levels of *MUTYH*. This finding may act as

an independent predictor of poor survival in GC patients (250). It has been proposed that *MUTYH* plays a tumor suppressor role in ulcerative colitis as well as in MAP patients (251). Tumors observed among the index case's family are uterine and breast cancer in the maternal aunt, breast cancer in maternal cousin, which might suggest that the variant of *ERCC3* was inherited from the maternal lineage. Although, the maternal grandfather had colon cancer, which is typical for *MUTYH* mutation, the paternal grandfather had also a *MUTYH* specific gastric cancer. Unfortunately, DNA from the family members was not available for segregation analysis.

Mendelian causes of inherited susceptibility of cancer are rare and characterized by variable expression and incomplete penetrance (252). Our two digenic cases are potential examples of the so called Multilocus Inherited Neoplasia Alleles syndrome (MINAS) which defines the presence of multiple pathogenic germline mutations in rare inherited high and/or moderate-risk-associated cancer syndrome genes in one individual. It was first described by Whitworth and colleagues in 2016 (252). These deleterious variants appeared to act independently in many cases, however no consistent effect was observed. Although the frequency of such occurrences appears to be low in the literature, such cases have probably been under ascertained because the standard clinical practice was to test candidate inherited cancer genes sequentially until a pathogenic mutation is detected. As we carried out WES on a highly selected group of patients, we were able to identify two probands (18-048, A2049, see above), who perfectly fit to the MINAS phenomenon. A third subject could be considered also as MINAS phenotype, the mother of proband A2301, from whom the *MLH3* variant was inherited, and who carry a *BRCA1* pathogenic mutation as well. The mother had characteristic tumor types for both genes, since endometrial cancer is typical for *MLH3* dysfunction, and breast cancer is associated with *BRCA1* alterations. In both our digenic (or MINAS) cases remains an open question which mutation caused the development of TGCT. Somatic analysis of the tumor tissue might help to elucidate which variant influenced more the tumor manifestation and had the second hit mutation.

Finally, we found a frameshift duplication (NM_001100119 c.978dupC) in gene *XRCC3.* Worth to state that, *XRCC3* interacts with *CHEK2,* and it is linked to an established TGCT-associated risk locus embracing *RAD51C.* Pathogenic variants within this gene are mainly associated with breast cancer and melanoma, but polymorphisms within the gene could significantly increase the risk for the development of prostate cancer and acute or chronic myeloid leukemia (253–255). Significant association was detected between *XRCC3* rs1799796 polymorphism and an increased risk of prostate cancer, in a study with 99 prostate cancer men

and 205 cancer-free controls, using PCR-RFLP method for SNP genotyping (253). Considering leukemia, the association is controversial, and might be found only in selected populations (254). In a Chinese meta-analysis, Yan et al. (254) systematically analyzed the association between *XRCC3* Thr241Met (rs861539) polymorphism and the risk of leukemia and found a statistically significant risk only in the Asian population. However, further large-scale, well-designed studies are needed to confirm these results. Regarding the patient's family, on the paternal lineage the patient's grandfather and great-grandfather had prostate cancer, whereas the maternal grandmother was affected by leukemia. Interestingly, we did not identify *CHEK2* mutations in our highly selected cohort of TGCT patients, not even in this case, where the family members were affected by prostate cancer.

VII. CONCLUSIONS AND WIDER IMPLICATIONS

TGCT is highly heterogeneous and challenging malignancy. Our multicenter case-control analysis of 592 individuals with TGCTs, 352 with oncohematological (OH) malignancy and 463 cancer-free controls suggest a familial aggregation of cancer in men with TGCT, and with OH. Furthermore, we observed that subjects with impaired spermatogenesis have more neoplasms among their relatives and that oncological patients (TGCT and OH) have less siblings than their healthy counterparts. Therefore, we propose that there might be a link between spermatogenic defects and a more systemic problem leading to higher morbidity including cancer development. Among the biological explanations for such relationship there could be an overall genomic instability in the family, which is a known mechanism in both oncogenesis and male infertility.

In order to explore the genetic basis leading to genomic instability in patients with TGCT and a general familial predisposition to cancers, we performed Whole Exome Sequencing (WES) followed by a targeted look up for mutations in DNA Repair genes. We found that DNA Repair genes might have a role in the etiopathogenesis of TGCT in the context of familial aggregation of neoplasms. A previous study (188)provides evidence for a DNA Repair gene, *CHEK2,* as a novel moderate-penetrance TGCT susceptibility gene, with possible clinical utility. In addition to highlighting DNA-repair defect as a potential mechanism driving to TGCT susceptibility and oncogenesis in general, our analysis also provides new possibilities for the detection of high-risk individuals i.e. those who present multiple members with cancers in their family. Based on a previous case report (206) and on our results, we suggest to include TGCT in the Lynch syndrome (LS) disease spectrum as part of the LS-associated urological malignancies. We propose a more "family-tailored" surveillance protocols of LS patients, especially for those belonging to families presenting with "unusual tumors". Broaden our awareness of the spectrum of LS-related urological cancers is pertinent to refining the management course for LS families. Patients with LS would benefit from informed measures for early detection and onco-andrological screening of possible urologic cancers. On the other hand, in case of pathogenic variants in DNA Repair genes, later in life, other types of malignancies could occur, hence a careful follow-up of these patients is advised.

In conclusion, our findings provide novel insight about the evaluation of patients with TGCT and LS-associated malignancies in their families. In light of the results obtained in the present study, further analyses are planned:

i) targeted genetic analysis of the parents of the three remaining mutation carriers, in order to determine the variants' origin and its potential relationship with the observed tumor types.

ii) searching for the "second hit" mutation in the tumor tissue i.e. we will: i) analyze the tumor tissue for the presence of "loss of heterozygosity" (LoH) for the selected germline variants, ii) in case of non-LoH, we will sequence the entire gene in order to search for the second pathogenic variant. This will help to confirm the causal relationship between the variant and the disease.

iii) for the MMR genes, i.e. for *MSH6, MLH3, MLH1* genes, microsatellite instability (MSI) analysis will be performed by comparing DNA extracted from the tumor tissue with DNA extracted from peripheral blood lymphocytes (and if available from the healthy tissue).

iv) finally, for all the interesting variants with uncertain significance ("hot" VUS), specific functional studies should be performed with the aim of defining their deleterious effect on the function of the protein encoded by the given gene.

"L'imperfezione ha da sempre consentito continue mutazioni di quel meraviglioso e quanto mai imperfetto meccanismo che è il cervello dell'uomo. Ritengo che l'imperfezione sia più consona alla natura umana che non la perfezione."

"It is imperfection - not perfection - that is the end result of the program written into that formidably complex engine that is the human brain, and of the influences exerted upon us by the environment and whoever takes care of us during the long years of our physical, psychological and intellectual development."

Prof. Rita Levi-Montalcini

Thank you! Grazie! Köszönöm! ¡Gracias!

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