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Early detection and complex treatment of metastatic testicular germ cell cancer

PhD Thesis

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PUBLICATIONS RELATED TO THE SUBJECT OF THIS THESIS

I. **Fazekas FE,** Ujfaludi Z, Biro K et al. Complex treatment of residual metastatic germ cell cancer: A single center experience. *J Biotechnol.* 2024; 20:389:61-67. **IF: 4.1, Q2**

II. Ujfaludi Z, **Fazekas FE**, Biro K et al. miR-21, miR-29a, and miR-106b: serum and tissue biomarkers with diagnostic potential in metastatic testicular cancer. *Sci Rep*. 14, 20151 (2024). **IF: 3.8, D1**

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I. Géczi L, Budai B, Polk N, **Fazekas FE**, Bodrogi I, Biró K. Neutrophil-to-lymphocyte ratio in primary mediastinal germ cell tumors: A retrospective analysis of >20 years single institution experience. *Curr Plobl Cancer*. 2020; 44(4):100537. **IF: 3.187, Q2**

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1. INTRODUCTION

1.1 Epidemiology of testicular germ cell tumors

Testicular cancer accounts for 1% of adult neoplasms and 5% of urological malignancies; with 90-95% of cases being germ cell tumors (GCT). In young adult men between the ages of 15 to 40 years old, testicular cancer is the most common type of solid tumor. TGCTs have shown a modest rise in incidence rates over the past few decades, particularly in industrialised countries. The cause of increasing incidence is not entirely understood. [1] Geographically, incidences vary significantly ranging from 3 to 12 new cases per 100,000 men/per year in Western countries. The lowest figures are reported in African countries; ranging between 0.3 and 0.6 cases per 100,000 males. [2]

The risk for testicular cancer is thought to be determined mostly or entirely in utero. [2] Risk factors include the components of testicular dysgenesis syndrome: cryptorchidism, hypospadias, impaired spermatogenesis and male infertility, as well as disorders of sex developement and a positive family history among first-degree relatives.[3], [4] A history of GCNIS or testicular cancer in the contralateral testis also increases risk. Testicular cancer survivors have a five-fold increased risk (2% lifetime risk for all men diagnosed with GCT) for developing a second, contralateral testicular malignancy. [5], [6]

The peak incidence of testicular tumors is typically observed between the ages of 25–29 years for nonseminomas, and 35–39 years for seminomas. [7] There is no benefit to routine screening for TGCT due to the overall low incidence and favourable cure rates.[8] Monthly testicular self-examinations, starting at puberty, can be an effective way of recognizing early, localized disease.[9]

With the introduction of platinum-based chemotherapy in the late 1970s, mortality rates have declined drastically. Disease specific survival is excellent in localised disease, and favorable even in advanced, metastatic cases. [10], [11]

5% of germ cell tumors arise from an extragonadal location [12], and mediastinal GCTs represent 25% of them.[13] Extent of the disease was confirmed to be an important prognostic factor by several publications. [14], [15] 41% of mediastinal GCT are metastatic at detection. [13]

1.2 Pathology of testicular cancer

Pure seminoma represents approximately 40-50% of testicular GCT in men between 25 and 55 years of age. Additionally, 15% of seminomas are combined with other histological variants. The cells of seminoma generally feature clear cytoplasm and a large, single central nucleus characterized by irregular chromatin and nucleoli. These cells are arranged in solid or cord-like clusters, surrounded by a thin layer of connective tissue that is typically infiltrated by T-lymphocytes and macrophages/dendritic cells.[16] Concerning the immunophenotype of seminomas, a membranous expression of PLAP [17], and c-kit are found in most cases [18], and a nuclear expression of the OCT3/4 is seen in all cases. [19] Markedly, AFP levels will be normal in pure seminoma cases. AFP is a glycoprotein secreted by yolk sac tumors and embryonal carcinomas. [20] Trophoblastic cells can produce elevated ßHCG levels occasionally. Seminomatous GCTs are highly sensitive to chemotherapy and irradiation.[16]

Spermatocytic Seminoma, which represents 1-2% of TGCTs, has no relation to the aforementioned pure seminoma. The tumor typically presents in men over 50 years, almost always in localized form. Generally, no systemic treatment is needed following orchiectomy. [16], [21]

Nonseminomatous germ cell cancer - embryonal carcinoma, choriocarcinoma, yolk sac tumor, teratoma and mixed germ cell tumor – typically present in the third decade of life and are often associated with marker elevation (AFP, ßHCG). 62% of cases are mixed germ cell tumours.[22]

Sex-cord/gonadal stromal tumors account for 3–6% of testicular tumors in adults. These are usually benign, heterogeneous lesions that arise from the supportive and hormone-producing tissues of the testes. [23]

Germ cell neoplasia in situ (GCNIS) is known to be the precancerous form of invasive testicular cancer, both seminoma and nonseminoma. The developement of GCNIS is higher in patients with a history of contralateral testicular GCT, in undescended testis and in infertile men. [24]

1.3 Genetics of testicular germ cell cancer

Testicular germ cell cancer is associated with several genetic abnormalities that may contribute to its development. A gain in the short arm if chromosome 12 is the most consistent genetic abnormality found in testicular germ cell tumors, seen in around 50% of seminomas and 80% of non-seminomatous germ cell tumors.[25] The receptor tyrosine kinase c-KIT plays a key role in gonadal development and spermatogenesis. [26] c-KIT mutations are frequently present in seminomatous GCTs and bilateral testicular cancer. [27]The gain of 12p or c-KIT mutations are thought to be necessary for GCNIS cells to progress into invasive cancer. [28] Germline loss-of-function CHEK2 variants are 4 times more likely to be present in TGCT patients, than healthy controls. In a series, patients with the pathogenic CHEK2 loss-of-function variants developed cancer 6 years earlier than their counterparts with CHEK2 wild-type alleles. [29] Gain of chromosomes X, 7, 8, and 21 and loss of chromosomes Y, 1p, 11, 13, and 18 are also characteristic of post-pubertal TGCT. [30]

The embryonic origin of GCTs might explain their overall sensitivity to DNA damaging treatments (i.e., cisplatin-based chemotherapy and radiotherapy). [31] The histological composition of the tumor has significant impact on its respone to treatment: loss of embryonic features results in induction of treatment resistance.[32] A significant next-generation sequencing study involving a large cohort of therapy-resistant patients showed that resistance to cisplatin in advanced type II GCTs is significantly associated with inactivation of p53, amplification of the gene encoding the negative regulator of p53 *MDM2*, or *MYCN* amplification, which transcriptionally targets both *TP53* and *MDM2*. The genes RAC1 and FAT atypical cadherin 1 (FAT1), which are part of the RAS and WNT signalling pathways, were also commonly associated with chemotherapy resistance. [33]

1.4 Staging of testicular cancer

Accurate staging of a malignant tumor is crucial, as it guides treatment and helps predict prognosis. Proper staging involves assessing the anatomical extent of the primary tumor, the regional lymphatic spread and distal metastases. Staging examinations include a physical examination, imaging studies and serum marker tests.[5]

pT - Primary Tumour

pTX	Primary tumour cannot be assessed (see note[1])
pT0	No evidence of primary tumour (e.g., histological scar in testis)
pTis	Intratubular germ cell neoplasia (carcinoma *in situ*)[+]
pT1	Tumour limited to testis and epididymis without vascular/lymphatic invasion; tumour may invade tunica albuginea but not tunica vaginalis
pT2	Tumour limited to testis and epididymis with vascular/lymphatic invasion, or tumour extending through tunica albuginea with involvement of tunica vaginalis
pT3	Tumour invades spermatic cord with or without vascular/lymphatic invasion
pT4	Tumour invades scrotum with or without vascular/lymphatic invasion

N - Regional Lymph Nodes – Clinical

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis with a lymph node mass 2 cm or less in greatest dimension or multiple lymph nodes, none more than 2 cm in greatest dimension
N2	Metastasis with a lymph node mass more than 2 cm but not more than 5 cm in greatest dimension; or more than 5 nodes positive, none more than 5 cm; or evidence of extranodal extension of tumour
N3	Metastasis with a lymph node mass more than 5 cm in greatest dimension

Pn - Regional Lymph Nodes – Pathological

pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis
pN1	Metastasis with a lymph node mass 2 cm or less in greatest dimension and 5 or fewer positive nodes, none more than 2 cm in greatest dimension
pN2	Metastasis with a lymph node mass more than 2 cm but not more than 5 cm in greatest dimension; or more than 5 nodes positive, none more than 5 cm; or evidence of extranodal extension of tumour
pN3	Metastasis with a lymph node mass more than 5 cm in greatest dimension

M - Distant Metastasis

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
	M1a Non-regional lymph node(s) or lung metastasis
	M1b Distant metastasis other than non-regional lymph nodes and lung

S - Serum Tumour Markers (Pre chemotherapy)

SX	Serum marker studies not available or not performed		
S0	Serum marker study levels within normal limits		
	LDH (U/l)	*hCG (mIU/mL)*	*AFP (ng/mL)*
S1	< 1.5 x N and	< 5,000 and	< 1,000
S2	1.5-10 x N or	5,000-50,000 or	1,000-10,000
S3	> 10 x N or	> 50,000 or	> 10,000

Table 1: TNM classification for testicular cancer (adapted from UICC, 2016, 8th edn.) [34]

1.5 Prognostic risk group classification of metastatic testicular germ cell cancer

A prognostic risk-factor system for metastatic GCT has been established by the 1997 International Germ Cell Cancer Collaborative Group based on clinically independent adverse factors. [35] The classification system has been revalidated in 2021 both for nonseminomatous and seminomatous tumors. [11], [36]

	Seminoma	**Non-seminoma**
Good-prognosis group	All of the following criteria: • Any primary site • No non-pulmonary visceral metastases • Normal AFP • Any hCG • Any LDH	All of the following criteria: • Testis/retro-peritoneal primary • No non-pulmonary visceral metastases • AFP < 1,000 ng/mL • hCG < 5,000 IU/L (1,000 ng/mL) • LDH < 1.5 x ULN
Intermediate-prognosis group	All of the following criteria: • Any primary site • Non-pulmonary visceral metastases • Normal AFP • Any hCG • Any LDH	Any of the following criteria: • Testis/retro-peritoneal primary • No non-pulmonary visceral metastases • AFP 1,000 - 10,000 ng/mL or • hCG 5,000 - 50,000 IU/L or • LDH 1.5 - 10 x ULN
Poor-prognosis group	No patients classified as “poor-prognosis”	Any of the following criteria: • Mediastinal primary • Non-pulmonary visceral metastases • AFP > 10,000 ng/mL or • hCG > 50,000 IU/L (10,000 ng/mL) or • LDH > 10 x ULN

Table 2: Prognostic-based staging system for metastatic germ cell cancer (IGCCCG) [11], [36]

1.6 Diagnosis of testicular cancer

Patients most often present with an enlarged testicle, or a painless lump in the testicle. Testicular pain or tenderness is not an uncommon presentation, and may be mistaken for inflammation, delaying diagnosis. Occasionally, testicular cancer can be an incidental finding on ultrasound. Gynecomastia may be seen in a small number of patients.[37] [38], [39] Abdominal or back pain can occur in delayed diagnosis as a consequence of pathological lymph node metastases.[37] Monthly testicular self-examination is encouraged for young men, especially testicular cancer survivors and their first-degree male relatives. [40]

After physical assessment, high-frequency (>10 MHz) testicular ultrasound examination is recommended to confirm the presence of a mass, assess its volume and location and characterize the contralateral testicle. Serum tumor markers are determined before and after orchiectomy, and then during follow-up, as they provide staging and prognostic information. [37] The precise frequency of tumor marker testing is not well defined, due to the lack of high quality evidence. [41] Classical tumor markers (AFP, ßHCG, LDH) have limitations due to their low sensitivity; as much as 40% of cases are marker negative. If, after orchiectomy they stay elevated or increase, metastatic dissemination is likely. [42]

Variable	**AFP**	**hCG**	**LDH**
Assay techniques (as recommended by NACB) [43]	2-site immunometric assays with mAbs ± polyclonal antisera	Double-antibody immunometric assays that measure total hCGβ (intact α/β dimer plus free β monomer)	Enzymatic activity assays measuring conversion of lactate to pyruvate or vice versa
ULN	10-15 µg/L (≈9 if < 40 years of age; ≈13 if > 40 years of age)	5-10 U/L (0.7 U/L in men < 50 years of age; 2.1 U/L if > 50 years of age)	Highly variable and laboratory-specific; depends on assay conditions; elevated if > 1.5 times lab-specific ULN
Units (and conversion factors, if applicable)	International units (kU/L) or mass units (µg/L); 1 U = 1.21 ng	International units (U/L; 5 U/L of hCG corresponds to 15 pmol/L)	U/L and fold-increase over ULN
Detection limit (as	< 1 µg/L (0.8	< 1 U/L of serum	Highly dependent

recommended by NACB) [43]	kU/L) of serum or plasma	or plasma (and < 2% cross-reactivity with LH)	on assay method and conditions
Approximate biologic half-life	5-7 days	1.5-3 days	Not reported
Seminomatous GCT (approximate proportion of patients with elevations)	Never elevated in pure seminoma	Yes (15%–20% in advanced disease)	Yes (in 40%–60% of patients)
Nonseminomatous GCT (approximate proportion of patients with elevations)	Yes (10%–20% in stage I, 20%–40% in low-volume stage II, 40%–60% in advanced disease)	Yes (10%–20% in stage I, 20%–30% in low-volume stage II, 40% in advanced disease)	Yes (in 40%–60% of patients)
Other malignancies sometimes associated with elevations	Hepatocellular carcinoma, gastric, lung, colon, and pancreatic cancer	Neuroendocrine, bladder, kidney, lung, head, neck, GI, cervix, uterus and vulva, lymphoma	Lymphoma, small-cell lung cancer, Ewing sarcoma, osteogenic sarcoma
Nonmalignant conditions sometimes associated with elevations	Alcohol abuse, hepatitis, cirrhosis, biliary tract obstruction, hereditary persistence	Marijuana, hypogonadism	Many (processes that involve cell or tissue damage, eg, myocardial infarction, liver or muscle disease), hemolysis of blood sample

Table 3: Summary of key information for serum tumor markers of GCTs [44]

According to the EAU Guidelines, physical assessment, testicular ultrasound examination and serum marker test are sufficient to confirm the clinical diagnosis of testicular cancer. There is no evidence supporting any size criteria for a testicular lesion to be safely followed-up.[37] Contralateral biopsy is not recommended in patients > 40 years without risk factors. [45]

Contrast enhanced computerised tomography or MRI of the chest, abdomen and pelvis needs to be obtained to determine anatomical extent of the disease. In choriocarcinoma patients a brain MRI scan is also recommended. [37]

Micro RNAs are being recognized as promising new biomarkers. Elevated levels of microRNA-371a-3p has been observed before radical orchiectomy in 80-90% of both seminomatous and non-seminomatous cancers. [46] Several studies indicate that

miRNAs, especially miR-371a-3p, show greater accuracy compared to conventional GCT markers in diagnosis, clinical staging, treatment monitoring, and predicting the presence of residual or recurrent viable disease. [47][48][49] According to current literature, miRNA have low level or absent expression in teratoma, which will limit its utility in nonseminatous GCT. [49], [50], [51]

1.7 MicroRNA

MicroRNAs (miRNAs) are small, non-coding RNA molecules, typically 20-22 nucleotides long, that play a crucial role in the post-transcriptional regulation of gene expression. In the recent years they have garnered significant interest due to their involvement in normal cellular and disease processes, e.g. cancer. miRNAs are key regulators of gene expression, influencing various cellular processes such as proliferation, differentiation, apoptosis, and metabolism.

The biogenesis of miRNAs begins in the nucleus with the transcription of a pri-miRNA precursor, which is subsequently processed by endonuclease enzymes into a 80-100 nucleotides long pre-miRNA sequence. pre-miRNAs are transported from the nucleus to the cytoplasm, where a cytoplasmic ribonuclease cleaves them into double stranded mature miRNA. The duplex binds to Argonaute proteins resulting in the formation of the RNA-induced silencing complex (RISC), which subsequently regulates the translational repression or degradation of target messenger RNA (mRNA). [52], [53]

MiRNAs were also revealed to act as a ligand to activate various signaling pathways. Fabbri et al. were the first to demonstrate that tumor cell-secreted miR-21/miR-29a bind to murine Toll-like receptor 7 or human Toll-like receptor 8, initiating a Toll-like receptor-mediated prometastatic inflammatory response. This mechanism of action of miRNAs is implicated in tumor growth and metastasis. [54] Additionally, miRNA has been shown to affect the nuclear factor κB signaling pathway in natural killer cells, which could influence host defense against infection and malignant transformation. [55] Altered miRNA expression in tumors could affect several of the cancer hallmarks for tumor initiation and progression, e.g. evading growth suppressors and sustaining proliferative signaling [56], [57], resisting cell death, [58] activating invasion and metastasis [59], [60] and inducing angiogenesis.[52] [61] Emerging evidence has suggested miRNAs to be promising novel biomarkers for detection,

prognosis and monitoring of human cancers. Studies using minimally invasive techniques, such as liquid biopsy to collect samples are crucial for advancing reliable and cost-effective miRNA-based technology for routine clinical use. [52]

1.8 Treatment and survivorship

Since the implementation of platinum-based chemotherapy as introduced by Einhorn and Donohue [62], the mortality of testicular cancer has significantly decreased. According to the population-based Thames Cancer Registry, in 1960-1969 the 10-year relative survival for seminoma and nonseminoma patients was 78% and 55%, respectively.[63] With current standard-of-care management 90-95% of testicular tumors are cured.[64] Modern treatment strategies now focus on risk-adapted therapy, which reduces the number and severity of side effects while improving the patient's quality of life, without compromising the chance of recovery.[65] Given that these patients are typically young and have many years of life ahead of them, addressing -survivorship issues is crucial even before treatment is initiated. Potential late effects include cardiovascular toxicities, hearing loss, peripheral neuropathy, secondary cancers, hypogonadism, infertility, psychological impacts, and long-term surgical complications. These survivorship issues emphasize the necessity for long-term follow-up as well as careful risk stratification and limiting therapy to just what is needed to achieve a cure. [5], [66], [67], [68], [69]

1.8.1 Localized seminoma

The primary treatment for localized testicular cancer is radical inguinal orchiectomy. It is known, based on large, unselected patient series on surveillance, that 85% of patients with localized disease are cured by orchiectomy alone. [70], [71] A risk-adapted management strategy is recommended in localized seminoma. Patients with a tumor measuring 4 cm or less and without stromal invasion of the rete testis are recommended surveillance. Those with a tumor larger than 4 cm and/or evidence of stromal invasion of the rete testis should receive one course of adjuvant carboplatin. Survaillance can also be offered in high risk localized cases if the appropriate resources are available and the patient is motivated.[37] [72] Adjuvant radiotherapy is

recommended only for patients who are not candidates for adjuvant chemotherapy or surveillance. [71]

1.8.2 Localized nonseminoma

High-risk patients with evidence of lymphovascular invasion in the orchiectomy specimen, are recommended one cycle of adjuvant BEP. Patients without lymphovascular invasion are candidates for surveillance or adjuvant BEP.[73] For patients with localized disease and malignant somatic transformation in the tumor, bilateral nerve sparing RPLND is the treatment of choice.[74]

1.8.3 Metastatic testicular germ cell cancer and RPLND

In managing metastatic disease, the TNM stage and IGCCCG classification of the disease guides treatment. In bulky, metastatic, life-threatening cases, chemotherapy should start without delay, followed by orchiectomy. [10], [34], [36]

For intermediate and poor prognosis metastatic testicular cancer, there is international consensus on treatment with 4 cycles of cisplatin-based combination chemotherapy. Patients with an intermediate or poor prognosis should always be referred to a center experienced in managing advanced germ cell tumors prior to the initiation of treatment. Patients in the good prognosis IGCCCG group receive 3 series of BEP. Dose reductions and delays in treatment should be avoided.[37] [75]

According to current guidelines, surgical resection is mandatory in all non-seminoma patients harbouring postchemotherapy residual masses of ≥ 1 cm in transaxial long axis at cross-sectional CECT imaging. With residuals below 1 cm, salvage RPLND or surveillance are both appropriate alternatives. [37][75][76] The lymphatic drainage of the testicle was first described by Jamieson and Dobson in 1910. The most frequent landing sites of metastases are next to the major vessels located in the retroperitoneum. [77] Based on the lymphatic mapping above, the technique of retroperitoneal lymph node dissection was described by Hinman at John Hopkins Hospital in 1914. Operative mortality was 11% at the time, which was considered „surprisingly low”, given the newness and radicality of the procedure. [78] Extended bilateral RPLND and the nerve-sparing technique with preservation of ejaculation were both reported by Donohue et al. in 1977 and 1990, respectively. [79], [80]

Seminoma germ cell tumors demonstrate a high sensitivity to both radio- and chemotherapy, resulting in a low incidence of viable disease post-treatment. For patients with pure seminomas who have non-regressing lesions of ≥ 3 cm, an FDG-PET scan can be utilized, offering a high negative predictive value of 95% to rule out active disease [81]. The use of chemotherapy is associated with potential significant morbidity and mortality in short- and long-term survivors, e.g. leukaemia and cardiovascular disease. The SEMITEP trial has reported the de-escalation of chemotherapy to be feasible in men with good-prognosis metastatic seminoma based on negative interim FDG PET/CT results.[82] The currently ongoing SAKK 01/10 trial[83] combined de-escalated chemotherapy with de-escalated involved node radiotherapy, with the aim of reducing toxicity while preserving efficacy. Favourable 3-year progression-free survival was observed in case of patients with stage IIA or IIB classic seminoma. Salvage retroperitoneal lymph node dissection can be technically challenging due to the significant desmoplastic reaction caused by seminomas, and it is associated with an increased rate of complications [84]. Overall, salvage RPLND is rarely indicated in pure seminomatous cases. [75]

The role of primary RPLND in the management of metastatic testicular cancer has changed significantly during the last 15 years, and is still controversial. In 2008 Peter Albers recommended one course of adjuvant BEP instead of primary RPLND in clinical stage I nonseminoma cases. Their recommendation was based on a 7,59% difference in the 2-year recurrence-free survival rate between chemotherapy and surgery.[85] However, long-term survival analysis has proven that treatment-induced late effects, (eg. secondary tumors and cardiac problems) of BEP or radiotherapy were associated with significant excess risk of non-testicular cancer related mortality after >10 years follow up. [86] In their retrospective study Mousa et al. suggested adjuvant chemotherapy to be overtreatment with no survival benefit for most pathological stage II nonseminoma patients treated with primary RPLND. [87] According to a multicenter retrospective cohort study presented at the 2024 ASCO GU, primary RPLND should be considered in marker negative clinical stage 2b nonseminomatous GCT as well. [88]

The results of the PRIMETEST trial of primary retroperitoneal lymph node dissection in stage II A/B seminoma were presented by Peter Albers at the 2022 GU ASCO. According to their new evidence, both open and minimally invasive surgical resection of small volume, unilateral seminoma metastasis is feasible with acceptable

toxicity. RFS was 69% in a cohort of 31 eligible patients with a median follow-up of 30 months. [89]

1.9 Stage migration

Huddart et al. [90] observed that although the gold standard for systemic treatment of testicular tumors remained largely unchanged from the 1980s to 2000, patient survival rates in the UK improved during this time. This increase in survival was attributed to awareness campaigns about testicular cancer conducted by various UK organizations in the early 1990s, which encouraged patients to consult doctors sooner regarding their symptoms. Their analysis revealed that the average size of tumors at diagnosis was 1.5 cm smaller in the period from 1999 to 2002 compared to the years from 1984 to 1995. Additionally, when comparing these two time frames, the percentage of patients diagnosed with stage I tumors rose from 57% to 77%.

Early detection has led to a significant reduction in the size of tumours at diagnosis, and an increase in the proportion of tumours detected at an early stage - a phenomenon known as stage migration. Both tumour size and stage are important prognostic factors, it is therefore logical that smaller tumours detected at an earlier stage lead to better survival outcomes. [22], [91]

Stage migration of testicular GCT was reported in Canada during the COVID-19 pandemic. 7.8% patients identified before 2019 presented with stage III disease, compared to 15.4% diagnosed during the pandemic. It is elementary, that the detection and treatment of testicular cancer proceed without delay, even amid a global pandemic. [92], [93]

2. AIMS OF THE STUDIES

Our aims were the following:

I. Pilot study on testicular cancer stage migration and patient delay at a single site

- to assess size, stage and histology type of testicular tumors of 12 years of patients at a single urooncology center

- to report time from onset of first symptoms suggestive of testicular tumor to first consultation with a physician

II. Assessment of postchemotherapy RPLND outcomes and complications at a single high volume referral center in Hungary

- to compare different treatment groups based on the number of BEP cycles received.
- considering the extent of retroperitoneal resection.
- considering residual tumor stage and histopathology.

III. Assessment of pre-selected sera and/or tissue miRNA expression profiles in testicular cancer patients with postchemotherapy residual retroperitoneal lesions and healthy controls

- to differentiate between testicular germ cell cancer patients and healthy individuals.
- to develop more reliable tumor markers for TGCT management to monitor treatment responses and assess post-chemotherapy residual lesions
- to correlate with chemotherapeutic response, thus faciliating the follow-up and monitoring after systemic therapy.

3. MATERIALS AND METHODS

3.1 Pilot study on testicular cancer stage migration and patient delay at a single site

3.1.1 Study group characterisation

A retrospective chart review of patients (n= 143) who underwent radical inguinal orchiectomy for testicular cancer at a single hungarian urooncology referral center between 2007 and 2018 was performed. The first and the last 3-year period (2007-2009, n= 44 and 2016-2018, n=16) were compared in the study. Patients with no evidence of malignancy in the orchiectomy specimen were excluded from the analysis.

In the period from 2007 to 2009 pure seminomatous tumors were found in 56,8% (n= 25) of removed testes, while nonseminatous/mixed GCT were described in 43,25 (n=19) of specimen. Salvage RPLND was performed in 6,8% (n= 3) of cases during the follow-up period.

Between 2016 and 2018, pure seminomas, nonseminomas/mixed GCT and postchemotherapy burned-out GCT were identified in 37,5% (n= 6), 50% (n= 8) and 12,5% (n= 2) cases, respectively. 18,75% (n=3) of patients underwent salvage RPLND and in one case second-look RPLND was performed.

3.1.2 Statistical analysis

Descriptive statistical methods, correlation and regression analysis and contingency table analysis was used to examine the relationship between the two selected time periods and the characteristics of the removed tumors.

3.2 Assessment of postchemotherapy RPLND outcomes and complications at a single high volume referral center in Hungary

3.2.1 Study group characterisation

In a retrospective cross-sectional study, medical records of 127 patients who underwent salvage or second-look RPLND between 2007-2023 in a single RPLND referral center were reviewed. 114 of them were salvage and 13 were second look cases. All surgeries were performed by two high volume surgeons. Mean age at surgery was 35 years (ranged 19-69). Primary tumor was right side testicular cancer in 63, left side in 58 and retroperitoneal GCT in 6 cases. Primary GCT pathology was, non-seminoma/mixed GCT, pure seminoma or fibrosis/necrosis in 110, 13 and 4 patients, respectively. 100 patients received standard chemotherapy regimen, while 11 and 16 men underwent 1-2 or 5-6 cycles of BEP, respectively. Serum tumor markers after chemotherapy were normal. Further patient parameters including residual mass size (N stage) and histopathology, adjunctive vascular and visceral surgeries and complications as well as long term patient outcomes were recorded and analyzed.

Patient were classified into three systemic treatment subgroups according to the number of BEP cycles they received; “standard” (3-4 BEP cycles), “less than standard” (1-2 BEP cycles) and “more than standard” (5-6 BEP cycles) subgroups. [94]

3.2.2 Statistical analysis

In our survival analysis, we utilized the survminer package. The statistical analysis considered the date of surgery and the end of oncological follow-up. Time was represented in annual intervals on the graphs. For the statistical analysis, we applied the survival package, where we calculated the survival probabilities and used 95%

confidence intervals. To compare the survival curves of different groups, we used the log-rank test to determine statistically significant differences among the examined groups, with significance determined at $p < 0.05$. For data analysis and visualization, we utilized survminer package in the R programming environment (R version 4.2.1).[94], [95], [96], [97]

3.3 Assessment of pre-selected sera and/or tissue miRNA expression profiles in testicular cancer patients with postchemotherapy residual retroperitoneal lymph nodes and healthy controls

3.3.1 Study group and specimens

The test cohort was composed of 27 blood serum samples of post-chemotherapy, clinically lymph node positive testicular GCT patients. The sample size of the cohort was not determined statistically prior to experimentation. The control group of 27 blood serum samples from healthy subjects were obtained by volunteers. The blood samples were handled as described below.

Tissue samples were collected from the cancer patient group by salvage RPLND (n=18), second look RPLND (n=3) or radical inguinal orchiectomy (n=1). Right after surgical removal representative areas of the removed tumors were resected, snapfrozen and saved for further investigation.

All histological slides were assessed by an experienced uropathologist, and staged in accordance with the TNM classification 8th edition. Histology of resected tumors was organised into 3 groups; necrosis/fibrosis only, teratoma (mature or immature) or viable GCT. Cases with a combination of viable GCT and teratoma were classified as viable GCT, while a mixed histology of necrosis and teratoma was defined as teratoma.

3.3.2 Preparation of blood sera

Collection of blood samples was carried out on cancer patients one day prior to surgery. Blood collection tubes with separating gel were used, and the tubes were stored at 4 °C for 45 to 60 min right after the phlebotomy. Sera samples were separated with centrifugation (2000 rpm, 10 min, 4 °C). Upper phase, which contained the blood serum was aliquoted to sterile, DNase-, RNase-free cryogenic storage tubes and snapfrozen and stored in liquid nitrogen.

3.3.3 Serum miRNA purification, reverse transcription and qPCR

Hemolysis of sera was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Serum miRNAs were purified using ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, USA) adhering to the manufacturer's protocol.

targeted RNA	**sequence (5'-3')**
hsa-miR-19a	GCA GTG TGC AAA TCT ATG C
hsa-miR-21	TAGCTTATCAGACTGATGTTGA
hsa-miR-29a-3p	TAG CAC CAT CTG AAA TCG G
hsa-miR-106b	TAA AGT GCT GAC AGT GCA G
hsa-miR-155-5p	CGCAGTTAATGCTAATCGTGATAG
hsa-miR-199a-5p	GCCCAGTGTTCAGACTAC
hsa-miR-376-3p	GCA GAA TTG CAC TTT AGC AAT G
hsa-miR-371-3p	GTG CCG CCA TCT TTT GAG
hsa-miR-373-3p	GAA GTG CTT CGA TTT TGG G
U6	CTC GCT TCG GCA GCA CAT A
SNORD44	GATGATGATAAGCAAATGCTGACTGAAC

Table 4. Sequences of PCR primers used in qPCR reactions

Equal volumes of extracted miRNA samples underwent reverse transcription by MystiCq microRNA cDNA Synthesis Kit (Sigma-Aldrich, USA). Oligonucleotides for miRNA specific PCR amplification were designed using the miRprimer_2 software. [98] Primer sequences are provided in Table 4. The qPCR reactions were conducted on Rotor-Gene Q 5plex HRM Real-Time PCR Cycler Platform (Qiagen, Germany) using SYBR Green chemistry (GoTaq qPCR Master Mix, Promega, USA). Normalized serum levels of the miRNAs were calculated by the -dCT method using the Cq values of U6 as endogenous control and are indicated as log2 fold-change in the figures.

All tissue samples were embedded in Cryomatrix (Thermo Fisher Scientific, USA) and cut into 10 μm sections using a CryoStar NX50 microtome (Thermo Fisher Scientific, USA). The initial and final sections were hematoxylin–eosin stained[99] and evaluated by expert uropathologists. For miRNA extraction, five sections from each

lymph node sample were processed using the ReliaPrep miRNA Cell and Tissue Miniprep System (Promega, USA) in accordance with the manufacturer's instructions.

3.3.4 Biostatistics

Statistical analyses of the control and tumorous datasets were conducted using the SigmaPlot 12.5 software package (Systat Software Inc., USA). The distribution of datasets was assessed using the Shapiro-Wilk normality and Equal Variance tests. The values of variances were determined by ANOVA or Kruskal-Wallis ANOVA on Ranks methods followed by multiple comparisons using the Holm-Sidak test or the Tukey-test, respectively. Diagnostic abilities and cutoff values were established by the receiver operating characteristic curve (ROC curve) analysis tool of SigmaPlot 12.5 software applying a 99% confidential interval. Variance-based statistics were performed with the ClustVis online toolset (https:// biit. cs. ut. ee/ clust vis/). For heatmap generation, data were clustered based on Euclidean distance and average linkage methods.

4. RESULTS

4.1 Pilot study on testicular cancer stage migration and patient delay at a single site

4.1.1 Tumor size and stage at detection, and patient delay

Between 2007 and 2009, the average size of testicular tumors was 55.4 mm, and 67.6% of the cases were classified as stage I at detection. In contrast, during the period from 2016 to 2018, the average tumor size decreased slightly to 49.3 mm, and the detection rate for stage I cases dropped to 40%. Within both time intervals half of the patients saw their physician within 1 month of developing symptoms suggestive of testicular cancer. Between 2007-2009 11% of patients waited more than 6 months before seeking help, in the years 2016-2018 this rate was 25%.

Overall, looking at the period 2007-2009 and 2016-2018, the size of detected testicular tumours were comparable, and the proportion of localized disease at detection slightly decreased. These findings were not significant on statistical analysis. Patient delay was longer in the period from 2016 to 2018. Considering the whole cohort, a weak but significant positive association was observed between stage and tumour size: **Spearmans Rho, r= 0.198; p<0.02 (N=143).**

4.2 Assessment of postchemotherapy RPLND outcomes and complications at a high volume referral center in Hungary

4.2.1 Systemic treatment groups and outcomes

	Less than standard 1-2 BEP cycles (n = 11)	Standard 3-4 BEP cycles (n = 100)	More than standard 5-6 BEP cycles (n = 16)
Mean age (y)	34	35	36
Primary GCT origin *Right testis* *Left testis* *Retroperitoneum*	 7 (63,6%) 4 (36,4%) 0	 48 (48%) 49 (49%) 3 (3%)	 8 (50%) 5 (31,25%) 3 (18,75%)
Primary histology *Nonseminoma (110)* *Seminoma (13)* *Fibrosis, necrosis (4)*	 9 (81,8%) 2 (18,2%) 0	 86 (86%) 10 (10%) 4 (4%)	 15 (93,75%) 1 (6,25%) 0
N stage *cN1 (13)* *cN2 (53)* *cN3 (61)*	 1 (9,1%) 4 (36,4%) 6 (54,5%)	 12 (12%) 40 (40%) 48 (48%)	 0 9 (56,25%) 7 (43,75%)
Prognostic group *Good (70)* *Intermediate (43)* *Poor (13)*	 9 (81,8%) 2 (18,2%) 0	 55 (55%) 32 (32%) 12 (12%)	 6 (37,5%) 9 (56,25%) 1 (6,25%)
Residual mass histology *Viable tumor (26)* *Teratoma (67)* *Fibrosis/necrosis (34)*	 2 (18,2%) 6 (54,5%) 3 (27,3%)	 18 (18%) 56 (56%) 26 (26%)	 6 (37,5%) 5 (31,25%) 5 (31,25%)
Adjunctive surgery (29) Nephrectomy (6)	1 (9,1%) 0	22 (22%) 3 (3%)	6 (37,5%) 3 (18,75%)
Progression after RPLND	4 (36,4%)	25 (25%)	7 (43,8%)
Alive to date	8 (72,7%)	85 (85%)	9 (56,3%)

Table 5: Demographic and clinical parameters considering chemotherapy regimen groups

The "more than standard" subgroup had the highest rate of viable tumor in the specimen (37,5%), post-RPLND disease progression (43,8%), adjunctive surgeries (37,5%) and the lowest rate of survival (56,3%), although these findings were not significant on statistical analysis. In the "less than standard" and "standard" subgroups teratoma was the most prevalent lymph node histology. 50% of patients with primary retroperitoneal GCT received "more than standard" BEP courses.

Significantly more nephrectomies were performed in the "more than standard" treatment group (p= 0,0166, Pearson's Chi-squared test).

4.2.2 GCT clinical parameters and outcomes

Following chemotherapy, residual retroperitoneal masses were classified as cN3 in 49.6%, cN2 in 40.2%, and cN1 in 10.2% of cases. cN3 residual tumors were the most common across all histological subgroups. Modified template surgeries were conducted more often than bilateral procedures, with 84 cases (66.1%) compared to 43 cases (33.9%). The use of bilateral template was most prevalent in cN3 nonseminoma cases. Surgical complications were reported in 44 cases, with the majority (36 cases, or 81.8%) classified as Clavien-Dindo grades 1-2. Additional procedures were necessary in 29 cases, with repairs made to the aorta, inferior vena cava, or renal artery in 4, 9, and 2 cases, respectively. In 6 cN2-3 nonseminoma patients nephrectomy was performed. A double-J stent was placed in 5 cases postoperatively, where an ureter lesion was confirmed.

	Seminoma			**Non-seminoma**			**Necrosis/fibrosis**		
	n=13			**n=110**			**n=4**		
	N1	**N2**	**N3**	**N1**	**N2**	**N3**	**N1**	**N2**	**N3**
n=	2	4	7	11	45	54	-	2	2
%	14,3%	35,7%	50%	10,0%	40,9%	49,1%	-	50%	50%
Extent of RPLND									
Bilateral	-	1	1	4	13	24	-	-	-
Modified	2	3	6	7	32	30	-	2	2
Type of RPLND									
Salvage	2	4	5	11	43	46	-	1	2
Second look	-	-	2	-	2	1	-	1	-
Nephrectomy	-	-	-	-	1	5	-	-	-
Ureter end-to-end anastomosis	1	-	-	-	-	1	-	-	-
Ureteral injury	-	-	-	-	1	4	-	-	-
Aortic injury	-	-	-	-	1	2	-	-	1
IVC injury	-	-	1		4	3	-	-	1
Renal artery injury	-	-	-	-	1	1	-	-	-

Table 6: Surgical complications by primary tumor histology and cN stage groups

4.2.3 Survival analysis of patient subgroups

Survival was lower for patients with primary retroperitoneal GCT and in cases of viable tumor in the removed metastasis. Disease specific survival was higher for patients with teratoma in the specimen. In case of progression after salvage RPLND (n= 37 or 29,13%) cancer-specific survival was lower. **These findings were significant on statistical analysis.**

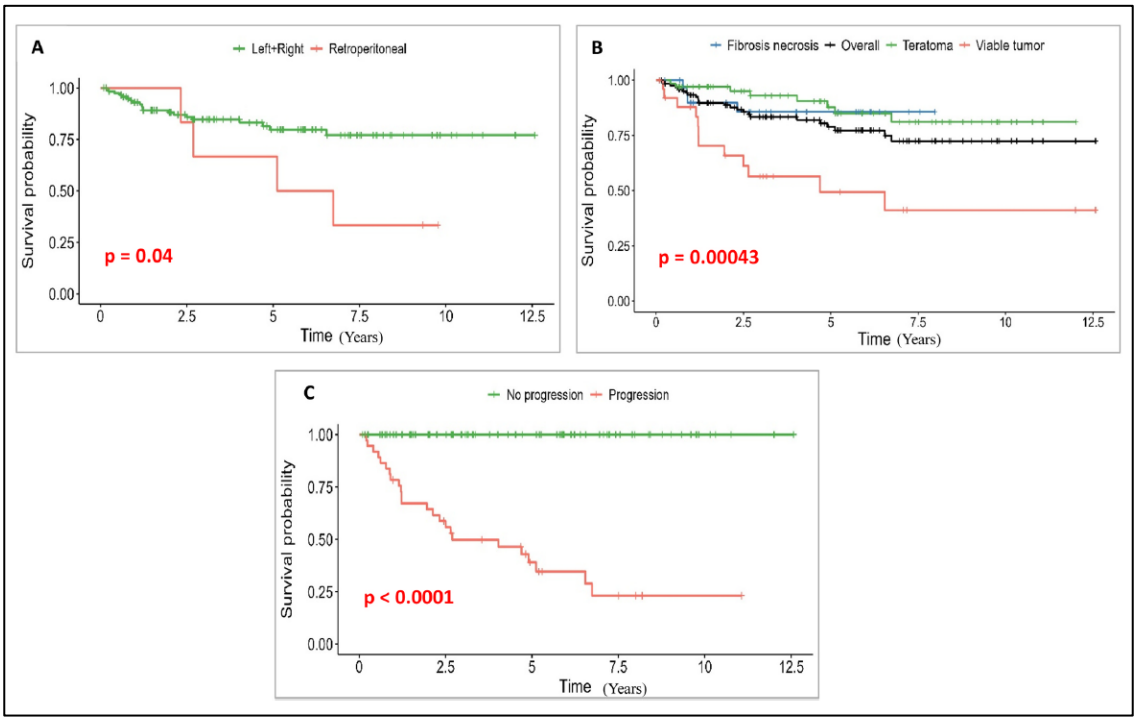

Figure 1:
A) Survival considering primary tumor origin.
Green: left or right testicular GCT. Orange: retroperitoneal GCT.
B) Survival considering residual mass histopathology.
Black: overall survival. Green: teratoma. Blue: fibrosis, necrosis. Orange: viable tumor.
C) Survival considering progression after salvage RPLND.
Green: no progression after RPLND. Orange: disease progression after RPLND.

In patients with retroperitoneal progression after RPLND a higher rate of primary retroperitoneal neoplasms, cN3 stage tumors, viable residual tumor and adjunctive surgeries, higher mean age and lower rate of IGCCCG good prognostic group cases and disease-specific survival were observed. These findings were not significant on statistical analysis. The majority of retroperitoneal recurrence (n= 18 or 90%) was found inside the surgical field, while 2 (10%) cases were localized outside the surgical field but still within the bilateral template. Considering distant metastases, 12 (75%) of lung, 2 (66,6%) of mediastinal and all patients with liver, spinal, brain, pelvic or intestinal progression died during the follow up period.

4.2.4 Second look RPLND outcomes

The rate of primary retroperitoneal GCT, cN3 metastases, additional surgeries, poor IGCCG prognostic parameters and disease specific mortality were higher in the second look RPLND group compared to the salvage surgery group. Residual mass pathology was comparable between the two groups. These findings were not significant on analysis.

	Second look (n=13)	**Salvage RPLND (n = 114)**
Mean age (y)	39	34
Primary GCT origin *Right testis* *Left testis* *Retroperitoneum*	 6 (46,2%) 4 (30,7%) 3 (23,1%)	 57 (50%) 54 (47,4%) 3 (2,6%)
Primary histology *Nonseminoma* *Seminoma* *Fibrosis, necrosis*	 9 (69,2%) 2 (15,4%) 2 (15,4%)	 101 (88,6%) 11 (9,6%) 2 (1,8%)
N stage* *cN1* *cN2* *cN3*	 0 2 (15,4%) 11 (84,6%)	 13 (11,4%) 49 (43%) 52 (45,6%)
Prognostic group *Good* *Intermediate* *Poor*	 5 (38,5%) 5 (38,5%) 3 (23%)	 65 (57%) 39 (34,2%) 10 (8,8%)
Residual mass histology *Viable tumor* *Teratoma* *Fibrosis/necrosis*	 3 (23,1%) 6 (46,2%) 4 (30,7%)	 23 (20,2%) 61 (53,5%) 30 (26,3%)
Adjunctive surgery	4 (30,7%)	25 (21,9%)
Alive to date	8 (61,5%)	94 (82,5%)

Table 7: Comparing clinical and outcome data in second look and salvage RPLND Patients. *Postchemotherapy.

4.1 Assessment of pre-selected sera and/or tissue miRNA expression profiles in testicular cancer patients with postchemotherapy residual retroperitoneal lymph nodes and healthy controls

4.1.1 Pre-selected sera miRNA expression profiles can differentiate between TCa patients and healthy individuals

Based on literature data, nine candidate diagnostic miRNAs were selected according to their general oncogenic, tumor-suppressing, or dual role in tumorigenesis, including their specific dysregulation in GCT.[100] After assessing the serum expression levels of each circulating miRNA, the differential expressions were calculated and statistically analyzed. Five candidates - miR-19a, miR-21, miR-29a, miR-106b, and miR-155 – demonstrated significant levels in the testicular cancer patient group compared to the control group. Although miR-199a was significantly upregulated in the tumorous group; however, the difference was much less pronounced than that of the first five pre-selected miRNAs (Figure 2). The expression pattern was similar between patients and healthy controls in the case of miR-371a and miR-373 (Figure 2).

To develop a comprehensive serum expression pattern of the candidate miRNAs, our research group has used the medians of normalized values for each sample. When including all nine miRNAs in our calculations (referred to as the "median sum"), we observed a significant difference between the two cohort groups (Figure 3A), though the tumorous category exhibited a relatively high standard deviation. Focusing on those miRNAs, that demonstrated the highest significant difference between the two studied cohorts, two overlapping groups of miRNAs have been established: (I) "median 3m" included miR-21, miR-29a, and miR-106b and (II) "median 4m" implicated miR-19a, miR-21, miR-29a, and miR-106b. Both indicators exhibited significant differences between the control and testicular cancer patient cohorts (Figure 3A). miR-155 showed quite diverse distribution in the control samples, in many cases without any detectable serum levels. Consequently, it was deemed an unreliable factor and excluded from median analyses, despite showing a significant difference between the two examined cohorts (Figure 2).

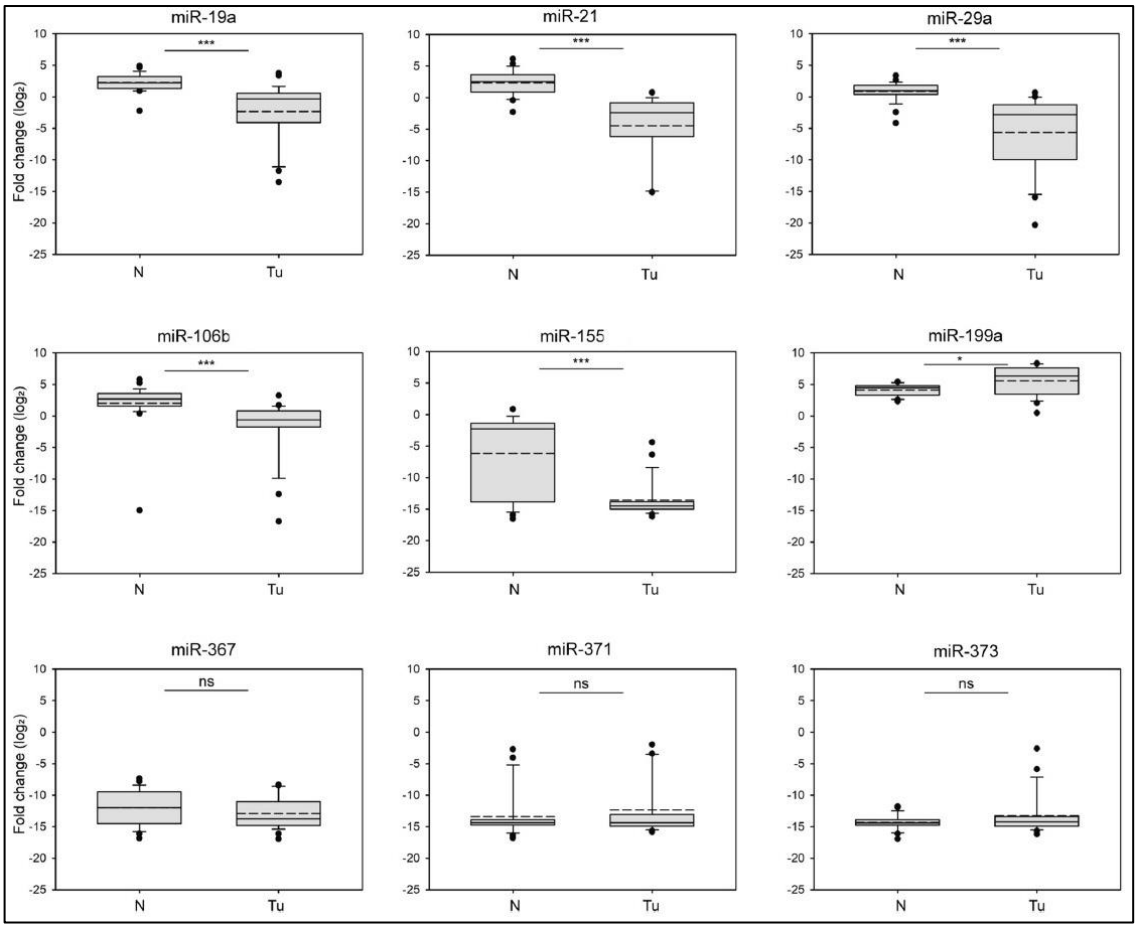

Figure 2: Pre-selected miRNAs display different serum levels in testicular germ cell cancer patients than in healthy individuals. The relative amounts of circulating miRNAs in testicular GCT patients were compared to those of healthy donors and statistically evaluated with ANOVA. The normalized levels of circulating miRNAs are displayed on box plots and indicated as log2 fold change. Abbreviations stand for the following: N = normal (healthy donors), Tu: tumor (TCa patients serum sample). Stars indicate significant differences: *P < 0.05, ***P < 0.001, and ns = non-significant. Solid lines represent means, and medium dashed lines indicate the medians of datasets.

To further assess the possible diagnostic ability of the candidate markers, we performed Principal Component Analysis (PCA) based on the normalized serum levels of the nine miRNAs. The two studied cohorts formed discreet, slightly overlapping clusters (PC1+PC2 components depict 69.3% of the total variance, Figure 3B). Hierarchical clustering of the miRNAs and samples showed a similar separation (Figure 3C, Y-axis). The candidate markers formed three distinct clusters: (I) miR-367, miR-371a, and miR-373, (II) miR-19a, miR-21, miR-29a, and miR-106b, and (III) miR-155 (Figure 3C, X-axis). The median values of the first two clusters were significantly different in the tumorous group than in the healthy individuals (Figure 3C). However,

miR-155 demonstrated a unique intensity pattern across the samples, reflecting the high variance its detected serum levels.

ROC curve analyses were conducted to evaluate the diagnostic potential of our pre-selected marker miRNA panel. The medians of all nine candidate markers (median sum) were able to discriminate between the normal and testicular cancer category with 93% possibility (Area Under the ROC Curve (AUC) 0.93, Figure 3D). The specificity of this test was 78% with a threshold set at 0.0275 (Figure 3D). The discriminative abilities were even higher (96%) when considering the summarised expression patterns of the significantly altered miRNAs (median 3m and median 4m vs. median sum, Figure 3E). However, the specificity of these indicators as diagnostic markers varied: "median 3m" exhibited a specificity of 78%, while "median 4m" had a lower specificity of 52% (Figure 3E). These findings suggest that using the serum expression patterns of miRNAs—particularly that of miR-21, miR-29a, and miR-106b—may have a role in monitoring testicular GCT patients after chemotherapy.

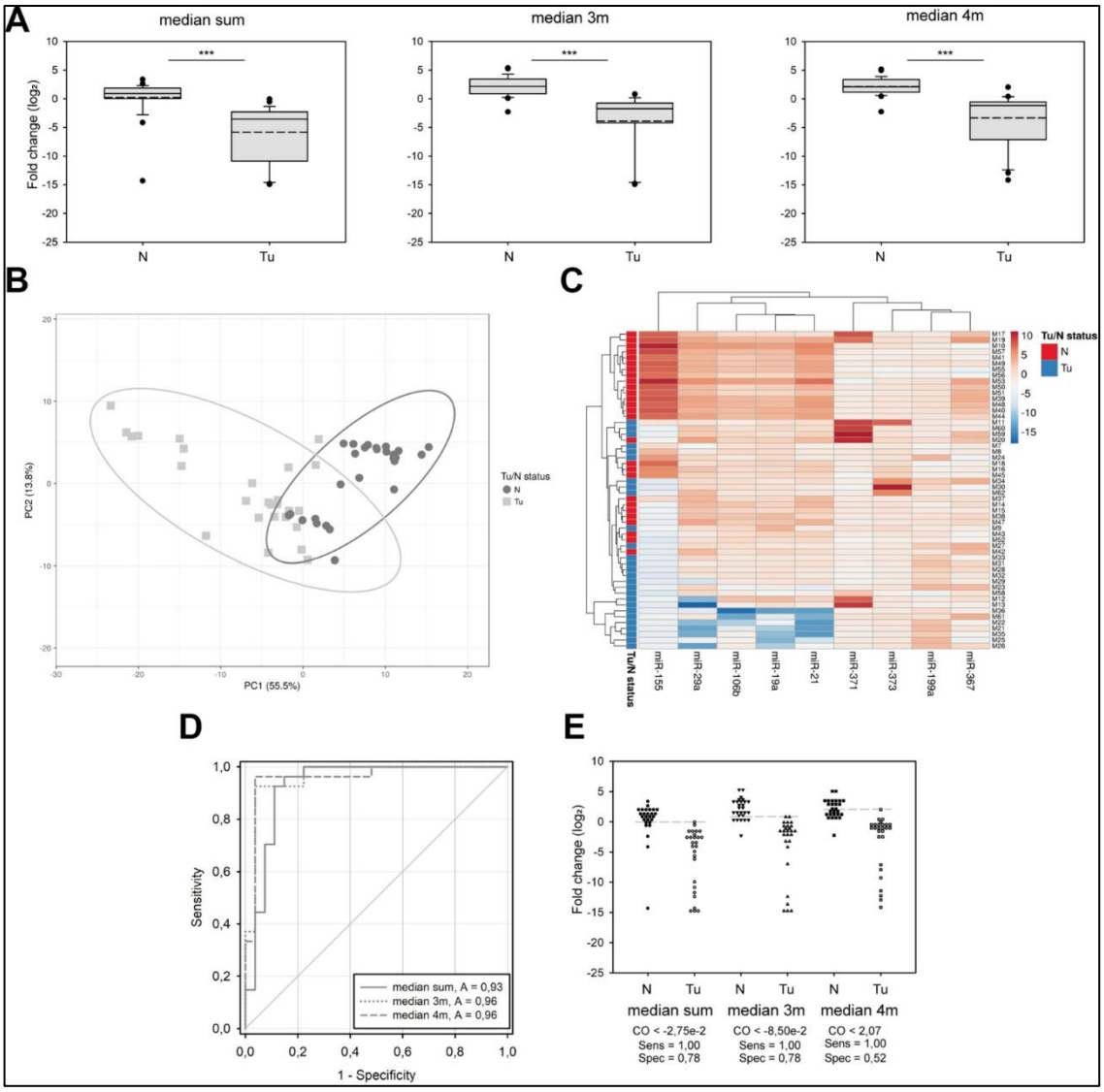

Figure 3: Serum expression patterns of selected oncomiR combinations have a diagnostic ability in TCa postchemotherapy monitoring. Medians of normalized expressions of all or selected miRNAs were calculated and compared in the two-sample groups and indicated as log2 fold change. (A) Left: Box plots of medians of all nine miRNAs (median sum). Middle: Box plots of medians of miR-21, miR-29a, and miR-106b (median 3 m). Right: Box plots of medians of miR-19a, miR-21, miR-29a, and miR-106b (median 4 m). Solid lines represent means, and the medium dashed lines indicate the medians of datasets. (B) The principle component analysis (PCA) plot of the 9 miRNA datasets generated with the ClustVis online tool. (C) Hierarchical clustering of the nine oncomiRs and the enrolled individuals' samples represented by a heat map and generated with the ClustVis online tool. (D) A receiver operating characteristic curve (ROC) analysis of the median all miRNAs, median 3 m, and median 4 m indicators, area under the curves (AUCs) are highlighted. E) Scatter plots of the 9 miRNAs, 3 m, and 4 m in the control and TCa samples. The grey dashed line shows the cut-off value of the tests. Abbreviations stand for the following: N = normal (healthy donors), Tu: tumor (TCa patients serum samples). Stars indicate significant differences: ***P < 0.001, and ns = non-significant, calculated using ANOVA. Solid lines represent means, and medium dashed lines indicate the medians of datasets.

4.1.2 3.3 miR-21, miR-155, and miR-371 oncomiRs express differentially in teratoma-infiltrated metastatic lymph nodes

The tissue levels of the pre-selected miRNAs have been measured in the resected pathologic lymph nodes. To explore whether the expression of any of the marker miRNAs correlates with the statuses of the LNs, testicular GCT patients were classified into three groups considering the histological result of the removed metastases. 15.2% of the cohort, were scored in the "reactive lymph node" (RNL) group. In 33.3% of the patients, only necrotic or scar tissue was detected in the removed lymph node due to chemotherapy, standing for the "no living tumor" (NLT) group. The largest sub-group, representing 51.5% of the cohort, included the teratoma-containing LNs ("teratoma group", TCa). None of the nine evaluated miRNAs demonstrated significantly altered expression in the NLT group compared to the other two histologic categories (Figure 4). However, there were slightly significant differences observed between the RLN and teratoma groups for miR-21, miR-155, and miR-373 (Figure 4).

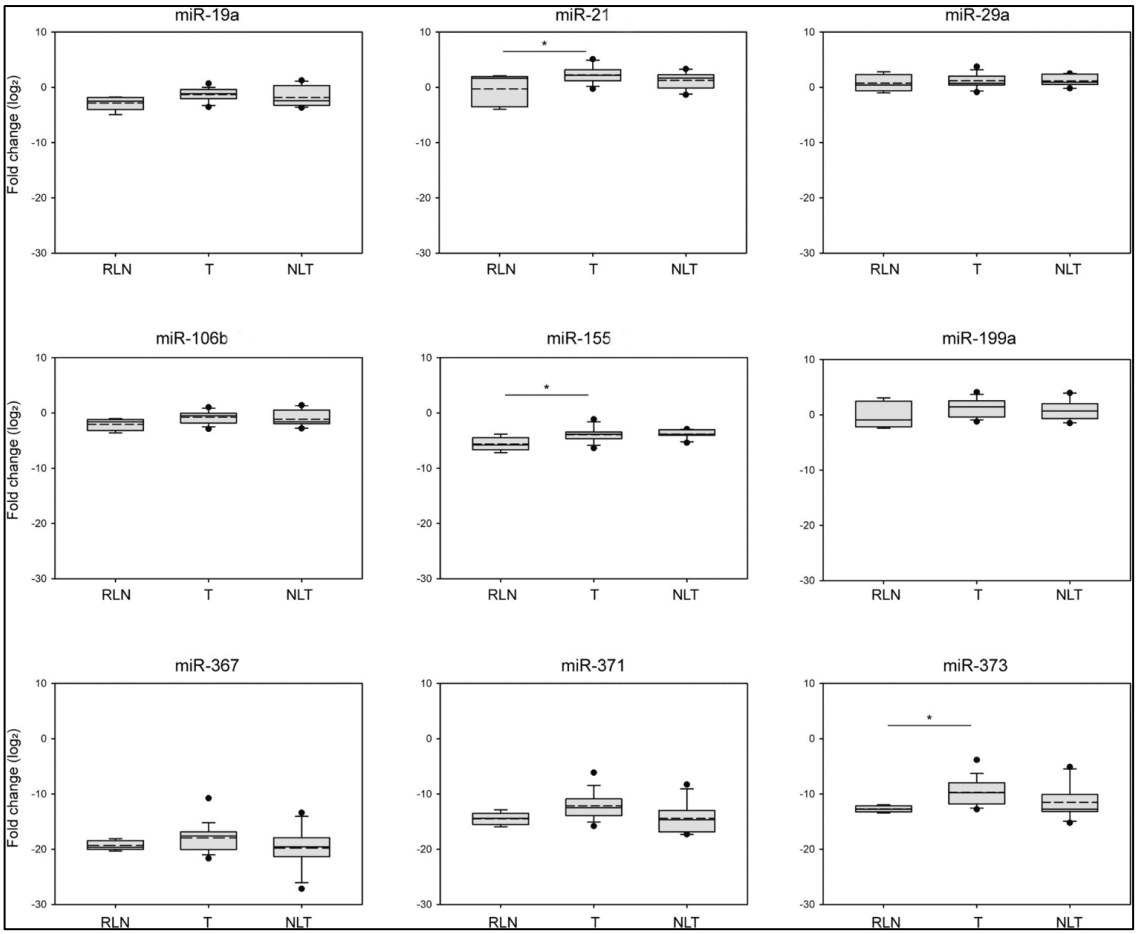

Figure 4: miR-21, miR-155, and miR-373 oncomiRs are expressed differentially in teratoma-infiltrated metastatic lymph nodes. The relative tissue expression levels of miRNAs in LNs derived from TCa patients were compared to those of healthy donors and statistically evaluated with ANOVA. The normalized levels of miRNAs are displayed on box plots and indicated as log2 fold change. Abbreviations stand for the following: RLN = reactive LNs, T = teratoma

(metastatic LNs with living teratoma cells), NLT = no living tumor (LNs with scar tissue or necrosis). Stars indicate significant differences, *P < 0.05, calculated using ANOVA. Solid lines represent means, and the medium dashed lines indicate the medians of datasets.

Using variance-based statistical analyses, the expression pattern of the nine pre-selected markers was not specific for any of the three tissue categories, as none of the sample groups clustered together in PCA analysis nor hierarchical clustering displayed by a heat map [101]. However, when a similar assessment was performed on the scored sample sets, three groups of miRNAs were separately clustered (Figure 5A, Y-axis): (I) miR-367, miR-371a, and miR-373, (II) miR-21, miR-29a, and miR-155, and (III) miR-199, miR-19a and miR-106b.

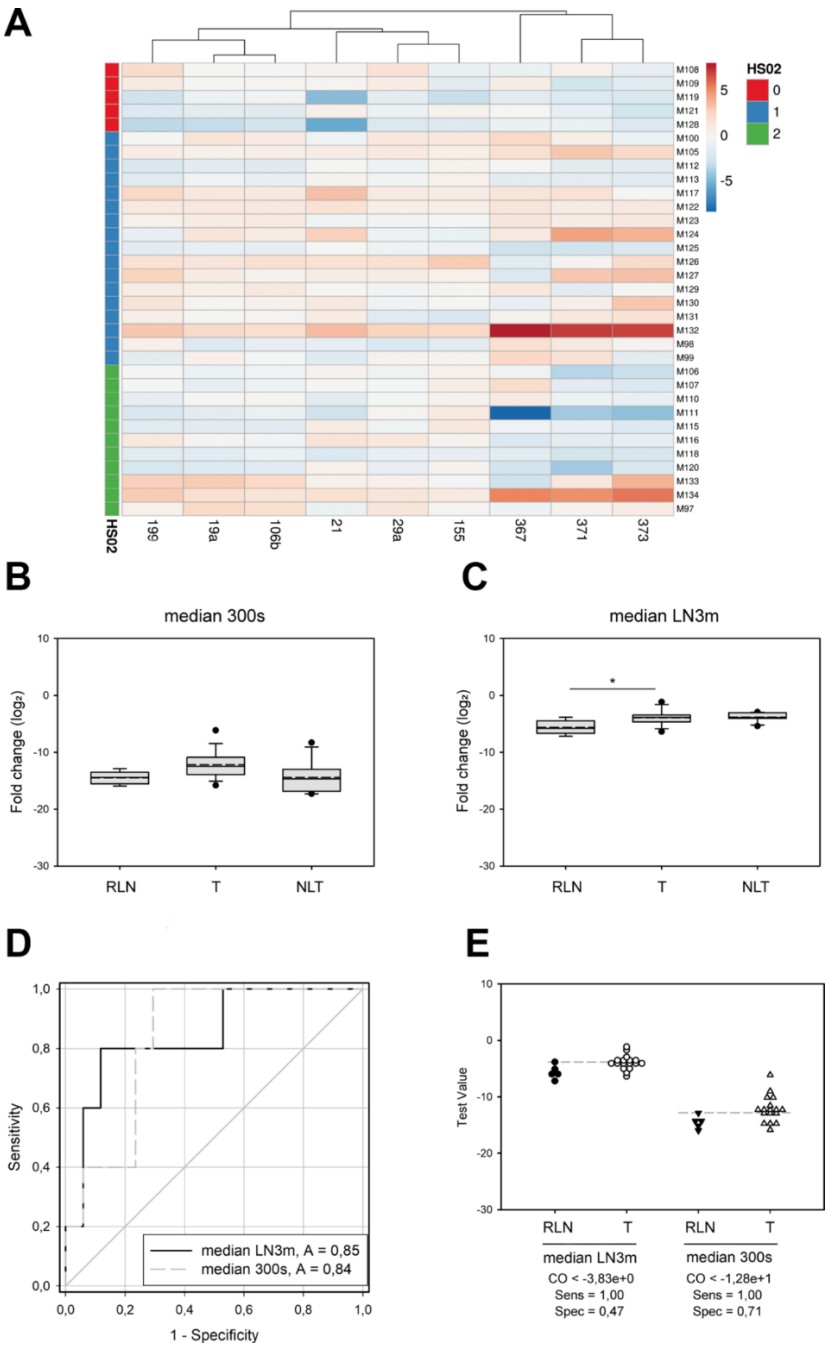

Figure 5: The tissue expression patterns of unique combinations of specific miRNAs differ in inflamed LNs from teratomainfiltrated metastatic TCa lesions. (**A**) Heat map of the nine oncomiRs in RLNs, teratoma-infiltrated metastatic LNs, and necrotic LNs/scar tissue generated with the ClustVis online tool. Histology scores (HS) are indicated on the Y-axis as 0: RLNs, 1: teratoma, 2: scar tissue or necrotic LNs. (**B**) Box plots of medians of miR-367, miR-371, and miR-373 (median 300 s). (**C**) Box plots of medians of miR-21, miR-29a, and miR-155 (median LN3m). Solid lines represent means, and the medium dashed lines display the medians of datasets. The normalized tissue expression levels of miRNAs are indicated as log2 fold change. (**D**) ROC curve analysis of the median LN3m and median 300 s indicators; AUCs are highlighted. (**E**) Scatter plots of the median LN3m and median 300 s indicators in the control and TCa samples. The grey dashed line shows the cut-off value of the tests. Abbreviations stand for the following: HS = histology score, RLN = reactive LNs, T: teratoma (metastatic LNs with living teratoma cells), NLT: no living tumor (LNs with scar tissue or necrosis), A = AUC value, CO = cut-off value, Sens = sensitivity, and Spec = specificity. Stars indicate significant differences, *P < 0.05, calculated using ANOVA and post-hoc tests.

The clusters miR-367–371a–373 and miR-21–29a–155 were analysed separately, and referred to as "median 300s" and "median LN3m", respectively. The differences in between the summarized expression patterns of the three-sample indicators were greater than would have been expected by chance (ANOVA, P = 0.021), nevertheless, the pairwise comparisons did not reveal any unique characteristics specific to sub-groups (post-hoc test by Dunn's Method, P > 0.05) (Figure 5B–C). A similar test of the median LN3m cluster affirmed a significant diversity of teratomatous samples compared to the reactive lymph nodes (post-hoc test by Dunnett's Method, P < 0.05) but not to the NLT group. These results suggest that the tissue expression patterns of specific combinations of miRNAs may potentially distinguish reactive lymph nodes from teratoma-infiltrated residual lesions.

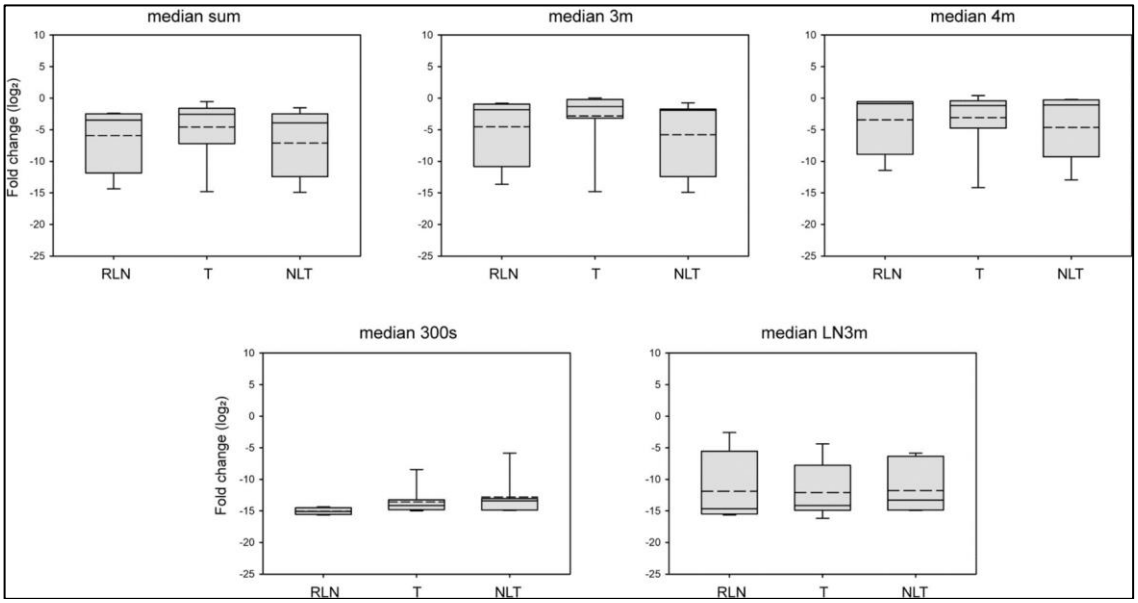

Figure 6: The serum levels of circulating miRNA indicators in distinct chemotherapy responders. The serum levels of the five previously defined indicators are represented in box plots. Solid lines represent means, and the medium dashed lines display the medians of datasets. Abbreviations stand for the following: RLN = reactive LNs, T: teratoma (metastatic LNs with living teratoma cells), and NLT: no living tumor (LNs with scar tissue or necrosis).

ROC analyses were performed to explore the potential diagnostic ability of median 300s and LN3m indicators, including only the RLN and teratoma-infiltrated sub-groups of samples. The AUC values for the two indices were 0.84 and 0.85, respectively; albeit with relatively weak specificity, 71% and 47%, respectively (Figure 5D). Our data demonstrated distinct differences in the tissue expression levels of sets of oncomiRs between inflamed lymph nodes and teratoma-infiltrated metastases. Furthermore, assessing the potential diagnostic value of these indices is limited in this

cohort due to the lack of retroperitoneal lymph nodes from healthy individuals. Additionally, the sample sizes for the scored sub-groups were uneven and relatively small.

4.1.3 3.4 The serum levels of circulating miRNAs show no differences between the distinct chemotherapy responders

The patient sera samples were classified according to the donors' histological result into RLN, TCa, and NLT sub-groups. Our research team has then re-analyzed the serum levels of the nine pre-selected circulating miRNAs. Patients without LN tissue samples were excluded from these analyses. None of the miRNAs showed significant differences between the three-sample categories.[101] The serum expression patterns of all the indicators determined by the preceeding examinations on sera and tissue samples, - median sum, 3m, 4m, 300s, and LN3m - were also analyzed. For the median 300s, all indicators were unevenly distributed and varied across the samples (Figure 6). The overall serum expression patterns of miR-267, miR-371, and miR-373 exhibited a more narrow dispersion and showed an increasing trend in the teratomatous subgroup compared to the RLN category; however, this difference was not statistically significant. These findings suggest that using the serum level patterns of circulating miRNAs, whether individually or as combined indicators, has strong limitations in evaluating the therapeutic responses of metastatic testicular GCT patients. To enhance and improve the diagnostic utility of this approach, additional candidate miRNAs should be included in future research endeavors.

5. DISCUSSION

5.1 Pilot study on testicular cancer stage migration and patient delay at a single site

IARC's 2022 report shows 554 new testicular cancer cases and 46 TGCT-related deaths in Hungary. The age-standardized rate of testicular cancer mortality was the 5th highest in Europe, below Slovakia, Bosnia-Herzegovina, Poland and Bulgaria.[102] Several factors may be associated with the alarming rate of mortality. Some of these factors were investigated by our colleges at the Hungarian National Institute of Oncology. In the prospective study of Küronya Z et al., TGCT patients completed

questionnaires on subjective social status, objective socioeconomic position and on patient's delay. DSS was significantly lower in the highest social quartile (1.56%) than in the lowest social quartile (13.09%). Küronya et al. also found that a longer patient delay was associated with higher stage in nonseminoma tumors, and 57,2% of deceased patients waited longer then a year before consulting a physician with their symptoms. The most important factors associated with patient delay were the patient's and their mother's education level. They concluded that raising testicular cancer awareness in the lower SES population could improve survival. [103]

Inspired by the findings of Küronya et al. we conducted a pilot study on testicular cancer stage migration and patient delay.[90] A retrospective chart review of patients who underwent orchiectomy for testicular cancer at a single site between 2007 and 2018 was performed. Overall, comparing the period 2007-2009 and 2016-2018, neither the diameter, nor the TNM stage of testicular tumours showed a significant change at diagnosis. The proportion of advanced disease at first presentation increased, but this finding was not significant on statistical analysis. Unlike Huddard et al. in the UK, our assessment did not detect a stage migration of testicular cancer in the study cohort. A weak but significant positive association was observed between tumor diameter and stage at detection. This finding is not surprising, as primary tumor size is a known prognostic factor in localized seminomatous GCT. [91] In nonseminomatous GCT lymphovascular invasion was found to be only factor with considerable prognostic value.[104] Patient delay was alarming between 2016 to 2018, as 25% of patients waited more than 6 months to seek help with their symptoms.

A significant limitation of this pilot study is that it was confined to a single center, which may restrict the generalizability of our findings to the broader population. To learn more, our research group initiated a nationwide multicenter study on the stage migration of testicular cancer and patient delay. At one of the designated sites, approval from the Internal Ethics Comittee has already been obtained.

Testicular cancer has a cure rate of 96% when diagnosed in the early stages.[9] Raising awareness in the most affected age group (young men aged 15-35) and their family practitioners could save lives and prevent lifelong complications. In 2019 our research group proposed a prospective, questionnaire based study and educational campaign about testicular self examination in secondary schools. Even though many schools would have been happy to host the campaign, our research proposal was declined by the Hungarian Medical Research Council. The Council was opposed to the

idea that the questionnaire would have been made available for teenagers online without parental consent. After that our plans to visit secondary schools were cut short by the COVID-19 pandemic.

5.2 Assessment of postchemotherapy RPLND outcomes and complications at a high volume referral center in Hungary

Apart from timely detection of localized testicular cancer, up-to-date management of advanced, metastatic disease is also known to improve outcomes. In 2023-2024 our research team has conducted a retrospective cross-sectional study on the complex treatment of residual metastatic germ cell cancer at a single hungarian referral center. The medical charts of 127 postchemotherapy RPLND (salvage or second look) patients were reviewed. 100 patients received standard chemotherapy regimen (3-4 series of BEP), while 11 and 16 men underwent 1-2 or 5-6 cycles of BEP, respectively. Patients in the „Less than standard" systemic treatment group had their BEP regimen interrupted by adverse events. In 72,7% of them harboured viable tumor or teratoma. Salvage surgery was the only potentially curative option for these patients.

In the "More than standard" group, 16 patients (12.6%) underwent 5–6 cycles of BEP treatment. A significant portion (62.5%) fell into the poor or intermediate prognostic category according to the IGCCCG classification. A higher rate of viable tumor on LN pathology (37,5%), progression after RPLND (43,8%) and lower survival (56,3%) was observed compared to the „Standard" treatment group. Additionally, the rate of adjuvant surgery, particularly nephrectomy, was elevated in the "More than standard" group. Since the residual mass stage, pathology, and the rate of surgical complications remained unchanged, and the long-term toxicity of platinum-based chemotherapy is well-documented, [86]these patients would likely have benefited from undergoing surgery earlier, rather than more doses of chemotherapy.

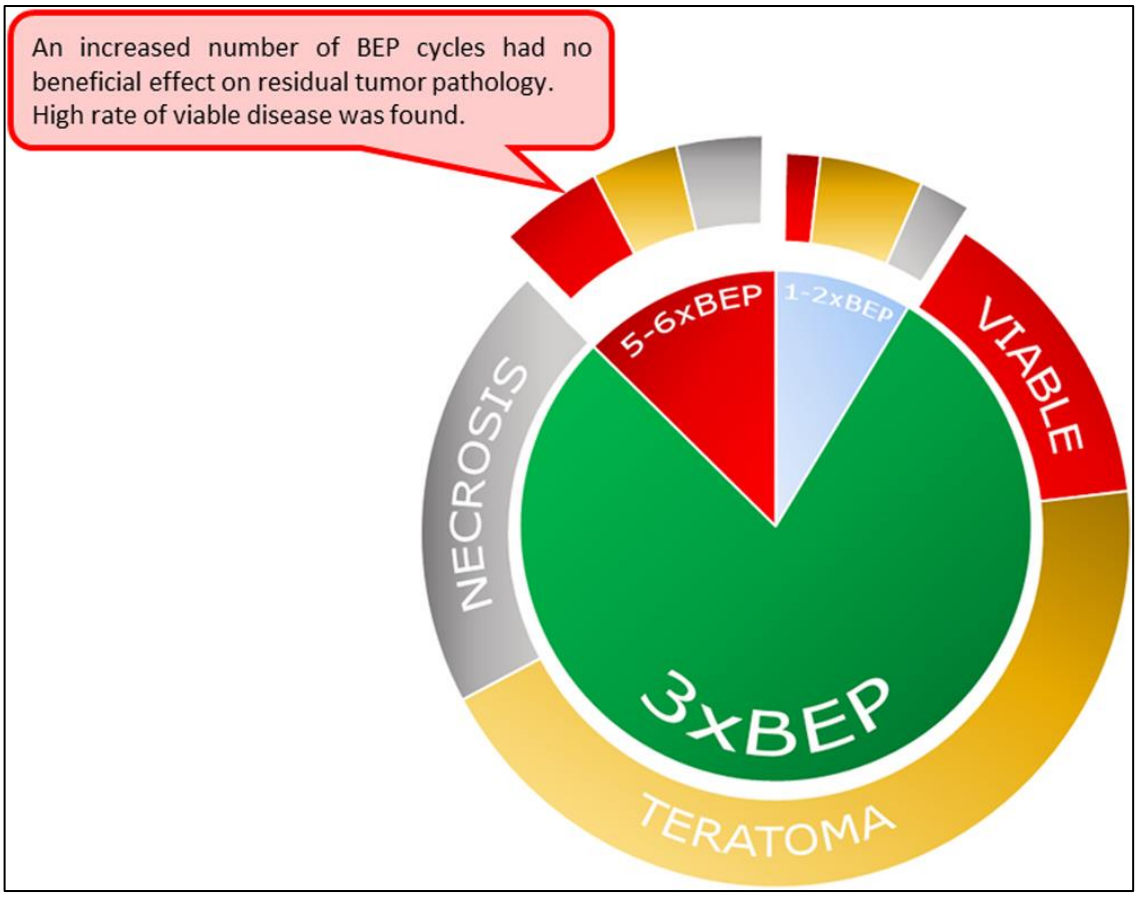

Figure 7: Residual lymph node histopathology comparing systemic treatment groups.

Inner circle: systemic treatment groups. Green: standard BEP chemotherapy regimen (3–4x BEP). Blue: less BEP cycles than standard (1–2x BEP). Red: more BEP cycles than standard (5–6x BEP).

Outer ring: residual LN histology. Red: viable tumor. Yellow: teratoma. Gray: fibrosis, necrosis.

A correlation was observed by Heidenreich et al. between the number of operations performed by an individual surgeon and the frequency of complications. For optimal results it is imperative to refer TGCT patients with resectable residual tumors to a high volume surgical center.[105] In the past annually an average 9 RPLNDs have been performed at our referral center by 2 surgeons.

In our cohort 29 patients (22.8%) required adjunctive surgeries, such as nephrectomy or reconstruction of major vessels and the ureter. In 6 nonseminoma patients with bulky (cN2-3) tumors nephrectomy was performed to ensure complete tumor removal. Ureteral injury was noted postoperatively in 5 nonseminoma patients, and in these cases, a double-J stent was placed to prevent urine leakage. The stent was removed after 4 to 6 weeks. Ureteral resection and reconstruction were carried out in two cases involving tumor-invaded ureters. Ureteral injury was reported to be the most

common surgical complication of PC-RPLND by the Swedish Norwegian Testicular Cancer Group.[106] Previous reports indicate that approximately 7% of cN2-3 patients will need vena caval resection during RPLND ([107], [108]. Heidenreich et al. reported a higher incidence of postoperative complications in patients undergoing complex RPLND (41.7% versus 7.2%, P = 0.02), with most complications classified as Clavien-Dindo grade I–IIa. In their research, 79.1% of patients who required complex surgeries had bulky disease, while only 26.58% of those who underwent standard surgery presented with extensive residual masses.[109] In our cohort, 93.7% of patients experienced no or minor surgical complications (Clavien-Dindo I–II).

Retroperitoneal progression following salvage resection occurred in 20 cases (15.7%). On one hand, this progression can be attributed to the biological characteristics of the tumors; 60% of these cases were classified as cN3 stage, 20% were of retroperitoneal origin, and 75% were in the intermediate or poor prognostic groups according to the IGCCCG classification. Teratoma and viable tumor were identified in 55% and 35% of cases, respectively. On the other hand, surgical errors may also contribute to progression within the operative field or RPLND template. For example, a modified template RPLND was performed in 5 of the 12 cN3 cases instead of a bilateral approach. [110]Surgical records indicated that unresectable disease was present in 3 cases, making intervention on the contralateral, clinically negative side unnecessary. In 2 other instances, the procedure was halted due to significant blood loss. At the conclusion of the study, 9 (45%) of the patients with retroperitoneal progression were still alive. [94]

5.3 Assessment of pre-selected sera and/or tissue miRNA expression profiles in postchemotherapy residual retroperitoneal lymph nodes and healthy controls

Currently utilized tests have only modest fidelity in the management of GCT.[42] Establishing more dependable tumor markers could be an essential asset in the management of germ cell malignancies, aiding in everything from diagnosis and early staging to monitoring treatment effectiveness and evaluating post-chemotherapy residual lesions. The introduction of new markers could influence follow-up procedures and reduce the burden CT imaging. Using highly accurate markers could help avoid

unnecessary surgeries in patients harbouring no viable tumor or teratoma after chemotherapy.[49] [111] A hungarian study has reported neutrophil-to-lymphocyte ratio to be an independent marker for overall survival in primary mediastinal germ cell cancer.[112] Compared to classical markers of TGCT, microRNAs offer several advantages as biomarkers, including their detectability in the bloodstream, exceptional stability, and short half-life.[49], [113], [114]

Our prospective assessment has established six individual miRNAs (miR-19a, miR21, miR-29a, miR-106b, miR-155, and miR-199a) with significant expression in post-chemotherapy TCa patients. The miR-21+miR-29a+miR-106b cluster demonstrated the highest sensitivity (96%) and specificity (78%) for identifying testicular cancer (TCa). This result highlights the cluster's potential as an important resource for early detection and post-chemotherapy monitoring. [101]

While most oncomiRs, such as miR-21, are typically overexpressed in human cancers[115], [116], it was intriguing to find that many of them were reduced in the serum of testicular cancer patients. This seemingly contradictory result may be attributed to the characteristics of the cohort, as the testicular cancer patients included in the study has undergone orchiectomy and chemotherapy. Consequently, they did not bear the primary tumor, and the metastatic lymph nodes responded -at least partially - to systemic treatment. [101]

Additionally, our study examined the tissue levels of the selected miRNAs in post-chemotherapy residual tumors, uncovering distinct expression patterns among the reactive lymph node, no living tumor tumor, and teratoma groups. MiR-21, miR-155, and miR-373 were found to be slightly elevated in teratoma compared to reactive lymph nodes. Two clusters of microRNAs (miR-367+miR-371a+miR-373 and miR-21+miR-29a+miR-155) effectively distinguished between the RLN and T groups, albeit with relatively low specificity. The minimal or absent expression of miRNA in teratoma has been reported by several recent publications. Our findings are subject to further validation in future research endeavors. Future analyses should aim to adress the limitations of sample size and subgroup inequality, including viable GCT sera and tissue samples. [101]

5.4 Patient education

According to literature, 70% of patients would like their doctor to recommend a trusworthy internet source for their condition, but only 4% of patients receive such advice. Unfortunately, most websites have been designed primarily as promotional and marketing platforms for specific practices or industries. The medical information provided by these platforms is more often than not outdated, innaccurate or outright misleading. In recommending reliable medical websites, healthcare professionals must consider many parameters, e.g. the ethical standards of the platform and the quality of the information provided. [117] It is also important to recognize that patients may have a difficulty understanding health care information as a result of the medical terminology and jargon that is often used. The entire healthcare system is based on the premise that patients can comprehend complex written and spoken information. Limited health literacy effects health status, health outcomes, health care use, and health costs. [118]

Our research team has created an educational website and a multiplatform application to provide reliable information in plain hungarian language about urological cancers and other urological diseases for patients. Several expert-edited articles, short films, patient interviews and educational posters are available on the website, ***www.urodoki.hu***. Alongside content on urological conditions, the topics include physiotherapy, healthy lifestyle, mental health, patients' letters and a FAQ section.

The ***URODOKI application*** aims to empower patients to be a more active and well informed participant of their health care journey. The application presents expert-reviewed content on common urological diseases, necessary diagnostic test and modern therapeutic options in an accessible way. It also has a calendar funcion, which helps patients keep track of scheduled diagnostic and follow up appointments, and other impontart events.

In March 2024, Urodoki staff has conducted a one month campaign to raise awareness about testicular cancer. Several new articles and short films with information on risk factors, symptoms and treatment methods of the disease have been published. Social media platforms and paid advertisements were used to bring the educational content to as many people as possible. During the campaign period our website had 19.130 individual visitors. Our Facebook ads, Youtube and TikTok videos had 251.500, 107.329 and ~500.000 views, respectively.

Figure 8: Testicular Cancer Awareness Campaign powered by urodoki.hu (March, 2024)

5.5 Clinical overview

Testicular cancer leads to the unnecessary death of too many young patients in Hungary. The nations mortality rates are one of the highest in the region. Our research team has recognized several potential points of intervention.

Longer patient delay is associated with higher stage of tumors, ultimately leading to increased mortality. We have done extensive work in **patient education** to improve health literacy in the hungarian population and reduce patient delay by providing information about significant symptoms of cancer.

Novel, highly sensitive tumor markers have also been found with the ability to differenciate between testicular cancer patients and healthy controls. The exact role of microRNA in the detection and monitoring of tecticular cancer is yet to be established by urooncology guidelines. The concept of identifying cancer from liquid biopsy samples is very appealing. Financing issues and the absent expression of tumor markers in teratoma may limit the utility of miRNAs at this time.

Metastatic testicular cancer patients should receive oncologic and surgical treatment at **centers of excellence**. The experience of the medical oncologist and the operating surgeon influences treatment outcomes substantially. Adherence to clinical practice guidelines, e.g. the Heidenreich criteria is essential.

Managing testicular germ cell cancer is a rewarding endeavor, as the patients are young, the treatments are effective and a lot of healthy life years can be won by practicing risk-adapted standard of care.

6. CONCLUSIONS

6.1 Pilot study on testicular cancer stage migration and patient delay at a single site

a) The size and pathological stage of testicular tumors detected during 2007-2009 and 2016-2018, remained essentially unchanged. No stage migration was observed.
b) Patient delay was alarming; between 2016 to 2018 25% of symptomatic patients waited for more than 6 months before seeking professional help.
c) These results highlight the necessity of patient education, especially among young men.

6.2 Assessment of postchemotherapy RPLND outcomes and complications at a high volume referral center in Hungary

a) Survival was significantly lower in cases of primary retroperitoneal GCT *(p=0,04),* viable disease in residual mass *(p=0,00043)* and progression after RPLND *(p=0,0001).*

b) An increased number of BEP cycles in metastatic GCT had no beneficial effect on residual lymph node pathology, surgical outcome or survival. In fact, the rate of nephrectomy was significantly higher in the "more than standard" treatment group (*p= 0,0166, Pearson's Chi-squared test*). The "more than standard" subgroup had the highest rate of viable tumor (37,5%), post-RPLND disease progression (43,8%), adjunctive surgeries (37,5%) and the lowest survival rate (56,3%), although these findings were not significant on statistical analysis.

c) For intermediate and poor prognosis metastatic TGCT, there is international consensus on treatment with 4 cycles of cisplatin-based combination chemotherapy. This patient group should always be referred to a center experienced in managing advanced germ cell tumors for oncologic as well as surgical management in order to achueve optimal outcomes. Patients with good prognostic group metastatic disease should receive 3 cycles of BEP.

d) Ideally, Heidenreich criteria should be used to determine surgical template, although this is not always feasible in frailer patients and very bulky metastases.

6.3 Assessment of pre-selected sera and/or tissue miRNA expression profiles in postchemotherapy residual retroperitoneal lymph nodes and healthy controls

a) We found six individual miRNAs (miR-19a, miR21, miR-29a, miR-106b, miR-155, and miR-199a) with significant expression in post-chemotherapy TGCT patients.

b) The miR-21+miR-29a+miR-106b cluster had the highest sensitivity (96%) and specificity (78%) for TGCT.

c) Tissue levels of miR-21, miR-155, and miR-373 were slightly elevated in teratoma compared to reactive lymph nodes. The clusters (miR-367+miR-

371a+miR-373 and miR-21+miR-29a+miR-155) differentiated between reactive LN and teratoma groups with low specificity.

d) Circulating miRNA expression in sera samples did not exhibit significant differences among the patients of the three histological groups.

7. ABBREVIATIONS

AFP	alpha fetoprotein
ASCO GU	Amesican Society of Clinical Oncology, Genitourinary Cancers
AUC	Area Under Curve
BEP	bleomycin, etoposid, cisplatin
CECT	contrast-enhanced computed tomography
CHEK2	checkpoint kinase 2
DNA	deoxyribonucleic acid
DSS	disease-specific survival
EAU	European Association of Urology
FAQ	frequently asked questions
FAT1	FAT atypical cadherin 1
GCNIS	germ cell neoplasia in situ
GCT	germ cell tumor
hCG	human chorionic gonadotropin
IARC	International Agency for Research on Cancer
IGCCCG	International Germ Cell Cancer Collaborative Group
IVC	inferior vena cava
LDH	lactate dehydrogenase
MDM2	Mouse Double Minute 2
MHz	Megahertz
miRNA	microRNA
MRI	Magnetic Resonance Imaging
MYCN	N-myc proto-oncogene
NACB	National Academy of Clinical Biochemistry
OCT 3/4	Octamer binding transcription factor 3/4
PCA	principle component analysis
PCR	polymerase chain reactio

PLAP	Placental-like alkaline phosphatase
qPCR	quantitative polymerase chain reaction
RAC1	RAS-related C3 botulinum toxin substrate 1
RAS	rat sarcoma
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROC	Receiver operating characteristic
RPLND	retroperitoneal lymph node dissection
SES	socioeconomic status
TGCT	testicular germ cell tumor
TP53	Tumor Protein P53
TNM	Tumor, Node, Metastasis classification
UICC	Union for International Cancer Control
ULN	upper limit normal
WNT	Wingless and Int-1

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