University of Szeged
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Doctoral School of Interdisciplinary Medicine

Early detection and complex treatment of metastatic testicular germ cell cancer

Summary of the Ph.D. 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**

PUBLICATIONS NOT RELATED TO THE SUBJECT OF THIS THESIS

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**

II. **Fazekas FE**, Biró K, Ágoston P et al. Az osztott dózisú trimodális kezelés első hazai alkalmazása nagy kockázatú húgyhólyagdaganat esetében. *Orv Hetil.* 2021; 162(50):2017–2022. **IF: 0.707, Q4**

III. **Fazekas FE**, Maráz A, Lakosi F, Buzogány I, Beöthe T. A prosztatarák elsővonalbeli kezelése stádium- és rizikóbeosztás szerint. Összefoglaló közlemény. *Magy Urol.* 2021; 33(4):163–170.

IV. **Fazekas FE**, Márványkövi F, Lajos M et al. Pyelonephritis apostematosa miatt operált, transzfúziót elutasító betegünk esete. *Magy Urol.* 2023; 35(1):39-41.

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VI. Szőnyi M, **Fazekas FE**, Beöthe T, Biró K. Prehabilitáció és rehabilitáció hólyagdaganat miatt operált betegeknél. *Magy Urol.* 2024; 36(2):79–84.

PRESENTATIONS/POSTERS RELATED TO THE SUBJECT OF THIS THESIS

I. **Fazekas FE**, Bíró K, Buzogány I, Páhi Z, Ujfaludi Z, Oláh NO, Pankotai T, Beöthe T. Salvage RLA heredaganatos betegeknél. MKOT XXVIII., Budapest, 12-14 Nov. 2023.

II. **Fazekas FE**, Bíró K, Buzogány I, Beöthe T. Salvage RLA műtéteink az elmúlt 15 évben. MKOT XII., Budapest, 10-12 Nov. 2022.

III. **Fazekas FE**, Bíró K, Buzogány I, Beöthe T. Mi változott az elmúlt 12 évben? Heredaganat-méretek és stádiumok a felismeréskor. MUT XXV., Budapest, 8-10 Oct. 2020.

1. INTRODUCTION

1.1 Epidemiology of testicular germ cell tumors

Testicular cancer represents approximately 1% of adult tumors and 5% of urological malignancies, with germ cell tumors (GCTs) accounting for 90-95% of these cases. This cancer type is the most prevalent solid tumor among young adult men aged 15 to 40 years. Over recent decades, there has been a modest increase in incidence rates, particularly in industrialized countries, although the reasons remain unclear. Incidence rates vary significantly worldwide, with Western countries reporting 3 to 12 new cases per 100,000 men each year, in contrast to African countries, where rates are as low as 0.3 to 0.6 cases per 100,000 men.

The risk factors associated with testicular cancer include components of testicular dysgenesis syndrome (e.g., cryptorchidism and hypospadias), disorders of sex development, family history, and previous testicular cancer or germ cell neoplasia in situ (GCNIS) that increase the risk of contralateral testicular malignancy. Peak incidence for nonseminomas occurs in men aged 25-29 years, while seminomas peak between 35-39 years. Due to low incidence rates and high cure rates, routine screening is not recommended, but monthly self-examinations are beneficial for early detection.

1.2 Pathology of testicular germ cell cancer

Seminoma constitutes 40-50% of testicular GCTs in men aged 25-55. Immunophenotypically, seminomas express PLAP, c-KIT and OCT3/4. In pure seminomatous tumors AFP levels remain normal. Seminomas are highly sensitive to chemotherapy and irradiation. Spermatocytic seminoma, comprising 1-2% of GCTs, usually requires no systemic treatment post-orchiectomy. Nonseminomatous GCTs, including embryonal carcinoma, yolk sac tumor, teratoma and choriocarcinoma, typically present with elevated tumor markers and are often mixed histologies. Sex-cord/gonadal stromal tumors account for 3-6% of testicular tumors and are generally benign. Germ cell neoplasia in situ (GCNIS) is known to be the precancerous form of invasive testicular cancer, both seminoma and nonseminoma.

1.3 Genetics of testicular germ cell cancer

Several genetic abnormalities are associated with the development of TGCTs, with a notable gain in chromosome 12p being consistent across both seminomas and non-seminomatous tumors. c-KIT mutations are frequently present in seminomatous GCTs and bilateral tumors. The gain of 12p or c-KIT mutations are thought to be necessary for GCNIS cells to progress into invasive cancer. Germline loss-of-function CHEK2 variants increase cancer risk. Gain of chromosomes X, 7, 8, and 21 and loss of chromosomes Y, 1p, 11, 13, and 18 are also characteristic of post-pubertal testicular cancer.

1.4 Diagnosis of testicular germ cell cancer

According to the EAU Guidelines, physical assessment, testicular ultrasound examination and serum marker test are sufficient to confirm the clinical diagnosis of testicular cancer. Classical tumor markers (AFP, ßHCG, LDH) provide staging and prognostic information, but they have limitations due to their low sensitivity; as much as 40% of testicular tumor cases are marker negative. Contrast enhanced computerised tomography or MRI of the chest, abdomen and pelvis needs to be obtained to determine anatomical extent of the disease. Several studies indicate that microRNAs, 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. 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, and are involved in various cellular processes such as proliferation, differentiation, apoptosis, and metabolism. The utility of miRNAs are limited by their low level or absent expression in teratomas.

1.5 Treatment and survivorship

The introduction of platinum-based chemotherapy has significantly improved the survival rates for testicular cancer. According to the Thames Cancer Registry, the 10-year relative survival for seminoma and non-seminoma patients was 78% and 55%, respectively, between 1960 and 1969. Currently, about 90-95% of testicular tumors can be cured with standard-of-care treatment. Modern risk-adapted therapy aims to minimize side effects while enhancing the quality of life without compromising the

chance of recovery. As many patients are young and have many years ahead, addressing survivorship issues is essential even before treatment starts. Potential late effects can include cardiovascular complications, infertility, secondary cancers, psychological effects, and more, emphasizing the importance of long-term follow-up and personalized treatment strategies.

For localized seminoma, the primary treatment entails radical inguinal orchiectomy, which cures approximately 85% of patients. A risk-adapted management strategy is employed, recommending surveillance for tumors 4 cm or less without stromal invasion of the rete testis, while larger tumors may require one course of adjuvant carboplatin. For localized non-seminoma, high-risk patients with lymphovascular invasion are recommended one cycle of adjuvant BEP chemotherapy.

In managing metastatic disease, the TNM stage and IGCCCG classification of the disease guides treatment. Immediate chemotherapy is essential for life-threatening bulky cases, followed by orchiectomy. Patients with intermediate or poor prognosis are offered 4 cycles of BEP chemotherapy, with timely referral to specialized centers critical for managing advanced tumors. For non-seminoma patients with post-chemotherapy residual masses of 1 cm or more, surgical resection is mandatory.

Salvage retroperitoneal lymph node dissection (RPLND) is infrequently used in pure seminomatous cases due to complications and technical challenges. Recent research supports both open and minimally invasive techniques for small-volume unilateral seminoma metastasis, showing favorable outcomes.

Huddart et al. observed a stage migration in testicular tumors in the period from 1999 to 2002 compared to the years from 1984 to 1995. This change was attributed to awareness campaigns leading to earlier detection and lower tumor sizes at diagnosis, ultimately contributing to better survival rates. However, recent data from Canada during the COVID-19 pandemic indicated a rise in advanced stages at diagnosis, underscoring the critical need for timely diagnosis and treatment in testicular cancer, even amid a global pandemic.

2. AIMS OF THE THESIS

In the present thesis, our aims were to:

- assess testicular germ cell cancer stage migration and patient delay at a single urooncology center in Hungary
- assess postchemotherapy RPLND outcomes and complications at a high volume referral center in Hungary
- assess of pre-selected sera and/or tissue miRNA expression profiles in testicular cancer patients with postchemotherapy residual retroperitoneal lesions and healthy controls
- develop more reliable tumor markers for testicular germ cell cancer management to facilitate early detection, monitor treatment responses and assess post-chemotherapy residual lesions

3. MATERIALS AND METHODS

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

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. The size, stage and histological composition of the removed testicular tumours were obtained from histopathology results. Patient delay (the time from onset of testicular cancer symptoms to the first medical examination) was determined from the patient history described in the medical records. 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

In a retrospective cross-sectional study patients (n=127) who underwent postchemotherapy RPLND between 2007 and 2023 a high volume referral center were evaluated. The patients received systemic treatment at various oncology centers. 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. Treatment outcomes and complications were assessed. In our survival analysis, we utilized the survminer package. 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).

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

Serum and tissue samples from postchemotherapy metastatic testicular germ cell cancer patients were collected, prepared and stored in liquid nitrogen at Péterfy Sándor Hospital, Dept. of Urology in Budapest. Histology of resected tumors were assessed by an experienced uropathologist, and organised into 3 groups; necrosis/fibrosis only, teratoma or viable GCT.

Serum miRNAs were purified and equal volumes of extracted miRNA samples underwent reverse transcription. Oligonucleotides for miRNA specific PCR amplification were designed using the miRprimer_2 software. The qPCR reactions were conducted using SYBR Green chemistry. Five sections from each lymph node sample were used for miRNA extraction. We developed a standardized serum-based measurement protocol and conducted comprehensive statistical analyses on the dataset to underscore the diagnostic accuracy of the miRNA pool. A detailed description of the techniques used is beyond the scope of this summary.

4. RESULTS

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

Overall, looking at the period 2007-2009 and 2016-2018, the size of detected testicular tumours were comparable. The rate of tumors classified as stage I at detection has dropped from 67.6% to 40%. These findings were not significant on statistical analysis. Between 2007-2009 11% of patients waited more than 6 months before seeking help, in the years 2016-2018 this rate was 25%. Within both time intervals half of the patients saw their physician within 1 month of developing symptoms.

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

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

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.

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.**

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.

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

Based on literature data, nine candidate diagnostic miRNAs were selected according to their role in tumorigenesis. 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. 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 two cohorts. The miR-21+miR-29a+miR-106b cluster had the highest sensitivity (96%) and specificity (78%) for testicular cancer on ROC curve analysis.

We examined the tissue levels of the previously selected miRNAs in lymph nodes removed by salvage RPLND surgeries. Lymph nodes were classified into three histological groups - the "reactive lymph node" (RNL), the "no living tumor" (NLT) and the "teratoma-containing group", (TCa). There were slightly significant differences observed between the RLN and teratoma groups for miR-21, miR-155, and miR-373. 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. Our data thus showed distinct differences in the tissue expression levels of sets of oncomiRs between reactive and metastatic LNs with living teratoma.

The serum levels of the nine pre-selected circulating miRNAs have been re-analyzed considering the donors' histological result (RNL, TCa, NLT). Neither any of the individual miRNAs, nor any of the previously established indicators showed significant differences between the three-sample categories. These data indicated that using the serum level patterns of circulating miRNAs individually or as combined indicators has substantial limitations in assessing metastatic TCa patients' therapeutic responses.

5. **PATIENT EDUCATION**

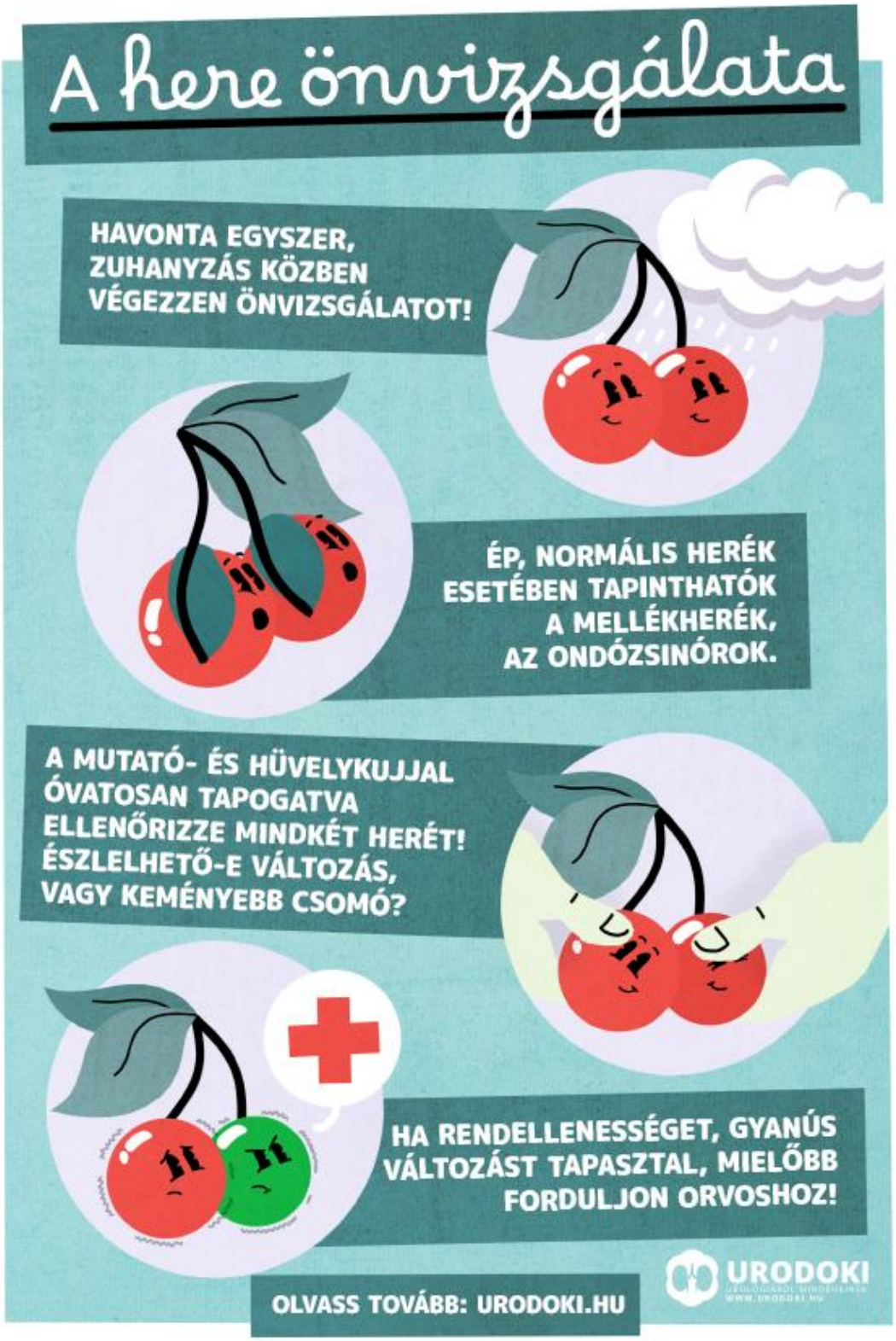

Figure 1: Testicular Cancer Awareness Campaign powered by **urodoki.hu** (March, 2024)

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.

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.

6. CONCLUSIONS

1. The size and pathological stage of testicular tumors detected during 2007-2009 and 2016-2018, remained essentially unchanged. No stage migration was observed.

2. Patient delay was alarming; between 2016 to 2018 25% of symptomatic patients waited for more than 6 months before seeking professional help.
3. 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).*
4. An increased number of BEP cycles in metastatic GCT had no beneficial effect on residual lymph node pathology, surgical outcome or survival.
5. 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.

6. 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.
7. The miR-21+miR-29a+miR-106b cluster had the highest sensitivity (96%) and specificity (78%) for TGCT.
8. 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.
9. Circulating miRNA expression in sera samples did not exhibit significant differences among the patients of the three histological groups.

7. ABBREVIATIONS

AFP	alpha fetoprotein
BEP	bleomycin, etoposid, cisplatin
CHEK2	checkpoint kinase 2
EAU	European Association of Urology
GCNIS	germ cell neoplasia in situ
GCT	germ cell tumor
hCG	human chorionic gonadotropin
IGCCCG	International Germ Cell Cancer Collaborative Group
LDH	lactate dehydrogenase
MRI	Magnetic Resonance Imaging
OCT 3/4	Octamer binding transcription factor 3/4
PLAP	Placental-like alkaline phosphatase
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROC	receiver operating characteristic
RPLND	retroperitoneal lymph node dissection
TGCT	testicular germ cell tumor
TNM	Tumor, Node, Metastasis classification

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