MicroRNA-371a-3p as a Serum Biomarker for the Early Detection of Recurrences in Patients with Testicular Germ Cell Tumors

MicroRNA-371a-3p als Serum-Biomarker zur Früherkennung von Rezidiven bei Patienten mit testikulären Keimzelltumoren

Dissertation

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1 Introduction

1.1 Cancer

Cancer is defined as a large group of complex and multifactorial diseases resulting from a combination of genetic and epigenetic alterations of somatic cells (You and Jones, 2012). It is characterized by the uncontrolled cell division of abnormal cells, leading to a balance disturbance between cell proliferation and apoptosis (Gonzalez et al., 2018). According to the World Health Organization (WHO) estimates from 2019, cancer is the second leading cause of mortality worldwide following heart diseases with around 19 million new cancer cases and approximately 10 million cancer-related deaths in 2020 (Sung et al., 2021; Deo et al., 2022).

Cancer development typically involves the accumulation of genetic abnormalities over time, which provide the cells with several growth advantages, allowing them to evade normal regulatory mechanisms that control cell growth and cell division (Tomasetti et al., 2017; Mbemi et al., 2020). Several factors can lead to these genetic alterations, including mutations, environmental factors as well as lifestyle choices (Parsa, 2012). Mutations can arise from both environmental factors, such as exposure to radiation, and certain chemicals, as well spontaneous factors which occur during processes of DNA replication (Parsa, 2012; Tomasetti et al., 2017). The regulation of signaling pathways, especially in proliferation, and apoptosis, is crucial to maintain cellular function and to prevent disease (Feitelson et al., 2015). Mutations in these pathways can lead to uncontrolled cell division, providing cells with a distinctive set of functional capabilities, which are essential to the formation of malignant tumors (Williams et al., 2012). These characteristics are recognized as the hallmarks of cancer and were initially proposed by Hanahan and Weinberg in 2000, which include characteristics like *sustained proliferative signaling*, *evading growth suppressors, resistance to cell death*, *enabling replicative immortality inducing angiogenesis,* and *activating invasion* and *metastasis* which contribute to the development and progression of malignant tumors (Williams et al., 2012; Hanahan and Weinberg, 2000). A decade later, the hallmarks were validated and expanded to introduce two emerging hallmarks into the classification, including *deregulating cellular energetics* and *avoiding immune destruction*, as well as two enabling characteristics *genome instability* and *tumor promoting inflammation* (Hanahan and Weinberg 2011; Fouad and Aanei, 2017). Tumor development generally involves four main stages: Initiation, promotion,

progression and metastasis stage (Frank, 2007). The initiation step involves alteration in the nucleic acids caused either by environmental mutagens or spontaneous mutations which typically occur in key genes, including oncogenes and tumor suppressor genes, contributing to tumor progression (Parsons, 2018). During tumor promotion and progression, the cells undergo additional genetic changes which contribute to enhanced survival of cancer cells. The final stage in cancer progression is metastasis, where tumor cells spread through the lymphatic vessels or the bloodstream to distant sites (Basu, 2018; Parsons, 2018). Based on their growth patterns, tumors could be either classified as benign or malignant. Benign tumors are localized, and they tend to proliferate slowly whereas malignant tumors shows invasive features that can spread into surrounding tissues or to distant areas of the body (Jang et al., 2011). Benign tumors are often enclosed by a capsule, which separates the tumor tissue from surrounding tissue (Lubkin and Jackson, 2002). Heredity may also play a role in the development of some tumors (Huo et al., 2021). Genetic predisposition, such as inherited mutations in genes like *BRCA1* or *BRCA2*, significantly increase the risk of certain types of cancer, particularly breast and ovarian cancers. (Parsa, 2012; Cani et al., 2023).

1.2 Testicular tumors

Testicular cancer is considered relatively rare accounting for approximately 1-2% of all malignancies and 5% of urological tumors in males (Rosen et al., 2011). However, despite the lower overall incidence, it represents the most common solid malignancy in men aged 15 to 40 years (Giona, 2022). With early diagnosis and effective treatment, testicular cancer has an excellent prognosis with an overall 5-year survival rate of about 95% (Albers et al., 2015; Nappi et al., 2017; Ding et al., 2022). Testicular cancer comprises a heterogeneous group of neoplasms, involving both benign and malignant forms and is characterized by the abnormal growth of cells in the testes, which are responsible for producing sperm and testosterone (Looijenga et al., 2020). The testes are in a pouch of skin called the scrotum which has a crucial role in maintaining the temperature of the testes for an optimal sperm production (Suede et al., 2024). Testicular cancer commonly affects one testicle, but it can potentially spread to the other testicles, although the risk is relatively rare (Dax et al., 2022). While testicular cancer is considered rare, its incidence has indeed increased by a factor of 1.80 over the last 25 years, especially notable in Caucasian males (Yazici et al., 2023).

Worldwide, there are approximately 72,000 new diagnoses and 9,000 deaths per year due to testicular cancer (Fitzmaurice et al., 2017; Altunkurek, 2020). In Germany, specifically, there are around 4,000 new testicular cancer cases each year according to the Robert Koch Institute (RKI 2020). There is distinct geographic distribution of testicular cancer world-wide, with the highest incidence observed in Northern Europe (6.7%), Western Europe (7.8%), Australia, (6.5%) and North America (5.1%), while considerably lower rates are observed in South Europe and America (Rosen et al., 2011). Additionally, Asia and Africa tend to report lower incidence rates which can be attributed to a limited healthcare infrastructure resulting in delayed diagnoses and lower reporting of cases (Wang et al., 2021). Moreover, reduced awareness or limited knowledge about testicular cancer can also affect the accurate reporting of cases (Rosen et al., 2011; Omotoso et al., 2023). In addition, several other factors contribute to the geographic clustering in the incidence of testicular cancer, including genetic, environmental and lifestyle factors (Sonneveld et al., 1999). Conversely, despite the incidence being low in Africa and Asia, the high mortality rate in these countries demonstrates a lack of effective detection programs, healthcare infrastructure and treatment resources (De Toni et al., 2019). Nevertheless, the overall mortality from testicular cancer has attenuated since 1970, primary attributed to crucial advancements and improvements in therapy, particularly after the introduction of cisplatin-based chemotherapy regimens (Giona, 2022).

The etiology of testicular cancer are not well understood, but several risk factors have been identified which are important for early detection and treatment (Richiardi et al., 2007; Yazici et al., 2023). Cryptorchidism (undescended testicle) is the most common risk factor for testicular cancer which increases the risk three to four-fold (Gurney et al., 2017). Further important risk factors include previous history of testicular cancer (Hoshi et al., 2020), testicular trauma (Guth et al., 2023), and family history which can contribute to a genetic predisposition. Certain genetic conditions such as Klinefelter's syndrome are also associated with an elevated risk of testicular cancer (Williams and Stoeber, 2012).

1.3 Testicular germ cell tumors

Histopathologically, testicular cancer can be divid into two main groups, one comprising testicular germ cell tumors (TGCT) and the other consisting of non-germ cell tumors (Aschim et al., 2006). The majority of testicular tumors (approx. 90-95 %)

originate from germ cells and are malignant (Giona, 2022). The remaining 5-10% are typically benign and include stromal tumors, such as Leydig cell tumors and Sertoli cell tumors (Boccellino et al., 2017). Testicular germ cell cancer can develop from germ cell neoplasia in situ (GCNis), formerly known as Carcinoma in situ (CIS), Intratubular Germ Cell Neoplasia Unclassified (IGCNU) or Testicular Intraepithelial Neoplasia (TIN) (Dieckmann and Loy, 1993). According to the WHO classification, testicular cancers are subdivided into two major subgroups (60% seminomas and 40% non-seminomas), which plays an essential role for the diagnosis and treatment options (Katabathina et al., 2021). Histologically, classification of TGCT was based on morphological features (Stang et al., 2019). According to the updated 2016 edition of the WHO classification, the classification is also based on pathogenetic features and TGCTs are divided into two main pathogenetically distinct groups: GCNis-related and non–GCNis-related tumors (Moch et al., 2016; Williamson et al., 2017; Berney et al., 2022). The non-GCNis related tumors can be categorized into type I and III TGCTs, while the GCNIS-related tumors are associated with type II germ cell tumors (Looijenga et al., 2019). Type I TGCTs are often referred to as non–GCNis associated tumors and are usually diagnosed in early childhood (< 14 years) or rarely in elderly patients (Ronchi et al., 2019). Type I TGCTs include subtypes such as yolk sac tumors, which are typically malignant and teratomas which can be either benign or malignant tumors based on their characteristics (Ronchi et al., 2019). Most testicular tumors (type II TGCTs) typically arise from GCNis and are malignant, which are histologically further subdivided into seminomatous germ cell tumors (SGCTs) and non-seminomatous germ cell tumors (NSGCTs) (Jimenez-Rojo et al., 2021; Katabathina et al., 2021). Seminomas are typically the most common type, accounting for 60% of testicular germ cell tumors, while non-seminomas make up the remaining 40% (Terbuch et al., 2022; Jansson et al., 2023). Type II TGCTs show a distinct age distribution pattern (Ghazarian et al., 2018). The incidence of NSGCTs peaks around the age of 25, whereas SGCTs have a peak incidence about ten years later, around the age of 35 (McGlynn and Cook, 2009; Ghazarian et al., 2018). Seminomas represents usually morphologically homogeneous tumor types, while NGGCT involve a heterogeneous group of cell types which are further classified into several subtypes based on their histological features including embryonal carcinomas, yolk sac tumors, choriocarcinomas, teratomas as well as mixed tumors (Chen and Amatruda 2013; Marko et al., 2017). NSGCTs are clinically considered more aggressive than

seminomas which require often a more intensive treatment approach (Scandura et al., 2021). Seminomas, in contrast, tend to grow more slowly and are usually confined to the testicle at the primary diagnosis. Moreover, they are generally more sensitive to radiation therapy and the overall prognosis for seminoma is often better compared to NSGCTs (Siverino et al., 2016). The recurrence rate for stage I seminoma and non-seminoma ranges from 15-20%, during the frst two or three years of follow-up (Lobo et al., 2019).

Type III TGCTs are spermatocytic seminomas, which are considerably less frequent compared to classical testicular seminomas, comprising 4–7% of all seminomas. Moreover, they are most frequently diagnosed in elderly men, usually over the age of 50 (Wetherell et al., 2013). The pathogenesis of TGCTs is a complex process involving various genetic and environmental factors (Facchini et al., 2018). Most TGCTs arise in a precursor lesion referred to as GCNis, which is believed to originate from undifferentiated primordial germ cells (PGC), the precursors of both male and female gametes (Nicu et al., 2022). The differentiation of PGCs starts during early embryogenesis, when PGCs migrate to the gonadal ridge, which later differentiate into gonocytes (Bharti et al., 2021; Nicu et al., 2022). Once at the gonadal ridge, they undergo several epigenetic modifications, losing their embryonic characteristics which may lead to testicular cancer (Nicholls and Page, 2021; Urbini et al., 2021). TGCTs arise from abnormalities in the differentiation process of germ cells, which fail to mature into spermatogonia. Instead, they remain dormant in their differentiation process and underwent genetic alterations, acquiring features that promote their survival and may contribute to fertility issues or the formation of GCTs in the testicles (Baroni et al., 2019; Fink et al., 2021). Failure in maturation might result from several factors involving genetic abnormalities, epigenetic changes or environmental influences (Nicu et al., 2022). In the context of testicular cancer, alteration of the short arm of chromosome 12 play an essential role in tumor progression, specifically the formation of isochromosome 12p and other forms of 12p amplification, which represent hallmark features of type II TGCTs (Oldenburg et al., 2013; Bharti et al., 2021; Müller et al., 2021). The presence of GCNis is associated with an increased risk of progression to invasive malignancy, possibly around 70% after 7 years (Pierconti et al., 2019). Therefore, regular monitoring is important for patients diagnosed with GCNis to reduce the risk of developing testicular cancer (Sheikine et al., 2012).

1.4 Diagnosis and treatment of testicular germ cell tumors

Testicular cancer usually presents as a painless unilateral mass in the testicle that might be noticed incidentally by the patient (Marko et al., 2017). Acute testicular pain is less common occurring in about 10% of patients, often associated with metastasis (Gaddam and Chesnut, 2024). The initial evaluation of suspected testicular cancer is based on history and physical examination along with scrotal ultrasound (Chovanec and Cheng, 2022). Further diagnostic process includes measuring serum tumor markers, including α-fetoprotein (AFP), β-subunit of human chorionic gonadotropin (β-HCG or bHCG) and lactate dehydrogenase (LDH), which serve as effective prognostic tools, assisting in timely diagnosis, staging, risk assessment, monitoring treatment success, and also to detect relapse during follow-up (Beyer et al., 2013; Dieckmann et al., 2019a). In almost all cases, the primary treatment for testicular cancer patients is radical orchiectomy, which includes removal of the affected testicle and a part of the spermatic cord through an inguinal incision followed by a subsequent histologic examination of the tumor (Stephenson et al., 2019; Gaddam and Chesnut, 2024). Further treatment strategies depend on clinical staging by computed tomography (CT) of chest, abdomen and pelvis, and serum tumor marker (Albers et al., 2015; Baird et al., 2018). Persistently elevated serum tumor markers after radical orchidectomy, might indicate the presence of occult metastases and the need for subsequent treatment. Therefore, it is vital to evaluate both pre- and post-orchiectomy tumor marker levels to effectively monitor the disease progression and asses the response to treatments (Pedrazzoli et al., 2021). During the follow-up after treatment, monitoring tumor markers are also essential in detecting any potential cancer recurrence. An elevation of these tumor markers can serve as an indicator of relapse (Gilligan et al., 2010; Krege et al., 2023).

The staging for testicular cancer is still performed according to the Tumor, Node, Metastasis (TNM) classification developed by the the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) (Kandori et al., 2019). The TNM system incorporates data specifically regarding the primary tumor, and imaging to identify the presence of any lymph node or distant metastases (N or M stage) (Amin et al., 2017). Additionally, for testicular cancer, a serum tumor marker category (S) is included in the TNM status which is based on post-orchiectomy tumor marker levels (Pierre et al., 2022). Clinical stage I is defined as the primary tumor is

confined to the testicle. Metastatic cases are classified as CSII or CSIII, determined by the location and extent of the spread (Krege et al., 2023). Stage II indicates spread to nearby lymph nodes in the retroperitoneum, while stage III involves distant metastasis, often associated with elevated tumor marker levels (Pierre et al., 2022; Krege et al., 2023). For metastatic germ cell tumour patients, three additional prognostic groups were introduced into the TNM classification by the International Germ Cell Cancer Collaborative Group (IGCCCG) that classifies patients into good, intermediate and poor risk groups based on the level of tumor markers, location of the primary tumor and the presence of non-pulmonary visceral metastases (Beyer et al., 2013; Pierre et al., 2022; Dubey et al., 2023). Thus, management strategy of testicular GCTs depends on both the TNM classification and the IGCCCG-prognostic system (Beyer et al., 2013). The treatment of testicular cancer has changed considerably over the last thirty years and is associated with long-term survival now (Feldman, 2008). In general, TGCTs have an excellent prognosis with cure rates exceeding 95% for the localized stages (Dieckmann et al., 2016) and are also still good even for the advanced stages ranging from 70–90%, attributed to the multimodal treatment involving surgery, radiotherapy, and chemotherapy, especially cisplatin-based combination therapy (Hale et al., 2018; Kliesch et al., 2021). Early diagnosis and treatment of testicular cancer is essential, as it can significantly reduce mortality rates and increase the overall prognosis of testicular cancer (Pietrzyk et al., 2020). The vast majority of testicular cancer patients are diagnosed at clinical stage I (seminoma 85% and non-seminoma 70%-75%), which represents a localized form of testicular cancer, usually confined to the testicle without evidence of spread to distant organs (Ruf et al., 2022; Krege et al., 2023). Most patients with CSI are cured with orchiectormy alone and the overall cure rate approaches 100% regardless of the treatment after orchiectomy (Kollmannsberger et al., 2011; Dieckmann et al., 2016). Therefore, the main goal of the treatment is to maintain long-term survival rates while minimizing unnecessary interventions (Alexander et al., 2010; Tandstad et al., 2010). Figure 1 illustrates the treatment options for both seminoma and non-seminoma patients. According to European Association of Urology (EAU) guidelines (2024), standard management for CSI seminoma patients includes radical inguinal orchiectomy followed by different treatment options including active surveillance, adjuvant chemotherapy, or adjuvant radiation therapy depending on prognostic factors for the risk of relapse (Ehrlich et al.,

2015). In seminoma CSI, a tumor size of greater than 4 cm and rete testis invasion are considered as important prognostic factors (Dieckmann et al., 2016).

According to EAU (2024), active surveillance is considered the preferred management for CSI seminoma due to concerns about overtreatment in a young patient population and the risk of potential long-term toxicity associated with other treatment approaches (Mahmoud Sayed et al., 2023; McHugh et al., 2024). Active surveillance involves close monitoring with imaging scans and assessment of serum tumor markers at regular intervals for early identification of potential relapse (Warde et al., 2002; Dieckmann et al., 2016). Decisions regarding adjuvant treatment should involve discussion with patients about risks and individual circumstances (Bumbasirevic et al., 2022). For CSI seminoma patients with a tumor size of greater than 4 cm, adjuvant treatment may be considered to reduce the risk of recurrence or address any psychological patient concerns (Krege et al., 2023). In patients without these risk factors (tumour size < 4 cm and no rete testis invasion), the 5 year relapse rate under surveillance is up to 6-8%, respectively (EAU, 2024). According to EAU guidelines (2024), adjuvant therapy options include the administration of one dose of carboplatin chemotherapy or radiotherapy (Figure 1). Both treatments can reduce the risk of relapse to approximately 5%. However, despite the excellent cure rates associated with radiotherapy, this management has been associated with possible long-term risk of secondary malignancies, such as pancreatic, gastric, bladder, and kidney cancers, and thus no longer particularly in young patients with a long life expectancy (Zengerling et al., 2018; McHugh et al., 2024). Since relapsed patients during surveillance can still be cured with adjuvant chemotherapy, the main goal of the treatment is minimizing toxicity (Cathomas et al., 2011). Therefore, active surveillance is still the preferred strategy for CSI seminoma patients in relevant guidelines (Oldenburg et al., 2022; EAU Guidelines, 2024) which spares 60% to 75% of CSI patients from unnecessary toxicity after orchiectomy (Alexander et al., 2010; Kollmannsberger et al., 2015). For seminoma, the majority of relapses are observed in the retroperitoneum, which will be detected on abdominal/pelvic CT scans (Pierre et al., 2022). CSI seminoma patients who relapse after first treatment should receive cisplatin-based chemotherapy which include 3x BEP or 4x etoposide and cisplatin (EP) or are mostly salvaged by radiotherapy (Figure 1) (Crocetti et al., 2021).

Treatment options for CSI NSGCT include active surveillance, adjuvant chemotherapy (BEP x 1–2), or retroperitoneal lymph node dissection (RPLND) (Heidenreich et al.,

2018). As shown in Figure 1, CSI non-seminoma patients are divided into low-risk (15-20% relapse rate) and high-risk groups (40-50% relapse rate) depending on the absence or presence of lymphovascular invasion, which is the most important prognostic factor for developing metastatic disease (Schmoll et al., 2010). Therefore, a risk-adapted treatment of stage I NSGCT is an alternative for patients with CSI NSGCT, recommended by the EAU and the German S3 guidelines. When discussing treatment options with patients, it is essential to consider their specific circumstances including disease risk factors, and personal preferences (EAU, 2024). Similar to stage I seminoma, active surveillance is the preferred approach for low-risk patients which avoid risks of acute and potentially long-term toxicities recommended by EAU guidelines and German S3 guidelines, while the management of high-risk non-seminoma patients is more controversial (Winter and Hiester, 2021). The most common treatment for patients with stage I high-risk NSGCTs is the administration of one cycle of BEP according to the German S3 guidelines (Heidenreich et al., 2018; Winter and Hiester, 2021). In case of recurrence after adjuvant treatment or active surveillance, first-line treatment consists of three or four cycles of BEP according to the IGCCCG followed by resection in case of residual tumour (Gilligan, 2023).

For stage IIA/B seminoma patients, radiotherapy or alternatively chemotherapy (3x BEP or 4x EP) is considered standard (EAU, 2024). Management of stage II non-seminoma patients depends on tumor marker levels. According to EAU guidelines, RPLND is recommend as a treatment option for tumor marker negative stage II non-seminoma patients to reduce the risk of adjuvant treatment related toxicity (Albers et al., 2015). In case of persistently elevated tumor markers; chemotherapy is the initial treatment of choice (Katdare et al., 2023). For patients with metastatic disease (clinical stage IIC–III) chemotherapy is the initial line of treatment. The standard regimen for good-risk patients is three courses of BEP and four cycles of BEP for intermediate and poor risk patients depending on stage and IGCCCG prognosis group (Stephenson et al., 2019; Beyer et al., 2021).

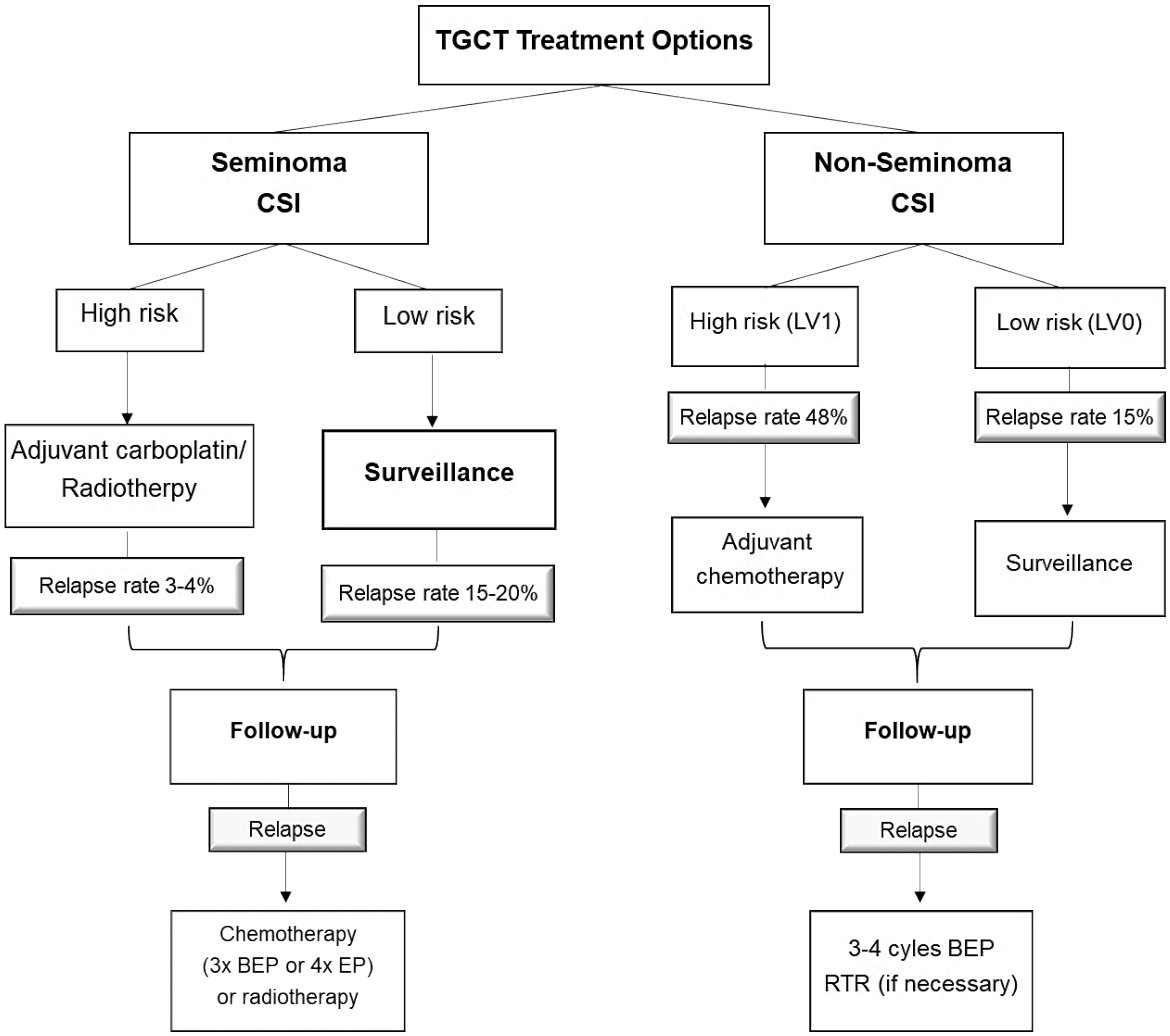

Figure 1: Management strategy after orchiectomy based on individual risk factors in testicular cancer patients with clinical stage I seminoma and non-seminoma. BEP: Bleomycin, Etoposide, Cisplatin; EP: Etopside, Cisplatin; LV1: Lymphovascular Invasion; LV0: Without Lymphovascular Invasion; RTR: Residual Tumor Resection. The figure is based on a model by (mod. Krege et al., 2008).

1.5 Evaluation of microRNA-371a-3p as a new biomarker for the detection of recurrences during follow-up of CSI TGCTs

The overall survival for patients with stage I testicular cancer is excellent with over 98% regardless of treatment strategy (Daugaard et al., 2003; Wagner et al., 2019). However, despite this progress in treatment, concerns have emerged regarding the long-term side effects of therapies like chemotherapy and radiotherapy, including an elevated risk of secondary malignancies and cardiovascular disease (Haugnes et al., 2012; Wagner et al., 2019). Therefore, it is crucial not only to achieve successful treatment but also to minimize the potential long-term side effects, especially because of the young age of patients (Nappi et al., 2017). Moreover, considering the 15–50%

risk of relapse of TGCT patients within 5 years, follow-up care after treatment is essential for early relapse detection and accurate disease monitoring (Wagner et al., 2019; Fischer et al., 2023). Follow-up involves three primary objectives: Early diagnosis of recurrence, monitoring of treatment-related toxicity, and detection of secondary diseases (Dieckmann et al., 2022a). Currently, no biomarkers are available to reliably identify recurrence in CSI patients managed with surveillance (Nappi et al., 2017; Lobo et al., 2020; Belge et al., 2024). The risk of recurrence often depends on various morphologic features of the primary tumor, which play a crucial role in determining the most effective treatment strategy (Belge et al., 2024). In seminomas, the risk of relapse is approximately 15-20% for patients with primary tumor size of over 4 cm, while smaller primary tumors generally have progressively lower relapse rates (Beyer et al., 2013; Belge et al., 2024). In non-seminoma patients, the presence of lymphovascular invasion is associated with a higher risk of recurrence, ranging from 40-50%. Patients without this histologic feature have a lower recurrence rate with approximately 15-20% (Oldenburg et al., 2022). The current EAU guidelines recommends follow-up protocols for different patient groups. For CSI seminoma patients undergoing active surveillance or after adjuvant treatment, abdomino-pelvic CT is recommended every 6 months for the initial 2 years, once in the third year and once at 5 years (Pierre et al., 2022; Katdare et al., 2023). Tumor markers are evaluated at 6 monthly intervals during the first 3 years and then once at 5 year (Katdare et al., 2023). For non-seminoma stage I on active surveillance, abdominopelvic CT is recommended at 6 monthly intervals in the first year, then once for the next 2 years, followed by a final scan at 5 years (Pierre et al., 2022; Katdare et al., 2023). Follow-up strategies for CSI testicular cancer cases include a combination of regular physical examinations, imaging studies with CT or MRI, and assessment of serum tumor markers AFP, bHCG and LDH (Oldenburg et al., 2022; Belge et al., 2024). While elevation of tumor markers serves as the initial indication of cancer recurrence, they show several limitations due to their low sensitivity with approximately 60% positive detection rate in all TGCT cases, leaving 40% without a definitive indication (Kollmannsberger et al., 2015; Dieckmann et al., 2016; Nicholson et al., 2019). The tumor markers AFP and bHCG are also produced by various other malignancies (Gilligan et al., 2010). For instance, bHCG elevations are commonly seen in diverse carcinomas affecting the bladder, kidney, lung, and gastrointestinal tract. Similarly, AFP elevations are typical in hepatocellular carcinoma and certain liver diseases

(Gilligan et al., 2010). Moreover, LDH is considered highly nonspecific and may be found in different conditions unrelated to cancer (Dieckmann et al., 2019a) and is more useful as a prognostic factor because it often correlates with tumor burden (Pedrazzoli et al., 2021). Additionally, the frequencies of elevated tumor marker levels largely depend on the histological composition of the tumor and higher clinical stages (Dieckmann et al., 2019a). AFP and bHCG serve as valuable tools in managing TGCTs, exhibiting typically high expression rates in non-seminomas and less frequent occurrence in seminoma (Dieckmann et al., 2018). Overall, about 70% of non-seminoma patients show elevated levels of either bHCG or AFP at primary diagnosis, however during a relapse, about only 41%-61% of cases exhibit elevated markers, depending on the presence of lymphovascular invasion (Daugaard et al., 2014; Kollmannsberger et al., 2015; Belge et al., 2024). Elevated bHCG levels are commonly associated with certain subtypes of NSGCTs, including choriocarcinoma and embryonal carcinoma (Wang et al., 2020). Even in seminoma patients, bHCG expression occurs in around 30% of cases, but only 11%-22% of relapses exhibit elevated levels of this tumor marker (Belge et al., 2024). AFP elevation is usually linked to yolk sac tumors thus showing low sensitivity for detecting embryonal carcinoma components (Dieckmann et al., 2019a). Moreover, AFP is generally not elevated in seminomas and thus it is recommended that pure seminomas with increased AFP levels should be treated as cases of NSGCT, except an alternative explanation for the AFP elevation is present, such as liver disease (Iwatsuki et al., 2016). Consequently, the early identification of disease relapse is hampered by the limited accuracy of current tumor markers, potentially resulting in false positive or negative results (Fischer et al., 2023; Nestler et al., 2023). Therefore, a comprehensive follow-up strategy involves a combination of various imaging modalities like CT scans or MRI examination, to enhance the detection of potential recurrences early (Lobo et al., 2021a). CT scans play an important role in the initial accurate staging of disease and determining recurrence in patients after treatment completion (Kreydin et al., 2013). For non-seminoma patients, more frequent abdominal CT scans are recommended than for patients with stage I seminoma, especially during the first year of follow-up (Kollmannsberger et al., 2015). Although CT scans are valuable tools for diagnostic purposes, they expose relatively young patients to ionizing radiation, since they use X-rays to create detailed cross-sectional images of the body (Busch et al., 2022). Thus, frequent and unnecessary CT scans can pose potential risks over time associated with

infertility, especially in reproductive-age individuals (Busch et al., 2022). Therefore, MRI could be considered as an alternative to CT scans. However, MRI examinations are cost-intensive, and the long-term toxicity of the gadolinium-based contrast agent remains unclear (Busch et al., 2022; Matulewicz et al., 2023). Moreover, imaging techniques show limited sensitivity required for early detection of occult disease (Charytonowicz et al., 2019). In light of this, there is an urgent need for improved tools for relapse detection during the follow-up of TGCT patients, for more effective monitoring of disease progression, while reducing the need for frequent routine imaging scans (Charytonowicz et al., 2019; Lobo et al., 2021a; Tavares et al., 2023). Recently, microRNAs (miRNAs) have emerged as potential non-invasive biomarkers for implementation in the clinic (Belge et al., 2012; Dieckmann et al., 2012; Wang et al., 2018). MiRNAs are endogenous, small, non-coding RNA molecules, typically consisting of around 20-25 nucleotides, that play a significant role in post-transcriptional gene expression, which is essential for various cellular processes, including development, differentiation and apoptosis (Dieckmann et al., 2019a). The alteration in miRNA expression can be measured in body fluids, providing a non-invasive approach of diagnosis and monitoring (Chen et al., 2012). Their unique characteristics in various types of cancer, make them valuable markers for cancer diagnosis, prognosis, and therapy (Chakrabortty et al., 2023). The first evidence of the involvement of miRNAs in cancer can be attributed to the research by Calin et al. (2002) which showed that patients diagnosed with chronic lymphocytic leukemia (CLL) display significant dysregulation of miR-15a and miR-16-1.

The first association of miRNAs with testicular cancer was made in 2012 (Belge et al., 2012; Dieckmann et al., 2012). Accumulated evidence has shown, that circulating miRNA-371a-3p (abbr. M371) can reliably identify TGCTs (with the exception of teratoma) (Belge et al., 2012; Dieckmann et al., 2012; Gillis et al., 2013; Syring et al., 2015; Dieckmann et al., 2019a; Leão et al., 2019). A large, prospective multicentre study by Dieckmann et al. (2019a) demonstrated that M371 has the best discriminative capability for the primary diagnosis of GCT with both a sensitivity and a specificity greater than 90%, outperforming AFP, β-HCG, and LDH which combined showed a sensitivity of 50%. Several independent studies further underline its excellent sensitivity and specificity, ranging from 85% to 90.1% and 89.1%-99%, respectively (Van Agthoven et al., 2017; Nappi et al., 2019; Lobo et al., 2021a; Myklebust et al., 2021). Despite the promising data concerning the diagnosis and treatment of TGCTs,

there is little information regarding its role in TGCT recurrence detection (Ditonno et al., 2023). The integration of the M371 test in routine follow-up protocols of testicular cancer patients offers several advantages (Tavares et al., 2023; Belge et al., 2024). The high sensitivity and specificity of the M371 test suggests the potential for early detection of recurrence, which allows for timely intervention, thus reducing the need for serial CT scans and associated healthcare costs (Charytonowicz et al., 2019; Christiansen et al., 2022). Ongoing studies aim to understand the importance of M371 in detecting disease recurrence, exploring aspects such as the origin of M371, its association with risk factors for progression, and also its association with various clinical parameters like tumor size, histology, clincal stage and tumor marker expression rates (Belge et al., 2020; Dieckmann et al., 2022b, 2022c, 2023). Additionally, the potential of M371 in identifying disease recurrence among TGCT patients has been underscored by various studies, indicating its promising role as an optimal biomarker for early and reliable detection of recurrences in patients under active surveillance (Terbuch et al., 2018; Fankhauser et al., 2022). However, due to limited data and conflicting results it remains uncertain whether M371 is capable of predicting recurrences. Moreover, there are controversial results regarding the M371 expression rate in both recurrent and primary tumors (Lobo et al., 2021a). In a recent Swiss study by Fankhauser et al. (2022) the ability of M371 to detect recurrence in TGCT patients with CSI in surveillance was evaluated. The results of the study revealed promising findings, indicating that M371 showed high sensitivity and specificity in detecting recurrences in patients under active surveillance. Moreover, recurrences were detected at a median of 2 months earlier with M371 than with standard follow-up investigations. However, the study faced some limitations, including a relatively small cohort size and lack of long-term follow-up (Fankhauser et al., 2022). Therefore, further extensive research is important to establish the effectiveness of M371 test as a diagnostic marker in TGCT monitoring.

1.6 Aim of the thesis

The overall aim of this thesis is to comprehensively investigate the potential of microRNA-371a-3p (M371) as a biomarker for early recurrence detection in patients with testicular germ cell tumors, with a specific focus on patients in clinical stage I during follow-up. Specifically, the thesis aims to answer three key questions:

(i) Is the M371 test capable of detecting relapses during follow-up of CSI patients undergoing active surveillance?
(ii) Can the test detect recurrence earlier than conventional methods?
(iii) Do elevated postoperative M371 levels predict future recurrence?

Therefore, M371 was comprehensively investigated in multiple studies, with a focus on determining its origin and assessing correlations with various risk factors and other clinical characteristics to provide a deeper understanding of the implications of M371 in follow-up of TGCT patients. In detail, the aims of the publications included in this thesis were the following:

Publication I. The main objective was to investigate the origin of circulating M371 by measuring its levels in the serum, tumor tissue, and contralateral testes of TGCT patients. Additionally, the aim was to determine whether elevated M371 levels in TGCT tissue correlate with higher levels of the miRNA in the serum.

Publication II. The aim of this study was to investigate the associations between M371 levels with clinical risk factors, such as LV1 and predominance of EC, in CSI testicular nonseminomatous germ cell tumors, and especially to evaluate its predictive value for disease progression.

Publication III und IV. Clinical characteristics of testicular cancer and the role of M371 were investigated in two separate studies. The focus of publication III was on analyzing tumor size and its associations with tumor histology, clinical staging, serum tumor marker expression rates, and patient age.

Publication V The primary objective of my thesis was addressed in publication V. The aim of this study was to investigate the diagnostic accuracy of M371 serum levels in detecting recurrence among CSI TGCT patients during active surveillance in a prospectively long-term study, and to evaluate its potential to improve current follow-up strategies.

2 Materials and methods

2.1 Sample materials

The thesis involves a diverse patient cohort with detailed registrations, histopathological evaluations, and regular follow-up visits involving imaging and M371 measurements. Five publications included in this thesis have received ethical approval from respective committees. Initial approval was granted by the Ethical Committee of Ärztekammer Bremen (#301/11, May 30, 2011), with subsequent approvals from Ärztekammer Bremen (#301/15, July 08, 2015; #301/17, September 21, 2017; #301/18, July 21, 2018), and Ärztekammer Hamburg (MC 152/19, July 15, 2019). Additionally, ethical approval was received by the Ethikkommission der Ärztekammer Hamburg (PV7288, March 2, 2020). Written informed consent was obtained from all participating patients, and the study strictly adhered to the principles outlined in the Declaration of Helsinki by the World Medical Association as amended by the 64th General Assambly, October 2013.

2.1.1 Serum samples

The majority of the serum samples were taken from patients with GCTs in clinical stage I managed with active surveillance. The serum samples were obtained from 23 urological institutions in Germany, Austria and Italy. In Germany specifically, the serum samples were provided by institutions including Albertinen-Krankenhaus Hamburg, Asklepios Klinik Altona, Bundeswehrkrankenhaus Hamburg, and Klinikum Bremen-Mitte. Serum samples were obtained through blood aspiration during routine examinations, and were collected in 9 ml serum separation tubes (Sarstedt, Nümbrecht, Germany). After centrifugation at 2,500 x g for 10 minutes, serum aliquots were stored deep frozen at -80°C until further analysis. The primary focus of the study was the analysis of M371 expression over a period of 3,5 years. Thus, M371 measurements for patients with seminomas were performed every six months, while nonseminoma patients underwent M371 assessments every three months during initial two years and six-monthly thereafter.

Furthermore, preoperative serum samples were collected from patients undergoing surgery for testicular tumor, to identify GCNis, following institutional guidlines. Control serum samples were obtained from patients without GCT undergoing orchiectomy for epididymitis.

Serum tumor markers bHCG, AFP, and LDH were measured in hospital laboratories according to institutional guidelines. Histopathological parameters such as lymphatic or vascular invasion, tumor size and histology of tumor mass, were sourced from hospital-based electronic case files, offering a comprehensive view of the patient cohort.

2.1.2 Tissue samples

Preoperative tissue samples were collected from patients undergoing surgery for testicular tumors. Moreover, corresponding contralateral testis tissue was extracted from biopsy specimens. All tissue samples were provided by the Albertinen-Krankenhaus Hamburg and were deep frozen at -80°C for subsequent processing. For control, testicular tissue was obtained from patients without GCT. Additionally, surrounding testis tissue and the epididymis were analysed.

2.1.3 FFPE tissue

Formalin-fixed paraffin-embedded (FFPE) tissue samples were taken from six patients with testicular GCTs to examine the presence of miR-371a-3p, in tumor tissue. The GCTs histologically consisted of three mixed nonseminomatous tumors (embryonal carcinoma, yolk sac tumor, and chorio carcinoma), one pure seminoma, one pure embryonal carcinoma, and one teratoma. Additionally, a tissue specimen from the contralateral testis was analyzed as part of the study. The FFPE samples underwent sectioning to obtain thin slices (approximately 3-5 µm thickness), which were then mounted on glass slides. The analysis includes both immunohistochemistry and in situ hybridization (ISH) techniques.

2.2 Methods

All studies presented in this thesis were conducted centrally at the University of Bremen.

2.2.1 RNA isolation from tumor tissue and serum

Total RNA was extracted from 200 µl cubital vein serum using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer´s instructions. For tissue, 10-50 mg of tumor and contralateral testis tissue were homogenized in 1000 µl TRIzol® Reagent according to the manufacturer’s instructions (Fisher Scientific, Schwerte, Germany) using a TissueLyser (Qiagen, Hilden, Germany) with 5 mm steel beads for

10 min at 30 Hz. Finally, the extracted RNA was resuspended in 50 µl nuclease-free water.

2.2.2 cDNA synthesis

In *publication I and* II, reverse transcription was performed for all samples using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). For the reverse transcription of microRNA into cDNA, 6 µl total RNA were added to the master mix of the kit including specific stem-loop primers for miR-371a-3p (assay ID 002124) and miR-30b-5p (assay ID 000602). The reaction, with a final volume of 15 µl, was incubated in the a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) at 16 °C for 30 min, followed by 42 °C for 30 min and then 85 °C for 5 minutes, before cooling to 6°C. The cDNA was stored at -20 °C.
In *publications III-V*, reverse transcription was carried out for both miR-371a-3p and miR-30b-5p using the M371-Test (mir|detect, Bremerhaven, Germany) which include miRNA assay for the target miRNA as well as for the endogenous control. The cDNA synthesis was carried out in a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) following the previously described procedure.

2.2.3 Preamplification

Preamplification was performed to enhance the concentration of miRNA due to the initially low amounts. Standard PCR was conducted for preamplification of the cDNA using 1:100 diluted TaqMan Assays (Applied Biosystems, Darmstadt, Germany) for miR-371a-3p and miR-30b-5p. Each reaction consisted of 20 µl final volume. The cycling conditions on the Mastercycler (Eppendorf, Hamburg, Germany) were 95 °C for 1 min, followed by 14 cycles of 95 °C for 15 s and at 60 °C for 4 min, and hold at 6°C. The preamplification product was diluted 1:2 in nuclease-free water and used for qPCR *(publication I and II)*. In *publications III-V*, the preamplification process involved the use of M371 test (mir|detect, Bremerhaven, Germany), which involves specific primers targeting the miR-371a-3p and miR-30b-5p. The cycling conditions were the same as described previosly.

2.2.4 Quantitative real-time PCR

For the quantitative real-time PCR, 5 µl of the preamplification product was added to thhe FAST Start Universal Probe Master Mix (Roche, Mannheim, Germany) along with the TaqMan microRNA assay (Applied Biosystems, Darmstadt, Germany) using the

7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The relative quantification was performed with miR-93-5p (assay ID 000432) as endogenous control *(publication I)* and miR-30b-5p *(publication II-V)*. All PCR experiments were carried out in triplicates. A negative control without reverse transcriptase was added to detect contamination with genomic DNA. PCR conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The relative quantity (RQ) of miR-371a-3p was calculated according to the comparative ΔΔCT method (Livak and Schmittgen, 2001). In *publications III-V*, the measurement of M371 was performed by qPCR as described previously using the M371 test (mir|detect, Bremerhaven, Germany).

2.2.5 Immunohistochemistry

The histological types of testicular germ cell tumors were identified through various staining techniques, including hematoxylin and eosin (H&E stain), OCT4, PLAP, and glypican 3. These methods were used for the distinction between tumor-free areas and tumor tissue, with specific stains targeting embryonal carcinomas (OCT4), seminomas (PLAP), and yolk-sac tumors (glypican 3) according to institutional standard operating procedures *(publication I)*.

2.2.6 MicroRNA *in situ* hybridization in GCTs

Following the morphological identification of the particular GCT-subtypes, microRNA analysis was conducted using in situ hybridization (ISH) with a miRCURY LNA probe (Exiqon, Vedbaek, Denmark; probe ID 38555-15) specific for miR-371a-3p. The ISH protocol was performed according to manufacturer´s recommended conditions, including a proteinase-K concentration of 15 µg/ml, a hybridization temperature of 51° C, and a probe concentration of 80 nM. Microscopic assessments were performed using an Axioskop 2 plus microscope (Zeiss, Göttingen, Germany). Histological observations were documented using AxioCam HRc digital camera (Zeiss, Göttingen, Germany) and edited via AxioVision Software v.4.8 (Zeiss, Göttingen, Germany). MiR-371a-3p presence within GCTs was determined by distinct blue staining. Consequently, only cells exhibiting this staining were classified as miR-371a-3p positive. The evaluation solely focused on determining the presence or absence of as miR-371a-3p in the specimen, without any quantification *(publication I).*

2.2.7 Statistical analysis

In this study, various statistical methods were applied for a comprehensive data analysis. Patient data were initially stored in MS Excel (MS Excel version 2019), Microsoft Corp., Redmond, USA) and later SPSS version 26 (IBM, Armonk, NY, USA) was used for final evaluation.

The Wilcoxon signed rank test and Mann-Whitney *U* test were utilized to compare median M371 expression levels in releated subgroups and among the various subgroups, respectively. All tests were two-sided and statistical significance was considered at $p < 0.05$ *(publication I, II, and V).* Receiver Operating Characteristic (ROC) curve analysis was utilized to evaluate the diagnostic performance of serum M371 levels in predicting relapses, calculating sensitivity, specificity, and the Area Under the Curve (AUC) with corresponding 95% confidence intervals. Additionally, performance characteristics including positive predictive value (PPV) and negative predictive value (NPV) were calculated for the entire patient cohort and separately for seminomas and nonseminomas. The Youden Index analysis was calculated for the ROC analysis to determine the optimal cut-off value for serum M371 levels in effectively identifying *relapses (publication I, III, and V)*. Additionally, the Kaplan-Meier plot was utilized to compare the median time to relapse detection between M371 and traditional markers. The Log rank test was performed to determine the significance of any observed differences *(publication V)*.

The association between preoperative M371 expression and tumour size in either LVo or LV1 was determined with linear regression analysis. Multiple regression analysis was used to examine the dependence of M371 expression from various factors, like tumor size, age, EC content, classical marker expression, and LV status. Furthermore, the Chi-squared test was used for statistical comparison of different categorial variables *(publication II)***.** Moreover, a detailed analysis of continuous variables were examined, including median, quartiles, and standard deviation, visually presented through box and whisker diagrams. Different statistical tests, such as Kruskal–Wallis, Wilcoxon, Chi-squared, Cochran–Armitage trend, and Jonckheere–Terpstra, were applied to investigate relationships between tumor sizes, clinical characteristics, and other factors in the context of testicular cancer *(publication III and IV)***.**

3 Results

The findings obtained during the preparation of this thesis have been published in five scientific articles, the results of which are summarized below.

3.1 Publication I: Graded expression of microRNA-371a-3p in tumor tissues, contralateral testes, and in serum of patients with testicular germ cell tumor

Previous studies established the diagnostic potential of microRNAs, especially miR-371-373 and miR-302/367 clusters as potential biomarkers for TGCTs, outperforming the classical markers AFP, bHCG, and LDH with a sensitivity of 90.1% and specificity of 94.1% (Belge et al., 2012; Gillis et al., 2013; Murray et al., 2016a; Lobo et al., 2019). Among these, miR-371a-3p has shown promising diagnostic value, correlating with clinical stages, tumor sizes, therapy response, and presence in relapsing GCT cases (Van Agthoven et al., 2017; Dieckmann et al., 2019a). Although clinical data suggest a strong correlation between tumor burden and miR-371a-3p serum expression, the specific origin of serum-based miRNAs from GCT cells remains unresolved and the correlation between miR-levels in tissue and serum is unclear.

Based on these findings, this publication aimed to investigate the origin of miR-371a-3p by measuring its expression in various testicular tissue, contralateral testicular tissue, healthy tissue from controls, and non-testicular tissue from the tunica vaginalis. The second goal was to explore whether increasing M371 levels in GCT tissue correlate with higher serum levels. The study included a total of 38 patients undergoing surgery for testicular tumors, comprising of 29 seminomas and 9 non-seminomas. Additionally, preoperative serum samples were obtained from 36 of the 38 patients.

The results of the study showed a significant elevation of miR-371a-3p in tumor tissue compared to both contralateral testicular tissue and controls. Similarly, miR-371a-3p expression levels were significantly elevated in tumor tissue compared to healthy testicular tissue ($p < 0.001$), while no significant difference was observed in healthy testicular tissue and contralateral testicular tissue ($p = 0.985$).

Overall, the study reveals five key findings:

(1) M371 levels were significantly higher in GCT tissue compared to corresponding contralateral testes and normal testicular tissue ($p < 0.001$). Moreover, even non-

testicular tissue from the tunica vaginalis displays significantly lower miR-371a-3p expression levels.

(2) Insitu hybridization confirmed the intracellular localization of M371 in all GCT subtypes, except teratoma.

(3) There is a significant positive correlation between miR-371a-3p levels and corresponding serum levels ($p < 0.05$) ($r^2 = 0.181$). This correlation was stronger in the cohort of CSI patients ($p < 0.05$) ($r^2 = 0.257$), while it was not significant in CS2 and CS3 cases ($p > 0.05$) ($r^2 = 9.5 \times 10^{-5}$).

(4) The study reveals a baseline expression of miR-371a-3p in healthy testicular tissue, such as the contralateral testis, providing a standard comparison with the notably increased levels detected in testicular GCTs.

(5) The teratoma subtype of GCT does not express miR-371a-3p, consistent with its absence in serum.

These findings indicate that circulating miR-371a-3p primarily originates from GCT tissue, making it a specific tumor marker for TGCT patients. The correlation between tissue and serum levels, enhances the potential clinical utility of miR-371a-3p as a valuable biomarker for diagnosis, therapy response, and follow-up, especially in localized cases.

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Graded expression of microRNA-371a-3p in tumor tissues, contralateral testes, and in serum of patients with testicular germ cell tumor

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Own contribution

- Experimental concept and study design: 25%
- Experiment execution and acquisition of experimental data: 60%
- Data analysis and interpretation: 30%
- Preparation of Figures and Tables: 25%
- Drafting of manuscript: 25%

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Research Paper

Graded expression of microRNA-371a-3p in tumor tissues, contralateral testes, and in serum of patients with testicular germ cell tumor

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ABSTRACT

Background: Serum levels of microRNA-371a-3p represent a specific tumor marker of testicular germ cell tumors (GCTs) but the origin of circulating miR-371a-3p is not finally resolved. The correlation between miR-levels in tissue and serum is unclear.

Results: MiR-levels in GCT tissue are 399-fold higher than in contralateral testicular tissue and 5843-fold higher than in non-testicular tissue. MiR tissue levels correlate with corresponding serum levels ($r^2 = 0.181$). ISH detected miR-371a-3p intracellularly in GCT cells except teratoma. A low expression was also detected in normal testicular germ cells.

Conclusions: Circulating miR-371a-3p is specifically derived from GCT tissue. The miR is present in GCT cells except teratoma. A low expression is also found in normal testicular tissue but not in non-testicular tissue. MiR-371a-3p levels in tissue and serum correlate significantly. This study underscores the usefulness of serum miR-371a-3p as tumor marker of GCT.

Patients and methods: Expression levels of miR-371a-3p were concurrently measured in tissues of GCT, contralateral testes ($n = 38$), and in serum ($n = 36$) with real time PCR. For control, 5 healthy testicles and 4 non-testicular tissue samples were examined. MiR-levels were compared using descriptive statistical methods. We also performed *in situ* hybridization (ISH) of GCT tissue with a probe specific for miR-371a-3p.

INTRODUCTION

Serum levels of microRNAs (miRs) of the clusters miR-371-373 and miR-302/367 have been suggested as novel biomarkers of testicular germ cell tumors (GCTs) [1–3]. Of the candidate miRs, miR-371a-3p appears to be the most promising serum marker of GCT with a sensitivity of 90.1% and specificity of 94.1% [4, 5] outperforming the classical markers (alpha fetoprotein, beta human chorionic gonadotropin, lactate dehydrogenase) with their sensitivities of less than 50% [6]. Apparently, miR-371a-3p features almost all of the qualities a valuable tumor marker is supposed to have [7] since it correlates with clinical stages, and tumor sizes, it highlights response (or non-response) to therapy, and it is present in cases with relapsing GCT suggesting a prominent role of this miR upon follow-up examinations [8–15]. Preliminary data also suggest a possible role of the test upon evaluation of residual masses after chemotherapy [13, 16, 17].

While lots of clinical data suggest a strong correlation between tumor burden and miR-371a-3p serum expression, only limited evidence is available to show that serum-based miRs do primarily originate from GCT cells and do not represent any unspecific side reaction of the testis to invasive GCT.

Testicular vein blood sampling had demonstrated that the tumor-bearing testis is most likely the source of circulating miR-371a-3p [18].

Early experiments had provided evidence for the presence of miRs 372-373 in GCT tissue [19]. Later, high-throughput screening and microarray expression profiling documented miRs 371-373 to be present in tissue of GCTs [20–24]. A study using RNA extraction from formalin-fixed paraffin embedded GCT tissue again demonstrated the presence of miR-371a-3p in tumor tissue with different expression levels in the various histological subtypes of GCT [25]. All of these studies did not directly compare the miR-expression levels in tumor tissue with corresponding serum levels in the individual patients. The only study to date that evaluated both tissue expression levels and corresponding serum levels did not find a clear correlation between these levels [26].

The aim of the present study was to further clarify the origin of circulating miR-371a-3p by measuring this miR in serum of patients with GCT and concurrently in tissues of the tumor and of the contralateral testes in the same patients. The second goal was to explore if increasing levels of miR371 in GCT tissue would translate into higher serum levels of the miR.

RESULTS

Results of microRNA expression investigations in tissues

The median miR-371a-3p expressions in tumor, corresponding contralateral testicular tissue, testicular tissue of healthy controls, and non-testicular tissue of testis-surrounding tunica vaginalis were RQ = 7,040,480.1 (IQR 4,713,672.2–13,518,390.0), RQ = 40,974.1 (IQR 30,119.7–50,549.9), RQ = 37,081.7 (IQR 31,617.2–53,543.4), and RQ = 1,204.9 (IQR = 237.5–7,809.9) respectively. Thus, the individual miR-371a-3p levels found in tumor tissue are on average 399-fold higher than those of the corresponding contralateral testicular tissue ($p < 0.001$) (Figures 1, 2). Likewise, expression levels are significantly higher in GCT tissue than in healthy testicular tissue ($p < 0.001$). MiR-371a-3p expression in healthy testicular tissue is not significantly different from that in contralateral testicular tissue ($p = 0.985$). The miR-371a-3p expression in non-testicular tissue (tunica vaginalis) showed 30.8-times lower values than testicular tissue of healthy controls ($p < 0.05$) (Figure 2). In addition, one sample of epididymis was analyzed with the lowest miR-371a-3p expression of all tissue samples (RQ = 42.03) (Table 1). There is no difference detectable between the miR-371a-3p expression of seminomas and nonseminomas ($p = 0.941$). Likewise, there is no difference between miR-371a-3p expressions in tissues of CS1 and CS 2/3 cases ($p = 0.262$) (Supplementary Figures 1 and 2).

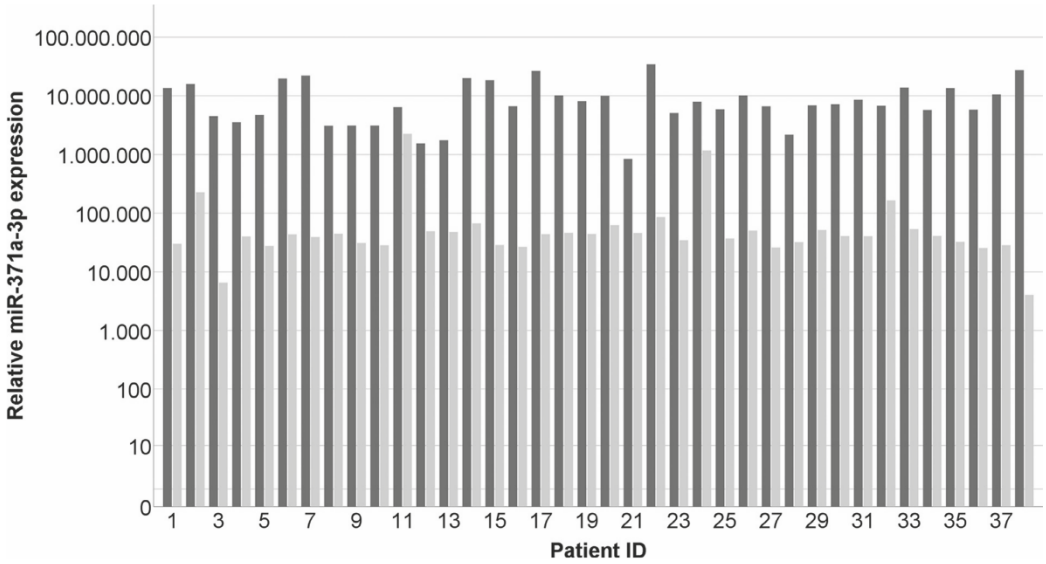

Figure 1: Individual results of measuring miR-371a-3p expressions in GCT tumor tissue samples (dark grey) and the corresponding contralateral testicle (light grey). $n = 38$. The patient ID is identical with data sets in Table 1. The y-axis is displayed in a logarithmic scale.

The ROC analysis based on miR measurements of GCT tissue and the corresponding contralateral tissue in 38 patients revealed an area under the curve of 0.997. GCT tissue can thus be discriminated from the corresponding contralateral tissue with a diagnostic sensitivity of 100% and a specificity of 94.7% (Supplementary Figure 3).

Comparison of miR-371a-3p expression in tissue with serum levels

MiR-371a-3p expression levels in GCT tissue are significantly higher than corresponding serum levels ($p < 0.001$) (Figure 3). There is a significant positive correlation between tissue levels and those found in serum ($p < 0.05$) ($r^2 = 0.181$) (Figure 4A). The correlation is stronger in the sub cohort of CS1 patients ($p < 0.05$) ($r^2 = 0.257$) (Figure 4B) than in cases with CS2 and CS3 where it is not significant ($p > 0.05$) ($r^2 = 9.5 \times 10^{-5}$) (Figure 4C).

Results of *in situ* hybridization

Figures 5 and 6 show the ISH results in the various GCT subtypes. The blue stain highlights cells expressing miR-371a-3p intracellularly (Figures 5A, 5B, 6A and 6B). An expression of miR-371a-3p was found in all subtypes of GCT except teratoma (Supplementary Figure 4). Identification of the different subtypes was achieved by additional staining with OCT4 for EC (Figure 5C), PLAP for seminoma (Figure 6C) and Glypican 3 for YST (Supplementary Figure 4). In contralateral tissue only isolated germ cells showed blue ISH signals.

DISCUSSION

There are five main results of this study: (1) The miR-371a-3p expression levels are markedly higher in GCT tissue than in the tissue of the contralateral testis and in normal testes, while non-testicular tissue from testis-surrounding layers (tunica vaginalis) has even much lower expression. (2) There is clear evidence from ISH studies that miR-371a-3p is localized within GCT tumor cells. (3) The miR expression level in GCT tissue is much higher than in serum. (4) In patients with localized disease (CS1), the GCT tissue miR-levels significantly correlate with the corresponding serum levels. (5) There is a baseline miR-371a-3p expression in tissues of contralateral testes of GCT patients and also in normal testes.

MiR-371a-3p levels in the tissues of tumor and contralateral testis

A pair-wise comparison of the expression of miR-371a-3p in GCT tissue with corresponding contralateral testicular tissue is a unique opportunity to look for the cellular origin of circulating miR-371a-3p molecules. The miR-level in GCT tissue is 399-fold higher than in contralateral testis tissue and the difference between GCT tissue and testicular tissue of control patients is almost the same. This result clearly points to the GCT tissue as the

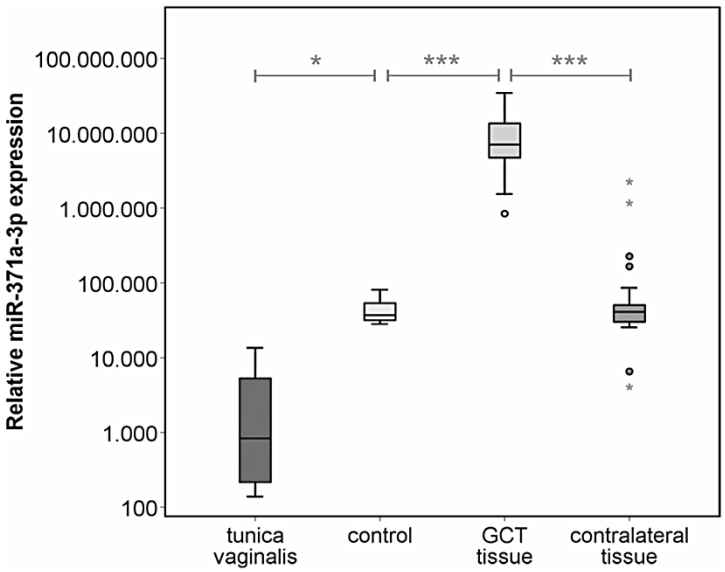

Figure 2: Relative miR-371a-3p expression in GCT tissue ($n = 38$) and corresponding contralateral testicular tissue ($n = 38$) of the same patients, healthy controls ($n = 5$) and non-testicular tissue samples of the tunica vaginalis ($n = 4$). The y-axis is plotted in a logarithmic scale. $^{***}p < 0.001$.

Table 1: Clinical data of analyzed patients

Patient ID	Age [yrs]	Diameter [mm]	Histology	Clinical Stage	site	RQ miR-371a-3p
					Contralateral tissue	30,119.70
1	38	46	Seminoma	CS2	GCT tissue	13,518,389.95
					Serum	2,881.17
					Contralateral tissue	226,385.18
2	34	36	Seminoma	CS1	GCT tissue	15,854,840.19
					Serum	5,509.17
					Contralateral tissue	6,555.18
3	38	19	Seminoma	CS1	GCT tissue	4,490,423.24
					Serum	669.09
					Contralateral tissue	40,019.62
4	28	31	Non-Seminoma	CS2	GCT tissue	3,547,619.87
					Serum	6,537.03
					Contralateral tissue	27,715.78
5	29	24	Non-Seminoma	CS1	GCT tissue	4,713,672.19
					Serum	3,287.19
					Contralateral tissue	43,490.72
6	36	54	Seminoma	CS1	GCT tissue	19,655,367.47
					Serum	39,571.18
					Contralateral tissue	39,196.03
7	32	42	Seminoma	CS1	GCT tissue	22,113,437.94
					Serum	41,500.32
					Contralateral tissue	44,404.55
8	51	18	Seminoma	CS1	GCT tissue	3,088,382.47
					Serum	1,096.01
					Contralateral tissue	31,181.87
9	45	67	Seminoma	CS1	GCT tissue	3,109,863.87
					Serum	7,068.64
					Contralateral tissue	28,494.98
10	41	45	Seminoma	CS1	GCT tissue	3,109,863.87
					Serum	6,313.85
11	25	26	Seminoma	CS1	Contralateral tissue	2,245,211.62
					GCT tissue	6,439,065.97
					Contralateral tissue	49,612.64
12	36	31	Seminoma	CS1	GCT tissue	1,544,191.24
					Serum	2,131.85
					Contralateral tissue	47,922.65
13	40	37	Seminoma	CS1	GCT tissue	1,749,389.37
					Serum	10,019.87
					Contralateral tissue	66,839.81
14	27	41	Non-Seminoma	CS1	GCT tissue	19,929,746.17
					Serum	120,356.55
					Contralateral tissue	28,693.18
15	36	33	Non-Seminoma	CS1	GCT tissue	18,466,664.88
					Serum	4,629.83
					Contralateral tissue	26,586.75
16	37	52	Non-Seminoma	CS1	GCT tissue	6,620,092.75
					Serum	40,760.69
					Contralateral tissue	43,793.22
17	30	22	Seminoma	CS2	GCT tissue	26,480,371.01
					Serum	2,652.53
					Contralateral tissue	46,290.23
18	34	73	Seminoma	CS3	GCT tissue	10,103,977.53
					Serum	56,414.42
					Contralateral tissue	44,036.74
19	31	68	Seminoma	CS1	GCT tissue	8,110,840.54
					Serum	4,772.14
					Contralateral tissue	62,623.65
20	49	18	Seminoma	CS1	GCT tissue	9,930,397.24
					Serum	288.5
					Contralateral tissue	46,002.35
21	47	30	Seminoma	CS1	GCT tissue	841,392.83
					Serum	100.78
					Contralateral tissue	85,902.95
22	34	34	Seminoma	CS1	GCT tissue	34,412,276.52
					Serum	16,913.42
					Contralateral tissue	34,646.45
23	34	33	Seminoma	CS1	GCT tissue	5,083,603.22
					Serum	469.32

					Contralateral tissue	1,167,847.12
24	38	50	Non-Seminoma	CS3	GCT tissue	7,899,993.31
					Serum	4,434.08
					Contralateral tissue	37,210.44
25	34	11	Seminoma	CS1	GCT tissue	5,859,800.02
					Serum	290.67
					Contralateral tissue	50,549.88
26	35	75	Seminoma	CS2	GCT tissue	10,076,002.16
					Serum	3,402.60
					Contralateral tissue	25,931.54
27	31	n. a.	Non-Seminoma	CS2	GCT tissue	6,601,763.38
					Serum	32,461
					Contralateral tissue	32,214.44
28	41	6	Seminoma	CS1	GCT tissue	2,191,397.85
					Serum	445.85
					Contralateral tissue	51,647.83
29	66	29	Seminoma	CS2	GCT tissue	6,891,659.54
					Serum	2,007.83
					Contralateral tissue	40,860.52
30	53	42	Seminoma	CS1	GCT tissue	7,189,300.62
					Contralateral tissue	40,550.16
31	47	37	Seminoma	CS2	GCT tissue	8,585,198.14
					Serum	231.91
					Contralateral tissue	166,068.62
32	52	48	Seminoma	CS1	GCT tissue	6,777,961.45
					Serum	840.1
					Contralateral tissue	53,692.02
33	40	56	Seminoma	CS1	GCT tissue	13,792,876.25
					Serum	4,638.43
					Contralateral tissue	41,087.73
34	30	45	Non-Seminoma	CS2	GCT tissue	5,743,186.58
					Serum	11,405.33
					Contralateral tissue	32,709.45
35	47	14	Non-Seminoma	CS1	GCT tissue	13,499,662.47
					Serum	516.07
					Contralateral tissue	25,468.39
36	34	47	Seminoma	CS1	GCT tissue	5,799,190.10
					Serum	9,597.35
					Contralateral tissue	28,593.90
37	51	22	Seminoma	CS1	GCT tissue	10,547,662.66
					Serum	3,634.19
					Contralateral tissue	4,060.44
38	47	16	Seminoma	CS2	GCT tissue	27,376,221.46
					Serum	18,247.61
39	47	0	Control	normal	Control	31,617.16
40	63	0	Control	normal	Control	53,543.36
41	19	0	Control	normal	Control	37,081.71
42	60	0	Control	normal	Control	28,298.15
43	54	0	Control	normal	Control	81,156.55
44	78	0	Control	normal	*Tunica vaginalis*	138.88
45	20	0	Control	normal	*Tunica vaginalis*	336.12
46	52	0	Control	normal	*Tunica vaginalis*	42.03
47	33	0	Control	normal	*Tunica vaginalis*	13,546.11
48	46	0	Control	normal	Epididymis	2,073.66

Abbreviations: CS: Clinical stage, GCT: Germ cell tumor, mm: millimeter, RQ: Relative quantity, yrs: Years.

source of circulating miR-371a-3p. Previous reports had already pointed to the presence of the miR-371-373 cluster in GCT tissue, without quantitative measurements [20], [21]. Our result is consistent with data from measuring the miR in testicular vein blood where a 195-fold higher level was found than in systemic circulation [18]. The present finding of a base-line expression of miR-371a-3p in contralateral testis tissue and in healthy testicular tissue mirrors the finding of a 4-fold higher miR-expression in testicular vein blood compared to peripheral blood in healthy males [18]. These data suggest the presence of this particular miR even in normal testicular tissue though in low quantity and not exclusively in GCT tissue. This assumption is supported by the findings of Boellaard *et al.* who recently documented the presence of miR-371a-3p in normal testicular tissue and its absence in other parts of the urogenital tract [27]. As miR-371 has been shown to be specifically associated with human stem cells [28–30] and as it is found in seminal plasma, too [31, 32], it is rational to assume that this miR is specifically generated by normal testicular germ cells and most probably even more by the cells of GCT.

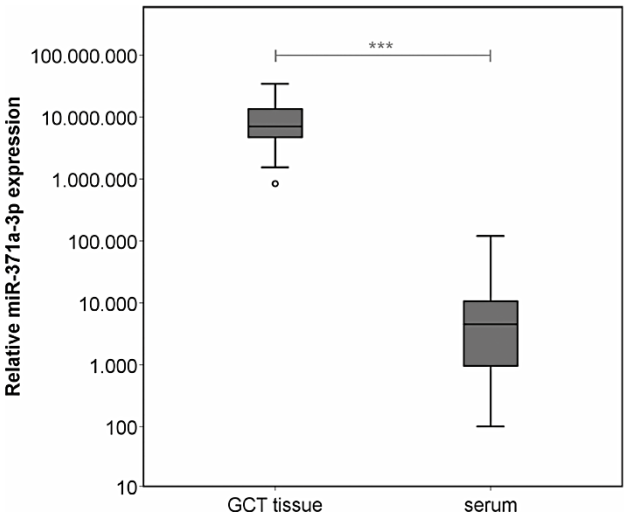

Figure 3: Relative miR-371a-3p expression in GCT tissue (n = 38) and corresponding preoperative serum samples of the same patients (n = 36). The y-axis is plotted in a logarithmic scale. $^{***}p < 0.001$.

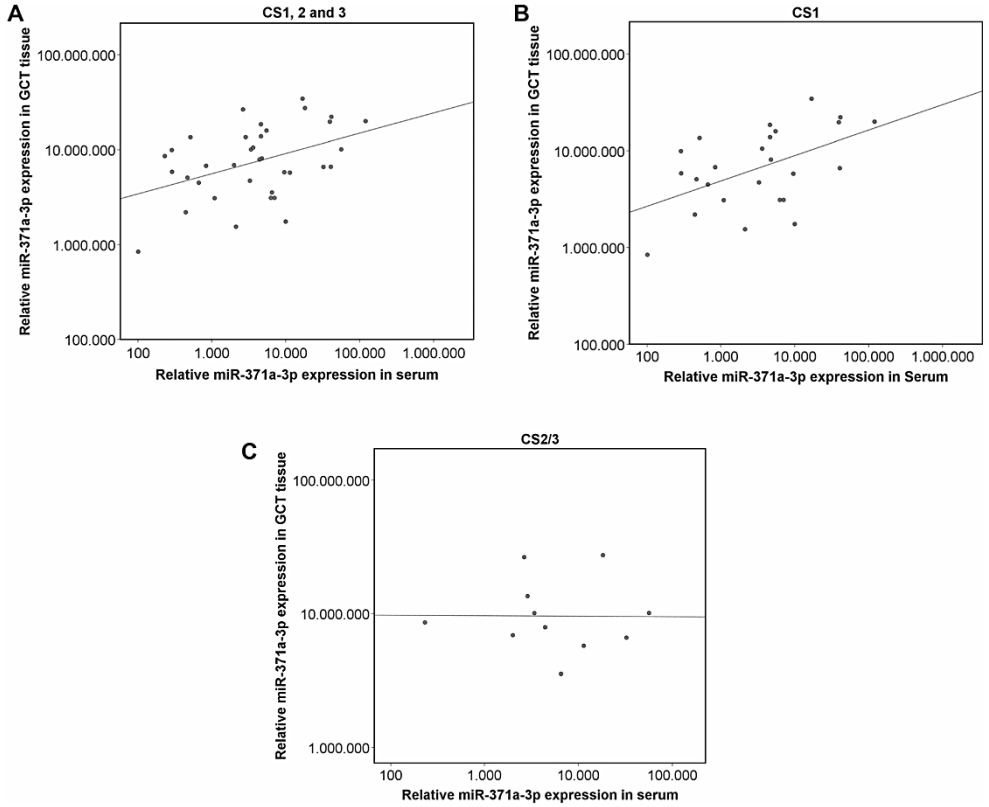

Figure 4: Scatterplot of the relative miR-371a-3p expression in GCT tissue and corresponding serum levels. The axes in all parts are depicted in a logarithmic scale. (**A**) Entire GCT cohort (n = 36) ($p \leq 0.05$) ($r^2 = 0.181$). (**B**) Only CS1 patients (n = 25; $p \leq 0.05$) ($r^2 = 0.257$). (**C**) Only CS2/3 patients (n = 11; $p \geq 0.05$) ($r^2 = 9.5 \times 10^{-5}$).

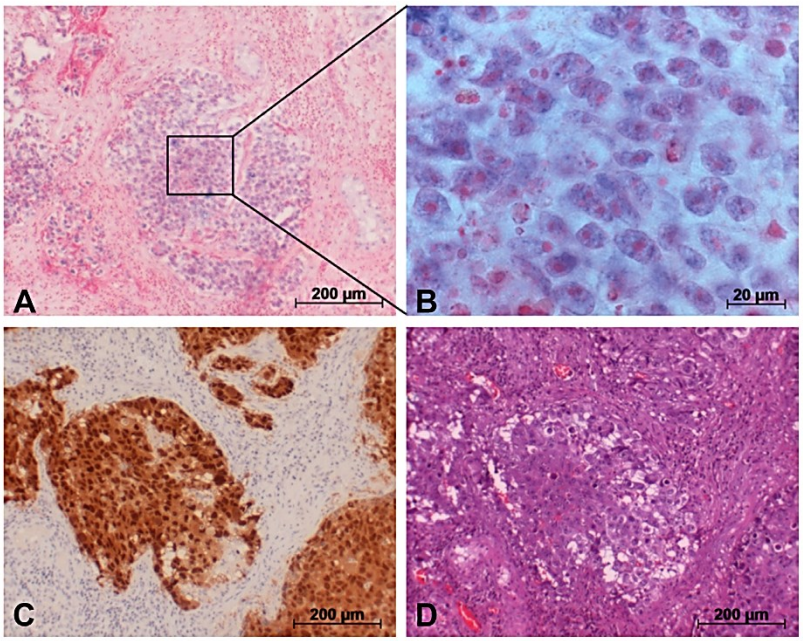

Figure 5: Detection of miR-371a-3p in GCT tumor (EC) via *in situ* hybridization. (**A**) *In situ* hybridization with a probe against miR-371a-3p causes blue staining in cells. (**B**) Section from A. (**C**) Immunohistochemical staining of the same area with an OCT4 antibody for identification of EC cells. (**D**) H&E staining of the same area.

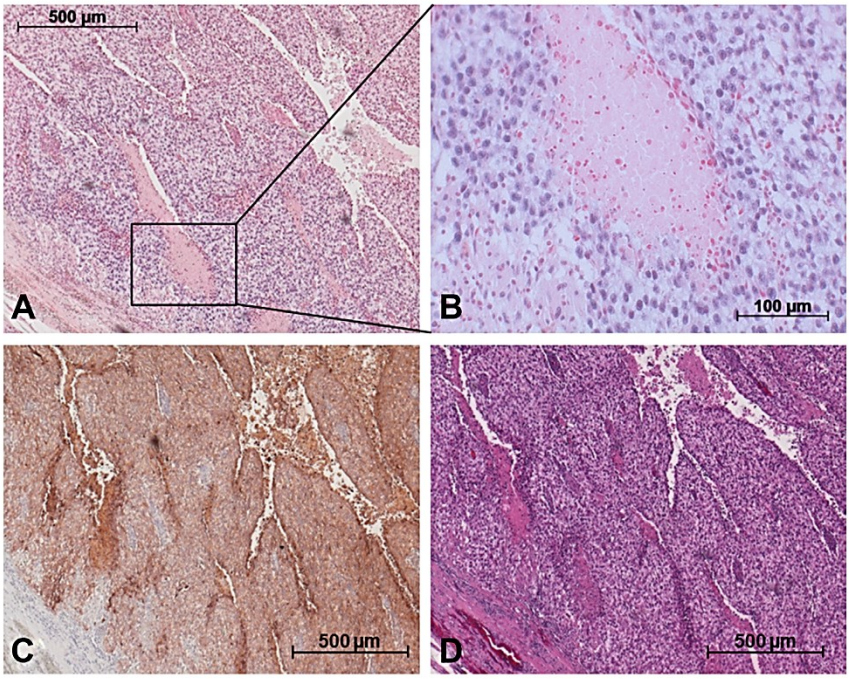

Figure 6: Detection of miR-371a-3p in GCT mixed tumor including SE and YST via *in situ* hybridization. (**A**) *In situ* hybridization with a probe against miR-371a-3p causes blue staining in cells. (**B**) Section from A. (**C**) Immunohistochemical staining of the same area with PLAP antibody for identification of SE cells. (**D**) H&E staining of the same area.

In serum, patients with nonseminomas showed significantly higher miR-371a-3p expression, and CS2/3 patients had higher levels than those with CS1 [5]. In tissue no difference was detectable between the miR-371a-3p expression of seminomas and nonseminomas nor between tissues of CS1 and CS 2/3 cases. Yet, these results should be interpreted with caution as only a small patient collective was examined.

Intracellular localization of miR-371a-3p by ISH

In situ hybridization clearly demonstrated microRNA-371-3p to be localized intracellularly in the cells of GCT. Thus, the ISH experiments morphologically supplement the data obtained by measuring the miR in homogenized GCT tissue.

In conjunction with previous reports on the presence of the miR-371-373 cluster in GCT tissue [20, 21, 25, 33] there is now ample evidence for the origin of circulating miR-371a-3p from testicular tumor cells.

Of note, all subtypes of GCT except teratoma stained positive for the miR-371a-3p probe. The absence of miR-371a-p staining in teratoma cells is consistent with the non-expression of this miR in serum of GCT patients [4, 5, 16]. Likewise, in an evaluation of miR-371a-3p expression levels in GCT subtypes by RNA extraction from formalin fixed paraffin embedded orchiectomy specimens, Vilela-Salgueiro *et al.* revealed a strong expression in all GCT subtypes except teratoma [25]. The reason for the non-expression of the miR by teratoma is probably related to the analogies of GCT biology and the human embryonal development [34, 35]. While most of the GCT subtypes mimic early developmental stages of embryonal development and accordingly retain their biochemical characteristics including the microRNA profile of stem cells, the teratoma subtype represents a more advanced and more mature histological subtype that has lost all of the biochemical characteristics of stem cells particularly the typical expression of miR-371a-3p [36].

Correlation of miR-expression levels in tissue with serum levels

Systematic paired measurements of miR-expression levels in GCT tissue and corresponding serum levels had not been reported so far. One early pilot study had indicated that miR-expression levels of tissue and serum do not correlate [24]. However, the present systematic analysis of 36 patients revealed a significant correlation of the expression levels of the two compartments. It is of note that this correlation was not significant in cases with clinical stages CS2 and 3. As in advanced clinical stages the marker substance is released from both the primary tumor and metastatic seeds it is rational that the correlation of tissue expression with serum levels is only significant in cases confined to the testis. The serum level of circulating miR-371a-3p is obviously a product of the number of miR-producing tumor cells (tumor bulk) as shown previously [5] and of the specific secreting capacity of the individual GCT as shown herein. Most probably, additional biological determinants e. g. direct vascular invasion of the tumor and other hitherto unknown factors do also affect the serum level of miR-371a-3p.

In all, the present evaluation confirms the understanding that circulating miR-371a-3p-are specifically derived from cells of testicular germ cell neoplasms.

This miR, thus represents a specific tumor marker for GCTs, which is not expressed in other diseases. By contrast, the specificity of the classical tumor marker AFP is considerably hampered by its association with non-GCT related conditions, such as liver diseases [37].

MATERIALS AND METHODS

Patients for tissue and serum investigations

Preoperative serum samples and corresponding tissue specimens were collected from patients (median age 36.5) undergoing surgery for testicular tumor. GCT tissue was taken from 38 orchiectomy specimens, and contralateral testis tissue was taken from corresponding contralateral biopsy specimens. This surgical procedure was routinely performed on all patients with suspected testis tumor to look for Germ cell neoplasia *in situ* (GCNis) according to institutional guide-lines. None of the patients enrolled in this study had contralateral GCNis. All tissue specimens were kept frozen at –80° C under further processing. Histologically, the GCT tissue specimens consisted of 29 seminomas and nine nonseminomas. Clinically, 27 patients had clinical stage 1 (CS1) and eleven CS 2 and 3 (CS2/3). Preoperative serum samples were obtained from 36 of the 38 patients. For control, testicular tissue was obtained from five men without GCT undergoing orchiectomy for epididymitis. Furthermore, four samples of testis surrounding tunica vaginalis and one specimen of the epididymis were analyzed. All patients had given informed consent prior to surgery. Ethical approval of the study was provided by Ärztekammer Bremen (reference No 301, 2011). All study activities had been conducted according to the Declaration of Helsinki of the World Medical Association (as amended by the 64th General Assembly, 2013). Individual data of the patients and controls are listed in Table 1.

Patients for histological investigation of presence of microRNAs in GCT cells

Formalin-fixed paraffin-embedded (FFPE) samples of six patients with testicular GCTs were analyzed by immunohistochemistry and *in situ* hybridization (ISH) to look for the presence of miR-371a-3p in tumor tissue. Histologically, the GCTs comprised of three mixed nonseminomatous tumors (embryonal carcinoma, yolk sac

tumor, and chorio carcinoma), one pure seminoma, one pure embryonal carcinoma and one teratoma. In addition, one tissue specimen of a contralateral testis was analyzed.

Extraction and measurement of miRNAs

Tumor and contralateral testis tissue (10–50 mg) was homogenized in 1000 µL TRIzol® Reagent following the manufacturer's instructions (Fisher Scientific, Schwerte, Germany) using a TissueLyser (Qiagen, Hilden, Germany) with 5 mm steel beads for 10 min at 30 Hz. The extracted RNA was resuspended in 50 µl nuclease-free water.

For the measurement of miR-371a-3p levels, RNA was isolated from 200 µL Serum using the miRNeasy mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's description. Reverse transcription (RT) was performed with 10–20 ng/µL RNA isolated from tissue and 6 µL RNA isolated from Serum, using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Standard PCR was carried out for preamplification of the cDNA with TaqMan Assays for miR-371a-3p (assay ID 002124) and the endogenous control miR-93-5p (assay ID 000432) in a 1:100 dilution. Measurement of the miRNA expression was performed with quantitative real-time PCR (RT-qPCR) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) using FAST Start Universal Probe Master (Roche Diagnostics, Mannheim, Germany) and the undiluted TaqMan Assays. The relative quantity (RQ) was calculated using the $2^{-\Delta\Delta CT}$-method [38].

Immunohistochemistry

For morphological identification of the histological type of GCTs sections of 5 µm of FFPE-blocks were analyzed with hematoxylin and eosin stain (H&E stain), OCT4, PLAP and glypican 3. Staining with hematoxylin and eosin with standard histological techniques were used to distinguish between tumor-free areas and tumor tissue. OCT4 staining for identification of embryonal carcinomas (EC), PLAP staining for seminomas (SE) and glypican 3 for yolk-sac tumors (YST) (Diagnostic BioSystems, Pleasanton, CA, USA) were then conducted according to institutional standard operating procedures [39, 40].

MicroRNA *in situ* hybridization

After immunohistochemical identification of the particular GCT-subtypes, the corresponding tumor sections were subsequently processed for *in situ* hybridization (ISH) with a miRCURY LNA probe (Exiqon, Vedbaek, Denmark; probe ID 38555-15) specific for miR-371a-3p. The protocol was performed according to the manufacturer's instructions using a proteinase-K concentration of 15 µg/ml, a hybridization temperature of 51° C and a probe concentration of 80 nM. Microscopic evaluations were performed on an Axioskop 2 plus microscope (Zeiss, Göttingen, Germany). Histological findings were documented using the AxioCam HRc digital camera (Zeiss, Göttingen, Germany) and then edited with AxioVision Software v.4.8 (Zeiss, Göttingen, Germany). Presence of miR-371a-3p within GCTs was defined by distinct blue staining of the cells, and accordingly, only these cells were considered miR-371a-3p positive. Only the presence or absence of the miR-371a-3p in the specimen was evaluated, no quantification was attempted.

Statistical methods

The Wilcoxon signed rank test was used for comparison of dependent subgroups. The Mann-Whitney U test was used to compare median miRNA expressions among the various subgroups. Receiver Operating characteristics (ROC) curves were calculated to analyze the sensitivity and specificity of tissue miR-levels to distinguish GCT tissue from non-tumorous tissue. Spearman's rank correlation coefficient was calculated to determine correlations. All tests were two-sided and significance was assumed at $p < 0.05$. Statistical analysis was performed using SPSS version 24 (IBM, Armonk, NY, USA).

LIMITATIONS

The results of the present study rest on 36 patients only, thus the statistical power is still limited. With respect to sample processing, the time for transfer of the tissue specimens from operation site to the laboratory i. e. the time-interval until conservation in the freezer (–80° C) varied from 12 hours to 36 hours depending on the conditions of surface mail. Thus, deterioration of some samples during transfer cannot entirely be excluded. We could not correlate the miR-measurements in contralateral testicular tissue specimens with the quality of spermatogenesis because these data were not available.

CONCLUSIONS

The present study provides much of evidence for the understanding that circulating miR-371a-3p molecules are specifically derived from testicular GCT cells. The inability of teratoma cells to produce miR-371a-3p is confirmed on the tissue level. There is a significant correlation of the miR-expression levels in GCT tissue with corresponding serum levels. Normal testicular tissue displays a low baseline expression of miR-371a-3p pointing to the role of the miR in human stem cells.

Abbreviations

AFP: Alpha fetoprotein; bHCG: Beta human chorionic gonadotropin; CS: Clinical stage; EC: Embryonal

carcinomas; FFPE: Formalin-fixed paraffin-embedded; GCNis: Germ cell neoplasia *in situ*; GCT: Germ cell tumor; H&E: Hematoxylin and eosin; IQR: Inter quartile range; ISH: *In situ* hybridization; LDH: Lactate dehydrogenase; miR: micro RNA; M371: miR-371a-3p; OCT4: Octamer-binding transcription factor 4; PLAP: Placental alkaline phosphatase; qPCR: Quantitative real-time PCR; RT: Reverse transcription; ROC: Receiver operating characteristics; RQ: Relative quantity; SE: Seminoma; SPSS: Statistical package for the social sciences; YST: Yolk-sac tumor.

Author contributions

GB, FH, CD, AR, KPD designed the study; KPD patient enrolment; FH, CD, FG executed the experiments; GB, FH, CD, KJ, AR, KPD analysis and interpretation of the data; FH, AR, FG statistical analysis; GB, FH, CD, AR, KPD drafting of manuscript. All authors critically revised and finally approved the manuscript.

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CONFLICTS OF INTEREST

Gazanfer Belge and Klaus-Peter Dieckmann each possess 9.7% ownership shares of miRdetect GmbH, Bremen, a start-up company aiming to develop a commercially available test for measuring microRNAs in serum. MiRdetect holds a patent for the measurement of miRNA in body fluids at the limit of detection. All other authors declare no competing interests towards this report.

A further patent for the use of miR-371a-3p as a marker for GCNis was filed by miRdetect/University of Bremen on November, 2nd 2016.

Arlo Radtke is an employee of miRdetect GmbH, Bremen since January 2019.

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3.2 Publication II: Associations of serum levels of microRNA-371a-3p (M371) with risk factors for progression in nonseminomatous testicular germ cell tumours clinical stage 1

The previous study by Belge et al. (2020) showed evidence that miR-371a-3p is a specific biomarker for testicular GCT. This study focuses on clinical stage I nonseminomatous tumors, comprising of 20% of all patients with TGCTs (Cheng et al., 2018). The study underscores that established risk factors like tumor size and stromal rete testis invasion in testicular GCTs have limitations. Notably, even small GCTs under 10 mm can induce elevated M371 serum levels, which gave rise to the hypothesis that persistently elevated postoperative M371 levels could indicate occult metastases in CS1 patients (Murray et al., 2016; Dieckmann et al., 2019b).
This study investigates the associations between M371 levels and histopathological factors like lymphovascular invasion (LVI) and embryonal carcinoma in CS1 testicular non-seminoma patients to test following hypotheses:

(1) Both risk factors, LV1 and predominance of EC are associated with postoperatively elevated M371 levels.
(2) Preoperative M371 levels are higher in patients with risk factors LV1 and/or EC than in those without these factors.
(3) The relative decrease of postoperative M371 levels is greater in patients with the factor (LV1) than in those without (LV0).

The study revealed that before orchiectomy, 84.7% of patients exhibited elevated M371 levels, which decreased to 29,4% postoperatively. Preoperative serum levels were significantly higher in the LV1 subgroup than in the LV0 group, with an AUC of 0.732 for predicting LV1 status. However, contrary to expectations, there was no difference in the postoperative median M371 level between the LV1 and LV0 subgroups. Conversely, postoperative levels were not associated with LV status, indicated by an AUC of 0.5 in the ROC curve. Regarding embryonal carcinoma, higher levels of EC (> 50% EC) are associated with elevated preoperative median M371 levels than that of the subgroup with < 50% EC ($p = 0.008$, Mann–Whitney U test). However, postoperatively, that difference was not statistically significant ($p = 0.112$).
Additionally, tumor size showed a clear association with preoperative M371 levels, with larger tumors being correlated with a higher M371 expression. Teratoma components in the primary tumor were associated with both LV1 and >50% EC.

The relative decrease of M371 after orchiectomy was not significantly different between LV1 and LV0 subgroups, however when analyzing absoulute differences (preoperative M371 minus postoperative M371) in a waterfall plot, LV1 patients exhibit significantly greater decreases compared to LV0 patients (p = 0,000059, Mann-Whitney U test).

In conclusion, the study found no clear association between postoperatively elevated M371 levels and the LV status, suggesting that persistent elevations could be linked to larger tumors with a slower M371 decay. If blood samples were obtained too early after orchiectomy, elevated M371 levels may still be attributed to the primary tumor, because the decay of the miRNA released by the tumor was still incomplete. Further research is needed to comprehensively investigate the clinical significance of preoperative M371 levels, particulary in relation to lymphovascular invasion and their potential as predictors for disease progression in CS1 testicular germ cell tumors.

-II-

Associations of serum levels of microRNA-371a-3p (M371) with risk factors for progression in nonseminomatous testicular germ cell tumours clinical stage 1

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Own contribution

- Experimental concept and study design: 25%
- Experiment execution and acquisition of experimental data: 80%
- Data analysis and interpretation: 30%
- Preparation of Figures and Tables: 50%
- Drafting of manuscript: 30%

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TOPIC PAPER

Associations of serum levels of microRNA-371a-3p (M371) with risk factors for progression in nonseminomatous testicular germ cell tumours clinical stage 1

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Abstract

Purpose Lymphovascular invasion (LV1) and presence of > 50% embryonal carcinoma (> 50% EC) represent risk factors for progression in patients with clinical stage 1 (CS1) nonseminomatous (NS) testicular germ cell tumours. As serum levels of microRNA-371a-3p (M371) are capable of detecting small amounts of GCT, we evaluated if LV1 and > 50% EC are associated with M371 levels.

Methods M371 serum levels were measured postoperatively in 153 NS CS1 patients and both pre- and postoperatively in 131 patients. We registered the following factors: age, tumour size, LV status, > 50% EC, teratoma in primary, preoperative elevation of classical tumour markers. M371 expression was compared among subgroups. The ability of M371 to predict LV1 was calculated by receiver operating characteristics (ROC) curves. Multiple regression analysis was used to look for associations of M371 levels with other factors.

Results Postoperatively elevated M371 levels were found in 29.4% of the patients, but were neither associated with LV status nor with > 50% EC. Likewise, relative decrease of M371 was not associated. ROC analysis of postoperative M371 levels revealed an AUC of 0.5 for the ability to predict LV1 while preoperative M371 had an AUC of 0.732. Multiple regression analysis revealed significant associations of preoperative M371 levels with LV status ($p = 0.003$), tumour size ($p = 0.001$), > 50% EC ($p = 0.004$), and teratoma component ($p = 0.045$).

Conclusion Postoperatively elevated M371 levels are not associated with risk factors for progression in NS CS1 patients. However, the significant association of preoperative M371 expression with LV1 deserves further evaluation.

Keywords Germ cell tumour · Tumour marker · MicroRNA · Vascular invasion · Embryonal carcinoma · Nonseminoma

Abbreviations

AFP	Alpha fetoprotein
bHCG	Beta human chorionic gonadotropin
CS	Clinical stage

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EC	Embryonal carcinoma
GCT	Germ cell tumours
IQR	Interquartile ranges
LDH	Lactate dehydrogenase
LV	Lymphovascular invasion
miRNA	MicroRNA
M371	MicroRNA-371a-3p
NS	Nonseminomatous
pT	Pathological stage
RLND	Retroperitoneal lymph node dissection
ROC	Receiver operating characteristics
RQ	Relative quantity
SPSS	Statistical package for the social sciences
ULN	Upper limit of norm

Introduction

Clinical stage 1 (CS1) nonseminomatous tumours (NS) comprise of 20% of all patients with testicular germ cell tumours (GCTs) [1]. However, accurate clinical staging is hampered by the inability of imaging procedures to detect micro-metastatic seeds [2, 3] and by the non-expression of tumour markers alpha fetoprotein (AFP), beta human chorionic gonadotropin (bHCG) and lactate dehydrogenase (LDH) in almost 50% of the cases [4, 5]. Approximately, 30% of NS cases classified as clinical stage 1 (CS1) do actually harbour microscopic neoplastic foci in the retroperitoneal nodes that will inevitably progress to overt metastatic disease [6]. Clinico-pathological risk factors may predict the presence of occult metastases and may, thus, aid decision-making with respect to prophylactic chemotherapy [7, 8]. Lymphovascular invasion (LV1, also characterised as pathological stage pT2 according to the TNM classification) is the most widely recognised risk factor that indicates a 50–60% risk of developing metastases if no treatment is administered [9]. Another promising risk marker is the presence of embryonal carcinoma (EC) in the primary tumour [10]. However, it is unresolved which proportion of EC is relevant for predicting occult metastases [11]. Other factors, e.g. tumour size and stromal rete testis invasion have shown some promise but have not yet reached international consensus. Noteworthy, lymphovascular invasion involves low specificity, since 15–20% of CS1 NS patients will progress despite the absence of vascular invasion (LVo) and conversely, about 40–50% with the factor (LV1) will not. Another problem of histology-based risk factors is inter-observer variation in the assessment of testicular pathological details which relates to the over-all rarity of testicular neoplasms [12, 13].

Serum levels of microRNA-371a-3p (M371) have been shown to be a promising novel biomarker of germ cell tumours outperforming the classical markers [14–16]. Even small GCTs of less than 10 mm of size can generate elevated M371 serum levels [17, 18]. Based on the high sensitivity of > 90%, it has been speculated that this marker would also be informative in patients harbouring small volume occult metastases [19, 20]. A prospective study had shown that M371 serum levels dropped after orchiectomy in 92% of CS1 patients, but around 20% of them had still elevated M371 levels postoperatively [17]. Although no follow-up of these particular cases was available, the hypothesis was raised that elevated M371 levels after surgery of CS1 patients could denote those with occult metastases [16]. If this hypothesis is correct, then a large number of patients with postoperatively elevated M371 levels are expected to have the hitherto known predictors of progression in their primary tumours. At present, there are no data to substantiate this possible association. We measured M371 serum levels in patients with CS1 testicular NS and tested the following hypotheses: (1) both, LV1 and predominance of EC are associated with postoperatively elevated M371 levels, (2) preoperative M371 levels are higher in patients with risk factors LV1 and/or EC than in those without these factors, and (3) the relative decrease of postoperative M371 levels is greater in patients with the factor (LV1) than in those without (LVo). In addition, we looked for associations of a number of other clinico-pathological factors with M371 levels in an exploratory approach.

Patients, methods

In a multicentric study, a total of 153 patients with CS1 NS testicular tumours aged 18–56 years, underwent measurement of postoperative serum levels of M371 of whom 131 also had measurements before orchiectomy. After blood aspiration serum aliquots were kept deep-frozen at minus 80 °C until processing. The laboratory measurement technique had been fully described earlier [17]. A serum level of RQ = 5 was considered the upper limit of norm (ULN). Patients were prospectively enrolled during 2016–2020, and 81 had been included in previous evaluations [17, 21]. In each case we registered patient´s age (years), presence of lymphatic or vascular invasion of the tumour (LV1: yes/LVo: no), presence of > 50% EC in the primary (yes/no), teratoma as component of the primary (yes/no), tumour size (mm), preoperative elevation of AFP, bHCG, and LDH, respectively (yes/no). Histopathological details were retrieved from local pathology reports without central pathological review.

Patients´ data were initially stored in a commercially available data base system (MS Excel, Microsoft Corp., Redmond, USA, version 2017) and transferred to SPSS software (SPSS Inc., IBM Corp, Armonk, NY, USA, version 24) for final evaluation. Statistical analysis comprised of calculating median and interquartile ranges (IQRs) with regard to age and tumour size, respectively. We calculated

relative frequencies of the presence of LV1, > 50% EC, teratoma as component, and elevations of classical tumour markers. Median and IQRs of the relative quantity (RQ) values of preoperative and postoperative M371 serum levels were calculated. To look for associations of M371 expression with vascular invasion, we compared the subgroups of LV1 and LVo with regard to the frequencies of M371 elevations and also regarding the median RQ values of serum levels of the two subgroups. Separate comparisons were done with preoperative and postoperative measurements. The same calculations were done with the subgroups with > 50% EC and < 50% EC, respectively. The subgroups of LV1 and LVo were compared to each other with regard to the absolute and relative decreases from preoperative M371 to postoperative levels. Receiver operating characteristics curves (ROC) were calculated to evaluate the sensitivity and specificity of preoperative and postoperative M371 expression to predict LV status. We also compared the subgroups LV1 and LVo with regard to frequencies of M371 elevations in marker-positive and marker-negative patients.

For statistical comparison of proportions (univariate categorical variables), the chi-squared test was used. For comparison of continuous variables, the Mann–Whitney *U* test and Wilcoxon signed-rank test were used. A $p < 0.05$ was considered significant. The association of preoperative M371 expression with tumour size in either LVo or LV1 was determined with linear regression analysis. Multiple regression analysis was used to look for the dependence of M371 expression from the factors tumour size, patients' age, EC content, classical marker expression and the LV status.

The study received ethical approval by Ärztekammer Bremen (#301, 2015). All patients gave informed consent prior to their inclusion in the study. All of the study activities conformed to the Helsinki Declaration of the World Medical Association (as amended by the 64th General Assembly, 2013).

Results

Elevated M371 levels were found in 111 patients before orchiectomy (84.7%), and postoperatively in 45 patients (29.4%). Lymphovascular invasion (LV1) was registered in 64 patients (41.8%). Further clinical and pathological details of the patients are listed in Table 1.

The preoperatively measured M371 serum levels are significantly higher in the LV1 subgroup than in the LVo group. The postoperative median M371 level of the LV1 subgroup is not different from that of the LVo subgroup (Table 2, Fig. 1). ROC curves revealed an AUC of 0.732 for the ability of preoperative M371 levels to predict the LV1 status (Fig. 2a). Postoperative M371 levels are not associated with the LV status as shown by an AUC of 0.5 in the ROC curve (Fig. 2b).

The relative decrease of serum M371 levels after surgery is not significantly different among the subgroups of LV1 and LVo. However, if absolute differences of RQ levels (preoperative M371 minus postoperative M371 level) are analysed in a waterfall plot (Fig. 3), it becomes obvious that LV1 patients have significantly greater decreases of M371 after surgery than LVo patients ($p = 0.000059$, Mann–Whitney *U* test).

Regarding embryonal carcinoma, the preoperative median M371 level of the > 50% EC subgroup is significantly higher than that of the subgroup with < 50% EC ($p = 0.008$, Mann–Whitney *U* test; Table 3). Also, the proportion of elevated M371 levels is significantly higher in the > 50% EC group (93.9%) than in the group with < 50% EC (75.4%)

Table 1 Patients' characteristics

	Eligible (*n*)	Results
Patients' age: median; IQR [years]	153	30; 25.5–37.0
M371—preoperative serum level elevated (*n*, %)	131	111 (84.7%)
M371—postoperative serum level elevated (*n*, %)	153	45 (29.4%)
With vascular invasion (LV1) (*n*, %)	64	64 (41.8%)
Without vascular invasion (LVo) (*n*, %)	89	89 (58.2%)
With > 50% EC in primary tumour (*n*, %)	152	79 (52.0%)
Teratoma component in primary tumour (*n*, %)	152	83 (54.6%)
Tumour size: median; IQR [mm]	149	27; 18.0–42.0
AFP—preoperative serum level elevated (*n*, %)	152	75 (49.3%)
bHCG—preoperative serum level elevated (*n*, %)	152	66 (43.4%)
LDH—preoperative serum level elevated (*n*, %)	147	13 (8.8%)
Any marker elevation preoperatively (*n*, %)	149	93 (62.4%)

LV1 lymphovascular invasion, *LVo* without lymphovascular invasion, *EC* embryonal carcinoma, *IQR* interquartile range

Table 2 Results of preoperative and postoperative of M371 measurements and comparisons of subgroups LVo versus LV1

	Eligible (*n*)		LVo	LV1	*p* value	Comparison LVo vs LV1
Preoperative M371 measurement (*n*)	131		80	51		
M371 elevated (*n*; %)	111	84.7%	64 (80.0%)	47 (92.2%)	0.081	Proportions of M371 elevation[a]
M371 elevated (median RQ; IQR)			76.74; 11.51–271.78	902.13; 74.70–2724.65	0.000012	Comparison of RQ values[b]
Postoperative M371 measurement	153		89	64		
M371 elevated (*n*; %)	45	29.4%	24 (27.0%)	21 (32.8%)	0.475	Proportions of M371 elevation[a]
M371 elevated (median RQ; IQR)			0.54; 0.00–5.49	0.26; 0.00–15.99	0.995	Comparison of RQ values[b]

RQ relative quantity, *IQR* interquartile range, *LV1* lymphovascular invasion, *LVo* without lymphovascular invasion

[a]Chi-squared test

[b]Mann–Whitney *U* test

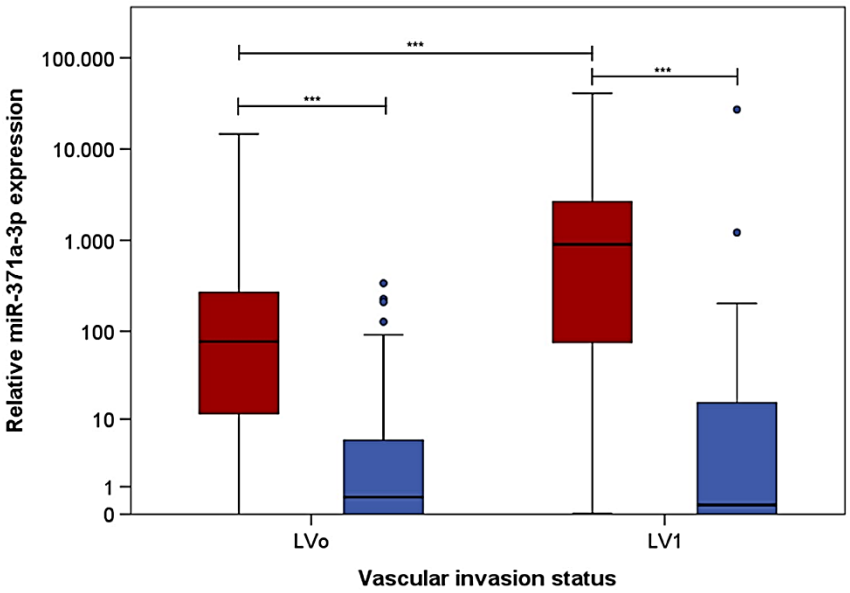

Fig. 1 M371 serum levels in patients with and without lymphovascular invasion. Box plots of the relative M371 expression in patients with lymphovascular invasion in primary tumour (LV1; $n=64$) and without (LVo; $n=89$). Comparison of preoperative median levels (red) with postoperative levels (blue). The *y* axis is plotted in a logarithmic scale. Horizontal bars highlight the significant statistical comparisons. All differences are significant (***$p<0.001$)

($p=0.003$). Postoperatively, the > 50% EC subgroup had more frequently elevated levels than the < 50% EC subgroup (35.4% versus 23.3%), however that difference is not significant ($p=0.112$). The median postoperative RQ value is higher in the > 50% EC group than in the < 50% EC group (1.3 versus 0.23, $p=0.061$); however, both values are below ULN (RQ = 5).

The elevation of classical tumour markers was not associated with preoperative M371 expression both in LV1 and LVo patients (Table 4). In marker-negative patients, the subgroups of LV1 and LVo revealed frequencies of M371 expression in 83.3% and 71.4%, respectively, while in marker-positive patients the frequencies were 96.7% and 84.3%, respectively. The differences between LV1 and LVo were not significant in both comparisons.

Preoperative M371 levels are associated with tumour size as revealed by linear regression analysis (scatter plot, Fig. 4). Regression curves show that the association is greater in LV1 patients than in LVo patients with a much greater coefficient of variation in the LV1 patients ($R^2=0.273$) than in those with LVo ($R^2=0.046$). In both subgroups the slopes of the regression lines are significantly different from zero ($p=0.0035$ and $p=0.0089$, respectively), thus providing evidence for the association of the M371 expression with tumour size in both subgroups, LVo and LV1, with a stronger association in LV1.

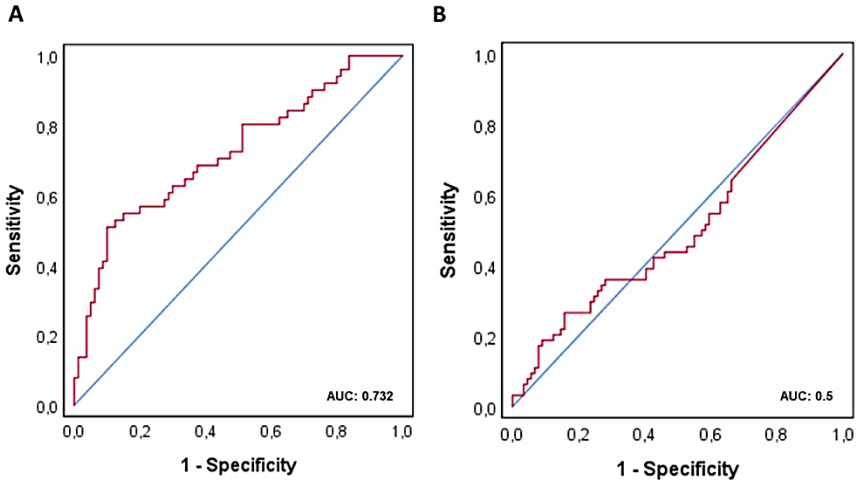

Fig. 2 **A** Ability of preoperative M371 serum level to predict lymphovascular invasion. Receiver operating characteristics curve (ROC) showing the ability of preoperative M371 levels to discriminate between LVo and LV1 status. This analysis involved 51 patients with LV1 and 80 with LVo; the AUC is 0.732. **B** Ability of postoperative M371 serum level to predict lymphovascular invasion. ROC curve showing the ability of postoperative M371 levels to discriminate between LVo and LV1 status. This analysis involved 64 patients with LV1 and 89 with LVo. The AUC is 0.5

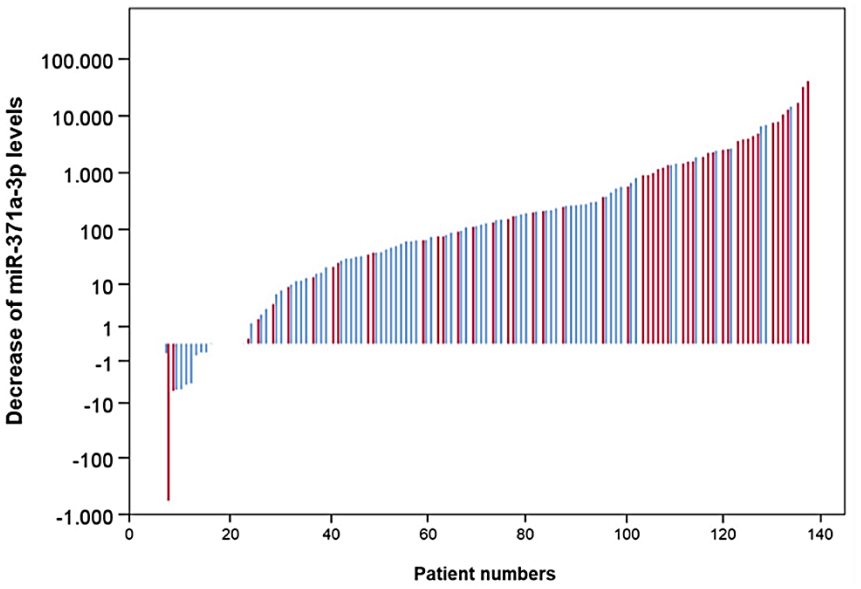

Fig. 3 Decreases of M371 serum levels after orchiectomy in individual patients with and without lymphovascular invasion. Waterfall plot showing absolute decreases of M371 levels following orchiectomy in 131 individual patients with paired measurements before and after orchiectomy, thereof 80 LVo patients (blue), and 51 LV1 patients (red). Each vertical bar represents one individual patient. The LV1 subgroup involves significantly greater declines than the LVo subgroup ($p = 0.000059$). The *y* axis is displayed in a logarithmic scale

Table 3 Impact of presence of embryonal carcinoma in the primary on M371 levels: preoperative and postoperative of M371 measurements and comparisons of subgroups

	Eligible (*n*)	< 50% EC	> 50% EC	*p* value	Comparison < 50% EC vs. > 50% EC
Preoperative M371 measurement (*n*)	131	65	66		
M371 elevated (*n*; %)	111 (84.7%)	49 (75.4%)	62 (93.9%)	0.003	Proportions of M371 elevation[a]
M371 elevated (RQ median; IQR)		63.1; 5.28–853.31	225.0; 67.24–1208.68	0.008	Comparison of RQ values[b]
Postoperative M371 measurement	152	73	79		
M371 elevated (*n*; %)	43 (28.9%)	17 (23.3%)	28 (35.4%)	0.112	Proportions of M371 elevation[a]
M371 elevated (RQ median; IQR)		0.23; 0.00–4.36	1.3; 0.00–17.19	0.061	Comparison of RQ values[b]

EC embryonal carcinoma, *IQR* interquartile range, *RQ* relative quantity

[a]Chi-squared test

[b]Mann–Whitney *U* test

Table 4 Comparison of subgroups LVo versus LV1 with regard to preoperative M371 expression in patients with and without elevation of classical markers ("marker-negative" and "marker-positive" patients)

	Eligible (*n*)	LVo	LV1	*p* value	Type of comparison
Marker-negative	46				
With M371 expression (*n*; %)	35	20 (71.4%)	15 (83.3%)		
No M371 expression (*n*; %)	11	8 (28.6%)	3 (16.7%)	0.486	Comparison of proportions[a]
Marker-positive	81				
With M371 expression (*n*; %)	72	43 (84.3%)	29 (96.7%)		
No M371 expression (*n*; %)	9	8 (15.7%)	1 (3.3%)	0.143	Comparison of proportions[a]

LV1 lymphovascular invasion, *LVo* without lymphovascular invasion

[a]Chi-squared test

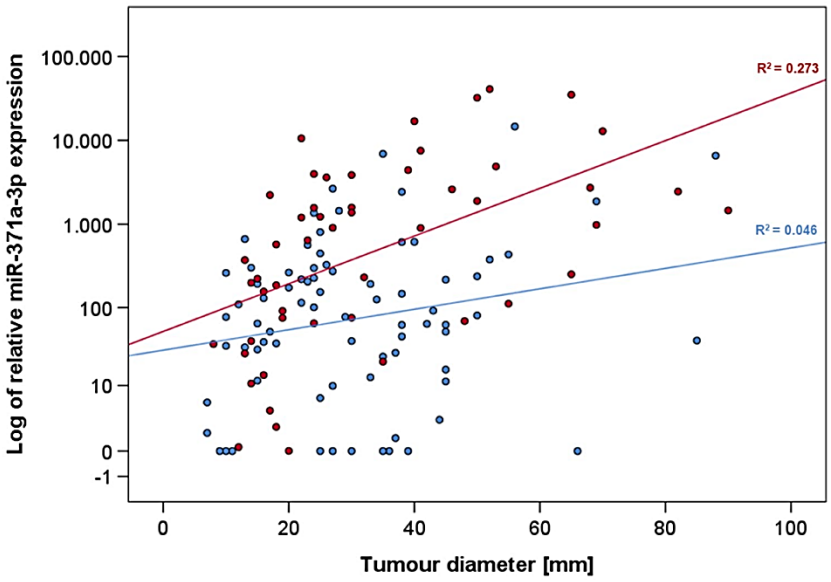

Fig. 4 Association of preoperative M371 levels with tumour size in patients with and without lymphovascular invasion. Scatter plot with regression lines showing the association between the preoperative M371 levels and tumour diameter in individual patients. The association in LV1 patients (red) is higher with $R^2=0.273$ ($p=0.0035$) than in LVo patients (blue) $R^2=0.046$ ($p=0.0089$). The *y* axis is plotted in a logarithmic scale

Multiple regression analysis showed > 50% EC ($p=0.004$), tumour size ($p=0.001$), teratoma components ($p=0.045$), and LV status ($p=0.003$) to be significant predictors of preoperative miRNA expression ($p=0.000014$; $R^2=0.239$) but not of postoperative expression ($p=0.063$; $R^2=0.083$) (Table 5).

Discussion

Elevated M371 serum levels after orchiectomy were found in 29.4% of CS1 testicular NS patients which is consistent with previous reports [17, 20]. The clinically important question is whether persistently elevated M371 levels after surgery in CS1 patients involve any biological significance, particularly, if postoperative M371 levels > ULN relate to the presence of occult metastases and may, thus, indicate the probability of progression. The central result of the present study is that both, the presence of lymphovascular invasion (LV1) and the presence of > 50% embryonal carcinoma in the primary tumour are not associated with postoperative M371 elevation. Accordingly, almost identical postoperative M371 expression rates are found in LV1 and LVo patients, and the ROC analysis clearly shows that postoperative M371 levels can predict the LV1 status only with chance probability (AUC = 0.5).

The proportions of patients with decreasing M371 levels secondary to orchiectomy are not different among the two subgroups with risk factors (LV1; > 50% EC) and those without (LVo; < 50% EC), respectively. The extent of reduction from preoperative to postoperative M371 level is greater in the LV1 subgroup than in the LVo subgroup, when absolute RQ values are considered. However, this difference relates to comparatively higher preoperative RQ values in

Table 5 Potential associations of preoperative and postoperative M371 serum levels elevations with clinico-pathological factors: multiple regression analysis

	Preoperative measurements *p* value	Postoperative measurements *p* value
Whole model	0.000014	0.063
Tumor size: > median; < median [mm]	0.001	0.019
Patient age: > median; < median [years]	0.335	0.056
EC in primary tumour [> 50%; < 50%]	0.004	0.105
Teratoma component in primary tumour [present; not present]	0.045	0.175
Classical markers [elevated; normal]	0.481	0.959
LV status (LVo; LV1)	0.003	0.245

Patients eligible: preoperatively $n = 124$; postoperatively $n = 142$

EC embryonal carcinoma, *LV1* lymphovascular invasion, *LVo* without lymphovascular invasion

LV1 patients. Thus, the percent decline of M371 levels does probably not involve any information about the probability of progression.

Evidence for the clinical significance of postoperatively elevated M371 levels can only come from systematic follow-up observations of patients who do not receive adjuvant treatment. However, we do not have information about the later course of the patients of the present investigation because the original study design had only involved M371 measurements before and after surgery for exploring sensitivity and specificity of the test with no further clinical observation. Moreover, as treatment decisions were made by local physicians, many of the patients of the present multicentric study have received some sort of adjuvant treatment, preventing any meaningful conclusions about persisting M371 expression after orchiectomy. First data providing insight into the biological significance of postoperatively elevated M371 levels in CS1 NS patients came from a recent study from Lobo et al. who longitudinally examined banked sera of CS1 patients under surveillance without adjuvant therapy. Noteworthy, the postoperatively measured M371 levels of patients destined to relapse were not different from those who remained disease-free [16]. Also, the percent decline of M371 (from preoperative levels to postoperative) was not predictive for recurrence. These results are widely in accordance with our data although a direct comparison is clearly not possible because in our present study the LV1 status was used as surrogate marker for relapses.

Another way of evaluating the biological significance of postoperatively elevated M371 levels would be to look histologically for occult disease in the abdominal lymph nodes obtained by retroperitoneal lymph node dissection (RLND). However, this management of nonseminomatous GCT used to be the standard of care only some decades ago [22]. Presently, RPLND has lost its significance and surveillance is the preferred way of care in NS CS1 patients [7]. Yet recently, the Dallas group measured data on post-orchiectomy M371 levels in patients undergoing primary RPLND and found that the levels measured before RPLND accurately predicted the presence of viable GCT in resected nodes with an AUC of 0.965 [23]. However, that study involved only 24 patients and that small patient sample comprised of both CS1 and CS2 patients. Furthermore, no information was given with regard to the intervals from orchiectomy to the time point of postoperative M371 measurement. Thus, that study undoubtedly confirmed the ability of M371 to detect small neoplastic GCT foci but did not really provide a clue to the enigma of postoperatively elevated M371 levels in CS1 patients.

Thus far, no clearly defined biological role of postoperatively elevated M371 levels in CS1 NS patients has been elucidated, however, a hypothesis may be raised. As shown in this study and earlier reports [17, 24], there is a highly significant association of preoperative M371 levels with tumour size, and more generally, with tumour bulk [25, 26]. Another important observation is the very rapid decay of M371 serum levels with an estimated half-life of less than 24 h and an association of the velocity of decay with tumour size with larger tumours needing more time to drop below the ULN [27, 28]. One may, therefore, hypothesise that postoperatively elevated M371 levels may be found mainly in those patients with large tumours because in these cases, the decrease of M371 is less rapid.

Another hypothesis to explain persistent elevated M371 after surgery could be a too short time interval between surgery and blood sample acquisition. Despite the known short half-life of M371, blood sampling on the first postoperative day may reveal still elevated miR levels particularly in those with large primary tumours.

Clearly, the true biological significance of postoperatively elevated M371 levels can only be assessed in a study where all patients will have blood aspirations for M371 measurement done within a clearly defined time-frame, preferably not earlier than 5 days after orchiectomy. In this study, the day of blood sampling was not specified by the protocol and, therefore, ranged from days 1 to 21, postoperatively. In light of the very short hospital stays of testicular cancer patients, a considerable number of whom will have had their postoperative M371 measurements at times when the postoperative decline of M371 levels have not yet dropped to the normal range despite complete eradication of GCT cells by orchiectomy. In all, postoperatively elevated M371 levels may first arise from large tumours with delayed decay of serum levels and secondly, from premature blood sampling. A novel information of this study is the strong association between preoperative M371 levels and LV1 as shown in the

ROC analysis that revealed an AUC of 0.732 for predicting the LV1 status by elevated M371 levels. No clear biological explanation for this relationship is at hand but it is rational to assume that neoplastic cells with direct communication to the vascular system (LV1) may drain their cellular products, e.g. microRNAs directly into the circulation, thus, causing higher M371 levels in these cases. This postulated pathway mirrors the observation of higher M371 levels in testicular vein blood than in the peripheral circulation [29]. A secondary factor causing higher preoperative M371 levels in patients with LV1 is the fact that lymphovascular invasion is more prevalent in larger tumours (Fig. 4). Thus, higher M371 levels in LV1 patients may relate to biological characteristics of vascular drainage and tumour bulk.

We also observed a significant association of preoperative M371 levels with the presence of > 50% EC in the primary. Clearly, the association of preoperative M371 levels with these two recognised risk factors needs to be further evaluated. It appears conceivable that M371 levels above a certain cut-off might quite accurately mirror biological features of the primary tumour including the LV1 status. As M371 levels are measurable by standardised laboratory techniques such measurements could represent a more robust risk marker for aggressive disease than observer-dependent histological markers. However, at present, the clinical significance of the association of preoperative M371 levels with histological risk factors remains elusive.

Another noteworthy finding is the inverse association of teratoma component in the primary with both the presence of lymphovascular invasion (LV1) and the presence of > 50% EC. This finding may point to the lower propensity of teratoma to metastasize and the result is consistent with results of clinical studies that reported lower incidences of relapses in NS CS1 patients who had teratoma components in their primary tumours [30].

In aggregate, we identified several histopathological features of the primary tumour that influence the preoperative M371 serum level. More evidence for this conclusion comes from the multiple regression model that revealed tumour size, > 50% EC, teratoma component, and the LV status to be significant predictors of preoperative M371 levels. According to this model, almost 24% of the variation of preoperative M371 expression ($R^2 = 0.239$) is related to these factors.

The major limitation of this study is the lack of follow-up data of the patients included. We used the LV1 status as surrogate marker for occult metastases but this marker has a known low sensitivity. Another limitation relates to the lack of information about the intervals of postoperative blood sampling from surgery. The lack of a central pathological review is clearly a weakness since the endpoints of our study relate to histopathological factors. Thus, inconsistencies regarding histopathological assessments of primary tumours cannot entirely be excluded. Also, exact quantification of the amount of embryonal carcinoma in the primary was not possible.

Strengths of the investigation may relate to the prospective multicentric enrolment of patients with minimising selection bias and also to the completeness of data sets in the majority of cases.

Conclusions

Against expectation, there is no association of postoperatively elevated M371 levels with the LV status. However, the hypothesis is offered that postoperatively elevated M371 levels may for the most part relate to premature blood sample acquisition after orchiectomy and delayed decay of M371 serum levels in large primary tumours. This assumption is based on the association of the velocity of decay of M371 serum levels with primary tumour size. However, it must also be considered that the M371 test with its present methodological features could possibly be too insensitive to detect microscopic foci of germ cell cancer. Noteworthy, preoperative M371 levels showed a remarkable association with the presence of both risk factors, LV1 and > 50% EC, and this relationship clearly deserves further evaluation.

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Declarations

Conflict of interest KPD and GB each possess 9.7% ownership shares of mirdetect GmbH, Bremerhaven, a start-up company aiming to develop a commercially available test for measuring miRs in serum. mirdetect holds a patent for the measurement of miR in body fluids at the limit of detection. AR is an employee of mirdetect GmbH, Bremerhaven, since the beginning of 2019. All other authors declare no competing interests towards this report.

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3.3 Publication III: Testicular Neoplasms: Primary Tumour Size Is Closely Interrelated with Histology, Clinical Staging, and Tumour Marker Expression Rates—A Comprehensive Statistical Analysis

The clinical status of testicular tumors depends on key factors such as tumor size, patient age, histology, serum markers, and clinical staging. However, the biological interrelationships between these factors, especially the influence of primary tumor size, are poorly understood (Leman and Gonzalgo, 2010). Despite various studies on tumor size, a comprehensive systematic analysis is lacking (Dieckmann et al., 2022c).

In this study, the tumor size was analyzed in a large patient cohort, exploring its associations with several factors, including histology, clinical staging, expression rates of classical tumor markers, and patient age. Specifically, the aim of the study was to assess four assumptions:

(1) The association between tumor size and histology, hypothesizing that a much higher proportion of benign tumours are found among primary testicular neoplasms sized < 1 cm compared to larger tumors, (2) the impact of primary tumor size on clinical staging in GCTs, to determine if smaller tumors are associated with with fewer advanced clinical stages while larger tumors are associated with more advanced stages, (3) the association between tumor size and expression rates of classical serum tumor markers in GCTs, as well as the novel marker M371, expecting lower marker levels in small tumour sizes, and higher expressions in larger tumors, (4) the interrelationship between patient age and primary tumor size, predicting that older patients would have larger tumors.

A total of 641 patients with a median age of 38 years were enrolled in this study. Tumor sizes ranged from ≤10 mm in 13.6% of patients to >30 mm in 45.2%, with an overall median tumor size of 30 mm.

(*Assumption 1)* Results have shown that median tumor sizes varied significantly among histological groups (Kruskal–Wallis test $p < 0.0001$), notably with the largest median tumor size (53 mm) observed in other malignant tumors, and the largest overall tumor measured 18.9 cm, classified as a non-seminoma giant tumor. While seminomas and non-seminomas show no significant size difference, the combined GCT median size was significantly larger than benign tumors. Overall, the distribution of histologic subgroup frequencies varied significantly among tumor size categories (p <0.0001, chi-square test). Moreover, in the subcentimeter category, benign tumors comprised over half of the cases. Additionally, a tumour size of 16 mm as threshold

between benign and malignant tumours, showed strong diagnostic performance, achieving high sensitivity and specificity (81.5% and 81.0%, respectively) and an AUC of 0.8912. (*Assumption 2)* Regarding clinical staging in GCTs, smaller tumor sizes (≤10 mm) were associated with clinical stage I, as nearly 98% of cases were classified as such. Overall, the study showed that the frequency of clinical stages significantly depends on the size of the tumors ($p < 0.0001$, Kruskal–Wallis test). Specifically, it was observed that smaller tumors were associated with a decrease in CSI cases, whereas an inverse trend was seen in stages with metastases ($p < 0.0001$; Cochran–Armitage trend test). *(Assumption 3)* Results have shown that larger tumors (>10 mm) consistently showed significantly higher elevated serum tumor marker levels, except for LDH, emphasizing a significant association between marker expressions and tumor bulk. Notably, the novel tumor marker M371 exhibited the highest expression rate among all tumor markers in both size categories. (*Assumption 4)* Regarding patient age and tumor size in GCT, the study found significant differences in median tumor size among age categories. However, there was no clear trend suggesting a association between specific age groups and varying tumor sizes.

In conclusion, the study emphasizes the significant impact of tumor size on the clinical course of testicular neoplasms, proposing its potential clinical use in diagnosing small neoplasms to prevent unnecessary orchiectomies. Furthermore, the novel marker M371 outperformed the classical markers and exhibited expression in around 40% of subcentimeter germ cell tumors, promising enhanced clinical applications and personalized treatment strategies in the future.

-III-

Testicular Neoplasms: Primary Tumour Size Is Closely Interrelated with Histology, Clinical Staging, and Tumour Marker Expression Rates-A Comprehensive Statistical Analysis

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Own contribution

- Experimental concept and study design: 10%
- Experiment execution and/or acquisition of experimental data: 50%
- Data analysis and interpretation: 25%
- Preparation of Figures and Tables: 10%
- Drafting of manuscript: 10%

cancers

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Article

Testicular Neoplasms: Primary Tumour Size Is Closely Interrelated with Histology, Clinical Staging, and Tumour Marker Expression Rates—A Comprehensive Statistical Analysis

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Simple Summary: The size of the tumour is of clinical relevance in testicular tumours. Our statistical analysis of 641 cases with testicular tumours revealed that 50% of cases with tumours smaller than 1 cm were of benign histology. The frequencies of both metastatic spread and elevations of serum tumour markers increase with growing tumour size. The novel tumour marker microRNA-371a-3p (M371) outperforms the classical markers and is measurable in 44% of germ cell tumours with tumour sizes below 1 cm.

Abstract: The role of primary tumour size (TS) in the clinical course of testicular tumours is incompletely understood. We retrospectively evaluated 641 consecutive patients with testicular neoplasms with regard to TS, histology, clinical stage (CS), serum tumour marker (STM) expression and patient age using descriptive statistical methods. TS $\leq$ 10 mm was encountered in 13.6% of cases. Median TS of 10 mm, 30 mm, 35 mm, and 53 mm were found in benign tumours, seminomas, nonseminomas, and other malignant tumours, respectively. In cases with TS $\leq$ 10 mm, 50.6% had benign tumours. Upon receiver operating characteristics analysis, TS of > 16 mm revealed 81.5% sensitivity and 81.0% specificity for detecting malignancy. In subcentimeter germ cell tumours (GCTs), 97.7% of cases had CS1, and CS1 frequency dropped with increasing TS. Expression rates of all STMs significantly increased with TS. MicroRNA-371a-3p (M371) serum levels had higher expression rates than classical STMs, with a rate of 44.1% in subcentimeter GCTs. In all, TS is a biologically relevant factor owing to its significant associations with CS, STM expression rates and histology. Importantly, 50% of subcentimeter testicular neoplasms are of benign nature, and M371 outperforms the classical markers even in subcentimeter tumours.

Keywords: testicular tumour; seminoma; tumour marker; microRNA; tumour size; germ cell tumour; alpha-fetoprotein; human chorionic gonadotropin

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1. Introduction

The clinical status of testicular tumours is characterized by the parameters primary tumour size, patient age, histology of the primary, serum tumour marker elevations and the extent and distribution of metastases (i.e., clinical staging). These features represent simple clinical factors, and the clinical usefulness of each factor has stood the test of time [1–5] despite ongoing incorporation of molecular genetic factors in other malignancies [6]. However,

only modest information is available regarding the biological interrelationships between the factors. In particular, the clinical and biological role of primary tumour size is still incompletely understood. The size of a testicular new growth is considered to have a bearing on histology found upon surgery [7]. This knowledge is of high clinical relevance, because in light of the continuously growing use of scrotal ultrasound in everyday urological practice and ongoing technical refinements of this technology [8–10], the incidence of small testicular neoplasms has been increasing in recent years. Tumour size also has a bearing on metastatic spread, at least in the subgroup of seminoma [11]. Likewise, primary tumour size is associated with the frequency of serum tumour marker elevations (expression rates) and the extent of elevations of classical serum tumour markers alpha fetoprotein (AFP), beta human chorionic gonadotropin (bHCG) and lactate dehydrogenase (LDH) [12]. Additionally, there is growing evidence for an association of the novel serum tumour marker microRNA-371a-3p (M371) with tumour bulk in general and tumour size in particular [13,14]. Finally, there is some suggestion of an association of patient age and tumour size, with a trend towards larger tumours in the elderly [15].

Obviously, there are manifold interrelationships between tumour size and other clinical characteristics of testicular neoplasms. A number of studies had analysed single issues regarding the clinical role of tumour size [12,16–18], but so far, there is no comprehensive systematic analysis regarding the clinical significance of this factor.

Therefore, we analysed tumour size in a large patient sample and looked for associations with histology of the primary, clinical staging, serum tumour marker expression rates, and with age. In particular, we sought to assess the following four assumptions, most of which are already supported by some pieces of evidence; however, the levels of evidence have not reached high levels so far: *(Assumption #1)* The size of a testicular new growth is associated with histology in the way that a much higher proportion of benign tumours are found among primary testicular neoplasms sized < 1 cm than among the larger ones. (*Assumption #2*) Primary tumour size has an impact on clinical staging in germ cell tumours (GCTs), with fewer advanced clinical stages in small tumours and more advanced stages in larger tumours. (*Assumption #3*) Tumour size is associated with the expression rates of the classical serum tumour markers in GCTs as well as with the novel marker M371. Lower frequencies of marker elevations are expected in small tumour sizes, and higher expressions in larger tumours. The new tumour marker M371 is much more frequently expressed than the classical markers bHCG and AFP. *(Assumption #4)* An interrelationship between patient age and primary tumour size is hypothesized in a way that older patients will have larger tumours.

2. Materials and Methods

2.1. Patient Recruitment, Data Procurement

All consecutive patients, aged 17–98 years, undergoing surgery for a newly diagnosed testicular mass in two Hamburg-based departments of general urology (Albertinen-Krankenhaus and Asklepios Klinik Altona) during 2012–2021, were included in the present retrospective study. Patients submitted to orchiectomy after previous chemotherapy were excluded. The following parameters were abstracted from archival case files: size of the testicular mass (mm) as recorded in the pathology reports, patient's age (years), histology of the surgical specimen categorized as seminoma (SE), nonseminoma (NS), benign tumour (BT), malignant tumour other than GCT (OM), clinical stage (CS) (only in GCTs); and preoperative expression of serum tumour markers bHCG, AFP, LDH, and M371. About one third of the patients had been included in previous investigations on various other issues [12,14,19].

In case of multiple separate tumours in one testicle, the diameters of each tumour were added up to the final tumour size. Simultaneous bilateral tumours were excluded from this analysis. Histological diagnoses were retrieved from pathology reports without central pathology review. Clinical stages were recorded as CS1; CS2a,b; CS2c; and CS3 according to current guide-lines [20]. Serum tumour markers bHCG, AFP, and LDH were measured in

hospital laboratories according to institutional guidelines. As during the observation period the commercially available test kits were changed for economic reasons with consecutive changes in upper limits of norm (ULN), we only recorded elevations of serum levels above ULN (yes/no). M371 serum levels were measured as detailed previously [14]. Briefly, RNA was isolated from aspirated serum, followed by reverse transcription to cDNA for both miR-371a-3p and the endogenous control miR-30b-5p. The quantitative polymerase chain reaction was performed after preamplification. M371 serum levels were originally provided as relative quantity (RQ) values. However, to be methodologically consistent with the analysis of classical markers, only dichotomized results were noted (elevated yes/no) by defining RQ = 5 as ULN.

Ethical approval was provided by Ethikkommission der Ärztekammer Hamburg on 2 March 2020 (PV7288). As solely anonymized patients' data were evaluated in the present study, the need for informed consent of patients was waived by the ethical committee. All study activities were conducted according to the Declaration of Helsinki of the World Medical Association as amended by the 64th General Assembly, October 2013.

2.2. Statistical Analysis

All patient data were originally filed in a commercially available data base (MS Excel, version 2017) and subjected to data validation prior to statistical analysis. For descriptive analysis of nominal variables, absolute frequencies, percentages and 95% confidence intervals (CIs) were presented.

Continuous variables were descriptively analysed by calculating measures of location and dispersion, such as median, first quartile (Q1), third quartile (Q3), interquartile range (IQR), minimum, maximum, arithmetic mean and standard deviation. For graphical presentation, box and whisker diagrams were created; the respective boxes were defined by Q1, median and Q3, and the whiskers were defined by the largest and lowest observed values that fell within the 1.5 times IQR measured from Q3 and Q1, respectively.

To test for any differences in the distribution of tumour sizes between more than two subgroups of patients defined by age and histology, Kruskal–Wallis tests were applied; these were replaced by Wilcoxon two-sample tests in case of two categories. Chi-squared tests were used to compare contingency tables of nominal variables. To assess whether malignancy rates of testicular neoplasms or tumour marker expression rates increased with tumour size, Cochran–Armitage trend tests were applied. The Jonckheere–Terpstra test served to assess whether tumour sizes increased with clinical stage.

To assess the ability of the tumour size to predict malignant histology, a receiver operating curve (ROC) was created. The area under the curve (AUC), together with its 95% Wald confidence interval, was calculated to quantify the goodness of prediction. Youden's index, defined as the maximum vertical distance between the ROC curve and the diagonal line, was used to identify the optimal cutoff of the tumour size for malignancy prediction. Additionally, the predicted probability curve for malignant histology with bounding 95% confidence intervals was calculated using a linear logistic regression model.

Due to the high completion rate of patient data sets, all patients were eligible for most of the analyses listed above. Only serum tumour marker expressions (especially for M371 marker) were not available in all patients. Thus, respective statistical analyses were performed with varying sample sizes according to the available entries.

p-values of less than 5% were considered statistically significant in this paper.

Statistical analysis was performed with SAS software package version 9.4 (SAS Institute, Cary, NC, USA) on the Windows platform.

3. Results

3.1. General Results

A total of 641 patients with a median age of 38 years were included in the present analysis. The frequencies of the four histologic subgroups with corresponding median ages are given in Table 1. Histologically, benign tumours (BT) comprised gonadal stromal

tumours for the most part and benign epidermoid cysts and other rare tumours to a lesser degree. Other, malignant tumours (OM) mainly comprised diffuse large B-cell lymphomas with few other forms of malignant testicular lymphoma. Age, tumour size, histology, and clinical staging were available in all patients. Marker elevations regarding AFP and bHCG were available in 640 patients, regarding LDH in 633 cases, and with respect to M371 in 451 patients. The median tumour size of the entire population (n = 641) was 30 mm (IQR 15–45 mm). Tumour sizes of ≤10 mm were found in 13.6% of patients, while 45.2% of patients presented with tumour sizes > 30 mm.

Table 1. Total patient population and frequency distribution of histologic subgroups and corresponding age distributions.

	n (%)	Age (Years)				
		Min	Q1	Median	Q3	Max
Total	641 (100%)	17	31	**38**	47	98
Seminoma (SE)	365 (56.94%)	17	33	**40**	48	78
Nonseminoma (NS)	179 (27.93%)	17	26	**31**	37	74
Benign tumours (BT)	79 (12.32%)	19	32	**41**	50	68
Other malignant tumours (OM)	18 (2.81%)	52	68	**72.5**	78	98

n: number of cases, Min: minimum, Q1: first quartile, Q3: third quartile, Max: maximum.

3.2. Assumption # 1 (Association of Tumour Size with Histology)

The median tumour sizes found in germ cell tumours and in the four histologic subgroups are delineated in Table 2 and in Figure 1. The largest median tumour size, of 53 mm, was observed in the other malignant tumours (OM). The largest tumour size overall was 18.9 cm in a patient with nonseminoma (so-called giant tumour). Overall, median tumour sizes were significantly different among the four histologic groups (Kruskal–Wallis test $p < 0.0001$).

Table 2. Distribution of tumour sizes stratified by histologic subgroups.

	GCT (mm)	Seminomas (mm)	Nonseminomas (mm)	Benign Tumours (mm)	Other Malignant Tumours (mm)
Mean	35.9	34.8	38.2	11.8	53.0
Std Dev	23.2	22.2	25.7	7.8	14.3
Min	3	4	7	3	16
Q1	15	19	20	6	49
Median	30	30	35	10	53
Q3	46	46	49	15	56
Max	189	170	189	42	78
n	544	365	179	79	18

Min: minimum, Std Dev: standard deviation, Q1: first quartile, Q3: third quartile, Max: maximum, Overall comparison: $p < 0.0001$ (Kruskal–Wallis Test).

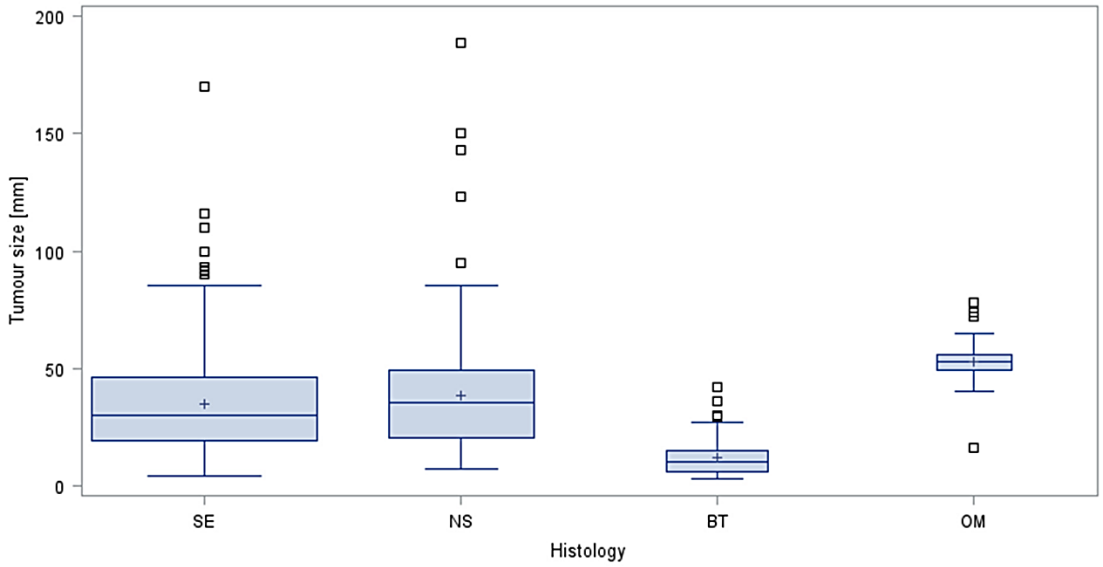

Figure 1. Tumour sizes observed in the four histologic groups. Box and whisker plots showing the distribution of tumour sizes stratified by histologic subtypes of testicular neoplasms. The boxes display the first quartile, median and third quartile. The whiskers are defined as the largest or lowest observed value that falls within 1.5 times the interquartile range measured from Q3 or Q1, respectively. Area of box relates to sample size. □ outliers; + denotes arithmetic mean; SE seminoma; NS nonseminoma; BT benign tumours; OM other malignancies.

Results of the comparisons of median tumour sizes between particular histological subgroups are listed in Table 3.

Table 3. Comparisons of median tumour sizes among particular histologic subgroups.

Histologic Groups	*p* *
SE vs. NS	0.1255
(SE + NS) vs. (BT + OM)	<0.0001
(SE + NS) vs. BT	<0.0001
(SE + NS) vs. OM	<0.0001
BT vs. OM	<0.0001

* Wilcoxon two-sample test.

Tumour sizes of seminomas and nonseminomas were not significantly different from each other. However, median GCT tumour size (SE + NS) was significantly larger than that of benign tumours (BT), while OM were significantly larger than GCTs (SE + NS) and BT. A typical example of a small benign tumour is given in Figure 2.

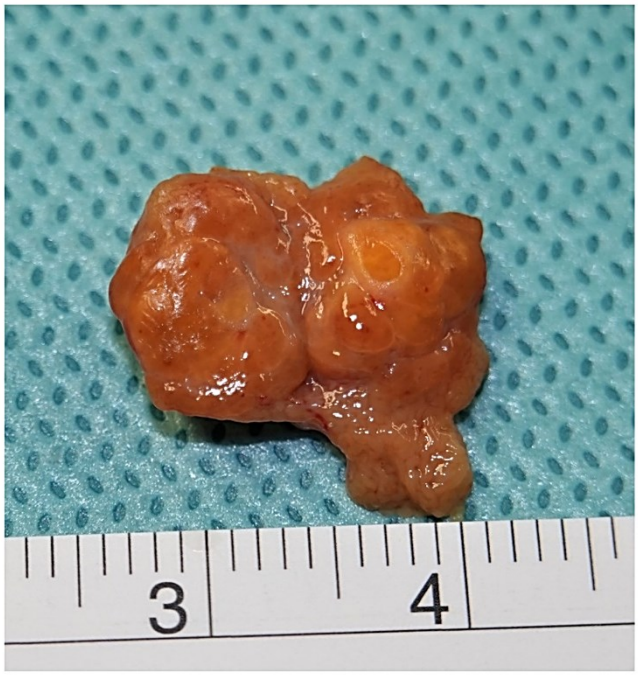

Figure 2. Typical subcentimeter testicular neoplasm. Surgical specimen of a 6 mm sized benign Leydig cell tumour excised by testis sparing surgery.

The frequencies of the four histologic subgroups in tumour size categories ≤ 10 mm and >10 mm, respectively, are shown in Table 4 and Figure 3. The distribution of histologic subgroups was significantly different between the two size categories.

Table 4. Frequencies of histologic subgroups in tumour size categories of ≤10 mm and >10 mm.

Size Categories	SE	NS	BT	OM	Total	*p* *
≤10 mm (*n* = 87)	35 (40.23%)	8 (9.20%)	44 (50.57%)	0 (0.00%)	100%	<0.0001
>10 mm (*n* = 554)	330 (59.57%)	171 (30.87%)	35 (6.32%)	18 (3.25%)	100%	

* Chi-squared test.

In the subcentimeter category, benign tumours represented more than half of the cases. In the larger tumour size category, seminomas predominated with 59.6%, while benign tumours comprised only 6.3%.

A more granular analysis with histologic subgroup frequencies in tumour size categories ≤ 10 mm; 11–20 mm; 21–30 mm; and >30 mm is provided in Table 5.

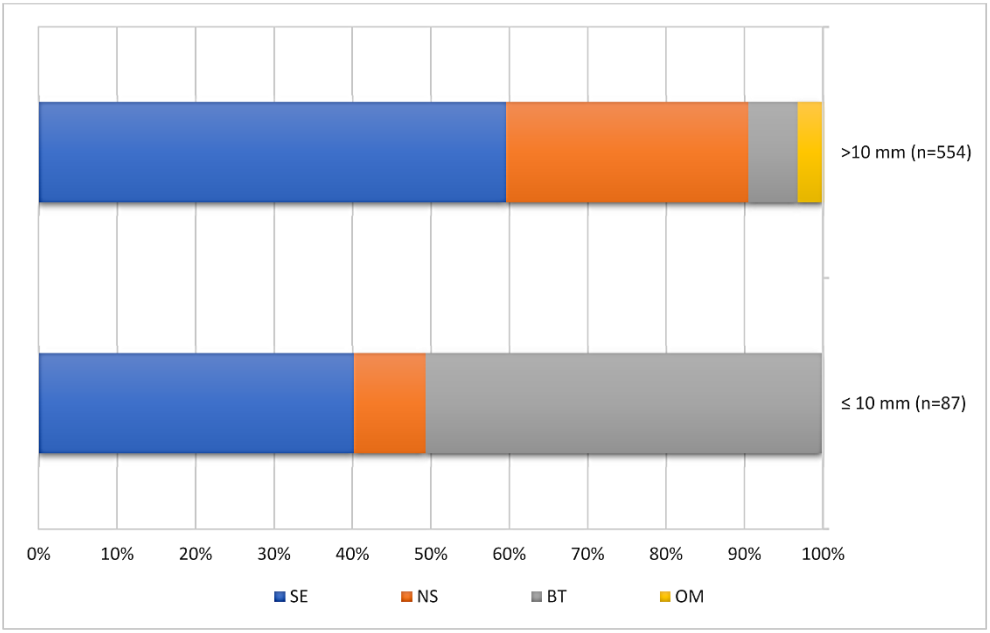

Figure 3. Proportions of histologic subgroups in testicular tumours sized >10 mm and ≤10 mm.

Table 5. Frequencies of histologic subgroups in four tumour size categories.

Size Categories	SE	NS	BT	OM	Total
≤10 mm (*n* = 87)	35 (40.23%)	8 (9.20%)	44 (50.57%)	0 (0.00%)	100%
11–20 (*n* = 141)	73 (51.77%)	41 (29.08%)	26 (18.44%)	1 (0.71%)	100%
21–30 (*n* = 123)	84 (68.29%)	32 (26.02%)	7 (5.69%)	0 (0.00%)	100%
>30 mm (*n* = 290)	173 (59.66%)	98 (33.79%)	2 (0.69%)	17 (5.86%)	100%

Overall comparison: $p < 0.0001$ (chi-squared test).

Overall, the distribution of histologic subgroup frequencies was significantly different among the tumour size categories ($p < 0.0001$, chi square test). If benign tumours (BT) were compared with all malignant tumours (GCT + OM), it became clear that in subcentimeter testicular neoplasms, more than half of all cases represented benign tumours, while decreasing relative proportions of this subtype were observed in categories with increasing tumour sizes (Table 6).

Table 6. Frequency distribution of malignant tumours and benign testicular tumours in four tumour size categories.

Size Categories	GCT + OM	BT	*p* *
≤10 (*n* = 87)	43 (49.43%)	44 (50.57%)	<0.0001
11–20 (*n* = 141)	115 (81.56%)	26 (18.44%)	
21–30 (*n* = 123)	116 (94.31%)	7 (5.69%)	
>30 mm (*n* = 290)	288 (99.31%)	2 (0.69%)	

* Cochran–Armitage trend test.

An inverse distribution of frequencies was found among the malignant tumours. The trend towards lower proportions of BT and higher frequencies of malignant tumours (GCT + OM) in increasing tumour size categories was highly significant ($p < 0.0001$, Cochran–Armitage trend test). The ROC analysis in Figure 4 exhibits the ability to diagnose a testicular neoplasm as a benign tumour by means of tumour size. Using a tumour size of 16 mm as threshold between benign and malignant tumours, the sensitivity and specificity of this factor were 81.5% and 81.0%, respectively (highest Youden index: 0.62507), with an area under the curve (AUC) of 0.8912 (95% Wald CI, 0.8569–0.9256).

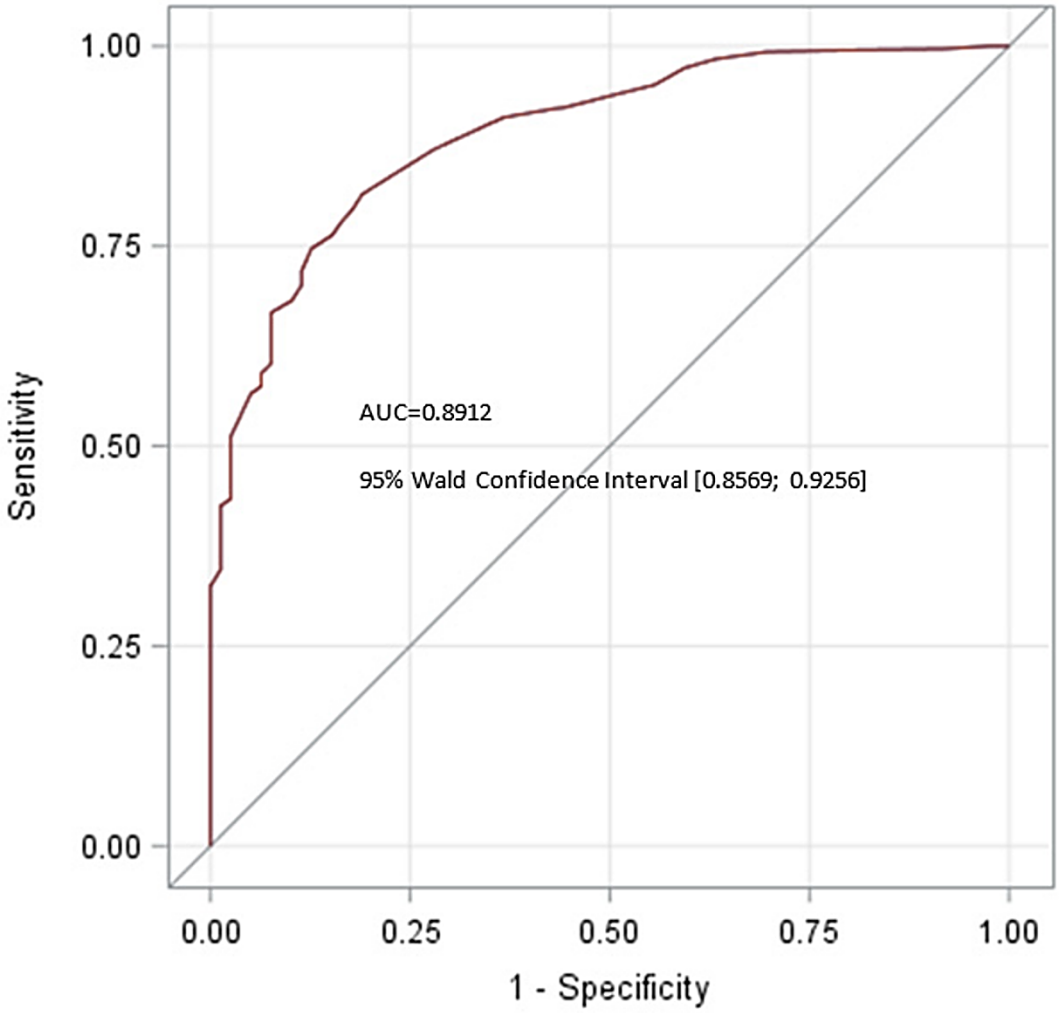

Figure 4. ROC analysis for predicting histology of a testicular neoplasm by its size.

The ROC curve with an AUC of 0.8912 showed that tumour size is in fact a useful tool for assessing the biologic behaviour of small testicular neoplasms. Using a tumour size of 16 mm as threshold between benign and malignant tumours, the sensitivity and specificity of this factor were 81.5% and 81.0%, respectively (Youden index: 0.62507).

Figure 5 presents a logistic regression analysis for predicting malignancy with tumour size. In this model, all tumour sizes ≥39 mm indicated malignancy, while tumours sized ≤8 mm involved a 50% chance of being malignant.

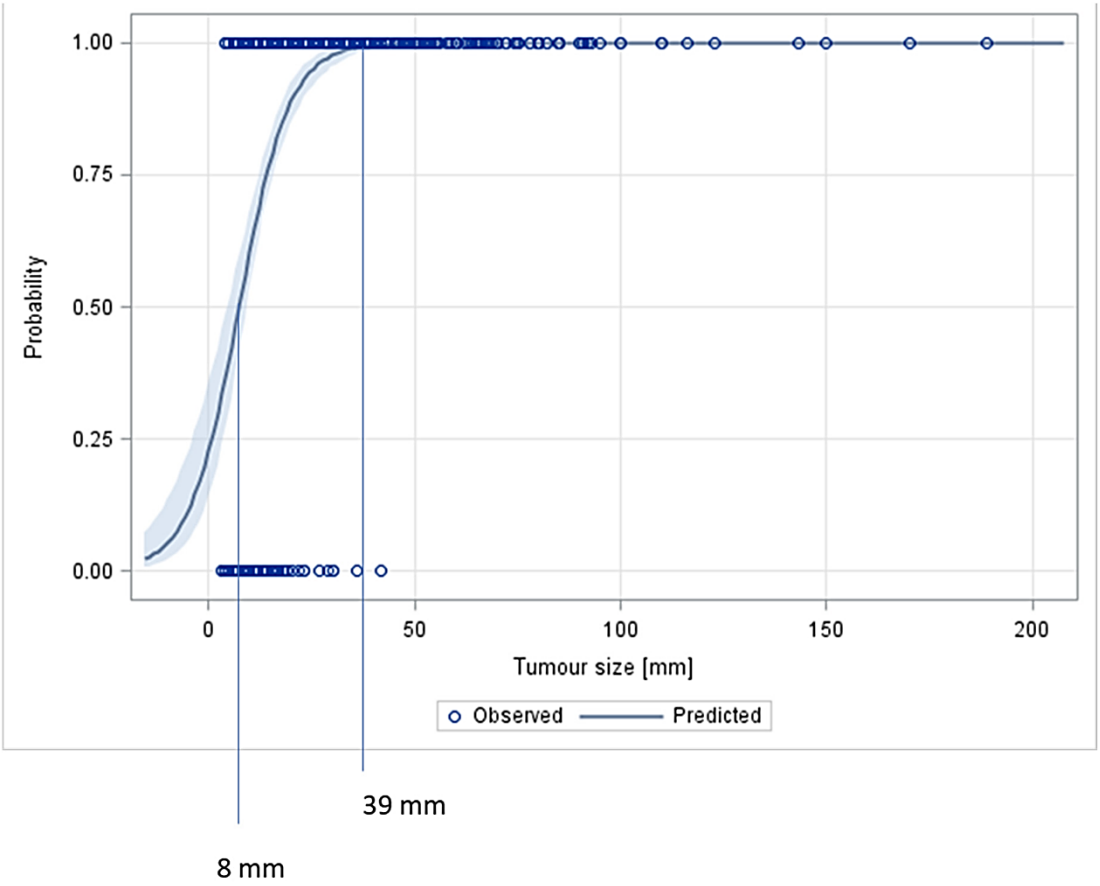

Figure 5. Probability curve for prediction of malignant histology of a testicular neoplasm. The logistic regression curve indicates the probability of a given tumour size to predict malignancy. Shadowed area represents 95% confidence intervals. Neoplasms with a size of ≤8 mm involve a 50% probability of malignancy, while tumour sizes of ≥25 mm, ≥33 mm, and ≥39 mm involve probabilities of 95%, 99%, and 100%, respectively.

3.3. Assumption #2 (Association of Tumour Size and Clinical Staging in GCTs)

Tables 7 and 8 list the frequencies of clinical stages in four tumour size categories of germ cell tumours. Notably, in the smallest tumour size category (≤10 mm), almost 98% of cases had clinical stage 1. Overall, CS frequencies were significantly different among the tumour size categories ($p < 0.0001$, Kruskal–Wallis test). Table 8 shows that among CS1 cases, there was a significant trend towards decreasing frequencies of cases with increasing tumour size, and an inverse trend was seen in stages with metastases ($p < 0.0001$; Cochran–Armitage trend test).

Table 7. Frequency distribution of clinical stages (CS) of germ cell tumours stratified by four categories of increasing tumour size.

	CS1 (*n*)	CS2a/2b (*n*)	CS2c (*n*)	CS3 (*n*)
≤10 mm	42 (97.67%)	1 (2.33%)	0 (0.00%)	0 (0.00%)
11–20 mm	98 (85.96%)	11 (9.65%)	3 (2.63%)	2 (1.75%)
21–30 mm	94 (81.03%)	19 (16.38%)	0 (0.00%)	3 (2.59%)
>30 mm	187 (69.00%)	53 (19.56%)	12 (4.43%)	19 (7.01%)

Jonckheere–Terpstra test to analyse increase in tumour sizes with increase in clinical stage: $p < 0.0001$.

Table 8. Frequencies of localized disease (CS1) and metastasized cases (>CS1) of germ cell tumours (SE, NS) in four categories of increasing tumour size.

	CS1 (*n*)	>CS1 (*n*)	*p* *
≤10 mm	42 (97.67%)	1 (2.33%)	<0.0001
11–20 mm	98 (85.96%)	16 (14.04%)	
21–30 mm	94 (81.03%)	22 (18.97%)	
>30 mm	187 (69.00%)	84 (31.00%)	

* Cochran-Armitage trend test.

The median tumour sizes with IQR and ranges in the four clinical stages are provided in Table 9 and Figure 6. The median tumour size increased with clinical stage, and overall, median tumour sizes were significantly different among the four CS ($p < 0.0001$, Kruskal–Wallis test).

Table 9. Distribution of tumour sizes stratified by clinical stages (CS) in germ cell tumours (SE, NS).

	CS1 (mm)	CS2a,b (mm)	CS2c (mm)	CS3 (mm)
Mean	32.6	41.2	53.2	65.5
Std Dev	20	19.5	30.1	48.3
Min	4	10	13	15
Q1	17	27	35	35.5
Median	30	38	49	50
Q3	43	52	72	77.5
Max	123	92	110	189
n	421	84	15	24

Min: minimum, Q1: first quartile, Q3: third quartile, Max: maximum, Std Dev: standard deviation.

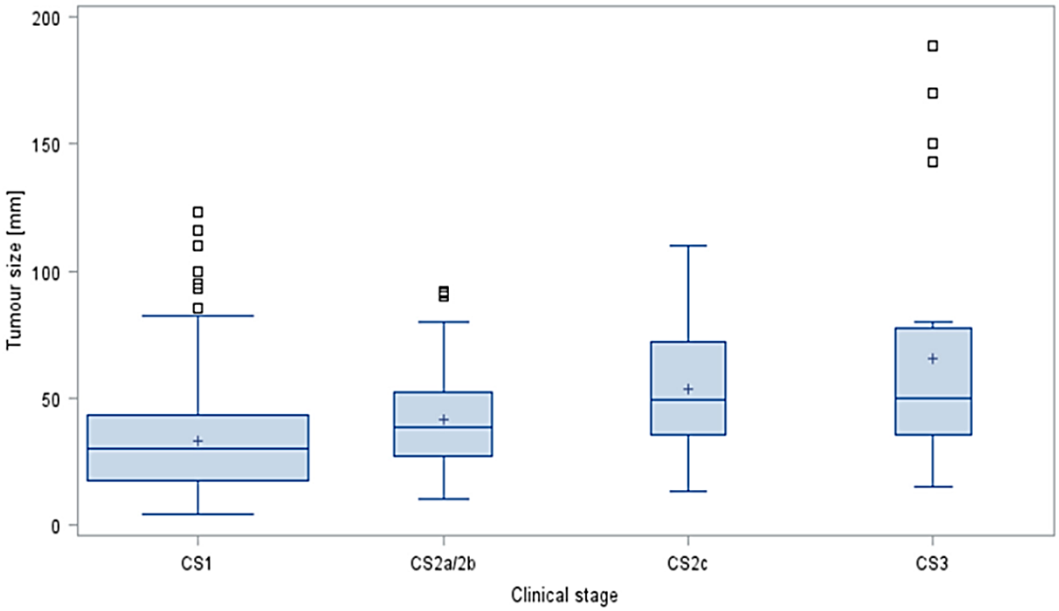

Figure 6. Tumour sizes in clinical stages in germ cell tumours. Box and whisker plots showing the distribution of tumour sizes stratified by clinical stages of testicular neoplasms. The boxes display the first quartile, median and third quartile. The whiskers are defined as the largest or lowest observed value that falls within 1.5 times the interquartile range measured from Q3 or Q1, respectively. Area of box denotes sample size. □ outliers; + denotes arithmetic mean; CS clinical stage.

3.4. Assumption #3 (Tumour Size Is Associated with Frequencies of Tumour Marker Expression in GCTs)

Table 10 outlines the frequencies of elevations of the serum tumour markers bHCG, AFP, LDH, AFP and/or bHCG, and M371 found in the entire population of patients stratified for tumours sized ≤10 mm and >10 mm, respectively. All of the markers were significantly more frequently expressed in larger than in smaller tumour sizes (all comparisons $p < 0.0001$, chi square test).

Table 10. Serum tumour marker expression rates stratified by tumour size categories in the entire population of patients with testicular tumours (SE, NS, BT, OM).

	bHCG	AFP	LDH	AFP/bHCG	M371
	***n*/N**	***n*/N**	***n*/N**	***n*/N**	***n*/N**
≤10 mm	6/87 (6.90%)	2/87 (2.30%)	3/87 (3.45%)	8/87 (9.20%)	16/75 (21.33%)
>10 mm	202/553 (36.53%)	116/553 (20.98%)	122/546 (22.34%)	241/553 (43.58%)	309/375 (82.40%)
p-value *	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

* Chi-squared test, AFP/bHCG elevation of AFP and/or bHCG; N: number of eligible patients.

M371 had the highest expression rate of all tumour markers in both size categories. Somewhat higher expression rates were observed if only germ cell tumours were evaluated (Table 11), but again, all markers had significantly higher expression rates in patients with tumour sizes >10 mm than in those with smaller sizes. The highest expression rate overall was 90.15%, found for M371 in GCTs sized >10 mm.

Table 11. Serum tumour marker expression rates stratified by tumour size categories only in patients with germ cell tumours (SE, NS).

	bHCG	AFP	LDH	AFP/bHCG	M371
	n/N	*n*/N	*n*/N	*n*/N	*n*/N
≤10 mm	5/43 (11.63%)	1/43 (2.33%)	3/43 (6.98%)	6/43 (13.95%)	15/34 (44.12%)
>10 mm	299/500 (59.80%)	115/500 (23.00%)	119/493 (24.14%)	239/500 (47.80%)	302/335 (90.15%)
p-value *	0.0002	0.0015	0.0101	<0.0001	<0.0001

* Chi-squared test, AFP/bHCG elevation of AFP and/or bHCG. N: number of eligible patients.

Table 12 shows the results for the tumour marker expression levels in the subgroup of GCT patients with CS1 stratified by tumour sizes ≤10 mm and >10 mm, respectively. Again, marker expression levels were higher in the category with larger tumour sizes. However, regarding LDH, the difference was no more significant ($p = 0.1039$, chi-squared test). Notably, 44% of CS1 patients with tumours ≤10 mm showed elevations of M371 levels, while each of the other markers were expressed in less than 10%.

Table 12. Serum tumour marker expression rates stratified by tumour size categories only in patients with germ cell tumours (SE, NS) and with clinical stage 1 (CS1).

	bHCG	AFP	LDH	AFP/ bHCG	M371
	n/N	*n*/N	*n*/N	*n*/N	*n*/N
≤10 mm	4/42 (9.52%)	1/42 (2.38%)	3/42 (7.14%)	5/42 (11.90%)	15/34 (44.12%)
>10 mm	132/378 (34.92%)	70/378 (18.52%)	63/375 (16.80%)	158/378 (41.80%)	240/273 (87.91%)
p-value *	0.0008	0.0081	0.1039	0.0002	<0.0001

* Chi-squared test, AFP/bHCG elevation of AFP and/or bHCG. N: number of eligible patients.

Table 13 delineates the expression rates of all tumour markers stratified by five tumour size categories in the entire study population and in the histologic subgroups of seminoma and nonseminoma (Figure 7). In all subpopulations, the expression rates of all tumour markers increased with increasing tumour sizes, except for AFP in the seminoma subgroup (Cochran–Armitage trend test, all except seminoma $p < 0.0001$; seminoma $p = 0.9133$). M371 revealed the highest expression rates in all size categories.

Table 13. Expression rates of serum tumour markers stratified by tumour size categories.

	bHCG	AFP	LDH	AFP/bHCG	M371
	n/N	*n*/N	*n*/N	*n*/N	*n*/N
Total population					
≤10 mm (*n* = 87)	6/87 (6.90%)	2/87 (2.30%)	3/87 (3.45%)	8/87 (9.20%)	16/75 (21.33%)
11–20 mm (*n* = 141)	23/141 (16.31%)	14 /141 (9.93%)	12/140 (8.57%)	30/141 (21.28%)	66/107 (61.68%)
21–30 mm (*n* = 123)	47/122 (30.33%)	21/122 (17.21%)	15/121 (12.39%)	43/122 (35.25%)	67/81 (82.72%)
31–50 mm (*n* = 179)	76/179 (42.46%)	52 /179 (29.05%)	45/176 (25.57%)	93/179 (51.96%)	107/114 (93.86%)
>50 mm (*n* = 111)	66/111 (59.46%)	29/111 (26.13%)	50/109 (45.87%)	75/111 (67.57%)	69/73 (94.52%)
p-value *	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Seminoma					
≤10 mm	2/35 (5.71%)	1/35 (2.86%)	3/35 (8.57%)	3/35 (8.57%)	11/28 (39.29%)
11–20 mm (*n* = 73)	8/73 (10.96%)	3/73 (4.11%)	8/72 (11.11%)	11/73 (15.07%)	36/53 (67.92%)
21–30 mm (*n* = 84)	18/83 (21.69%)	5/83 (6.02%)	9/83 (10.84%)	22/83 (26.51%)	48/56 (85.71%)
31–50 mm (*n* = 107)	35/107 (32.71%)	8/107 (7.48%)	27/106 (25.47%)	41/107 (38.32%)	74/77 (96.10%)
>50 mm (*n* = 66)	43/66 (65.15%)	2/66 (3.03%)	35/66 (53.03%)	45/66 (68.18%)	46/46 (100%)
p-value *	<0.0001	0.9133	<0.0001	<0.0001	<0.0001
Nonseminoma					
≤10 mm (*n* = 8)	3/8/37.50%)	0/8 (0.00%)	0/8 (0.00%)	3/8 (37.50%)	4/6 (66.67%)
11–20 mm (*n* = 41)	14/41 (34.15%)	10/41 (24.39%)	3/41 (7.32%)	17/41 (41.46%)	27/29 (93.10%)
21–30 mm (*n* = 32)	19/32 (59.38%)	16/32 (50.00%)	6/31 (19.35%)	21/32 (65.63%)	19/20 (95.00%)
31–50 mm (*n* = 64)	41/64 (64.06%)	44/64 (68.75%)	17/62 (27.42%)	52/64 (81.25%)	32/34 (94.12%)
>50 mm (*n* = 34)	23/34 (67.65%)	27/34 (79.41%)	14/32 (43.75%)	30/34 (88.24%)	20/20 (100%)
p-value *	0.0022	<0.0001	<0.0001	<0.0001	0.0745

* Cochran–Armitage trend test, AFP/ bHCG elevation of AFP or bHCG or of both markers, N: number of patients eligible in subgroup.

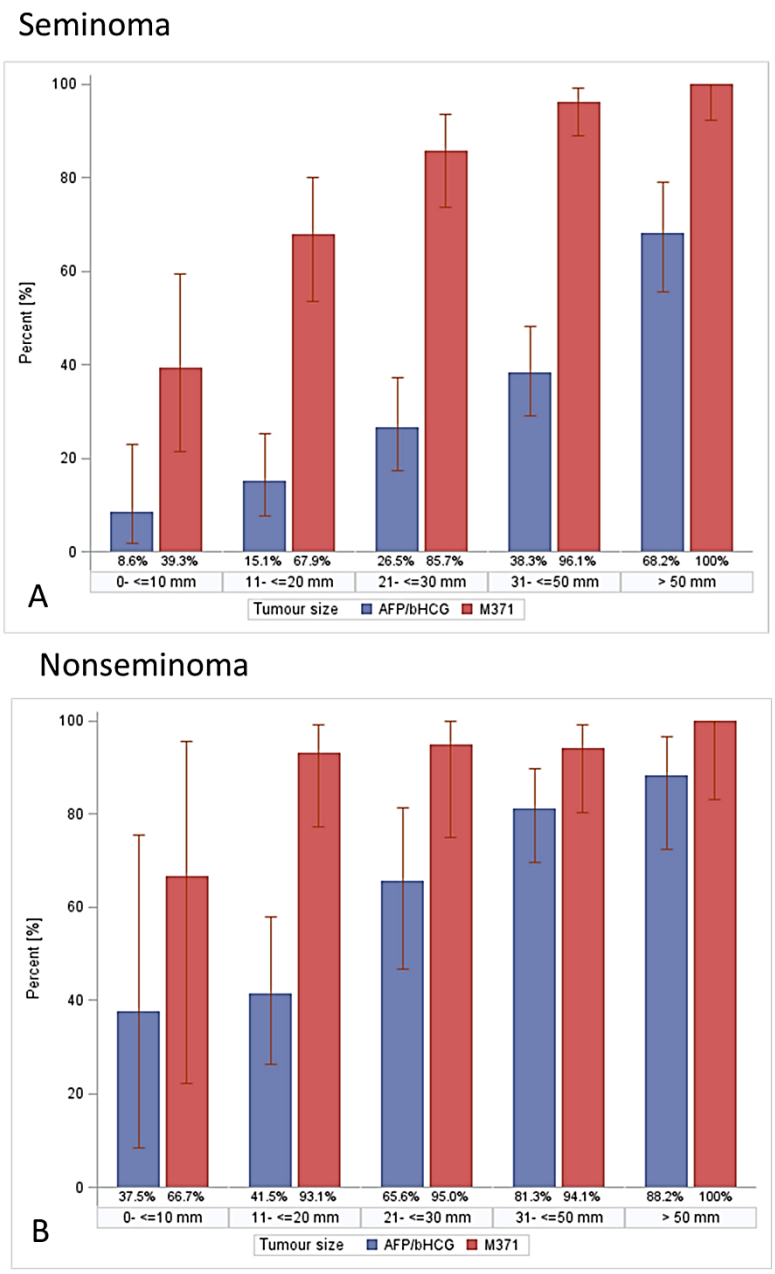

Figure 7. Expression rates of M371 and AFP and/or bHCG in germ cell tumours in relation to size of primary tumour in seminoma (**A**) and in nonseminoma (**B**). Blue columns denote expression rates of AFP and/or bHCG in five categories of tumour size, red columns indicate expression rates of M371. Overall, the expression rates of all tumour markers were higher in nonseminoma than in seminoma. All markers showed a significant trend towards lower expression rates with decreasing tumour size. M371 had higher expression rates than the other markers even in the smallest tumour size category. Error bars represent 95% CIs.

3.5. Assumption #4 (Association of Patient Age with Tumour Size)

Table 14 outlines the median tumour sizes in four age categories of the entire study population of patients with testicular neoplasms. Overall, the median tumour sizes were significantly different among the age categories, with the largest size in the age category >50 years (Kruskal–Wallis test $p = 0.0117$).

Table 14. Distribution of tumour sizes stratified by age categories in all patients with testicular neoplasms (SE, NS, BT, OM).

Age Categories (years)	Tumour Size (mm)					*p* *
	Min	Q1	Median	Q3	Max	
≤30	3	18	**32**	50	189	0.0117
31–40	4	15	**28**	43	95	
41–50	4	14	**24**	40	110	
>50	3	15	**35**	53	170	

Min: minimum, Q1: first quartile, Q3: third quartile, Max: maximum, * Overall comparison between age groups: $p < 0.0001$ (Kruskal–Wallis test).

However, there was no clear trend towards larger tumours in older patients or vice versa. Table 15 lists the median tumour sizes in the four age categories in patients with germ cell tumours. Here again, the largest median tumour size was observed in the oldest age category. Overall, the difference between the tumour sizes among the age categories was significant ($p = 0.0161$), but the difference between the youngest and the oldest age categories was only 1.5 years, and the inter quartile ranges widely overlapped. Thus, no clear association between age and tumour size could be documented.

Table 15. Distribution of tumour sizes stratified by age categories in patients with germ-cell tumours, only (SE + SE).

Age Categories (years)	Tumour Size (mm)					*p* *
	Min	Q1	Median	Q3	Max	
≤30	6	24	**35**	50	189	0.0161
31–40	4	20	**30**	45	95	
41–50	4	15	**27**	42	110	
>50	7	19	**36.5**	53	170	

Min: minimum, Q1: first quartile, Q3: third quartile, Max: maximum, * Overall comparison between age groups: $p < 0.0001$ (Kruskal–Wallis test).

4. Discussion

The present study thoroughly analysed the clinical influence of tumour size on presenting features of testicular neoplasms. One main result was that subcentimeter tumours comprised around 13.6% of all testicular new growths, which represented a frequency that had not been clearly specified before. Other central results were the significant associations of primary tumour size with the three factors histology of the primary, clinical staging, and serum tumour marker expression.

4.1. Tumour Size—General Considerations

The median sizes of seminomas and nonseminomas in the present study were 30 mm and 35 mm, respectively, with no significant difference between the two subtypes. This result is consistent with sizes of 35 mm found in both seminoma and nonseminoma in a Swiss study [21]. Similar results were reported in studies from Mannheim University with 37 mm (SE) and 38 mm (NS) [17,22], while a Cologne-based study reported a comparatively small mean size of 15 cm^3 for GCTs, which would correspond to a mean diameter of only

25 mm [23]. From Texas, a slightly higher mean diameter of 41 mm was reported for Americans of Caucasian descent [24]. However, as also shown in the present analysis, mean size may be somewhat larger than the median value. For decades, there has been a well-recognized shift towards decreasing sizes of primary testicular tumours [15,17,25–27]. This trend was also apparent when the present results were compared with the classical data reported from Toronto in 1966 with a median size of 5 cm in both seminoma and nonseminoma [28], and with the Dateca report from 1984 with mean sizes of 48 mm and 40 mm for seminomas and nonseminomas, respectively [29].

Of note, 13.6% of all tumours in the present study were within the subcentimeter range. During the last two decades, a plethora of studies reported on incidentally detected small testicular tumours; however, the relative frequency of such findings remained rather ill-defined [30]. A large study from the UK identified 81 (3%) subcentimeter masses among a total of 2681 patients with testicular neoplasms [16]. This figure is considerably lower than the 13.6% proportion observed in the present study. Selection bias may have contributed to the difference, as our institution represents a referral centre for testicular diseases. However, the difference may also partly relate to the well-documented time trend of decreasing tumour size, since the UK study recruited patients during 2003–2016, while our study included patients treated more recently (2012–2021). Differences regarding health system-related factors might have contributed to the difference as well. In a study from the U.S., 22 tumours (10.6%) out of 208 testicular neoplasms were shown to be smaller than 1 cm, which is quite close to our finding. However, that study reported lesion sizes measured sonographically, and the authors found that sonography may underestimate the true lesion size [31]. In a study from Turkey, a 13.9% proportion of subcentimeter tumours was reported, but, that study only evaluated tumours sized <3 cm [32]. Finally, a Swiss study reported a frequency of 3% of tumours sized <1 cm among 849 patients with GCT. However, that study enrolled only patients with germ cell tumours undergoing orchiectomy and excluded benign histologies and all cases with testis sparing surgery [33]. So, the true frequency of subcentimeter testicular tumours in that population was probably much higher. In light of the ever-growing clinical use of scrotal sonography in urologic practice and its ongoing technical refinements [8,9,34,35], the relative frequency of subcentimeter testicular tumours is probably in the range of 10% or more in contemporary series. This figure clearly underscores the clinical relevance of managing incidentally detected small testicular lesions.

4.2. *Association of Primary Tumour Size with Histology (Assumption #1)*

Benign tumours were shown to have a median size of only 10 mm, which is significantly smaller than that of GCTs (30 mm) and other malignant tumours (53 mm). More than 50% of all testicular tumours in the size category ≤10 mm consist of benign tumours, and we also noted a significant trend towards higher proportions of benign tumours with decreasing tumour size. Thus, small size of a testicular lesion appears to be a strong indicator of benign histology, and this result is in line with several previous reports [7,16,36–42]. The very high proportion of benign tumours among incidentally found small testicular masses had already been noted some decades ago [43], but this knowledge became clinically relevant only with the ever-growing number of small testicular neoplasms incidentally detected by improved ultrasonography technology. Accordingly, it was suggested to employ primary tumour size as a diagnostic tool for clinical assessment of incidentally detected small testicular masses [7,16]. A Turkish study of 252 patients including 35 cases with tumours ≤10 mm suggested a cutoff size of 15 mm to discriminate between benign and malignant tumours [32]. In the present study, the ROC analysis for prediction of malignancy by lesion size revealed a cutoff size of 16 mm to involve a sensitivity and specificity of 81.5% and 81%, respectively. Accordingly, the logistic regression curve revealed all tumours sized ≥ 39 mm to represent malignancy, while tumours ≤ 8 mm involved a 50% chance of being benign. Seven previous studies reporting results from ROC analyses are listed in Table 16 ([7,23,31,37,41,44,45]). Only one study reported on more than 100 patients [7].

Because of the divergent and mostly small sample sizes, the results are not consistent. Cutoff sizes ranged from 5 mm to 18.5 mm. Six studies reported sensitivities of >80%, while the others noted sensitivities of 55% or less, with AUC values ranging from 0.59 to 0.902.

Table 16. Sensitivity and specificity of tumour size as diagnostic tools for assessing small testicular masses—Synopsis of studies using ROC analysis.

		Patients	Cutoff	AUC	95% CI	Sensitivity	Specificity
First Author [#]	**Year**	**(*n*)**	**(mm)**			**(%)**	**(%)**
Shilo [37]	2012	11	18.5	0.902		87	83
Paffenholz [23]	2018	28	14	0.896		83	89
Gentile [7]	2019	108	8.5	0.75	0.63; 0.86	81	58
Staudacher [44]	2020	60	13.5	0.726	0.623; 0.828	55	85
Schwen [31]	2021	22	10	0.60		31.8	88.7
Gobbo [41]	2022	56	10	0.59	0.43; 0.75	25	92.9
Del Real [45]	2022	22	18.3	0.753		83	74
Present study	2022	79	16	0.8912	0.8569; 0.9256	81.5	81.0

n: patients with benign histology included in study; (#) number in list of references.

Though all of these investigations principally support the potential value of tumour size in predicting testicular histology, there is currently no consensus about the threshold sizes for clinical decision-making [46].

Caution comes from a recent study from Switzerland reporting metastatic disease in 5 of 25 patients (20%) with small germ cell tumours [33]. The authors, therefore, challenged the view that testicular masses < 1 cm are of benign nature without fail. Undisputedly, malignant testicular tumours may occur in the subcentimeter size category. In the present study, the proportion was 49%, and this finding is consistent with several other reports on small testicular tumours [7,16,38–40,45,47]. However, the authors of the Swiss study probably overestimated the risk of metastasized malignancy in newly detected testicular subcentimeter masses because they included only GCTs in patients undergoing full orchiectomy in their analysis, without considering benign tumours and neoplasms managed with TSS. In aggregate, tumour size is probably a valuable tool for clinically assessing small testicular masses, although it certainly needs to be employed with caution. In practical terms, conservative surgery using intraoperative frozen section examination appears to be appropriate in small testicular masses, since malignancy can principally be encountered among the incidentally detected lesions. Surveillance could be a solution in the very small masses (<5 mm) [9,35,48–50].

4.3. The Impact of Tumour Size on Clinical Staging in Testicular Germ Cell Tumours (Assumption #2)

The present study documented a clear association of tumour size with clinical stage. In the size category ≤10 mm, 97% of cases had localized disease (CS1). The median tumour size increased with increasing clinical stages, with a size of only 30 mm in CS1 and 50 mm in CS3. Very similar results were reported from the classical Dateca series where 84% of seminomas were sized <2.5 cm and had CS1 disease, whereas in the largest size category (>8.5 cm), only 43% had CS1. Likewise, in nonseminomas sized <2.5 cm, 71% of cases had CS1, as opposed to 42% in the largest size category [29]. The Mannheim group reported mean tumour sizes of 37 mm and 42 mm in seminoma cases with CS1 and in those with > CS1, respectively [17]. On the other hand, the present data also show that even in the size category >30 mm, as many as 69% of cases had CS1, and conversely, only 10% of all CS1 cases had tumour sizes ≤10 mm. Thus, the usefulness of tumour size as an aid for clinical decision making is probably limited because even patients with large primary tumours may have localized disease (CS1). Accordingly, the Indiana series did not observe different frequencies of lymph node metastases in tumour size categories <2 cm, 2–5 cm, and >5 cm, respectively, among nonseminoma patients undergoing retroperitoneal

lymph node dissection [51]. However, in seminoma, tumour size does appear to have a bearing on the metastatic potential of the tumour. In 2002, Warde et al. found a tumour size of >4 cm in testicular seminoma to involve a twofold risk of progression compared to smaller tumour sizes [52]. This significant association was confirmed by numerous clinical series and by two recent meta-analyses [11,53]. Accordingly, tumour size has been included as a prognostic factor for seminoma in all major guidelines [20,54,55]. It is unclear which particular biological mechanisms can induce metastatic spread (and thus higher clinical stages) in larger tumours. As tumour size is also associated with local pathological stage [19], it may be speculated that increasing tumour size may induce intratumoral processes promoting invasive tumour growth with metastatic spread.

4.4. The Association of Tumour Size with Serum Tumour Marker Expression Rates in Germ Cell Tumours (Assumption #3)

Significantly higher expression rates of all serum tumour markers were observed in the tumour size category >10 mm than in smaller tumours (≤10 mm). As serum markers are secreted from both the primary tumour and its metastases, the biological impact of tumour size on marker expression is best evaluated in localized disease (CS1). Here again, marker expression rates were significantly higher in the larger tumour size category (>10 mm), with the exception of LDH. The non-significant difference between the tumour size categories regarding LDH expression rate may relate to the low specificity of this marker for germ cell tumours.

In a more granular analysis evaluating marker expression rates in five tumour size categories, significant trends towards increasing marker expression rates with increasing tumour size were disclosed. This trend was found in both seminoma and nonseminoma, with the exceptions of AFP in seminoma ($p = 0.9133$) and M371 in nonseminoma ($p = 0.0745$). The two exceptions accord with expectations, since there was basically no AFP expression in seminoma, and hence no difference between tumour size categories regarding the expression of this marker. With respect to M371, the expression rate in nonseminoma was extremely high, with 66.7% even in the smallest size category; therefore, the difference relative to the larger size categories was comparatively small (100%). In light of the rather small sample size ($n = 109$, distributed over five size categories) the difference was no more significant, statistically. In all, these data clearly demonstrate the close association of serum-marker expressions with tumour bulk, and this result is consistent with the finding of higher serum levels of bHCG and AFP in patients with larger primary tumours than in those with smaller ones [12]. In another report, LDH expression rates were found to be higher in patients with larger tumours than in in those with small primaries [56]. In seminomas, bHCG serum levels were higher in patients with larger tumours than in those with small tumours [57]. In a small Spanish study, a significant correlation was found between tumour size and extent of elevations of bHCG and AFP [58]. With respect to clinical practice, the very low expression rates of bHCG and AFP (<10%) observed in the smallest tumour size category of germ cell tumours underscore the limited usefulness of the classical tumour markers for diagnosing subcentimeter testicular neoplasms. The novel marker M371 performed much better in that scenario, with expression rates of 66.7% and 39% in subcentimeter nonseminomas and seminomas, respectively. These results are widely in line with data reported from a multicentric study on 259 and 103 CS1 patients with seminoma and nonseminoma, respectively. In that study, M371 sensitivities of 56% and 98% were found for the diagnosis of subcentimeter seminomas and nonseminomas, respectively [14]. Support for the still-high sensitivity of M371 in small GCTs comes from a linear regression analysis reported from a Norwegian study on 131 patients with CS1 germ cell tumours. The authors found positive correlations between primary tumour size and M371 serum levels in both seminomas and nonseminomas, and quite a number of patients with subcentimeter primaries had measurable M371 levels. Notably, among the small tumours, a larger number of nonseminomas compared to seminomas had measurable M371 levels [59]. Overall, the sensitivity of M371 is better for nonseminoma than for seminoma

regarding small testicular masses. However, in light of the almost negligible sensitivity of the classical markers in subcentimeter testicular neoplasms, the sensitivity of around 40% for M371 in small seminomas still represents significant progress, and probably, the M371 test can be a valuable tool for diagnosing small testicular neoplasms.

4.5. No Association between Patient Age and Tumour Size (Assumption #4)

In the entire population of patients and also in the population of germ cell tumours, the largest median tumour sizes were observed in the oldest age category. Although the overall analysis revealed significant differences in median sizes among age categories, no clear trend of varying tumour sizes with age categories became apparent. Moreover, the youngest age category proved to present with the second largest tumour size, with only a 1.5 cm difference between the oldest and youngest category. These results are consistent with a previous investigation where no difference in tumour sizes had been found by comparing GCT patients <50 years with older ones [19]. Thus, the hypothesis of a trend towards larger tumours in the elderly, put forward by a UK-study, could not be confirmed by the present study [15].

4.6. Limitations of the Study

The retrospective study design could be a limitation to the present analysis because selection bias cannot entirely be ruled out. Small sample sizes in the histological subgroups BT and OM could have limited statistical power in spite of the overall large patient number in the study. Histological diagnoses relied on local pathological assessments only, with no central histopathological review. However, as both of the urologic departments participating in the present study had a clinical focus on testicular cancer, local pathologists clearly had suitable experience with testicular pathology. Misclassification of benign tumours cannot entirely be ruled out, since malignancy in some of these cases can only be documented by development of metastases during follow-up. However, as only one quarter of BTs had follow-up periods of less than 2 years, we believe that misclassification of BTs is probably only a minor problem. Missing information regarding therapeutic outcome might be another shortfall; however, the present investigation specifically aimed to analyse the biological role of tumour-size with regard to presenting features of testicular neoplasms. One possible strength of the study is the large number of patients with measurements of the novel tumour marker M371, featuring the utility of this test in clinical practice. Another asset could be the rather large number of patients with small testicular neoplasms, underscoring the utility of the factor of tumour size as a valuable tool in assessing such lesions.

5. Conclusions

The present study confirmed the significant biological role of tumour size in the clinical course of testicular neoplasms. In the diagnostic work-up of small testicular neoplasms, the factor of tumour size may be implemented clinically since more than 50% of cases with testicular new growths sized $\leq$10 mm had benign histologies. This knowledge may help in avoiding unnecessary orchiectomies and may open the door for a wider application of testis sparing surgery in these cases [60]. Increasing tumour sizes are associated with both increasing frequencies of metastatic disease (clinical stages > CS1) and increasing frequencies of elevation of serum tumour markers. Of note clinically, the novel marker M371 not only outperformed the classical markers in general, but was also expressed in around 40% of subcentimeter germ cell tumours. The present study did not look for the underlying molecular biologic processes that trigger the significant associations of tumour size with other clinical factors. However, it may be speculated that on the one hand, larger tumours involve a higher probability of the presence of marker-producing cells, and on the other hand, the larger number of proliferating cells involve a higher chance of developing cells with more aggressive features precipitating metastatic spread. The present study provides a multitude of data regarding descriptive features of testicular tumours that may

frame future molecular biological investigations and may thus aid in understanding the underlying biological characteristics of this disease.

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Data Availability Statement: The datasets generated and analyzed in this study are available upon reasonable request.

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Conflicts of Interest: K-PD and GB declare ownership of shares, each of 8.9%, in mirdetect GmbH, Bremerhaven, Germany. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

AFP	Alpha fetoprotein
AUC	area under the curve
bHCG	beta human chorionic gonadotropin
BT	benign tumours
CI	confidence interval
CS	clinical stage
GCT	germ cell tumour
IQR	inter quartile range
LDH	lactate dehydrogenase
M371	microRNA-371a-3p
mm	milli meter
NS	nonseminoma
n	number
OM	other testicular malignancies
ROC	receiver operating characteristic
SE	seminoma
STM	serum tumour marker
TS	tumour size
ULN	upper limit of norm
Q1	first quartile
Q3	third quartile

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3.4 Publication IV: Testicular neoplasms: the interrelationships of serum levels of microRNA-371a-3p (M371) and classical tumor markers with histology, clinical staging, and age-a statistical analysis

This study builds upon the previous research by (Dieckmann et al., 2022c), further investigating the interrelationships among clinical factors. As described in the prior study, testicular neoplasms are influenced by various clinical factors, which guide initial management and treatment strategies according to established guidelines (Kliesch et al., 2021; Dieckmann et al., 2022c; Oldenburg et al., 2022). Unlike some other malignancies where molecular genetic features play a significant role, in testicular tumors, the identification of isochromosome 12p remains the primary molecular tool for certain cases (Khoury et al., 2022). Additionally, the role of the novel tumor marker M371 in comparison to traditional markers is evolving (Dieckmann et al., 2019b), and patient age is recognized as a crucial factor in decision-making (Terbuch et al., 2019). The aim of this study was to systematically analyze four clinical scenarios: (1) the impact of patient age on histology, (2) the relationship between histology and serum marker expression, (3) the association of tumor marker expression frequencies with clinical stages, and (4) the association between marker elevations and patient age. A total of 641 patients with a median age of 38 years were enrolled in this study. The histologic groups analysed in this study include seminoma and non-seminoma, benign tumors, and other malignant tumors.

The results showed a significant association between patient age and histologic subgroups of testicular tumors ($p < 0.0001$, chi-square test), with non-seminoma prevalent in the youngest age category (61.5%), while seminoma being more prevalent in the 41–50 age range (73.1%).

The expression rates of tumor markers varied significantly among histologic subgroups. M371 exhibited the highest expression rate at 93.6% in non-seminoma. When considering overall, the expression rates of each markers differed significantly among the histologic subgroups. Specifically, a statistically significant difference in M371 expression was observed between seminoma and non-seminoma subgroups (82.7% versus 93.6%, chi-square test, $p = 0.0061$). In non-seminoma cases, M371 showed a higher expression rate (93.6%) than classical markers AFP/bHCG (68.7%). Benign tumors and other malignant tumors showed marker elevations only in isolated cases. Moreover, there was a significant correlation between tumor marker expression

rates and increasing clinical stages ($p<0.001$; Cochran-Armitage trend test). In metastasized cases, M371 displayed a remarkable 100% expression rate, surpassing the combined rate of AFP/bHCG (66,7%). In all clinical stages, M371 demonstrates a significantly higher expression rate compared to each of the classical markers and as well as when they are combined.

Regarding the age, the study reveals significant variations in the expression rates of tumor markers among different age categories. It indicates that younger age is associated with higher expression rates of tumor markers, particularly for bHCG, AFP, and M371. In contrast, LDH expression rates remained relatively consistent across age categories.

In summary, the study highlights significant correlations between patient age, histology, and clinical staging with serum tumor marker expression rates in testicular neoplasms, emphasizing the impact of tumor bulk. The novel marker M371 demonstrated superior performance compared to classical markers in a large patient sample, underscoring its clinical utility in enhancing diagnostic precision.

-IV-

Testicular neoplasms: the interrelationships of serum levels of microRNA-371a-3p (M371) and classical tumor markers with histology, clinical staging, and age - a statistical analysis

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Own contribution

- Experimental concept and study design: 10%
- Experiment execution and/or acquisition of experimental data: 50%
- Data analysis and interpretation: 25%
- Preparation of Figures and Tables: 10%
- Drafting of manuscript: 10%

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RESEARCH

Testicular neoplasms: the interrelationships of serum levels of microRNA-371a-3p (M371) and classical tumor markers with histology, clinical staging, and age—a statistical analysis

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Abstract

Purpose In testicular neoplasms, the interrelationship of elevations of the novel serum tumor marker microRNA-371a-3p (M371) and traditional markers with other clinical features is still incompletely understood. The present study evaluated marker expression rates in relation to various other clinical parameters.

Methods The following data were retrospectively registered from 641 consecutive patients with testicular neoplasms: histology, such as seminoma ($n = 365$), nonseminoma ($n = 179$), benign tumor ($n = 79$), other malignant tumor ($n = 18$); patients age (years); clinical stage (CS1, CS2a/b, CS2c, CS3); and preoperative elevation of beta HCG, AFP, LDH, M371 (yes/no). Descriptive statistical methods were employed with comparisons of various subgroups to disclose associations of marker expression rates with age, histology and CS, and of age with histology.

Results The histologic subgroups revealed significantly different expression rates of tumor markers. M371 performed best with expression rates of 82.69% and 93.58% in seminoma and in nonseminoma, respectively. In germ cell tumors, all markers had significantly higher expression rates in metastasized stages than in localized disease. All markers except LDH have significantly higher expression rates in younger than in older patients. Nonseminoma is most prevalent in the youngest age category, seminoma predominates in patients > 40 years, other malignancies were restricted to patients > 50 years.

Conclusion The study documented significant associations of serum marker expression rates with histology, age and clinical staging, with highest rates in nonseminomas, young age and advanced clinical stages. M371 showed significantly higher expression rates than other markers suggesting its superior clinical usefulness.

Keywords Testicular neoplasms · Seminoma · Nonseminoma · MicroRNA-371a-3p · Clinical stage · Alpha fetoprotein · Beta HCG · Lactate dehydrogenase

Abbreviations

AFP	Alpha fetoprotein
AUC	Area under the curve
bHCG	Beta human chorionic gonadotropin
BT	Benign tumors
CI	Confidence interval
CS	Clinical stage
GCT	Germ cell tumor
IQR	Inter-quartile range

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LDH	Lactate dehydrogenase
M371	MicroRNA-371a-3p
NS	Nonseminoma
n	Number
OM	Other testicular malignancies
SE	Seminoma
Q1	First quartile
Q3	Third quartile

Introduction

The factors histology, clinical stage (CS), serum tumor marker elevation, and patient age represent the traditional mainstay of clinical decision-making in testicular neoplasms according to recent guide-lines (Kliesch et al. 2021; Oldenburg et al. 2022; Gilligan et al. 2019). In contrast to other malignancies, particularly hematological neoplasms (Khoury et al. 2022), molecular genetic features have only gained marginal clinical importance in testicular tumors to date. Searching for isochromosome 12p for identification of germ cell tumors (GCTs) is the only molecular genetic tool employed in selected cases of testis tumors (Wyvekens et al. 2022). Histology forms the basis for the first strategic decision in the management of testicular neoplasms, because benign tumors do not need further treatment after surgery while the management of GCTs needs to be tailored to seminomatous or nonseminomatous histology and secondly to clinical stage (Cheng et al. 2018; Chovanec and Cheng 2022). Elevation of traditional serum tumor markers alpha fetoprotein (AFP), beta human chorionic gonadotropin (bHCG) and lactate dehydrogenase (LDH) is a typical feature of nonseminomatous GCTs, while it is less frequently found in seminomas and it is not observed in other testicular neoplasms (Leão et al. 2018). The extent of serum tumor marker elevation is paramount for prognostic grouping according to the International Germ Cell Cancer Consensus Group (IGCCCG) (Gillessen et al. 2021; Beyer et al. 2021). Recently, the novel serum tumor marker microRNA-371a-3p (M371) has been reported to outperform the traditional markers (Dieckmann et al. 2019a; Fankhauser et al. 2022). However, despite continuously increasing numbers of scientific reports (Leão et al. 2021; Almstrup et al. 2020; Regouc et al. 2020; Konneh et al. 2022), the full spectrum of features of this new marker is still little understood. Finally, patient age is a critical factor for clinical decision-making, because in the elderly, toxicity of treatment is much greater and cure rates are clearly inferior (Terbuch et al. 2019; Miller et al. 2017; Gillessen et al. 2021).

Most likely, the factors serum tumour marker expression, histology, clinical staging, and age represent a biological network with manifold interrelationships between each other. However, the interplay of these factors is only scantily explored (Dieckmann et al. 2019b). Accordingly, we evaluated four particular clinical scenarios, all of which represent associations of clinical factors that are frequently encountered in clinical practice, but the formal evidence derived from contemporary case series is still limited: *(#1)* Patient age has a bearing on histology of the primary, with quite divergent age predispositions of the various histologic subtypes of testicular neoplasms. *(#2)* Histology of testicular neoplasms impacts the frequencies of elevations of serum tumor markers with high expression rates in nonseminomas, moderate rates in seminoma and no expression in non-germ cell tumors. M371 is expected to be expressed in both subgroups of GCTs but not in non-germ cell tumors. *(#3)* Frequencies of tumor marker expression in GCTs are associated with clinical stages with higher frequencies of elevations in advanced clinical stages. *(#4)* Frequencies of marker elevations are inversely associated with age.

To test these clinical associations, we systematically analyzed the four parameters in a large series of consecutive patients with testicular tumors.

Materials and methods

Patients recruitment, data procurement

The patient population of this retrospective study consisted of all male subjects aged 17–98 years, diagnosed with testicular new growths while undergoing surgery in two Hamburg based urologic departments (Albertinen-Krankenhaus and Asklepios Klinik Altona) during 2012–2021. Patients with previous chemotherapy were excluded. The following data were secured from hospital-based electronic case files: patient's age (years); histology of the surgical specimen categorized as seminoma (SE), nonseminoma (NS), benign tumor (BT), malignant tumor other than GCT (OM); clinical stage (CS) at diagnosis (only in GCTs); and preoperative elevation of serum tumor markers bHCG, AFP, LDH, M371. The majority of the patients had been included in previous reports on other issues (Dieckmann et al. 2019a, 2019b, 2018, 2022a).

Histological diagnoses were derived from electronic documents without central pathology review. Clinical stages were filed as CS1; CS2a/b; CS2c; and CS3 according to modern guide-lines (Kliesch et al. 2021). Serum tumor markers bHCG, AFP, and LDH were measured in hospital laboratories according to institutional standard operating procedures. As test kits were repeatedly replaced for economic reasons during 2012–2021, the normal limits of tumor marker levels had to be adjusted, accordingly. Therefore, we restricted the study of serum tumor markers to a dichotomized analysis (i.e., elevation above the upper limit of norm [ULN] yes/no). The novel marker M371 was

measured by quantitative polymerase chain reaction with quantification in relation to endogenous miR-30b-5p, as detailed earlier (Dieckmann et al. 2019a). M371 measurement results were originally documented as relative quantity (RQ) values defining RQ = 5 as ULN. For reasons of methodological conformity, only dichotomized results were recorded (elevated yes/no).

The Ethikkommission der Ärztekammer Hamburg gave ethical approval (PV7288). The need for informed consent of patients was waived, because merely anonymized data were subject to the investigation. All study activities fully complied with the Declaration of Helsinki of the World Medical Association as amended by the 64th General Assembly, October 2013.

Statistical analysis

All case-related data were originally filed in a commercially available data base (MS Excel, version 2017) after thorough data validation. Final statistical analysis was conducted with SAS software package version 9.4 (SAS Institute, Cary, NC, USA) on windows platform.

Descriptive statistical analysis of nominal variables was accomplished by calculating absolute frequencies, percentages and 95% exact Clopper–Pearson confidence intervals (CIs).

Descriptive statistical analysis of continuous variables involved calculation of median, first quartile (Q1), third quartile (Q3), interquartile range (IQR), minimum, and maximum. Chi-square tests were used to compare contingency tables of nominal variables. Cochran–Armitage trend tests were applied to assess whether marker expression rates increase with clinical stages or with increasing age categories. The Kruskal–Wallis tests were applied to test for any differences in the distribution of ages between more than two subgroups of patients defined by histology. To assess marginal homogeneity as well as concordance of serum marker elevation rates, McNemar's test and Cohen's kappa statistics were employed. A kappa value below 0.60 indicates a significant level of disagreement. P-values less than 0.05 were considered as statistically significant in this paper. Data on serum tumor marker expressions particularly for M371 were missing in about one third of patients. Thus, the statistical analyses employed varying sample sizes according to the available entries.

Results

General results

A total of 641 patients with a median age of 38 years were enrolled. The frequencies of the four histologic subgroups with corresponding median ages are outlined in Table 1 and Fig. 1. Part of this descriptive analysis had been reported earlier (Dieckmann et al. 2022a). The distribution of ages is significantly different by overall comparison across histologic groups ($p < 0.001$, Kruskal–Wallis test). Benign tumors (BT) comprised of gonadal stromal tumors for the most part and benign epidermoid cysts and other rare neoplasms to a lesser degree. Other malignant tumors (OM) comprised of various forms of malignant testicular lymphoma. Age, histology, and clinical staging were available in all patients. Marker elevations regarding AFP and bHCG were available in 640 patients, regarding LDH in 633 cases, and with respect to M371 in 451 patients.

Association of patient age with histology

The frequencies of the histologic subgroups stratified in four categories of patient age are listed in Table 2. Overall, the frequencies of histologic subgroups are significantly different among the age categories ($p < 0.0001$, chi-square test). In the youngest age category, nonseminoma represents the most frequent subtype with 61.5%. In the age category 41–50 years, seminoma has the highest proportion with 73.1%. All patients of the OM subgroup are aged > 50 years. Table 3 lists the results of statistical comparisons of particular subgroups regarding the frequencies of age categories. Nonseminoma is more prevalent in the younger age categories than seminoma ($p < 0.0001$), while germ cell tumors as a whole (SE + NS) are not statistically different from benign tumors regarding the distribution of age categories.

Table 1 Patient population, frequencies of histologic subgroups and corresponding ages

	n (% of all)	min	Age (years) Q1	Med	Q3	max
Total population	641 (100%)	17	31	38	47	98
Seminoma	365 (56.94%)	17	33	40	48	78
Nonseminoma	179 (27.93%)	17	26	31	37	74
Benign tumours	79 (12.32%)	19	32	41	50	68
Other malignant tumours	18 (2.81%)	52	68	72.5	78	98

Kruskal–Wallis Test $p < 0.001$ (for overall comparison of groups regarding age)

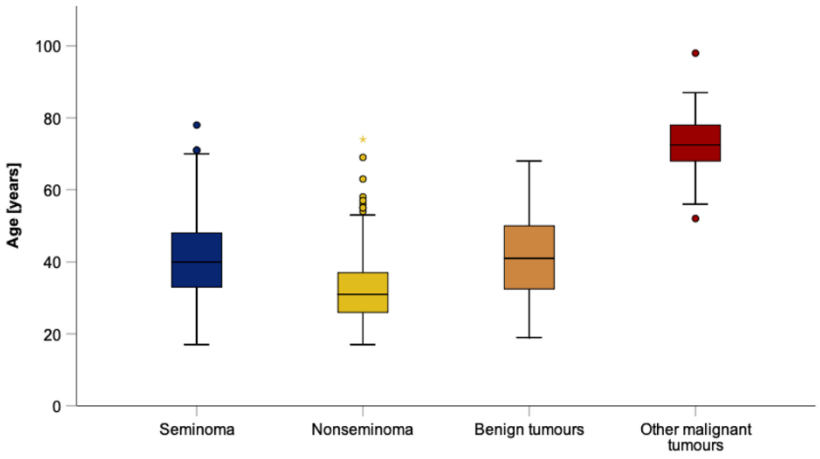

Fig. 1 Overview of age distributions in four histologic subgroups of testicular neoplasms. Box–Whisker plots showing the distribution of patient ages stratified by histologic subtypes of testicular neoplasms. The box displays the first quartile, median and third quartile. The whiskers are defined as the largest or lowest observed value that falls within the 1.5 times the interquartile range measured from Q3 or Q1, respectively. Dots represent outliers

Table 2 Frequencies of histologic subgroups in various age categories

Age categories (years)	*n*	SE (%)	NS (%)	BT (%)	OM (%)
≤30	143 (100%)	40 (27.97%)	88 (61.54%)	15 (10.49%)	0 (0.00%)
31–40	229 (100%)	147 (64.19%	58 (25.33%)	24 (10.48%)	0 (0.00%)
41–50	156 (100%)	114 (73.08%)	21 (13.46%)	21 (13.46%)	0 (0.00%)
> 50	113 (100%)	64 (56.64%)	12 (10.62%)	19 (16.81%)	18 (15.93%)

Chi-Square Test $p < 0.0001$ (for overall distribution of histologic subgroups among age categories)

SE seminoma, *NS* nonseminoma, *BT* benign tumors, *OM* other malignant tumors

Table 3 Comparisons of histologic subgroups with regard to distribution of age categories (*p* values) according to data listed

Histologic group	Compared with (histologic group)	*P* value*
Seminoma (SE)	Nonseminoma (NS)	< 0.0001
GCT (SE + NS)	Non-GCT (BT + OM)	< 0.0001
GCT (SE + NS)	Benign tumors (BT)	0.0977
GCT (SE + NS)	Other malignant tumors (OM)	< .0001

*Chi-Square Test, *GCT* germ cell tumor

Serum tumor marker expressions vary among histologic groups

Table 4 outlines the frequencies of elevations of each of the tumor markers (expression rates) in the four histologic subgroups. The highest observed rate represents 93.6% for M371 in nonseminoma. The expression rates of each of the markers are statistically different among the histologic subgroups by overall comparison. The results of statistical comparisons of marker expression rates among particular

Table 4 Tumor marker expression rates in histologic subgroups

	bHCG n/N (%) [95% CI]	AFP n/N (%) [95% CI]	LDH n/N (%) [95% CI]	AFP/ bHCG n/N (%) [95% CI]	M371 n/N (%) [95% CI]
SE (*n* = 365)	106/364 (29.12%) [24.50%, 34.08%]	19/364 (5.22%) [3.17%, 8.03%]	82/362 (22.65%) [18.44%, 27.32%]	122/364 (33.52%) [28.68%, 38.62%]	215/260 (82.69%) [77.54%, 87.09%]
NS (*n* = 179)	100/179 (55.87%) [48.27%, 63.27%]	97/179 (54.19%) [46.59%, 61.64%]	40/174 (22.99%) [16.96%, 29.96]	123/179 (68.72%) [61.37%, 75.42%]	102/109 (93.58%) [87.22%, 97.38%]
BT (*n* = 79)	2/79 (2.53%) [0.31%, 8.85%]	2/79 (2.53%) [0.31%, 8.85%]	1/79 (1.27%) [0.03%, 6.85%]	4/79 (5.06%) [1.40%, 12.46%]	5/71 (7.04%) [2.33%, 15.67%]
OM (*n* = 18)	0/18 (0.00%) [0.00%, 18.53%]	0/18 (0.00%) [0.00%, 18.53%]	2/18 (11.11%) [1.38%, 34.71%]	0/18 (0.00%) [0.00%, 18.53%]	3/10 (30.00%) [6.67%, 65.25%]

CI represent exact Clopper–Pearson confidence interval; N number of patients eligible

AFP/bHCG elevation of either AFP or bHCG or of both markers

histologic subgroups are listed in (Table 5). The difference between SE and NS regarding M371 expression is statistically significant (82.7% versus 93.6%, chi-square test, $p = 0.0061$).

In nonseminoma, the expression rates of AFP/bHCG and M371 are not much different from each other with 68.7% and 93.6%, respectively. To test for agreement of NS cases expressing M371 and AFP/ bHCG at the same time, we employed McNemar's test by analyzing 109 patients where both markers were available (Table 6). In this setting, the expression rates of AFP/bHCG and M371 were 69.72% and 93.58%, respectively. Testing for marginal homogeneity revealed a significant difference between the two rates ($p < 0.0001$, McNemar). The low Cohen's kappa coefficient of 0.11 further demonstrates the large disagreement between the two markers. Also, the 95% CIs of the frequencies of AFP/bHCG (61.37–75.42%) and M371 (87.22–97.38%) do not overlap. Thus, the M371 expression rate in nonseminoma is clearly superior to the combined measurement of classical markers (AFP/bHCG).

Benign tumors and other malignant tumors revealed elevations of tumor markers only in isolated cases. But of note, a 30% expression rate of M371 was found in other malignant tumors; however, the confidence intervals are extremely wide (6.67%—65.25%).

Table 5 Comparisons of tumor maker expression rates among histologic subgroups (*p* values) according to data listed

	bHCG *p*	AFP *p*	LDH *p*	AFP/ bHCG *p*	M371 *p*
overall	< 0.0001	< 0.0001	0.0001	< 0.0001	< 0.0001
SE vs. NS	< 0.0001	< 0.0001	0.9307	< 0.0001	0.0061
SE + NS vs. (OM + BT)	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

p values relate to chi-Square Test for comparison of frequencies of marker elevation rates across histologic subgroups; AFP/bHCG elevation of either AFP or bHCG or of both markers

Association of tumor marker expression rates with clinical staging in germ cell tumors

The expression rates of the serum tumor markers in the four clinical stages of GCTs are listed in Table 7. There is a clear and statistically significant increase of expression rates with increasing clinical stages with respect to each of the markers (all $p < 0.001$; Cochran–Armitage trend test). Noteworthy, in all metastasized stages (> CS1), M371 revealed a 100% expression rate (95% CIs 94.22 – 100%) while the rate of AFP/bHCG is 66.7% (95% CIs 58.27–74.94%). In all clinical stages, the expression rate of M371 is clearly superior to that of each of the classical markers and also to combined measurement of markers.

Association of age with serum marker expression rates

The expression rates of each of the tumor markers stratified by four age categories found in the entire study population (all histologic groups) are listed in Table 8. The expression rates of bHCG, AFP, and M371 are significantly different among age categories, but this not true for LDH (chi-square test). The trend of increasing expression rates in decreasing (younger) age is confirmed for bHCG, AFP, and M371, but not for LDH (Cochran–Armitage trend test). If only GCT patients are considered (Table 9), significantly different expression rates among age categories are only found for bHCG and AFP but not for LDH and M371 (chi-square test). However, the trend towards higher expression rates with decreasing age is found for bHCG, AFP, and also M371. This statistical trend is only weakly significant in M371 ($p = 0.04$), probably because all age categories revealed

Table 6 Detailed comparison of expression rates of AFP/bHCG and M371 in nonseminoma by analyzing the cases where both values are available ($n = 109$)

n (%)	M371		Overall AFP/ bHCG expression
AFP/bHCG	Negative	Positive	
Negative	4 (3.67%)	29 (26.61%)	33 /109 (30.28%)
Positive	3 (2.75%)	73 (66.97%)	76 /109 (69.72%)
Overall M371 express/ bHCGM371ion	7 /109 (6.42%)	102/109 (93.58%)	109 (109 (100%)
p value*	< 0.0001		
Agreement, *n* (%)	77 /70.64%)		
Disagreement, *n* (%)	32 (29.36%)		
κ**, 95% CI	0.1052 [-0.05; 0.26]		

*McNemar's test assessed the similarity of the overall (marginal) rates of marker positivity/negativity between the two markers

**Cohen's kappa assessed the agreement between/concordance of the expression of the two markers

Table 7 Frequencies of tumor marker expression rates in clinical stages of testicular germ cell tumors (SE + NS)

	bHCG	AFP	LDH	AFP/ bHCG	M371
	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]
CS1 ($n = 421$)	136/420 (32.38%) [27.92%, 37.09%]	71/420 (16.90%) [13.45%, 20.84%]	66/417 (15.83%) [12.46%, 19.69%]	163/420 (38.81%) [34.12%, 43.65%]	255/307 (83.06%) [78.39%, 87.08%]
CS2a/2b ($n = 84$)	43/84 (51.19%) [40.04%, 62.26%)	28/84 (33.33%) [23.42%, 44.46%]	27/80 (33.75%) [23.55%, 45.19%]	51/84 (60.71%) [49.45%,71.20%)	51/51 (100%) [93.02%, 100%]
CS2c ($n = 15$)	10/15 (66.67%) [38.38%,88.18%]	3/15 (20.00%) [4.33%, 48.09%]	10/15 (66.67%) [38.38%,88.18%]	12/15 (80.00%) [51.91%, 95.67%]	3/3 (100%) [29.24%, 100%]
CS3 ($n = 24$)	17/24 (70.83%) [48.91%, 87.38%]	14/24 (58.33%) [36.64%, 77.89%]	19/24 (79.17%) [57.85%, 92.87%]	19/24 (79.17%) [57.85%, 92.87%]	8/8 (100%) [68.77%, 100%]
p value*	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0009

CI represent exact Clopper–Pearson confidence interval; AFP/bHCG elevation of either AFP or bHCG or of both markers; *Cochran–Armitage Trend Test; N number of patients eligible; p values relate to comparisons of marker frequencies among clinical stages

Table 8 Frequencies of tumor marker expression rates in four age categories of all patients with testicular neoplasms (SE + NS + BT + OM)

	bHCG	AFP	LDH	AFP/bHCG	M371
Age (years)	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]
≤ 30 ($n = 143$)	68/143 (47.55%) [39.15%, 56.06%]	52/143 (36.36%) [28.49%, 44.25%]	34/142 (23.94%) [17.19%, 31.82%]	77/143 (53.85%) [45.68%, 62.02%]	75/96 (78.13%) [68.53%, 85.92%]
31–40 ($n = 229$)	67/229 (29.26%) [23.45%, 35.61%]	39/229 (17.03%) [12.40%, 22.54%]	35/226 (15.49%) [11.03%, 20.87%]	83/229 (36.24%) [30.02%, 42.47%]	128/166 (77.11%) [69.96%, 83.26%]
41–50 ($n = 156$)	46/155 (29.68%) [22.62%, 37.53%]	15/155 (9.68%) [5.52%, 15.46%]	36/154 (23.38%) [16.94%, 30.86%]	54/155 (34.84%) [27.37%, 42.90%]	76/109 (69.72%) [60.19%, 78.16%]
> 50 ($n = 113$)	27/113 (23.89%) [16.37%, 32.83%]	12/113 (10.62%) [5.61%, 17.82%]	20/111 (18.02%) [11.37%, 26.45%]	35/113 (30.97%) [22.61%, 40.36%]	46/79 (58.23%) [46.59%, 69.23%]
p value*	0.0002	< .0001	0.1296	0.0004	0.0085
p value**	0.0002	< .0001	0.6792	0.0001	0.0014

CI represent exact Clopper–Pearson confidence intervals; AFP/bHCG elevation of either AFP or bHCG or of both markers; *Chi-Square Test (relates to overall distribution of marker frequencies among age categories); **Cochran–Armitage Trend test (test for trend across age categories); N number of patients eligible

Table 9 Frequencies of tumor marker expression rates in four age categories of patients with testicular germ cell tumors (SE + NS)

	bHCG	AFP	LDH	AFP/ bHCG	M371
Age (years)	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]	n/N (%) [95% CI]
≤ 30 ($n = 128$)	68/128 (53.13%) [44.11%, 62.00%]	52/128 (40.63%) [32.04%, 49.66%]	34/127 (26.77%) [19.31%, 35.35%]	77/128 (60.16%) [51.13%, 68.70%]	75/81 (92.59%) [84.57%, 97.23%]
31 – 40 ($n = 205$)	67/205 (32.68%) [26.31%, 39.56%]	39/205 (19.02%) [13.89%, 25.08%]	34/202 (16.83%) [11.95%, 22.72%]	83/205 (40.49%) [33.71%, 47.55%]	126/146 (86.30%) [89.64%, 91.43%]
41 – 50 ($n = 135$)	44/134 (32.84%) [24.97%, 41.47%]	14/134 (10.45%) [5.83%, 16.91%]	36/133 (27.07%) [19.73%, 35.45%]	51/134 (38.06%) [29.82%, 46.84%]	74/91 (81.32%) [71.78%, 88.72%]
> 50 ($n = 76$)	27/76 (35.53%) [24.88%, 47.34%]	11/76 (14.47%) [7.45%, 24.42%]	18/74 (24.32%) [15.10%, 35.69%]	34/76 (44.74%) [33.31%, 56.5 9%]	42/51 (82.35%) [6 9.13%, 91.60%]
P value*	0.0008	< .0001	0.0818	0.0010	0.1629
P value**	0.0068	< .0001	0.7560	0.0098	0.0400

CI represent exact Clopper–Pearson confidence intervals; AFP/bHCG elevation of either AFP or bHCG or of both markers; *Chi-Square Test; **Cochran–Armitage Trend test; N number of patients eligible

high expression rates of M371 above 80%, yet with a slight increase to 92.6% in the youngest age category.

Discussion

The present study provides clear evidence for significant associations of serum tumor marker expression rates with histology, patient age, and with clinical staging in patients with testicular neoplasms. In GCTs, marker expression rates increase with clinical stages. The novel marker M371 significantly outperforms the classical serum markers AFP, bHCG and LDH. Also, younger patient age is significantly associated with higher serum marker expression rates. The present study confirmed current clinical experience but also expanded and corroborated existing knowledge by analyzing a large sample of contemporary and unselected patients and by employing comprehensive statistical analysis, thus providing a high level of formal evidence.

Association of age with histology of testicular tumor

The median ages of seminoma (SE), nonseminoma (NS), benign tumors (BT) and other malignant tumors (OM) are significantly different from each other. Conversely, the proportions of the four histologic subgroups vary significantly among age categories. Of note, malignancies other than germ cell tumors (OM) were only observed in patients aged > 50 years. This observation relates to malignant lymphoma, which represents the only histologic entity found in the OM group and this malignancy typically presents in patients aged > 60 years (Xu and Yao 2019; Koch et al. 2022). In the youngest age category (≤ 30 years), nonseminoma revealed the highest prevalence with 61.5%, while in the oldest age category (> 50 years), seminoma ranked first comprising of 56.6%. Benign tumors (BT) showed almost equal frequencies in all four age categories. These results are in accordance with the classical data reported from the large population of the British Testicular Tumour Panel ($n = 1812$) where mean ages of 41.2 years; 29.8; 59.8; and 33.3—47.5 years were noted in seminomas, nonseminomas, malignant lymphoma, and benign gonadal stromal tumors, respectively (Pugh 1976). The significantly younger median age of nonseminoma in comparison to seminoma is settled knowledge since decades and reflects the biologic difference between the two entities (Friedman and Moore 1946). The BT group of the present study consisted of Leydig cell tumors for the major part, and the median age of 41 years found in this group is identical with the median age of 41 years reported in a recent study on 208 cases with Leydig cell tumors (Ruf et al. 2020). However, this study also pointed out that Leydig cell tumors may occur at any age between 17 and 81 years and this observation is in line with the age distribution found in the present investigation. Likewise, a study from Wessex, UK, reported an arithmetical mean age of benign gonadal stromal tumors of 43 years but in view of the wide range of 18 – 79 years, no particular age predisposition was noted (Featherstone et al. 2009). An Italian study on small testicular tumors occurring in 64 infertile males reported a median age of 40 years in patients with benign tumors compared to the significantly younger age of 36 years in malignant tumors (Gobbo et al. 2022). Similarly, an Austrian study reported mean ages of 41.1 years and 32.5 years in benign and malignant tumors, respectively (Staudacher et al. 2020). However, the latter two studies probably involve selection bias. The Italian study had selectively enrolled patients with infertility which is rarely encountered in patients aged > 50 years. The Austrian study had solely included patients with tumors sized < 2 cm. By contrast, the present patient population comprises of unselected patients of all ages and of testicular new growths of all sizes. In all, patient age is significantly associated with histology of testicular neoplasms and this result suggests that age may be involved in pathogenetic processes of testicular neoplasms (Stang et al. 2023). With respect to diagnostic work-up of testicular masses, age is yet of limited value, since GCTs and benign tumors may occur at any age.

Association of tumor marker expression rates with histology

There are significant differences of expression rates of the markers bHCG, AFP, LDH, and M371 among the four histologic groups. The expression rate of bHCG in seminoma of 29.1% observed in this study is not much different from the rate of 35% observed in a pivotal series from 1997 (Weissbach et al. 1997) and also consistent with the rates of 18.8%–31.8% reported in a contemporary review (Dieckmann et al. 2019b). Noteworthy, AFP was found to be elevated in 5.2% of seminoma patients. Although AFP production in pure seminoma is biologically not possible, clinical series repeatedly reported slightly elevated AFP levels in pure seminoma with no changes despite curative treatment. The present results are in accordance the international consensus that mildly elevated AFP levels with no clinical significance may occur in isolated cases with seminoma (Dieckmann et al. 2017; Wymer et al. 2017; Brandt et al. 2022). In nonseminoma, bHCG and AFP expression showed rates of 55.8% and 54.2%, respectively. These results are consistent with rates of 52.9%–63.6% and 55.1%–70% for bHCG and AFP, respectively, reported in contemporary clinical series (Germa-Lluch et al. 2002; Dieckmann et al. 2019b; Neumann et al. 2011).

The novel marker M371 outperforms all classical markers with expression rates of 82.7% and 93.6% in seminoma and nonseminoma, respectively, and these rates are also

significantly higher than the combined rate of AFP and/ or bHCG in nonseminoma (68.7%).

This result is in line with all previous reports on M371 (Dieckmann et al. 2019a; Mørup et al. 2020; Sequeira et al. 2022; Syring et al. 2015; Murray et al. 2018; Piao et al. 2021) and it clearly underscores the usefulness of the M371 test reiterating the demand for its prompt clinical implementation (Leão et al. 2021). Noteworthy, we found a modest but significantly lower expression rate of M371 in seminoma compared to nonseminoma (82.7% versus 93.6%) which had been documented in most of the recent reports (van Agthoven and Looijenga 2017; Dieckmann et al. 2019a; Mørup et al. 2020) but not in all of them (Myklebust et al. 2021; Vilela-Salgueiro et al. 2018; Mego et al. 2019).

Expectedly, no significant elevations of serum markers were observed in benign tumors, since AFP, bHCG and M371 represent specific products of embryonic tissues that are only present in GCTs. Yet, we observed marker elevations in isolated patients with benign tumors, but there is no information about the extent of level elevations. This result is consistent with a previous series (Belge et al. 2020). Most probably, these elevations represent idiopathic unspecific elevations similar to the AFP elevations in seminoma (Dieckmann et al. 2017; Wymer et al. 2017). Of note is the elevation of M371 levels in 30% of other malignancies. Such elevations had been found previously in some cases with testicular malignant lymphoma (Belge et al. 2020; van Agthoven and Looijenga 2017). Most of the lymphoma cases with M371 elevations had extended disease. In the present study, only three of ten lymphoma cases had such elevations, and in light of the wide confidence limits (6.6%–65.3%), chance effects must be considered. However, the repeated detection of elevated M371 levels in malignant lymphoma may raise the hypothesis that M371 elevations may result not only from GCTs but possibly also from other neoplasms or from other diseases such as Covid 19 infection as recently reported (Goebel et al. 2022). In a previous study, isolated cases with non-testicular malignancies were shown to have M371 levels in the range of RQ 5–12, thus slightly above the ULN of RQ = 5. As some of the healthy controls also had levels of that extent, those elevations were not considered to represent a true-positive result for M371 (Spiekermann et al. 2015). In the present study, the value of RQ = 5 was considered as ULN by default. As shown in a recent study on residual masses subsequent to chemotherapy in seminoma, some patients without active disease had values slightly above the RQ = 5 threshold (Dieckmann et al. 2022b). Thus, it is currently unclear, if the ULN of 5 of the M371 test is uniformly appropriate. Conceivably, the ULN needs to be set somewhat higher than RQ = 5 and possibly, it needs to be adjusted to the clinical scenario examined. It may be speculated that some of the lymphoma cases of the present study could have had values minimally above the ULN of RQ = 5 and may, thus, represent unspecific marker elevations. Nonetheless, further investigation of M371 expression in malignant lymphoma is required.

Association of tumor marker expression rates with clinical stages in germ cell tumors

A significant trend towards higher expression rates with increasing clinical stages was shown for each of the four tumor markers. This result mirrors the significant association of marker expression rates with primary tumor-size reported earlier (Dieckmann et al. 2022a, 2019b). The most likely biological explanation for this association is the higher number of marker secreting neoplastic cells in both, increasing primary tumor sizes and increasing clinical stages.

With respect to the classical markers, higher sensitivities in metastasized cases than in localized disease (CS1) had first been documented by Skinner and Scardino in 1981 (Skinner and Scardino 1980) and were confirmed by many others thereafter (Lippert and Javadpour 1981; Bosl et al. 1981; Szymendera et al. 1983; Nørgaard-Pedersen et al. 1984; Fargeot 1990; Daugaard et al. 1990; Kausitz et al. 1992; Weissbach et al. 1997; Trigo et al. 2000; Rothermundt et al. 2018; Dieckmann et al. 2019b). As GCTs as a whole (SE + NS) were evaluated in the present investigation, AFP scored lower expression rates than bHCG which obviously relates to the non-expression of AFP in pure seminoma.

The present data revealed a very high sensitivity of M371 in comparison to the classical markers. In CS1, an elevation of serum levels of AFP and / or bHCG was found in 38.8%, whereas M371 scored more than double with 83.1%. In metastasized cases, M371 is expressed in 100% (95% CIs 94.22–100%) of cases opposed to 66.7% (95% CIs 58.27–74.94%) regarding AFP/bHCG. The confidence intervals do not overlap, and this result is consistent with the significant difference between the expression rates of M371 and AFP/bHCG in nonseminoma documented with McNemar's test. The very high sensitivity of M371 found herein is consistent with previous studies (Dieckmann et al. 2019a; Leão et al. 2021; van Agthoven and Looijenga 2017; Mego et al. 2019; Mørup et al. 2020; Myklebust et al. 2021; Lobo et al. 2019; Nappi et al. 2019; Badia et al. 2021; Ye et al. 2022; Sequeira et al. 2022) underscoring the great superiority of M371 to the classical markers.

Inverse association of age with serum tumor marker expression rates

There is a significant trend towards higher marker expression rates with younger age for bHCG, AFP, and M371, but not for LDH. This result is found both in the entire population (all histologic groups) and in the GCT subpopulation. The inverse association of marker expression rates with

age is probably related to the predominance of AFP- and bHCG-producing nonseminomatous germ cell tumors in the younger age categories. Conversely, seminomas with the majority of which being marker negative, do predominantly occur in older ages (Secondino et al. 2022; Cheng et al. 2018). Thus, the specific predispositions of the various histologic GCT subtypes to different age categories have likely caused the trend towards higher marker expression rates in younger patients. Accordingly, LDH expression is almost equally expressed in all age categories most probably because it is merely related to the number of cell damages and is not specific for any particular histologic GCT subtype.

In GCTs, the trend towards higher expression rate in younger age categories is also seen in M371. This association deserves some consideration, since very high sensitivities of this marker are documented in both seminoma and nonseminoma. However, the sensitivity of M371 is somewhat higher in nonseminoma than in seminoma, and this difference may translate into higher expression rates in younger patients. The inverse age trend is slightly weaker in M371 than in AFP and bHCG as can be noted by the numerically higher p value in M371 ($p = 0.04$) compared to 0.0068 and < 0.0001 regarding bHCG and AFP, respectively. The weaker age trend of M371 does also probably come from the still high M371 expression rate of 82.35% in patients aged > 50 years and the rather small difference to the rate of 92.59% in the youngest age category. The association of marker expression with age had been noted only in one previous report (Dieckmann et al. 2019b). As the association is well explained by the specific age predispositions of the GCT subtypes, that issue had obviously not attracted further investigations.

Limitations of the study

Selection bias cannot entirely be ruled out in view of the retrospective study design of the present investigation. As the main goal of the study was to examine marker expression rates in relation to clinical factors, the statistical analysis was possibly hampered by lacking marker measurements in a number of patients. With respect to tumor marker elevation rates, only a dichotomized analysis was available in the present study with no further information about the extent of elevations although that information could have endorsed the results. The histological subgroups BT and OM encompassed only small sample sizes which could have limited statistical power despite the overall large patient population. Tumor histologies were based on local pathological examination without expert histopathological review. Probably, a strong point of the study is the large number of 451 measurements of the next-generation tumor marker M371 featuring the usefulness of this diagnostic tool in the clinical management of testicular cancer. Another asset could be the thorough statistical analysis of a large and representative population of patients with testicular neoplasms.

Conclusions

There are strong interrelations of patient age, histology, and clinical staging with serum tumor marker expression rates in patients with testicular neoplasms. The rates of all markers correlate with tumor bulk. The superiority of the new marker M371 to the classical serum markers AFP, bHCG and LDH was confirmed in a large patient sample. Although sporadic elevations of M371 in non-germ cell tumors need further investigation, the present data underscore the exceptional clinical usefulness of the M371 test.

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Author contributions GB, HI, and K-PD contributed to study conception and design. CW supervised the whole project. JU, HI, K-PD, and FG performed the data collection and material preparation; GB, CD, MK, and FG performed measurements of M371; EMAS contributed tumor marker measurements; UP and FG contributed the statistical analysis. K-PD and GB wrote the first draft of the manuscript. HI, UP, and JU participated in manuscript editing and discussion. All authors have made a substantial, direct, and intellectual contribution to the work and approved the final version of the manuscript for publication.

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Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interests KPD and GB declare ownership of shares, each of 8.9%, in miRdetect GmbH, Bremerhaven, Germany. The remaining authors have no relevant financial or non-financial interests to disclose. All other authors declare no competing interests towards this report.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethical committee of Ärztekammer Hamburg (PV7288, July 15, 2019).

Consent to participate As solely anonymized patients' data were evaluated in the present study, the need for informed consent of patients was waived by the ethical committee.

Consent to publication Informed consent was obtained from all individual participants included in the study.

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3.5 Publication V: Detection of Recurrence through microRNA-371a-3p Serum Levels in a Follow-up of Stage I Testicular Germ Cell Tumors in the DRKS-00019223 Study

As a continuation of our previous investigation, in which the M371 test was proven to ba a beneficial diagnostic method, this study explores its potential in monitoring of patients with clinical stage I testicular GCTs, to enhance the early detection of relapses and optimize personalized treatment strategies.

Testicular germ cell tumors account for the majority of malignancies in young men, and advancements in their diagnosis and follow-up strategies are essential for effective management (McHugh et al., 2024). Despite effective treatment options for testicular GCT patients and a cure rate of over 90 %, the detection of relapses is hampered by low sensitivity and specificity of current biomarkers, especially in clinical stage I cases, the stage in which the mojaority of GCT cases are diagnosed (Chovanec and Cheng, 2022). Most of these patients are managed with surveillance alone after orchiectomy (Zengerling et al., 2018). The risk of recurrence in these cases depends on primary tumor characteristics, like tumor size and lymphovascular invasion (Blok et al., 2020). Current follow-up strategies, including imaging and measuring serum tumor markers, are hindered by low sensitivity, particularly in detecting lymph node metastases, emphasizing the urgent need for improved methods (Hudolin et al., 2012; Oldenburg et al., 2022). In our previous studies, we demonstrated that M371 is suitable as a novel and effective marker, exhibiting elevated sensitivity and specificity. However, the senitivity of M371 for the detection of recurrences remains to be evaluated.

This study aims to prospectively assess the utility of the M371 test in detecting relapses among CSI TGCT patients undergoing active surveillance, enabling timely interventions and more targeted treatments, such as primary retroperitoneal lymph node dissection or chemotherapy (Sigg et al., 2024). Specifically, the aim of the study was to answer the following three questions:

(1) Is the M371 test capable of identifying newly emerging GCT disease during follow-up of CSI patients under active surveillance?

(2) Can the test detect recurrence at an earlier stage compared to conventional methods?

(3) Do elevated postoperative M371 levels serve as a predictor of future recurrence?

In this study, we monitored 258 patients with testicular germ cell tumors in clinical stage I for up to 48 months. During follow-up, 39 patients relapsed with a median interval to relapse of 6 months (IQR, 3-12 months), comprising 15.1% of the total cohort. Relapses were detected through imaging techniques (64%), marker elevation (26%), or a combination of both (10%). The ROC curve yielded an AUC of 0.991 for miR-371a-3p, with an optimal cutoff (RQ = 15) distinguishing relapses from recurrence-free cases. Using this threshold, 47 patients showed elevated M371 levels during follow-up, but only 39 had clinically confirmed relapses.

Moreover, the M371 test exhibited 100% sensitivity and 96.3% specificity for detecting relapses in CSI cases, outperforming the classical tumor markers for TGCT. Additionally, when examining seminomas and non-seminomas separately, the M371 test showed excellent discriminative performance with AUC values of 0,993 and 0,985, respectively. Regarding the false positive cases, eight out of 219 patients without relapse were identified, with notable differences in median M371 expression between false-positive cases and clinically confirmed recurrences. Notably, false-positive cases showed significantly lower median M371 expression (RQ = 37,6) than confirmed recurrences (RQ = 153,7; p = 0,024). In 28.2% of cases, M371 elevations preceded relapse detection by 3 to 15 months, but the overall median time to relapse detection was 6 months for both M371 and traditional methods, with no significant difference (Log rank test; p = 0,956). Examining post-orchiectomy M371 levels, the data did not support the hypothesis that elevated levels can predict future relapse, as preoperative and postoperative levels were not significantly different between relapsing and non-relapsing patients.

This study highlights three key findings. Firstly, the M371 test demonstrates high sensitivity (100%) and specificity (96.3%) in detecting relapses during surveillance of CSI germ cell tumors, thus outperforming the classical protein-based markers. Secondly, the M371 test demonstrated a notably earlier detection of relapses in 28% of patients. In eleven cases, M371 elevations preceded relapse detection by 3 to 15 months compared to imaging and/or tumor marker elevations. However, the Kaplan-Meier curve revealed a consistent median time to relapse detection of 6 months for both methods. Thirdly, elevated M371 levels right after orchiectomy are not suitable to serve as reliable predictors for future relapses.

In conclusion, this study provides substantial evidence supporting the utility of the M371 test in detecting relapses among clinical stage I germ cell tumor patients.

Ongoing research will determine if the M371 test alone can accurately detect GCT relapses, to optimize diagnostic strategies that may reduce unnecessary imaging, minimize radiation exposure for young patients, and potentially contribute to cost saving.

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Detection of Recurrence through microRNA-371a-3p Serum Levels in a Follow-up of Stage I Testicular Germ Cell Tumors in the DRKS-00019223 Study

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Own contribution

- Experimental concept and study design: 25%
- Experiment execution and/or acquisition of experimental data: 80%
- Data analysis and interpretation: 50%
- Preparation of Figures and Tables: 50%
- Drafting of manuscript: 25%

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Detection of Recurrence through microRNA-371a-3p Serum Levels in a Follow-up of Stage I Testicular Germ Cell Tumors in the DRKS-00019223 Study

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ABSTRACT

Purpose: Surveillance of clinical stage I (CSI) testicular germ cell tumors (GCT) is hampered by low sensitivity and specificity of current biomarkers for detecting relapses. This study evaluated if serum levels of microRNA371a-3p (M371 test) can: (i) Accurately detect relapses, (ii) detect relapses earlier than conventional technology, and (iii) if elevated postoperative M371 levels may predict relapse.

Experimental Design: In a multicentric setting, 258 patients with testicular CSI GCT were prospectively followed by surveillance for a median time of 18 months with serial measurements of serum M371 levels, in addition to standard diagnostic techniques. Diagnostic characteristics of M371 for detecting relapses were calculated using ROC curve analysis.

Results: Thirty-nine patients recurred (15.1%), all with elevated M371 levels; eight without relapse had elevations, too. The test revealed the following characteristics: area under the ROC curve of 0.993, sensitivity 100%, specificity 96.3%, positive predictive value 83%, negative predictive value 100%. Earlier relapse detection with the test was found in 28%, with non-significant median time gain to diagnosis. Postoperative M371 levels did not predict future relapse.

Conclusions: The sensitivity and specificity of the M371 test for detecting relapses in CSI GCTs are much superior to those of conventional diagnostics. However, post-orchiectomy M371 levels are not predictive of relapse, and there is no significant earlier relapse detection with the test. In all, there is clear evidence for the utility of the M371 test for relapse detection suggesting it may soon be ready for implementation into routine follow-up schedules for patients with testicular GCT.

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Introduction

More than 90% of all patients with testicular germ cell tumors (GCT) can be cured with modern management (1). The majority of GCT cases present with clinical stage I (CSI) disease, and most of these patients are managed with surveillance after orchiectomy. The risk of recurrence in these cases depends on morphologic features of the primary tumor. In seminomas, approximately 20% of patients with primary tumor size of >4 cm will relapse, while smaller primaries involve gradually lower rates (2). In nonseminomas, lymphovascular invasion incurs a recurrence rate of 40% to 50%, while patients without this histologic feature will relapse in about 15% of cases (3). Follow-up (F/U) in CSI cases comprises cross-sectional abdominal imaging with CT or MRI, chest radiography, physical examination, and measurements of serum tumor markers alpha fetoprotein (AFP), beta human chorionic gonadotropin (bHCG), and lactate dehydrogenase (4). However, diagnosis of recurrence is hampered by low sensitivity of 37% to 60% of current imaging modalities for detecting lymph node metastases (5, 6). Only lymphadenopathies >1 cm may be characterized as metastases if located in the typical landing zones of testicular neoplasms (7). Similarly, serum tumor markers have a low sensitivity for detecting relapses (8, 9). bHCG is expressed in only 30% of all seminoma patients, but no more than 11% to 22% of relapses have elevations of this marker (9, 10). In nonseminoma, bHCG and/ or AFP are expressed in 70% of CSI cases (11), but relapse is detected by elevated markers in 41% to 61% of cases depending on the presence of lymphovascular invasion (12, 13). There is no expression of AFP in pure seminomas and no expression of either AFP or bHCG in pure teratomas.

Translational Relevance

MicroRNA-371a-3p (M371) is involved in cell pluripotency control in human embryonic stem cells. This miRNA is also highly expressed in tissue of testicular germ cell tumors (GCT), and serum levels of M371 have been shown to be superior to the classical protein-based tumor markers of testicular GCTs with regard to primary diagnosis. The current study prospectively evaluated the usefulness of M371 to detect relapses in 258 patients with clinical stage I GCTs managed by surveillance. The sensitivity and specificity of the novel marker to detect relapses is 100% and 96.3%, respectively, greatly outperforming the classical protein-based markers. Thus, microRNA-371a-3p, originally considered to represent an important player in early human embryogenesis has meanwhile secured much of evidence for its clinical utility as a powerful serum tumor marker of germ cell cancer awaiting clinical implementation.

In aggregate, the early detection of recurrences is considerably impeded by the low accuracy of currently available diagnostic modalities. Therefore, better tools for relapse detection are required. In recent years, evidence has accumulated suggesting the utility of serum levels of microRNA-371a-3p (M371) as a novel and highly sensitive serum biomarker for GCTs (14, 15). The sensitivity and specificity for diagnosing primary GCTs are reported to be 85% to 90.1%, and 89.1% to 99%, respectively (16–20). Notably, patients with recurrent GCT also revealed elevated M371 levels (21). However, the database regarding the sensitivity of M371 for detecting recurrence is still limited and there are also controversial results (22). Moreover, the expression rate in recurrences may be somewhat lower than that in primary GCT, as reported in one major series (19). However, other series reported equally high M371 sensitivities in both recurrences and in primary GCT (22–24).

In the current study, we prospectively evaluated the usefulness of the M371 test for detecting relapses in a series of patients with CSI GCT managed by active surveillance. In particular, the following three questions were addressed: (i) Is the M371 test capable of detecting any newly arising GCT disease during F/U of CSI patients under active surveillance? (ii) Can the test detect recurrence earlier than conventional methods? (iii) Do elevated postoperative M371 levels predict future recurrence?

Materials and Methods

Study design and participants

Between June 2019 and November 2022, 352 patients with CSI GCT were prospectively enrolled from 23 urologic institutions in Germany, Austria, and Italy. Ninety-one patients were excluded for various reasons (Details in **Fig. 1**). The majority of excluded participants had provided less than three serum samples during F/U. Three patients had to be excluded because they had contralateral germ cell neoplasia *in situ* proven by biopsy during orchiectomy. Confounding of M371 measurements might have resulted in these cases because elevations may occur in 50% of these patients in the absence of metastases (25).

A total of 258 patients were eligible and managed by active surveillance (**Table 1**).

Follow-up visits involved imaging procedures, clinical examinations, and measurements of traditional tumor markers according to contemporary guidelines (refs. 4, 26; Supplementary Tables S1 and S2). In addition, serum levels of M371 were measured at each visit. Thus, in seminomas, M371 measurements were performed every six months, while in nonseminomas, the test was employed every three months during the first 2 years and six-monthly thereafter. A subsample of 64 patients also underwent M371 measurement prior to orchiectomy. The first measurement was performed within 2 weeks after orchiectomy.

Reference standard for relapse was an unequivocal and continuous radiographic enlargement of regional lymph nodes to >1.0 cm short axis diameter or rising tumor marker levels (AFP, bHCG) beyond the upper limit of norm (ULN) in the course of two consecutive visits or by both criteria. Patients who developed contralateral GCT were also rated as relapses. We included such secondary cancers because the aim of the current study was to analyze the ability of the test to diagnose any newly arising GCT lesions irrespective of their location. The median follow-up time was 18 months [interquartile range (IQR), 9–27 months]. Local caregivers of study patients were blinded to measurement results of the M371 test, thus no clinical decision-making was based on study results.

Ethical approval was provided by the Ethical committee of Ärztekammer Hamburg (MC 152/19, July 15, 2019) and by Ärztekammer Bremen (#301/17, September 21, 2017). The study was registered at Deutsches Register Klinische Studien (DRKS-00019223). Written informed consent was obtained from all patients. All study activities were conducted in accordance with the Declaration of Helsinki of the World Medical Association, amended by the 64th General Assembly in October 2013.

Whole blood samples were collected in 9 mL serum separation tubes (Sarstedt, Nümbrecht, Germany). Serum for the measurement of M371 expression was obtained after centrifugation and aliquots were stored in 2 mL cryotubes (Th Geyer, Renningen, Gemany) at −80°C until processing.

Serum M371 expression levels were measured as detailed earlier (19). Briefly, total RNA was isolated from 200 μL cubital vein serum using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription was performed for both miR-371a-3p and endogenous control miR-30b-5p using the M371 test (miRdetect, Bremerhaven, Germany). The miRNA expression was quantified on a 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) using the M371 test (miRdetect, Bremerhaven, Germany). The serum levels of M371 were measured relative to the endogenous control miR-30b-5p. The relative quantity (RQ) of miR-371a-3p was calculated using the ΔΔCt method (27). The measurements of classical tumor markers were performed in routine hospital laboratories according to institutional standard operating procedures.

Statistical analysis

Patient data regarding the histology of testicular tumors and age were registered in a commercially available database (MS Excel, version 2019) at study entry. During F/U, the results of the measurement of M371, classical tumor markers, and imaging procedures were added to the files at each time point of F/U. Statistical evaluation was performed using SPSS version 26 (IBM, Armonk, NY, RRID: SCR_002865). For comparison of continuous variables, the Mann–Whitney U test and Wilcoxon signed-rank test were used. Statistical significance was assumed at $P < 0.05$. The following performance characteristics were calculated with corresponding 95% confidence intervals (CI) by employing the highest measurement value obtained in each patient during F/U: Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and AUC of ROC curve.

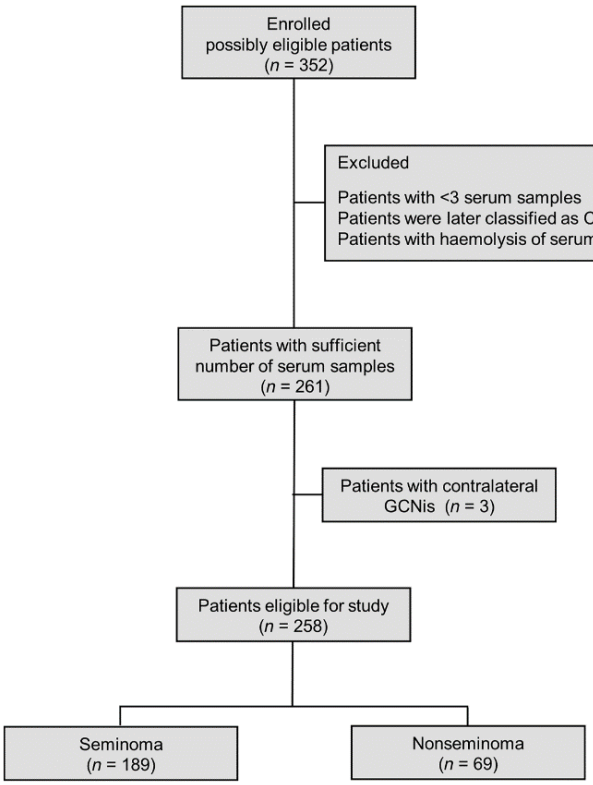

Figure 1.
Study profile. Outline of the selection process of patients with CSI GCT upon enrollment.

Table 1. Characteristics of patients.

Entire patient population	***(n)***	
Total number of patients included (*n*)	258	
Seminoma (*n*; % of all)	189	73.3%
Nonseminoma (*n*; % of all)	69	26.7%
Median age at diagnosis (years; IQR; range)	36	31–44; 18–70
Median duration of follow-up (months; IQR; range)	18	9–27; 3–48
Total number of M371 measurements (*n*)	1179	
Relapsing patients	(*n*)	
Number of relapses (*n*; % of all)	39	15.1%
Number of relapsing seminoma (*n*; % of all relapses)	17	43.6%
Number of relapsing nonseminoma (*n*; % of all relapses)	22	56.4%
Median interval to relapse, all patients (months; IQR)	6	3–12
Median interval to relapse, seminoma patients (months; IQR)	9	3–18
Median interval to relapse, nonseminoma patients (months; IQR)	6	3.75–9
Median age of all relapsing patients (years; IQR)	36	26–43
Median age of relapsing seminoma patients (years; IQR)	36	30–56
Median age of relapsing nonseminoma patients (years; IQR)	34.5	24.25–40.75
Elevation of bHCG at relapse (*n* elevated/*N* eligible; % of eligible)	11/31	35.5%
Elevation of AFP at relapse (*n* elevated/*N* eligible; % of eligible)	8/32	25.0%
Elevation of bHCG/AFP at relapse (*n* elevated/*N* eligible; % of eligible)	14/31	45.2%
Elevation of M371 at relapse (*n* elevated/*N* eligible; % of eligible)	39/39	100%

AFP/bHCG: elevation of bHCG or AFP or of both.

These characteristics were calculated for the entire cohort of patients with GCT and also separately for both seminomas and nonseminomas. Youden index analysis was used to determine the optimal cut-off value of serum M371 levels for identifying relapses.

On the basis of the currently available published data (22, 24), sensitivity and specificity of the M371 test both exceeding 90% and an AUC clearly above 0.9 would represent a positive result with respect to first goal of the study.

Data availability statement

The complete original data set consisting of all raw data pertaining to this study is attached in the electronic supplement (Supplementary Table S3). All results of this study, all tables, and all figures are derived from this data set.

Results

Sensitivity and specificity of miR-371a-3p for detecting relapses

Clinical characteristics of the 258 patients enrolled in this study are listed in **Table 1**. Thirty-nine patients (15.1%) developed relapse at a median of 6 months (IQR, 3–12 months), two of whom developed contralateral GCT; all others had retroperitoneal lymphadenopathies. Of the relapses, 64% were detected by imaging techniques only, 26% by marker elevation only, and 10% by both modalities. The ROC curve revealed an AUC of 0.991 (95% CI, 0.982–1.000; **Fig. 2**). Youden index analysis revealed an RQ of 15 as optimal cutoff for differentiating relapses from healthy patients. Using this threshold, 47 patients were found to have elevated M371 levels during F/U, but only 39 of them had clinically confirmed relapse (**Fig. 3A**; **Table 1**). Eight of the 219 non-relapsing patients had to be considered false-positive results, while 211 were true-negative. If the cutoff of RQ = 5 as used in our former studies (19, 21, 25) had been applied, as many as 28 additional cases would have been rated false positive. The M371 test has a diagnostic sensitivity of 100% (95% CI, 100.0%–100.0%), and it has a specificity of 96.3% (95% CI, 93.9%–98.8%) for detecting relapses of CSI cases upon surveillance. Separate analysis of seminomas and nonseminomas revealed an AUC of 0.993 (95% CI, 0.984–1.000) and 0.985 (95% CI, 0.961–1.000), respectively (Supplementary Fig. S1A and S1B). Of note, the median M371 expression of false-positive cases was significantly lower (RQ = 37.6) than that of clinically confirmed recurrences (RQ = 153.7; $P = 0.024$). **Table 2** provides details regarding the diagnostic performance characteristics of the test including PPV and NPV along with separate analyses of both seminomas and nonseminomas.

The classical markers bHCG and AFP attain much lower sensitivities for the detection of relapses than M371, but their specificities are in the same range as the M371 test (**Table 2**; Supplementary Fig. S2).

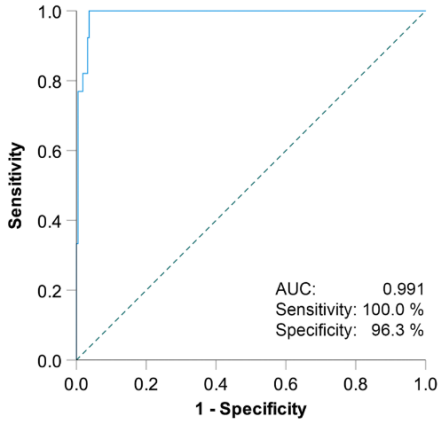

Figure 2.
Performance characteristics of M371 test. ROC curve graphically displays the excellent sensitivity and specificity of the M371 test to detect relapses in patients with CSI GCT.

Does the M371 test detect recurrences earlier than conventional methods?

In 11 cases (28.2%), elevations of the M371 test preceded relapse detection with imaging techniques and/or marker elevations by 3 to 15 months. The time points of the first detection of relapse with conventional methods and the M371 test in each of the relapsing patients are listed in Supplementary Table S4. The Kaplan–Meier plot (**Fig. 3B**) reveals no significant difference between the median time to relapse detection with the M371 and with traditional methods (Log rank test $P = 0.956$). Both diagnostic modalities detect relapses at a median of 6 months, with no significant diagnostic advantage of M371 over conventional techniques.

Do elevated postorchiectomy M371 levels predict future relapse?

Figure 4 illustrates the median M371 levels of relapsing patients and those without relapse at the time of orchiectomy, immediately after orchiectomy (median interval from surgery to miRNA test of 8 days), and at the last visit during follow-up. Serum levels at the last visit were much higher in relapsing patients than in non-relapsing ($P < 0.001$), as noted previously. However, both the preoperative and postoperative levels are not different among relapsing and non-relapsing patients ($P = 0.52$). Thus, the current data do not support the hypothesis that postoperative M371 levels can predict future relapse.

Discussion

This study revealed three crucial results. First, there is clear evidence for the utility of the M371 test for detecting relapses in patients with CSI GCT during surveillance, featuring a sensitivity and a specificity of 100% and of 96.3%, respectively. Second, the test does not offer a significantly earlier relapse detection than imaging techniques and/or serum marker elevations. Third, elevation of M371 immediately after orchiectomy failed to predict the future risk of recurrence.

Sensitivity and specificity of M371 for detecting relapses

The M371 test has previously been shown to greatly outperform the classical tumor markers bHCG and AFP with respect to the primary diagnosis of GCTs (28, 29). The current study revealed evidence for another feature of the test: the ability to accurately detect relapses in CSI GCTs managed with surveillance. In fact, the diagnostic ability of the test appears to be more accurate in this setting than in the primary diagnosis of GCTs, as the sensitivity and specificity of 100% and 96.3%, respectively, as found in the current study, seemingly surpass the sensitivity and specificity of 90.3% and 94.1%, documented in the primary diagnosis setting (19). This small difference in diagnostic accuracy between the two clinical scenarios may relate to the low sensitivity of M371 in detecting small volume seminomas with less than 50% sensitivity in primary seminomas sized <1 cm. As the vast

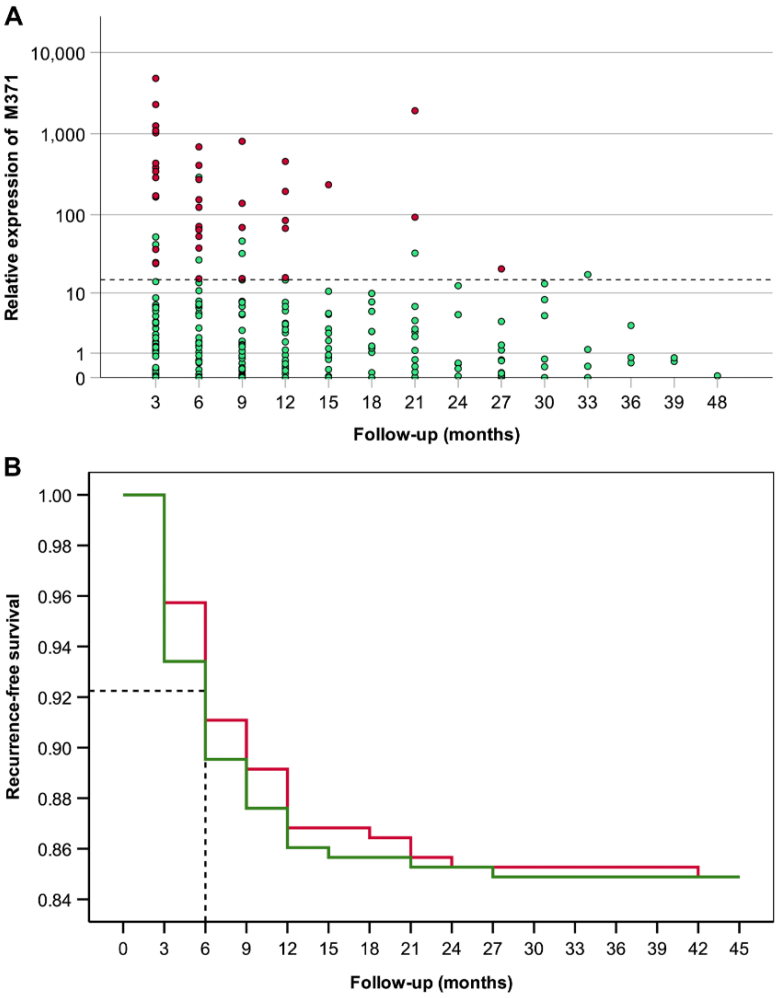

Figure 3.
Relapse detection with M371 during follow-up. **A,** Individual M371 measurements of relapsing and non-relapsing patients during follow-up ($n = 258$). Each dot represents the highest M371 measurement of each single patient at the corresponding time point of follow-up. Red dots denote relapsing patients, green dots non-relapsing. The dashed black line marks the cutoff. The y-axis is depicted in a logarithmic scale. Clearly, all relapsing patients are located beyond the cut-off line (elevated M371 levels), and none of the relapsing cases lies below the line, but very few non-relapses lie beyond the cut-off line (false-positives). **B,** Relapse-free survival curves showing time-points of relapse detection with M371 test and with conventional methods. The graph shows that time-points of relapse detection with the M371 test and with standard methodology are not different from each other ($P = 0.956$; Log rank test). Median time to relapse detection is 6 months with both methods as indicated by dashed lines. The hypothesis of earlier relapse detection with the M371 test is not substantiated by these data. Green curve: relapse detection with M371 test, red curve: relapse detection with imaging and classical tumor markers.

Table 2. Performance characteristics of M371 and serum markers for detecting relapses in CSI GCTs.

	Clinically confirmed relapses					Relapse-free cases								
	Eligible			Sensitivity		Eligible			Specificity		PPV		NPV	
Marker	(*n*)	TP	FN	(%)	95% CI (%)	(*n*)	TN	FP	(%)	95% CI (%)	(%)	95% CI (%)	(%)	95% CI (%)
bHCG	31	11	20	35.5	19.2–54.6	196	192	4	98.0	94.9–99.4	73.3	51.0–95.7	90.6	86.6–94.5
AFP	32	8	24	25.0	11.5–43.4	196	184	12	93.9	89.5–96.8	40.0	18.5–61.5	88.5	84.1–92.8
bHCG/AFP	31	14	17	45.2	27.3–64.0	196	180	16	91.8	87.1–95.3	46.7	28.8–64.5	91.4	87.4–95.3
M371 of all GCT	39	39	0	100	100–100	219	211	8	96.3	93.9 –98.8	83.0	72.2–93.7	100	100–100
M371 S	17	17	0	100	100–100	172	166	6	96.5	93.8–99.3	73.9	56.9–91.8	100	100–100
M371 NS	22	22	0	100	100–100	47	45	2	95.7	92.9–100.0	91.7	80.7–100.0	100	100–100

Abbreviations: TP, true positive; FN, false negative; TN, true negative; FP, false positive; bHCG/AFP, cases with either elevation of bHCG or AFP or both; S, seminoma; NS, nonseminoma.

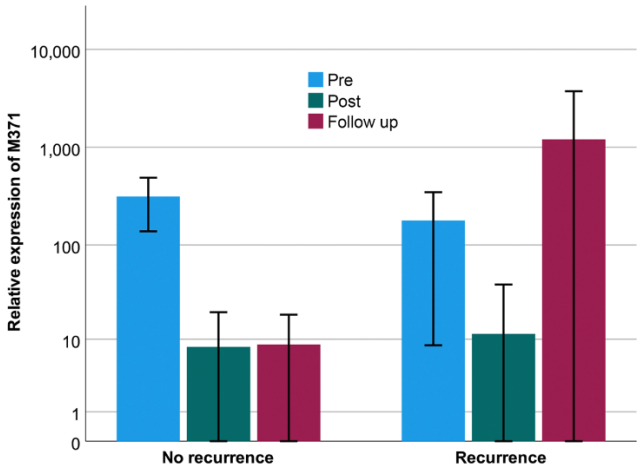

Figure 4.
Median M371 levels in patients with and without recurrence at different time points. The figure shows that M371 levels measured immediately after orchiectomy are not different among the subgroups of relapsing ($n = 5$, right side of image) and non-relapsing cases ($n = 59$, left side of image), respectively. The hypothesis that elevated M371 levels after orchiectomy may predict relapse is to be rejected by these data. As expected, last M371 measurements are significantly higher in patients with relapse than in those without. These data refer to a subsample of $n = 64$ cases of the entire study population. Blue boxes: median preoperative M371 levels; green boxes: levels measured immediately after orchiectomy; violet boxes: levels measured at last follow-up visit. The y-axis is depicted in a logarithmic scale. Error bars denote 95% CIs.

majority of relapsing cases in this study were confirmed radiologically with lymphadenopathies >1 cm, there were no particularly small tumor volumes among the relapsing seminoma cases in this study. Thus, the sensitivity of the test in this setting proved to be superior to the performance in the primary diagnosis, where usually around 10% of newly diagnosed seminomas are smaller than 1 cm (30).

A possibly low prevalence of teratomas among the relapsing cases might have indirectly contributed to the excellent performance of the test in this study. The insensitivity of the M371 test to detect teratomas is a well-documented weakness of the test (31), but this limitation obviously did not influence the overall results of this study because the number of teratomas was probably small. We hypothesize this, although we do not have histologic confirmation of relapsing nonseminomas. Teratomatous relapses typically tend to present late (32), but none of the relapses in the current study occurred later than 9 months after orchiectomy.

The separate analyses of seminomas and nonseminomas revealed widely equivalent performance characteristics of the test in both subgroups. Only PPV was lower in seminoma with 73.9% versus 91.7% in nonseminoma. Although the difference is still modest with widely overlapping 95% CIs of the two values, the disparity between the two subgroups does obviously relate to the higher number of false-positive findings in seminoma ($n = 6$) than in nonseminoma ($n = 2$) in conjunction with a higher number of seminomas ($n = 189$) than nonseminoma ($n = 69$) in the study population and the higher frequency of relapses in nonseminoma (32%) than in seminoma (9%).

Notably, in the current study, a cut-off value of RQ = 15 was used to identify relapses, which is somewhat higher than RQ = 5, the ULN currently used with the M371 test for detecting primary GCTs (19). Both threshold values were calculated using the Youden index analysis relating to the corresponding study populations; however, two reasons may account for the small difference regarding the cut-points, one clinical, and one technical. The patient population used for calculating the former cut-point consisted of patients with all clinical stages and a 58% proportion of seminomas from several European countries (19), whereas the current study population consisted of CSI patients only with a 73% proportion of seminoma from only Central European countries. Measurement of the M371 serum levels were performed with the commercially available M371 test kit (miRdetect, Bremerhaven, Germany) in this study. This test basically consists of the same technical steps as the formerly used method; however, due to minor refinements, it appears to detect its miRNA target with higher sensitivity than the former one. It is conceivable that the ULN of the M371 test should be adjusted to each clinical scenario in which the test is used. Such a methodology has recently been suggested for the assessment of post-chemotherapy residual masses of metastatic seminoma (33). However, for practical purposes, a uniform threshold value that fits all clinical scenarios is desirable. Such a uniform value would likely range around RQ = 10–12, but the ULN of M371 still needs to be determined.

Eight patients without relapse were found to have elevated M371 levels. Notably, the median M371 level found in false-positive cases was significantly lower than that of true-positives. Four cases were detected as M371 positive early during follow-up. They maintained mildly elevated levels at follow-up visits, but remained recurrence-free. Two other patients developed elevated M371 levels during later follow-up, but they have likewise remained recurrence-free ever since, although long-term follow-up is missing. Two others discontinued follow-up, and no further information was available. M371 elevations unrelated to GCTs have been reported in thyroid neoplasms (34), in COVID-19 infections (35), and in selected patients with testicular malignant lymphoma (36). However, it is unlikely that the false-positive patients in the current study were afflicted with such diseases. Thus, these eight cases had to be considered as false-positives, rendering the specificity of the test to 96.3% in the setting of relapse detection in patients with CSI GCT. As false-positive values may predominantly occur in elevations close to the ULN, it could be rational, practically, to repeat such measurements if no other evidence for relapse is given.

The excellent sensitivity of the M371 test to detect relapses in patients with CSI GCT during surveillance has been previously noted. The Toronto group reported a 94.1% sensitivity of M371 for detecting recurrences in 34 CSI patients using banked serum samples. In contrast to our study, the authors did not observe false-positive findings (22). A Swiss study reported 100% sensitivity of M371 in detecting relapses among 10 of 33 patients with CSI GCT prospectively followed. Notably, these authors also reported one false-positive finding (24).

In a small Dutch case series, 3 of 3 patients with CSI GCT were shown to have elevated M371 levels at the time of relapse (37).

The traditional tumor markers bHCG and AFP revealed sensitivities of 35% and 25%, respectively, in the current study, and even the combined application of both markers yielded only a 45.2% sensitivity in the current study. These results are in accordance with previous reports that demonstrated the modest performance of traditional markers in relapse detection (8, 38). Series with high proportions of nonseminomas reported somewhat higher sensitivities of traditional markers of 40% to 73% (13, 39). Thus, the particularly low sensitivity of bHCG and AFP found in the current study may relate to the high proportion of seminomas in this study.

In aggregate, there is much evidence for the utility of the M371 test in detecting recurrences in early stage GCTs. However, the ability to detect relapses is not restricted to CSI cases because M371 is a universal marker for GCTs (40). Accordingly, elevated M371 levels have been reported in recurrences arising from various clinical scenarios other than surveillance in CSI (19, 20, 23, 41, 42).

Earlier detection of relapses with M371

Early case reports gave rise to the hope that M371 elevations may offer significantly earlier detection of relapses than conventional diagnostic techniques (37, 43). The current study revealed that earlier diagnosis of relapse with the test was in fact observed in 28% of patients. Specifically, in eleven cases, M371 elevations preceded relapse detection with imaging and/or marker elevation by 3 to 15 months. However, as shown by the Kaplan–Meier curve, the median time to relapse detection is 6 months with both M371 measurements and with standard technology. Thus, there is no statistically significant time gain with the M371 test until relapse detection. The Swiss study reported a median time-shift of two months with the M371 test until detection of relapses (24). The Toronto group found higher M371 levels at time points closer to the time of relapse detection. However, that result did not reach statistical significance (22). Overall, there might be a weak trend towards earlier diagnoses with the test. However, presently there is no clear evidence for clinically relevant antedating of relapse diagnoses with the M371 test.

Postorchiectomy M371 levels are no predictors of future relapses

With the advent of miRNA testing in GCTs, it has been speculated that persistent elevated M371 levels after orchiectomy could predict future relapses (19, 44). The current study clearly shows that M371 levels measured within 2 weeks after orchiectomy were not significantly different between patients destined for relapse and those staying healthy. This result is in line with the findings of both the Toronto group (22) and the Swiss study (24). Further support for the non-association of postoperative M371 levels with recurrences comes from the lack of any association between postoperative M371 levels and the presence of established risk factors for recurrence in nonseminomas (45). In all, the level of evidence for the insignificance of post-orchiectomy M371 levels is sound, but recent data may contribute further pieces to the puzzle (46).

Limitations of the study

Eighty-two patients originally enrolled had to be excluded from study because they had provided less than 3 serum samples for analysis. This reduction of sample size may have reduced statistical power and may thus represent a possible weakness of the study. Also, timing of M371 testing deviated from F/U rosters with both additional and missed testings in about 40% of patients. Major reasons for this weakness were nonadherence of patients to F/U schedules and significant variability in adherence to recommended schedules. Non-compliance is a well-documented problem in the real world environment of surveillance of CSI patients (47, 48) and the current study was not free from it.

Another possible weakness of this study is the rather short median follow-up of 18 months. Although most relapses in patients with CSI GCT will arise within the first 2 years, some more may occur during the third year after surgery, and the latter were missed by the study. However, as the main goal of our study was to evaluate the diagnostic utility of the M371 test rather than analyze the frequency of relapses, the short follow-up period is probably a minor problem.

Lack of information regarding the clinical and histologic details of relapsing patients might be a shortcoming because patients with bulky disease could not be differentiated from those with low-volume tumor load.

A possible downside of the current study is that hemolysis of serum samples was examined macroscopically and not systematically using technical methods. Hemolysis may confound quantification of miRNAs in serum because high amounts of endogenous control miR-30b are released from damaged erythrocytes (49).

A methodologic weak-point could result from the fact that the cut-point of RQ = 15 used in this study had not been validated in an independent data set.

The strengths of the investigation relate to the prospective multicentric enrollment of patients and direct comparison of M371 with traditional markers in the majority of cases.

Conclusions

This study confirmed previous reports (22–24, 37) and thus significantly increased the level of evidence for the ability of the M371 test to detect relapses in patients with CSI GCT. Although some practical issues still need to be addressed such as validation of the cut-off level, the test now appears to be close to its implementation in follow-up schedules. Practically, M371 testing could be performed simultaneously with traditional tumor marker sampling. Positive test results are highly suggestive of the presence of active germ cell cancer, but clinical decision-making should presently be based on further diagnostic techniques, mainly imaging. Future research will show if the test alone is sufficient to accurately diagnose GCT relapses as suggested by the current data. One vision could be that part of the imaging procedures could be spared, thus reducing young subjects´ exposure to ionizing radiation, and even cost saving could come true.

Authors' Disclosures

G. Belge reports grants from Deutsche Krebshilfe during the conduct of the study; as well as reports ownership 8.3% of shares in miRdetect Company, Bremerhaven, Germany. J. Heinzelbecker reports grants from German Cancer Aid outside the submitted work. F. Zengerling reports personal fees from Janssen-Cilag GmbH, Pfizer Pharma GmbH, and Roche Pharma GmbH outside the submitted work. J. Frey reports personal fees from mirdetect GmbH during the conduct of the study; personal fees from mirdetect GmbH outside the submitted work; and is an employee of mirdetect GmbH. K.P. Dieckmann reports ownership 8.3% of shares of miRdetect Company, Bremerhaven, Germany. No disclosures were reported by the other authors.

Authors' Contributions

G. Belge: Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, writing–original draft, project administration, writing–review and editing. **C. Dumlupinar:** Formal analysis, investigation, writing–

original draft, writing–review and editing. **T. Nestler:** Resources. **M. Klemke:** Formal analysis, investigation, writing–original draft, writing–review and editing. **P. Törzsök:** Resources. **E. Trenti:** Resources. **R. Pichler:** Resources. **W. Loidl:** Resources. **Y. Che:** Resources. **A. Hiester:** Resources. **C. Matthies:** Resources. **M. Pichler:** Resources. **P. Paffenholz:** Resources. **L. Kluth:** Resources. **M. Wenzel:** Resources. **J. Sommer:** Resources. **J. Heinzelbecker:** Resources. **P. Schriefer:** Resources. **A. Winter:** Resources. **F. Zengerling:** Resources. **M.W. Kramer:** Resources. **M. Lengert:** Investigation. **J. Frey:** Formal analysis. **A. Heidenreich:** Resources. **C. Wülfing:** Resources, supervision. **A. Radtke:** Conceptualization, formal analysis, writing–original draft, writing–review and editing. **K.P. Dieckmann:** Conceptualization, resources, formal analysis, supervision, validation, investigation, writing–original draft, project administration, writing–review and editing.

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Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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4 Discussion

Testicular germ cell tumors are the most common cancer type in young men aged between 20 and 45 years (Dieckmann et al., 2018), with around 25,000 new cases diagnosed annually in Europe and around 74,000 worldwide each year (Sung et al., 2021). After the completion of treatment, long-term follow-up care is crucial to safely monitor for a potential recurrence of cancer and complications of therapy (Niedzwiedz et al., 2019). The majority of relapses during surveillance occur within the first three years for stage I seminoma after initial treatment, while for non-seminomas relapses are observed within two years post-orchiectomy (Lieng et al., 2017). Therefore, close monitoring during this period is essential to detect recurrences as early as possible and initiate appropriate treatment (Pierre et al., 2022). Given the very high survival rates of TGCTs (95% after 5 years as well as 95% after 10 years), it is crucial to carefully consider the potential short- and long-term side effects of radiotherapy and chemotherapy, such as thromboembolism, fertility issues, cardiovascular toxicity and the risk of secondary malignancies which can occur years after GCT treatment (Feldman, 2008; Kliesch et al., 2021; Giona, 2022). Thus, active surveillance is the preferred approach for CSI testicular cancer patients, which involves closely monitoring the disease status of the patient over time with regular imaging tests and other assessments, but without treatment unless disease progression is detected (Kollmannsberger et al., 2015). By closely monitoring the disease progression, not only the risk of overtreatment is minimized, but also psychological problems are minimized (Doyle et al., 2024), since each treatment, such as RPLND, chemotherapy, and radiotherapy, have significant impacts on rehabilitation (Fung et al., 2019). According to the EAU guidelines (2024), distinct follow-up groups can be defined based on the varying risks of relapse and the various treatment approaches for each subtype. This groups include patients with seminoma stage I, those with non-seminoma CSI on active surveillance, and patients with metastatic disease in complete remission. The current modalities for diagnosing and monitoring testicular tumors in follow-up, such as physical examination, ultrasound, cross-sectional abdominal imaging with CT and the measurement of serum tumor markers bHCG, AFP and LDH include several restrictions (Lobo et al., 2019; Busch et al., 2022; Belge et al., 2024). Relapse rates vary based on histology, disease stage and treatment modality. In patients with stage I disease, overall, about 30% of patients will

relapse during active surveillance, which shows the importance of frequent CT examinations for timely detection of recurrence (Pierre et al., 2022). However, given the frequency of follow-up, there are concerns regarding the radiation exposure and potential risk of secondary malignancies and cardio vascular disease associated with CT examinations (Pierre et al., 2022; Tavares et al., 2023). Therefore, several follow-up protocols have optimized the number of CT imaging studies to reduce radiation exposure (Busch et al., 2022). MRI may be considered as an alternative to CT to avoid radiation exposure, however MRI scans typically impose significant costs on the healthcare system (Matulewicz et al., 2023). The EAU recommends serum tumor measurements four times a year in the first 2 years and then twice a year for the next 3 years (EAU, 2024). However, their sensitivity is limited with only 60% of testicular cancer patients showing elevation overall (Dieckmann et al., 2016, 2019a; Lobo and Leão, 2022). Moreover, their diagnostic accuracy varies in different clinical scenarios, leading to significant uncertainty (Dieckmann et al., 2018). The detection of these tumor markers largely depends on the histological composition of the tumor, with higher sensitivities seen in NSGCTs compared to SGCTs (Nappi et al., 2021). Specifically, bHCG is expressed in less than 30% of seminoma patients, while AFP is consistently negative. For non-seminomas, bHCG and AFP are expressed in 50-60% of patients (Pedrazzoli et al., 2021). LDH expression is observed in other medical conditions and is thus not specific for TGCT (Dieckmann et al., 2019a; Nappi et al., 2021; Pedrazzoli et al., 2021). Additionally, the positive and negative predictive values (PPV and NPV) of current clinical assessments, which include imaging and measurement of serum tumor markers, offer limited clinical utility in accurately guiding treatment decisions for patients in clinical stage I or those with small-volume stage II disease (CSIIA) (Dalal, 2006; Dieckmann et al., 2019a). Considering that testicular cancer patients need to be continuously monitored for up to 10 years after initial diagnosis, which includes 2 to 4 imaging diagnostics (CT or MRT), the significant burden on both patients and healthcare system becomes evident (Shaw, 2008).

When focusing primarily on patient survivorship, the overall aim is to cure patients using the least harmful treatment options while minimizing patient burden and avoiding unnecessary interventions (Stephenson et al., 2019; Krege et al., 2023; Tavares et al., 2023). To achieve this, precise assessments are essential to prevent both undertreatment and overtreatment (Stephenson et al., 2019). Current risk assessments exhibit false positive rates ranging from 15% to 50% for CSI non-

seminoma and 10% to 25% for seminoma (Kollmannsberger et al., 2015; Nicholson et al., 2019). These findings underscore the need for more accurate and reliable assessment methods to optimize treatment decision-making and relapse detection in testicular cancer patients (Tavares et al., 2023; Belge et al., 2024).

To overcome the limitations associated with the low sensitivity of established serum tumor markers in TGCTs, researchers have explored numerous novel markers over the past few decades, including neuron-specific enolase, placental alkaline phosphatase, and others (Lajer et al., 2002; Milose et al., 2011; Lobo et al., 2021a). Despite extensive research, none of these markers was confirmed suitable for clinical use. However, in the last decades, research has predominantly focused on microRNAs (Dieckmann et al., 2019b; Lobo et al., 2020; Nestler et al., 2023). Unlike traditional protein-based serum tumor markers, microRNAs are small noncoding RNA molecules consisting of around 22 nucleotides (Nestler et al., 2023). They play an essential role in posttranscriptional regulation of gene expression and RNA silencing, influencing various cellular processes including proliferation, differentiation, apoptosis and tumor development (Bezan et al., 2014; Spiekermann et al., 2015; Nappi et al., 2021). The dysregulation of miRNAs has been linked to numerous diseases, including cancer, cardiovascular disorders, and neurological conditions, making them promising targets for diagnostic purposes (Chen et al., 2012; Doghish et al., 2023). MicroRNAs are highly stable in body fluids and are characterized by their short half-life and rapid decay in circulation, as reported by Radtke et al. (2018), which make them suitable as biomarkers.

Various studies have already shown that members of the miRNA cluster 371–373 (miR-371, miR- 372, and miR-373), and especially miR-371a-3p are considered the most promising noninvasive biomarkers with much higher sensitivity and specificity than the classical markers (Murray et al., 2011; Belge et al., 2012; Dieckmann et al., 2012; Gillis et al., 2013; Syring et al., 2015). The comprehensive prospective study by (Dieckmann et al. (2019b), including 616 TGCT patients and 258 male controls, revealed a diagnostic sensitivity of 90.1%, specificity of 94% and an AUC of 0.966 for M371, which clearly outperforms the combined sensitivity of classical serum markers. Several retrospective studies have also shown a similarly impressive sensitivity and specificity of M371 in detecting testicular cancers compared to traditional markers (Syring et al., 2015; Van Agthoven and Looijenga, 2017; Badia et al., 2021; Myklebust et al., 2021).

Besides the promising performance of M371 with respect to primary diagnosis, various studies have also shown the efficiency of M371 in residual disease detection, follow-up monitoring, and assessment of response to therapy (Van Agthoven et al., 2017; Dieckmann et al., 2021; Fankhauser et al., 2022). A study by Lobo et al. (2019) showed that M371 levels were higher at relapse compared to postorchiectomy levels for 94.1% of patient. In the future, the integration of M371 into follow-up protocols could enhance management strategies by combining it with imaging techniques, potentially leading to more effective and cost-efficient treatment options (Tavares et al., 2023).

Despite these advancements in follow-up care of, the primary treatment for both stage I seminoma and non-seminoma TGCT remains radical inguinal orchiectomy. Overall, approximately 80% of CSI patients are cured with radical orchiectomy alone, which involves surgical removal of the affected testis (Dearnaley, 2001). However, nearly 30% of testicular cancer patients will relapse within five years following orchiectomy (Kollmannsberger et al., 2015; Wagner et al., 2024). Therefore, long follow-up care after initial treatment is crucial, especially since recurrence risk varies depending on the morphologic features of the primary tumor. Seminomas with larger primary tumors (>4 cm) exhibit a higher risk of recurrence with around 20%, whereas smaller primaries are associated with lower rates of recurrence (Warde et al., 2002; Dieckmann et al., 2022c). In contrast, non-seminomas with lymphovascular invasion have a recurrence rate of 40-50%, while those without this feature relapse in about 15% of cases (Belge et al., 2024). Testicular cancer has an excellent prognosis, with cure rates over 90% with modern management (Kollmannsberger et al., 2011; Gaddam and Chesnut, 2024). The majority of testicular cancer patients are diagnosed with clinical stage I, and most of these patients are managed with active surveillance after orchiectomy due to the low risk of cancer recurrence with approximately 15-20% and also the long-term toxicities associated with adjuvant therapy (Nichols et al., 2013; Ruf et al., 2022). Active surveillance involves regular follow-up visits, including physical examinations, the measurement of serum tumor markers and imaging tests to detect any potential cancer recurrence. Early on, adjuvant radiation therapy was the preferred treatment for stage I seminoma, as this tumor is very radio-sensitive, which can reduce the risk of relapse to approximately 4% (Nappi et al., 2017). However radiotherapy is associated with an increased risk of secondary malignancies like pancreatic, gastric, bladder, and kidney cancers, and thus s no longer recommended by most guidelines (Nappi et al., 2017; Oldenburg et al., 2022; McHugh et al., 2024). Another alternative to surveillance is

single agent carboplatin but it has been linked to unknown long-term risks. Therefore, surveillance is still the preferred management option for stage I seminoma patients, because if relapse is identified during surveillance, it can be effectively managed with full-course chemotherapy (Ehrlich et al., 2015; Kollmannsberger et al., 2015; Nason et al., 2020). The cure rate for CSI non-seminoma is excellent with close to 99%, which is achieved through three main treatment strategies including active surveillance, RPLND, and adjuvant chemotherapy (Winter and Hiester, 2021). Due to the significant risk of overtreatment with adjuvant therapy or RPLND (50% of the high-risk patients and potentially up to 88% of low-risk patients), surveillance becomes essential in managing stage I testicular cancer patients (De Wit and Fizazi, 2006).

The current diagnostic tools for follow-up examinations are similar as for primary diagnosis, including clinical examination, serum tumor marker monitoring, and imaging with CT or MRT (Kreydin et al., 2013; Busch et al., 2022; Pierre et al., 2022). A rise of classical tumor markers during follow-up can serve as an indicator for relapse (Fischer et al., 2023). However, classical tumor markers demonstrate low sensitivity for detecting relapses (Lobo and Leão, 2022; Oldenburg et al., 2022; Belge et al., 2024). For instance, bHCG, a marker elevated in only 30% of seminomas, detects relapse in a relatively small proportion of cases, ranging from 11% to 22% (Mortensen et al., 2014; Conduit et al., 2023). Similar, in non-seminomas, where bHCG and/or AFP are elevated in 70% of cases, the ability to detect relapse ranges from 41% to 61% (Daugaard et al., 2014; Kollmannsberger et al., 2015). Moreover, AFP is absent in pure seminomas (Belge et al., 2024). Overall, the early detection of recurrences is significantly hindered by the low accuracy of current serum tumor markers. Moreover, imaging modalities often lack the sensitivity required for early detection of recurrences, ranging from 37% to 60%, for detecting lymph node metastases (Brunereau et al., 2012, Hudolin et al., 2012). Only lymphadenopathies larger than 1 cm can be classified as metastases if they are located in the typical landing zones of testicular neoplasms (Pierorazio et al., 2020). Thus, there is an urgent need for improved tools for early relapse detection.

Accumulated evidence has shown that M371 indeed holds promise as a robust non-invasive biomarker for early and reliable detection of recurrences in patients undergoing active surveillance of stage I GCT (Dieckmann et al., 2019b; Leão et al., 2021; Tavares et al., 2023). The integration of the M371 test in follow-up protocols for testicular cancer provides several advantages regarding treatment optimization as

shown in Figure 2.

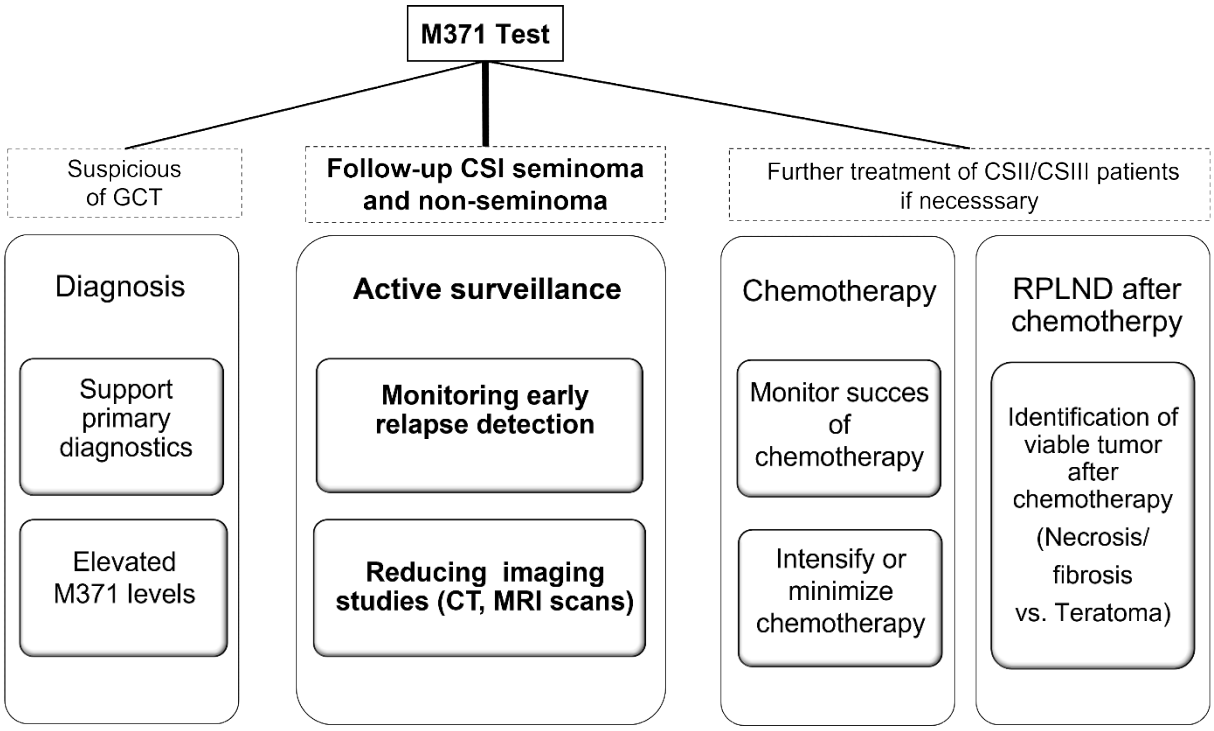

Figure 2: Proposed integration of the M371 test in testicular cancer management, demonstrating its role in diagnosis, treatment planning and follow-up (mod. www.uroday.com).

Primarily, it is known that the M371 test plays a significant role in the initial diagnosis of testicular cancer by detecting these tumors with high sensitivity (90,1%) and specificity (94%), as reported by Dieckmann et al. (2019b). The absence of M371 in other tumors enables a reliable differentiation between TGCTs and other testicle diseases (Belge et al., 2021). Moreover the M371 test offers a faster, more precise and reliable diagnosis compared to current biomarker standards (Tavares et al., 2023). Regarding the success of the M371 test in the initial diagnosis, integrating it into routine follow-up protocols holds promise for enhancing monitoring of GCT patients, thus leading to timely interventions (Tavares et al., 2023). Moreover, as relapse typically occurs in the retroperitoneum in 90% of patients, and most relapses are not identified solely through serum tumor markers, surveillance mainly focuses on retroperitoneal imaging, which includes CT scans and chest x-rays, which must be maintained for at least five years after initial trearmtent (Charytonowicz et al., 2019) (Figure 2). In the future, with the introduction of M371 test in the follow-up protocol for TGCT patients, imaging scans may only be necessary if there is a susception of recurrence based on elevated M371 levels, reducing the need for frequent imaging scans (Chavarriaga et

al., 2023; Matulewicz et al., 2023). Thus, the combination of the M371 test with a reduced number of CT scans could effectively reduce health-care costs (Lobo et al., 2021a; Busch et al., 2022; Tavares et al., 2023; Belge et al., 2024)

Another advantage of the M371 test is its utility in guiding chemotherapy intensity for testicular cancer treatment (Figure 2). Current guidelines recommend measuring serum tumor markers prior to each cycle of chemotherapy. The short half-life of M371, allows for rapid assessment of treatment effectiveness during chemotherapy and also enables timely intensification of therapy if required, thus minimizing unnecessary toxicity and side effects of chemotherapy (Nestler et al., 2023). It is also important to analyze the utility of the M371 test in assessing residual masses after chemotherapy in metastasized GCTs with the aim of avoiding overtreatment. Post-chemotherapy residual masses may consist of necrosis/fibrosis, teratoma, or viable cancer (Dieckmann et al., 2024). Since there is currently no reliable diagnostic tool to differentiate them, surgical resection is often performed on all cases (Nestler et al., 2023; Dieckmann et al., 2024). The integration of the M371 test into clinical practice would help to identify viable cancer cells and determine which patients require surgical resection and which can be managed by surveillance alone (Figure 2).

Besides the promising diagnostic potential of M371 test (Syring et al., 2015; Van Agthoven et al., 2017; Dieckmann et al., 2019b; Nappi et al., 2019), there is limited database regarding the sensitivity of M371 for detecting recurrence in GCT patients. While some studies suggest elevated M371 levels in recurrent cases, other studies show controversial findings (Lobo et al., 2021a; Belge et al., 2024). The promising diagnostic accuracy of M371 at the time of macroscopic recurrence and its short half-life, suggests the potential of M371 for early detection of relapse (Terbuch et al., 2018; Dieckmann et al., 2019b). Lobo et al. (2021) reported that M371 levels early after orchiectomy were not associated with disease relapse during follow-up. Therefore, M371 does not seem to offer any prognostic value.

Additionally, while one major series suggests a lower expression rate in recurrent cases compared to primary GCT (Dieckmann et al., 2019b), other studies indicate equally high M371 sensitivities in both recurrences and in primary GCT (Terbuch et al., 2018; Fankhauser et al., 2022). In a small prospective study, (Fankhauser et al., 2022) also evaluated the detection of recurrences using M371 during active surveillance for stage I GCT patients. After a median of 7 months, recurrence was detected in 10 of 33 patients (30%). Moreover, they reported, that M371 detected

recurrences at a median of 2 months earlier compared to standard follow-up investigations. While showing promising results regarding the M371 test in detecting recurrences, it is constrained by a limited sample size and short follow-up period, emphasizing the need for further research (Fankhauser et al., 2022). Thus, the overarching aim of this thesis was to prospectively evaluate the role of M371 in detecting relapses in a series of patients with CSI GCT managed by active surveillance. Through a series of studies, we investigated various aspects of M371, including its origin, interaction with several clinical factors, as well as its utility as a prognostic marker for tumor recurrence.

The first study of this thesis (Belge et al., 2020) focused on investigating the origin of miR-371a-3p within tissue samples from both the contralateral testis and germ cell tumor tissue, with the aim to understand the M371 expression patterns and its potential utility as a tumor biomarker for GCTs. The main results highlight several key points regarding the expression pattern of miR-371a-3p in GCT tissue compared to normal testicular tissue and its serum levels, as well as its intracellular localization within tumor cells. Firstly, the study demonstrates a significantly higher expression of miR-371a-3p in GCT tissue compared to both contralateral testicular tissue and normal testicular tissue, suggesting that GCT tissue serves as the primary source of circulating miR-371a-3p. The results align with previous studies indicating the presence of miRNAs of the miR-371-373 cluster in GCT tissue. This finding further highlights the specificity of miR-371a-3p as a marker for GCTs (Gillis et al., 2007; Palmer et al., 2010). Secondly, the in situ hybridization experiments, confirm the intracellular localization of miR-371a-3p within GCT tumor cells. In combination with previous studies on the expression of miR-371a-3p in GCT tissue, there is now abundant evidence for its origin from testicular tumor cells (Palmer et al., 2010; Rijlaarsdam et al., 2015; Vilela-Salgueiro et al., 2018). Thirdly, the association between microRNA expression in GCT tissue and serum levels was not investigated systematically in previous studies. The results of the study demonstrated that the miR expression level in GCT tissue was higher than in serum samples and a correlation was observed between miRNA expression levels in GCT tissue and corresponding serum levels in patients with localized disease. However, the correlation was not significant in advanced clinical stages (CS2/3), as factors like the release of marker substances from both the primary tumor and metastatic sites may influence miRNA serum levels in these cases. This finding suggests that serum level of miR-371a-3p reflects both the tumor bulk, and the specific

secreting capacity of the individual GCT, as previously observed by Dieckmann et al. (2019b). Additionally, other biological determinants such as direct vascular invasion of the tumor and potentially other unknown factors may also affect the serum level of miR-371a-3p (Belge et al., 2020). In all, the study confirms that circulating miR-371a-3p originates specifically from cells of testicular germ cell neoplasms, serving as a specific tumor marker for GCTs in contrast to AFP, which lacks specificity due to its association with non-GCT related conditions, such as liver diseases (Lembeck et al., 2020).

In the second study (Dieckmann et al., 2022b), the utility of M371 test was investigated as a potential biomarker for detecting occult metastases in CSI non-seminoma patients, particularly after orchiectomy. Despite being classified as CSI, some non-seminoma patients may have micro-metastatic seeds, which standard imaging procedures may fail to detect (Murray et al., 2016; Leão et al., 2019). While previous research has shown elevated M371 levels in 29.4% of CSI NS patients after orchiectomy, their biological significance remains unclear (Dieckmann et al., 2019b; Badia et al., 2021). However, against expectation, the study found no significant correlation between postoperative M371 elevation and risk factors such as LV status or the presence of >50% EC in the primary tumor, with an AUC of 0.5. Thus, postoperative 0M371 levels do not seem to serve as reliable predictors for disease progression in CS1 non-seminoma patients. Remarkable, the study demonstrated a significant association between preoperative M371 levels and both risk factors LV1 and >50% EC. Overall, the biological role of postoperatively elevated M371 levels in CSI NS patients remains unclear. However, there is a hypothesis suggesting that elevated postoperative M371 levels may primarily occur in patients with larger tumors due to a slower decrease of M371 associated with tumor size and with tumor bulk (Radtke et al., 2018). Another explanation for persistently elevated postoperative M371 levels could be premature blood sampling after orchiectomy (Dieckmann et al., 2022b). Further research is needed with comprehensive follow-up data of patients and consistent intervals of postoperative of blood sampling.

In the third study (Dieckmann et al., 2022c), included in this thesis, a comprehensive study was performed to analyze how tumor size influences clinical parameters such as histology, clinical staging, and tumor marker expression rates in patients with testicular cancer. Indeed, the findings from this study are crucial for advancing diagnostic, therapeutic, and surveillance strategies in testicular cancer. Notably, the study showed that subcentimeter tumors comprised approximately 13.6% of all tumors, indicating a

higher frequency compared to some previous research (Scandura et al., 2018), emphasizing the clinical importance of managing such lesions. Moreover, our findings are aligning with previous research that has explored the role of primary tumor size in the clinical course of testicular tumors. Specifically, the median tumor sizes in seminomas and non-seminomas observed in our study (Dieckmann et al., 2022c) are consistent with other studies (Heinzelbecker et al., 2011; Rothermundt et al., 2018). Regarding the association between tumor size and histology, our results are in line with previous findings indicating that smaller tumors are more likely to be benign (Scandura et al., 2018; Gentile et al., 2020; Wardak et al., 2023). Benign tumors were found to have a median size of 10 mm, significantly smaller than that of GCTs (30 mm) and other malignant tumors (53 mm) (Dieckmann et al., 2022c). The study showed a significant trend towards decreasing tumor size, consistent with other research in the field (Dieckmann et al., 2013; Galosi et al., 2016; Lagabrielle et al., 2018). In a Turkish study involving 252 patients, including 35 cases with tumors ≤10 mm, a cutoff size of 15 mm was suggested to discriminate between benign and malignant tumors (Keske et al., 2017). In our study, we identified a slightly higher cutoff size of 16mm, with a sensitivity and specificity of 81.5% and 81%, respectively (Dieckmann et al., 2022c). Although various studies support the potential utility of tumor size in predicting testicular histology, there is no agreement regarding the threshold sizes for clinical decision-making (Ates et al., 2016). Moreover, the study found that larger tumor sizes (>10 mm) were associated with significantly higher expression rates of serum tumor markers in GCTs, which is consistent with previous literature findings (Dieckmann et al., 2019a). Additionally, the study highlighted the limited usefulness of classical tumor markers like bHCG and AFP in diagnosing subcentimeter testicular neoplasms, emphasizing the better performance of the novel marker M371 in such cases. Regarding patient age and tumor size, there were significant differences in median sizes among age categories, with the largest meidan tumor size observed in oldest age category. However, there was no clear trend indicating that tumor sizes increased with age since the second largest tumor size was observed in the youngest age category.This findins are consistent with prior investigation that found no difference in tumor sizes between GCT patients <50 years and older ones (Dieckmann et al., 2018). Additionally, the study showed limited utility of classical tumor markers like bHCG and LDH in diagnosing subcentimeter testicular tumors, underscoring the impressive performance of the novel marker M371 in such cases, with expression rates of 66.7%

and 39% in subcentimeter non-seminomas and seminomas, respectively. These results are in line with previous research by (Dieckmann et al., 2019b), emphasizing the utility of the M371 test as a valuable tool with sensitivities of 56% and 98% for the diagnosis of subcentimeter seminomas and nonseminomas, respectively.

In the fourth study, the association between serum tumor marker expression rates and various clinical parameters, including histology, patient age, and clinical staging in GCT patients was investigated (Dieckmann et al., 2023). Consistent with previous reports, our results showed differences in median ages among histological subtypes, with non-seminomas being present predominantly in younger age categories and seminomas being more prevalent in older age groups (Xu and Yao, 2019; Koch et al., 2022). Furthermore, the study showed significant differences in marker expression rates among histological subtypes, a finding that aligns with previous literature (Weissbach et al., 1997; Dieckmann et al., 2019a). Notably, M371 showed superior sensitivity compared to traditional markers, consistent with previous reports (Syring et al., 2015; Murray et al., 2018; Dieckmann et al., 2019b; Sequeira et al., 2022). The findings regarding the association between tumor marker expression rates and clinical stages further emphasize the clinical relevance of markers assessment in prognostic evaluation. The results of our study indicate a significant trend towards higher expression rates with increasing clinical stages, highlighting the prognostic value of marker expression in predicting disease progression (Dieckmann et al., 2023). These findings are consistent with previous studies (Dieckmann et al., 2019a, 2022c). Overall, the results of this study contribute to a deeper understanding of the association between serum tumor marker expression, tumor histology, patient age, and clinical staging in testicular cancer, underscoring the clinical utility of M371 in testicular cancer management (Dieckmann et al., 2023).

Finally, the recent publication of our study on recurrence detection through M371 serum levels in follow-up of GCTs (Belge et al., 2024) represents a significant milestone in the evaluation of the clinical utility of M371. In this study, we monitored 258 patients with testicular germ cell tumors in clinical stage I for up to 48 months. The results of the study showed a remarkable sensitivity of 100% and a specificity of 96.3% for M371 in detecting relapses, thus clearly outperforming the classical serum markers bHCG and AFP for TGCTs (Belge et al., 2024). These findings underscore the importance of M371 in early and accurately identifying relapses, which is crucial for timely management. Moreover, the study indicates that M371 exhibits higher accuracy

in detecting relapses than in the initial diagnosis of GCTs, exceeding the sensitivity and specificity of 90.3% and 94.1%, respectively (Dieckmann et al., 2019b). This difference in diagnostic accuracy between the two clinical scenarios may be due to the limited sensitivity of M371 test in detecting small-volume seminomas. Since most relapses in this study involved lymph nodes with diameters above 1 cm, the M371 test was more effective at detecting relapses compared to its performance in the primary diagnosis, where about 10% of newly diagnosed seminomas are smaller than 1 cm (Dieckmann et al., 2022d). In both seminomas and non-seminomas, the M371 test showed similar performance characteristics. However, the PPV was higher in non-seminomas (91.7%) compared to seminomas (73.9%), likely due to a higher number of false-positive results in seminoma cases and the higher relapse frequencies in non-seminoma (32%) than in seminoma (9%) (Belge et al., 2024).

Notably, in the study (Belge et al., 2024), a cutoff value of RQ= 15 was used to identify relapses, which is higher than the RQ = 5 which was previously used as the upper limit of normal (ULN) for detecting primary GCTs with the M371 test (Dieckmann et al., 2019b). Both threshold values were determined through Youden index analysis relating to their corresponding study populations, however, this difference could be linked to variations in patient populations and technical improvements in the M371 test kit. Our study (Belge et al., 2024) focused specifically on CSI patients with a higher proportion of seminomas from Central European countries, while previous studies consisted of patients from various European countries and with various clinical stages (Dieckmann et al., 2019b). Therefore, the ideal cut-off for this particular scenario had to be determined separately. Moreover, in the study (Belge et al., 2024), the commercially available M371 test kit was utilized to measure serum levels, however, minor modifications in the M371 test methodology may have resulted in a higher sensitivity in detecting miRNA targets compared the former one, even though there are no significant differences between both methods. For practical purposes, the range of RQ=10-12 is recommended as a uniform threshold value across diverse clinical scenarios until further establish and a definitive ULN for the M371 test is determined (Belge et al., 2024). The excellent sensitivity of the M371 test in detecting relapses among patients with CSI GCTs during surveillance has been demonstrated in previous studies. Notably, studies by the Toronto group and a Swiss study reported high sensitivities of 94.1% and 100%, respectively, for M371 in detecting recurrences among CSI patients (Lobo et al., 2021b; Fankhauser et al., 2022). While the Toronto

group reported no false-positive findings, indicating high accuracy in detecting true relapses (Lobo et al., 2021), the Swiss study documented one false-positive result (Fankhauser et al., 2022). In addition, in a small Dutch case series, all three patients with CSI GCTs were found to have elevated M371 levels at the time of relapse (Van Agthoven et al., 2017).

In the study (Belge et al., 2024), the traditional tumor markers bHCG and AFP demonstrated sensitivities of only 35% and 25%, respectively, and even the combined application yielded only a 45.2% sensitivity, which are in accordance with previous reports (Nicholson et al., 2019; Chakiryan et al., 2021). These findings align with previous reports indicating the limited performance of traditional markers in detecting relapses, especially in cases with high proportion of seminomas (Daugaard et al., 2014; Trigo et al., 2000). Therefore, the notably low sensitivity of bHCG and AFP found in the current study, could be attributed to the high proportion of seminomas in this study. Overall, there is significant evidence supporting the utility of the M371 test in detecting recurrences after early stage GCTs. However, it is crucial to note that the ability of the M371 test in detecting recurrences is not limited to CSI cases, as it serves as a universal marker for GCTs (Murray and Coleman, 2019). As shown by various literature, elevated M371 levels have been observed in recurrences originating from various clinical scenarios other than surveillance in CSI (Terbuch et al., 2018; Dieckmann et al., 2019a; Nappi et al., 2019).

The current study (Belge et al., 2024) highlights the promising potential of the M371 test in the early detection of relapses among patients with CSI under active surveillance. In the study, recurrences were detected earlier in 28% of patients than with conventional diagnostics. Specifically, M371 elevations preceded relapse detection by 3 to 15 months in eleven cases, indicating potential for earlier relapse detection compared to imaging and/or marker elevation. However, the Kaplan–Meier curve did not demonstrate a statistically significant time advantage with the M371 test until relapse detection compared to standard imaging techniques. The Kaplan-Meier curve revealed that the median time to relapse detection was 6 months for both M371 measurements and standard methods which may be due to several reasons like sample size or variability in timing between M371 measurements and standard diagnostic tests (Belge et al., 2024). The Swiss study on the other hand, reported a median time of 2 months earlier with the M371 test for detecting relapses than standard follow-up investigations (Fankhauser et al., 2022). However, the study was limited by

its small sample size and relatively short duration of follow-up. Moreover, the Toronto group reported higher M371 levels closer to the time of relapse, but this finding did not achieve statistical significance (Lobo et al., 2021a). Contrary to expectations, the study showed that post-orchiectomy M371 levels do not serve as predictors of future relapses in GCTs. The M371 levels measured shortly after orchiectomy were not significantly different between patients who later experienced relapse and those who did not (Belge et al., 2024). This aligns with finding from previous studies by the Toronto group (Lobo et al., 2021a) and the Swiss study (Fankhauser et al., 2022).

Our study (Belge et al., 2024) presents some limitations, including a reduction in sample size due to exclusions, deviations from follow-up schedules observed in about 40% of patients, and a short median follow-up period of 18 months which represent a possible weakness of the study. Moreover, lack of detailed information regarding the clinical and histologic details of relapsing patients and the method of examining hemolysis in serum samples were additional weaknesses. However, the study had also notable strengths, including multicentric patient enrollment and direct comparison of M371 with traditional markers in most cases, enhancing the reliability of the study´s results (Belge et al., 2024).

Overall, this study provides significant evidence for the ability of the M371 test in detecting relapses among patients with CSI GCT. Despite practical issues such as the need to validate the cut-off level, the integration of M371 into follow-up schedules could potentially lead to early detection of relapses and improved management. However, clinical decisions should not depend solely on the M371 test but should also consider other diagnostic factors, especially imaging (Belge et al., 2024).

Future investigations will determine whether the M371 test alonce can accurately identify GCT relapses as suggested by the current data, thus reducing the exposure of young patients to ionizing radiation, and even lead to cost-savings in healthcare.

5 Summary

Testicular cancer represents a relatively rare neoplasia, accounting for 1.6% of all male cancer cases. However, it is one of the most common malignant diseases among young men aged between 15 and 40 years. Currently, about more than 4,000 men are diagnosed with testicular cancer in Germany each year. The first-line treatment for stage I GCT patients is surgical removal of the affected testicle, followed by active surveillance, which is often preferred over adjuvant chemotherapy to avoid overtreatment and minimize long-term side effects. After treatment is completed, follow-up care is crucial to detect cancer recurrence early. The recurrence rate during surveillance ranges from 15% to 20% for CSI seminoma, whereas for non-seminomas, the recurrence rate varies depending on risk factors, ranging from 15% to 50%. Tumor markers play an essential role in both diagnosis and follow-up. However, classical tumor markers face limitations due to their low sensitivity and specificity, with only 60% of all patients showing an elevation levels of these markers at initial diagnosis. Thus, additional imaging modalities such as CT or MRI a required during follow-up. However, the of CT is associated with radiation exposure in this young patient population. Therefore, additional markers are urgently needed for the early detection of recurrences in TGCT patients. Recently, microRNAs of the miR-371~373 cluster have been proposed as reliable serum markers for GCTs. MicroRNAs are short, single-stranded, non-coding RNAs between 18 and 24 nucleotides long. Among the three microRNAs of the miR-371~373 cluster (miR-371a-3p, miR-372-3p, and miR-373-3p), miR-371a-3p (M371) demonstrated the most favorable performance with higher specificity and sensitivity compared to traditional serum tumor markers.

The aim of this study was to evaluate the utility of M371 as a serum biomarker for the early detection of recurrences in CS1 patients with GCTs during active surveillance in a prospective long-term study. The results of the miRNA expression studies that were conducted to answer this question were published in five scientific reports, which are presented within this thesis. The first study investigates the origin of M371 and its correlation with tissue levels in testicular GCTs. The results showed that M371 levels in GCT tissue were significantly higher compared to contralateral testicular tissue and non-testicular tissue. Additionally, M371 levels in tissue correlated significantly with corresponding serum levels. The study showed that M371 is specifically derived from GCT tissue and could serve as a useful tumor marker for GCT. In the second study,

the association between M371 levels and progression risk factors in non-seminoma patients with clinical stage I was evaluated, specifically lymphovascular invasion (LV1) and the presence of >50% embryonal carcinoma (>50% EC). Results showed that postoperatively elevated M371 levels were not associated with LV status or >50% EC. In the third study, the role of the primary tumor size and its association with various clinical factors was investigated. Tumor size showed significant association with clinical stage, serum tumor marker expression levels and histology, underscoring that tumor size plays a significant role in disease progression. Additionally, the expression rates of all serum tumor markers increased with tumor size, with M371 showing superior performance, especially in smaller germ cell tumors. In the following study, the interrelationships of M371 levels and classical tumor markers in relation to various clinical parameters were evaluated. The findings showed distinct tumor marker expression rates among histologic subgroups, with M371 showing the highest rates in seminoma and non-seminoma patients. Additionally, all markers exhibited elevated expression in metastasized stages and in younger patients, except LDH. Overall, M371 demonstrated superior clinical utility to traditional markers. In the last study, the utility of M371 in detecting relapses in patients with clinical stage I TGCTs was evaluated, with a focus on accuracy and earlier relapse detection than traditional methods. Results showed that among the 258 GCT patients, 39 relapsed during the follow-up period of up to 48 months. All of these relapses were detected using the M371 test, emphasizing its potential as a highly sensitive biomarker for recurrence detection. The M371 test revealed impressive diagnostic characteristics with an area under the ROC curve of 0.993, sensitivity of 100% and specificity of 96.3%, thus outperforming the classical serum markers for TGCTs. Findings also demonstrated that relapses were detected earlier with the M371 test in 28% of cases.

The M371 test showed superior sensitivity and specificity for detecting relapses in CSI GCTs compared to classical tumor markers. Overall, the results show, that the M371 test holds promise for integration into clinical follow-up protocols, potentially leading to more effective and cost-efficient approaches, by reducing the need for frequent imaging scans and minimizing ionizing radiation exposure in young patient population.

6 Zusammenfassung

Hodenkrebs ist mit einem Anteil von 1,6% aller Krebsfälle des Mannes, eine eher seltene Neoplasie. Dennoch zählt der Hodenkrebs zu den häufigsten malignen Erkrankungen bei jungen Männern im Alter zwischen 15 und 40 Jahren.
Der erste Behandlungsschritt für Patienten im klinischen Stadium I ist die chirurgische Entfernung des betroffenen Hodens, gefolgt von aktiver Überwachung, die oft der adjuvanten Chemotherapie vorgezogen wird, um eine Übertherapie zu vermeiden und Langzeitfolgen zu minimieren. Nach Abschluss der Behandlung ist eine regelmäßige Nachsorge entscheidend, um Rezidive frühzeitig zu erkennen. Bei Patienten mit einem Seminom im CSI liegt das Rezidivrisiko bei 15-20%, wobei bei Nicht-Seminomen das Rezidivrisiko je nach Risikofaktoren ca. 15–50% beträgt. Tumormarker spielen eine wichtige Rolle sowohl bei der Diagnose also auch bei der Nachsorge. Allerdings weisen sie eine geringe Sensitivität und Spezifität auf, wobei sie nur bei etwa 60% aller Patienten mit Hodenkrebs erhöht sind. Daher sind während der Nachsorge zusätzliche Bildgebende Verfahren wie CT oder MRT erforderlich. Jedoch ist die Verwendung von CT mit einer Strahlenbelastung in dieser jungen Patientengruppe verbunden. Daher ist die Forschung nach neuen Biomarken zur Früherkennung von Rezidiven bei Patienten mit GCT besonders wichtig. Die microRNAs (miRs) des miR-371~373 Clusters sind in den letzten Jahren als verlässliche Serummarker für GCTs evaluiert worden. Bei miRNAs handelt es sich um nicht-kodierende, einzelsträngige RNA-Moleküle mit einer Länge von 18-24 Nukleotiden. In initialen Studien konnte gezeigt werden, dass Insbesondere miR-371a-3p (M371) geeignet ist, um Patienten mit testikulären Keimzelltumoren von gesunden Probanden zu differenzieren. Ziel dieser Arbeit war es, den Nutzen der M371 als Serum-Biomarker zur Früherkennung von Rezidiven bei Patienten mit GCT im CSI in der Nachsorge in einer prospektiven Langzeitstudie zu evaluieren. Die erste Studie untersuchte den zellulären Ursprung des Markers M371. Die Ergebnisse zeigen, dass die Expression der miR-371a-3p im Gewebe von Keimzelltumoren signifikant höher waren im Vergleich zu kontralateralen Hodengeweben. Darüber hinaus korrelierten die M371-Spiegel im Tumorgewebe signifikant mit den in den entsprechenden Patientenseren ermittelten Werten. Die Studie zeigt, dass M371 spezifisch aus GCT-Gewebe stammt und als spezifischerer und sensitiverer Tumormarker für testikuläre Keimzelltumoren dienen könnte. In der zweiten Studie wurde der Zusammenhang zwischen dem M371-Spiegel und

Risikofaktoren für eine okkulte Metastasierung bei CSI Patienten mit Nicht-Seminom untersucht, insbesondere das Vorhandensein von lymphovaskulärer Invasion (LVI) und ein Anteil von >50% embryonalem Karzinom (>50% EC). Die Ergebnisse zeigen, dass postoperativ erhöhte M371-Spiegel nicht mit dem LV-Status oder dem Anteil embryonalem Karzinom assoziiert sind. In der dritten Studie wurde die Rolle der Größe des Primärtumors und ihre Assoziation mit verschiedenen klinischen Faktoren untersucht. Es bestand eine signifikante Assoziation zwischen der Primärtumorgröße und dem klinischen Stadium, der Expression von Serum-Tumormarkern und der Histologie, was darauf hinweist, dass die Primärtumorgröße ein wichtiger Prognosefaktor ist. Darüber hinaus stiegen die Expressionraten aller Serum-Tumormarker mit zunehmender Primärtumorgröße an, wobei M371 eine überlegene Leistung zeigte, insbesondere bei kleineren Keimzelltumoren. In der darauffolgenden Studie wurden die Wechselbeziehungen zwischen der M371-Expression und klassischen Tumormarkern in Bezug auf verschiedene klinische Parameter analysiert. Die Ergebnisse zeigten unterschiedliche Tumormarker-Expressionraten zwischen histologischen Untergruppen, wobei M371 die höchsten Raten bei Seminom- und Nichtseminom Patienten aufwies. Insgesamt zeigte M371 eine überlegene klinische Nützlichkeit im Vergleich zu traditionellen Markern. In der letzten Studie wurde die Eignung von M371 bei der Erkennung von Rezidiven bei CSI GCT-Patienten untersucht, wobei der Schwerpunkt auf frühzeitiger Rezidiverkennung im Vergleich zu traditionellen Methoden lag. Die Ergebnisse zeigten, dass von den 258 GCT-Patienten 39 während des bis zu 48-monatigen Follow-up-Zeitraums ein Rezidiv entwickelten, die alle mit dem M371-Test erkannt wurden. Dies zeigt das Potenzial des M371-Tests als hochsensitiver Biomarker zur Rezidiverkennung. Die Sensitivität des M371-Tests betrug 100 %, die Spezifität 96,3 % und die Fläche unter der Kurve 0,993 in der ROC-Analyse und übertraf somit die klassischen Serummarker für TGCTs. Die Ergebnisse zeigten auch, dass eine frühere Diagnose eines Rezidivs mit dem M371-Test bei 28 % der Fälle beobachtet wurde. Der M371-Test zeigte eine überlegene Sensitivität und Spezifität bei der Erkennung von Rezidiven bei CSI-GCTs im Vergleich zu klassischen Tumormarkern. Insgesamt zeigen die Ergebnisse, dass der M371-Test vielversprechend für die Integration in klinische Nachsorgeprotokolle ist, was zu effektiveren und kostengünstigeren Ansätzen führen könnte, indem die Notwendigkeit bildgebender Untersuchungen verringert und die Strahlenbelastung in der jungen Patienten minimiert wird.

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8 Abbreviations

%	Percent
β-HCG	β-subunit of human chorionic gonadotropin
ΔΔCT	Delta delta CT
µl	Microliter
χ2	Pearson's Chi-square
AFP	α-fetoprotein
AUC	Area under the curve
AJCC	American Joint Committee on Cancer
BEP	Bleomycin, etoposide, cisplatin
BRCA1	Breast cancer 1, early onset
cDNA	Complementary DNA
CI	Confidence interval
CIS	Carcinoma in situ
CLL	Chronic lymphocytic leukemia
CS	Clinical stage
CT	Computed tomography
DNS	Deoxyribonucleic acid
EAU	European Association of Urology
EC	Embryonal carcinoma
EP	Etoposide and cisplatin
FFPE	Formalin-fixed paraffin-embedded
GCNis	Germ cell neoplasia in situ
h	Hour
H&E stain	Hematoxylin and Eosin
IGCCCG	International Germ Cell Cancer Collaborative Group
IGCNU	Intratubular Germ Cell Neoplasia Unclassified
IQR	Interquartile ranges
ISH	In situ Hybridization
LDH	Lactate Dehydrogenase
LV0	Without lymphovascular invasion;
LV1	Lymphovascular invasion
min	Minute

miR-302/367	MicroRNA cluster on chromosome 4
miR-371-3	MicroRNA cluster on chromosome 19
M371	miR-371a-3p
miRNA	MicroRNA
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
ml	Milliliter
OCT4	Octamer-binding transcription factor 4
PLAP	placental alkaline phosphatase
PPV	Positive predictive value
NPV	Negative predictive value
NS	Non-seminoma
NSGCTs	Non-seminomatous germ cell tumors
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
qPCR	Quantitative polymerase chain reaction
R^2	Pearson product-moment correlation coefficient
RKI	Robert Koch Institute
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RTR	Residual tumor resection
RPLND	Retroperitoneal lymph node dissection
RQ	Relative quantity
RT	Reverse transcription
SGCTs	Seminomatous germ cell tumors
sec	Second
TC	Testicular cancer
TGCTs	Testicular germ cell tumors
TIN	Testicular intraepithelial neoplasia
TNM	Tumor, Node, Metastasis
UICC	Union for International Cancer Control
UTR	Untranslated region
WHO	World Health Organizati

9 Complete list of publications

9.1 Peer-reviewed papers

1. Belge, G., Hennig, F., Dumlupinar, C., Grobelny, F., Junker, K., Radtke, A., & Dieckmann, K.-P. (2020). Graded expression of microRNA-371a-3p in tumor tissues, contralateral testes, and in serum of patients with testicular germ cell tumor. *Oncotarget*, *11*(16), 1462–1473.
   The first three authors contributed equally to this work.

2. Dieckmann, K.-P., Dumlupinar, C., Radtke, A., Matthies, C., Pichler, R., Paffenholz, P., Sommer, J., Winter, A., Zengerling, F., Hennig, F., Wülfing, C., & Belge, G. (2022). Associations of serum levels of microRNA-371a-3p (M371) with risk factors for progression in nonseminomatous testicular germ cell tumours clinical stage 1. *World Journal of Urology*, *40*(2), 317–326.
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3. Dieckmann, K.-P., Isbarn, H., Grobelny, F., Dumlupinar, C., Utschig, J., Wülfing, C., Pichlmeier, U., & Belge, G. (2022). Testicular Neoplasms: Primary Tumour Size Is Closely Interrelated with Histology, Clinical Staging, and Tumour Marker Expression Rates—A Comprehensive Statistical Analysis. *Cancers*, *14*(21), 5447.
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4. Dieckmann, K.-P., Dumlupinar, C., Grobelny, F., Utschig, J., Klemke, M., Ahmed Saad, E. M., Wülfing, C., Pichlmeier, U., Isbarn, H., & Belge, G. (2023). Testicular neoplasms: The interrelationships of serum levels of microRNA-371a-3p (M371) and classical tumor markers with histology, clinical staging, and age—a statistical analysis. *Journal of Cancer Research and Clinical Oncology*, *149*(10), 7079–7090.
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5. Belge, G., Dumlupinar, C., Nestler, T., Klemke, M., Törzsök, P., Trenti, E., Pichler, R., Loidl, W., Che, Y., Hiester, A., Matthies, C., Pichler, M., Paffenholz, P., Kluth, L., Wenzel, M., Sommer, J., Heinzelbecker, J., Schriefer, P., Winter, A., Zengerling, F., Kramer, M.W., Lengert, M., Frey, J., Heidenreich, A., Wülfing, C., Radtke, A., Dieckmann, K.-P. (2024). Detection of Recurrence through microRNA-371a-3p Serum Levels in a Follow-up of Stage I Testicular Germ Cell Tumors in the DRKS-00019223 Study. *Clinical Cancer Research*, *30*(2), 404–412.
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6. Dieckmann, K.-P., Grobelny, F., Soave, A., Che, Y., Nestler, T., Matthies, C., Heinzelbecker, J., Winter, A., Heidenreich, A., Niemzok, T., Dumlupinar, C., Angerer, M., Wülfing, C., Paffenholz, P., & Belge, G. (2024). Serum Levels of MicroRNA-371a-3p for Predicting the Histology of Postchemotherapy Residual Masses of Germ Cell Tumours. *European Urology Focus*, S2405456924000646.
   The first three authors contributed equally to this work.

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11 Erklärung

Versicherung an Eides Statt

Ich, Cansu Dumlupinar, versichere an Eides Statt durch meine Unterschrift, dass ich die vorstehende Arbeit selbständig und ohne fremde Hilfe angefertigt und alle Stellen, die ich wörtlich dem Sinne nach aus Veröffentlichungen entnommen habe, als solche kenntlich gemacht habe, mich auch keiner anderen als der angegebenen Literatur oder sonstiger Hilfsmittel bedient habe.

Ich versichere an Eides Statt, dass ich die vorgenannten Angaben nach bestem Wissen und Gewissen gemacht habe und dass die Angaben der Wahrheit entsprechen und ich nichts verschwiegen habe.

Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahren Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs. 1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

Erklärungen zur elektronischen Version und zur Überprüfung einer Dissertation

Hiermit betätige ich gemäß § 7, Abs. 7, Punkt 4, dass die zu Prüfungszwecken beigelegte elektronische Version meiner Dissertation identisch ist mit der abgegebenen gedruckten Version.

Ich bin mit der Überprüfung meiner Dissertation gemäß § 6 Abs. 2, Punkt 5 mit qualifizierter Software im Rahmen der Untersuchung von Plagiatsvorwürfen einverstanden.

Bremen, den 27. Juni 2024 Cansu Dumlupinar