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THERAPEUTIC ADVANCES in
Medical Oncology

Systematic Review

The utility of cfDNA in TGCT patient management: a systematic review

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Abstract

Background: Testicular germ cell tumors (TGCTs) are the most common young male malignancy with a steadily rising incidence. Standard clinical practice is radical orchidectomy of suspicious lumps followed by histopathological diagnosis and tumor subtyping. This practice can lead to complications and quality of life issues for the patients. Liquid biopsies, especially cell-free DNA (cfDNA), promised to be true surrogates for tissue biopsies, which are considered dangerous to perform in cases of testicular tumors. In this study, we have performed a systematic review on the potential of cfDNA in TGCT patient management, its potential challenges in translation to clinical application and possible approaches in further research.

Materials & Methods: The review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines on EuropePMC and PUBMED electronic databases, with the last update being on October 21, 2021. Due to the high heterogeneity in identified research articles, we have performed an overview of their efficacy.

Results: Eight original articles have been identified on cfDNA in TGCT patients published from 2004 to 2021, of which six had more than one TGCT patient enrolled and were included in the final analysis. Three studies investigated cfDNA methylation, one has investigated mutations in cfDNA, two have investigated cfDNA amount, and one has investigated cfDNA integrity in TGCT. The sensitivity of cfDNA for TGCT was found to be higher than in serum tumor markers and lower than miR-371a-3p, with comparable specificity. cfDNA methylation analysis has managed to accurately detect teratoma in TGCT patients.

Conclusion: Potential challenges in cfDNA application to TGCT patient management were identified. The challenges relating to the biology of TGCT with its low mutational burden and low cfDNA amounts in blood plasma make next-generation sequencing (NGS) methods especially challenging. We have also proposed possible approaches to help find clinical application, including a focus on cfDNA methylation analysis, and potentially solving the challenge of teratoma detection.

Keywords: biomarker, cfDNA, ctDNA, liquid biopsy, TGCT

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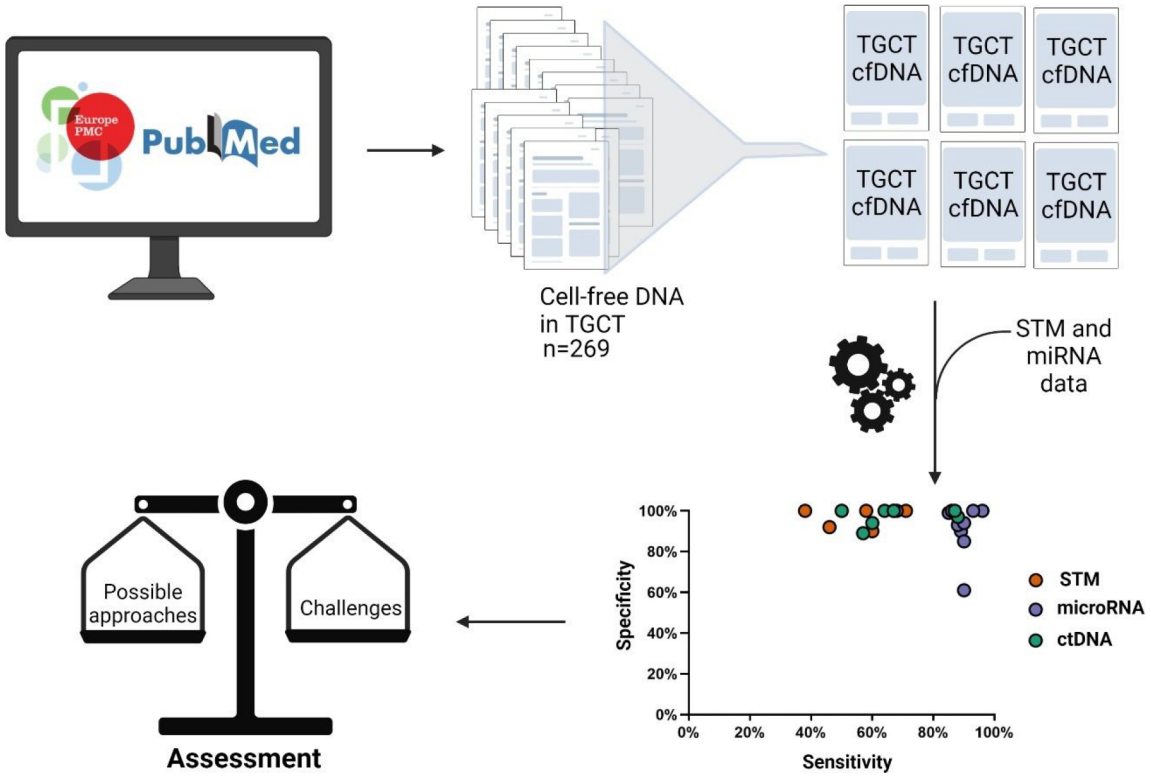


Background

Testicular germ cell tumors (TGCTs) make up around 95% of all testicular tumor cases and are the most common malignancy diagnosed in men (between 15 and 44 years of age), primarily of European descent.[1,2] The incidence of TGCT has been steadily rising over the last 20 years[3,4] with the highest predicted rise in East and South European countries.[5] There have been more than 71,000 cases of testicular cancer diagnosed in 2018 alone, of which over one-third were in Europe.[1] Reasons for this rising incidence are not fully understood but are hypothesized to be a combination of inherited and epigenetic factors.[3,6] The initial cure rate in TGCT patients is over 95% after the standard procedure of orchiectomy and, when indicated, cisplatin-based chemotherapy treatment.[1,4,6] However, 30% of cases will persist and 42% will recur after chemotherapy mainly due to gain of cisplatin resistance, with 50% of them dying from progressive disease.[3,7] In addition, reports of long-term morbidities due to the treatment are increasingly more common, with high rates of obesity, hypogonadism, erectile dysfunction, and cardiovascular disease.[2] Even the surgical techniques themselves can result in anejaculation and infertility.[2] This makes TGCTs an important sociodemographic issue, with each death resulting in 30 years of life being lost, more than in other cancers.[6] There is an evident need for improvement in TGCT patient management. In particular, better diagnostic and prognostic biomarkers are required to enhance diagnosis and treatment of TGCT resulting in an improved long-term health of survivors.[4]

According to the World Health Organization's (WHO) classification, TGCTs are divided into two types: germ cell neoplasia *in situ* (GCNIS)-derived TGCT (postpubertal TGCT/type II TGCT), and non-GCNIS-derived (prepubertal TGCT/type I TGCT and spermatocytic tumors/type III TGCT).[8] In this review, we will focus on the type II TGCT or the GCNIS-derived TGCT, which make up more than 90% of all adult TGCT cases.[9,10] GCNIS-derived TGCTs are a heterogeneous group of tumors that originate from the same precursor cell,[1,9] and are divided into seminomas (SEs) and non-seminomas (NSEs). SE and NSE components occur in patients in around the same ratio (1:1). However, in ~10% of cases, SE and NSE present together as mixed germ cell tumors.[1,4,9,11,12] NSEs are further subdivided into embryonal carcinomas, teratomas (TE),

choriocarcinomas, and yolk sac tumors.[3,8,9] The correct diagnosis of TGCT components and their ratio is important for treatment selection.[2,11]

Currently, the only population screening method is the patient's self-inspection. If a lump is detected, they are referred to their physician who instructs a scrotal ultrasound to detect a mass forming lesion. Finally, if a mass is present, a radical orchiectomy is performed. While in other cancers, a tissue biopsy is mandatory to confirm the diagnosis, testicular biopsies are not performed due to the risk involved (except in the case of patients with suspect contralateral TGCT).[6,13,14] Instead, after orchiectomy, the mass is analyzed and subtyped[6,13] using histology and immunohistochemistry.[9,11,15] While classical serum tumor markers (STMs: AFP, βHCG, and LDH) and imaging techniques are used in clinical practice, they offer limited sensitivity, and predictive value for TGCT. In addition, STMs are not fully TGCT specific, since they can be elevated in other cancers and pathologies.[16] In light of this, accurate and non-invasive biomarkers are needed for more precise diagnosis, follow-up for disease progression and relapse detection, as well as to spare patients from a possibly unneeded radical orchiectomy.[2,13,17] Biomarker research centered on liquid biopsies (LBs) has promised to solve the problems of tissue biopsies and high tumor heterogeneity.[18,19]

LB is defined by the U.S. National Cancer Institute as 'a test done on a sample of blood to look for cancer cells from a tumor that are circulating in the blood or for pieces of DNA from tumor cells that are in the blood'.[20] The application of LB allows both repetitive and minimally invasive biomarker analysis, regardless of tumor heterogeneity. This combined with the accessibility of LB material and the minimum of inconvenience to the patient[13,21,22] makes it of special interest in pathologies, such as TGCT.[13]

cfDNA was discovered in 1948 by Mandel and Metai in human blood plasma and has since then become one of the most prominent biomarkers in LB research.[23] cfDNA is thought to originate both, as a consequence of cell apoptosis/necrosis and from active secretion from cells,[23–25] in response to physiological processes (i.e. exercise, pregnancy), as well as malignant and non-malignant pathological conditions (i.e. inflammation, cancer, tissue damage).[23,25,26] Circulating tumor DNA (ctDNA) are tumor-specific DNA fragments detectable in body fluids.[18,21] Since cancer patients have an increased amount of cfDNA, specific analytical methods enable the detection of ctDNA in the larger cfDNA fraction,[13,27] namely, analysis of tumor-specific somatic mutations, structural variations, and changes in epigenetic patterns.[13,18,23,27] Of these, the most promise as cancer biomarkers has been shown by cfDNA methylation and mutation analysis.[21,28,29] cfDNA's non-genetic properties also vary between different biological states. The quantity of cfDNA can reflect tumor burden and progression, while differences in the fragmentation profile can discriminate cancer patients from healthy individuals, making cfDNA integrity a promising cancer diagnostic and prognostic biomarker in its own right.[18,29–31]

ctDNA has been widely investigated in multiple forms of cancer,[21,23] and promises to complement cancer diagnostics and monitoring of disease progression.[32] Molecular profiling using next-generation sequencing (NGS) genetic variation identification panels are already finding clinical application in treatment selection.[19,26,33] To evaluate the potential of cfDNA in TGCT management, we have performed a systematic review to identify all original research on cfDNA in TGCT. The studies will be assessed for clinical use, identify the potential benefits in relation to other biomarkers, as well as try and recognize potential challenges relating to real-world application.

Materials and methods

Search strategy

A systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[34]

A literature search of EuropePMC and PUBMED electronic databases was performed, with the last update being on October 21, 2021. Keywords were combined with Boolean operators into search terms and are listed in Supplementary Table 1.

A EuropePMC only search was performed to identify the number of original articles and review articles published on different cancer types.

TGCT systematic search results from EuropePMC and PubMed searches were initially combined, and duplicates were removed. The

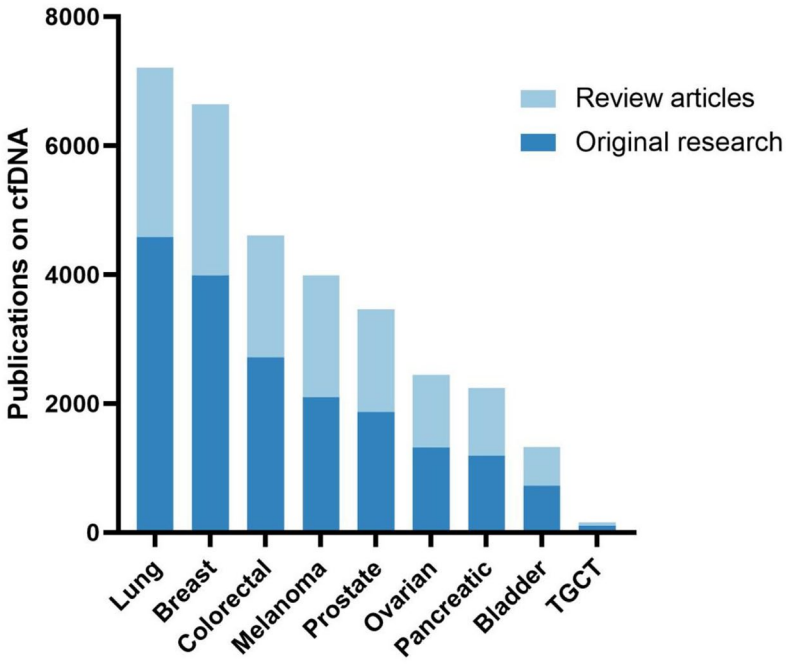


Figure 1. Number of publications in EuropePMC on cfDNA for different cancer types.

inclusion criteria were as follows: original research articles on cfDNA in patients with TGCT. All congress abstracts were removed from the search results. The remaining articles were all initially screened by title and then by abstract to progressively exclude articles not fitting the inclusion criteria. Finally, a full-text evaluation of the remaining articles was performed by two authors (JK and LS) to select for studies meeting the inclusion criteria. Debate over the inclusion of articles was resolved by consensus. Relevant information was extracted independently by the two authors from the final studies (number of participants, diagnosis, and cfDNA diagnostic parameters). In addition, the references of the studies included in the full-text search were manually searched by one author (JK) to identify additional potentially relevant studies.

Data analysis

A meta-analysis was intended if the reported cfDNA diagnostic data were homogeneous enough to allow for this. However, due to the heterogeneity in the parameters reported, the pooling of data was not appropriate. Therefore, an overview of the currently published articles on cfDNA in TGCT will be presented, summarizing the outcome measures. From the selected studies, the sensitivities and specificities of the analyzed cfDNA diagnostic parameters were extracted or calculated from the raw data by two authors (JK and LS).

Results

Search results

A literature search on EuropePMC for the rough estimate of publications on cfDNA in different types of cancer has returned 7210 results for lung cancer (4579 original and 2631 review articles), 6638 for breast cancer (3993 original and 2645 review articles), 4608 for colorectal cancer (2717 original and 1891 review articles), 3993 for melanoma (2094 original and 1899 review articles), 3467 for prostate cancer (1871 original and 1596 review articles), 2443 for ovarian cancer (1319 original and 1124 review articles), 1324 for bladder cancer (724 original and 600 review articles), and 155 for TGCT (102 original and 53 review articles) (Figure 1).

Our search has identified 249 publications on EuropePMC and 27 on PUBMED (Figure 2). Following the removal of 5 duplicates and 86 conference abstracts, 185 studies were selected for title and abstract search. Twenty-five studies were selected for full-text screening, from which six studies fit the criteria of original research on cfDNA of TGCT patients. However, one study had a single TGCT patient recruited in a larger cohort,[35] and another was performed on a single patient,[36] these were excluded from further analysis. From the reference search, two additional studies were selected for analysis, making the total number of included studies six.

Study characteristics

The heterogeneity among the included studies was high. Of the six included studies, three have investigated the methylation of TGCT cfDNA, one has investigated mutations in TGCT cfDNA using NGS, two have investigated cfDNA concentration (mitochondrial and total cfDNA) and one of the two has investigated cfDNA integrity.

Of the three studies that investigated cfDNA methylation one investigated the *XIST* gene methylation in blood plasma of 25 TGCT patients (14 SEs and 11 NSEs) and 24 non-TGCT

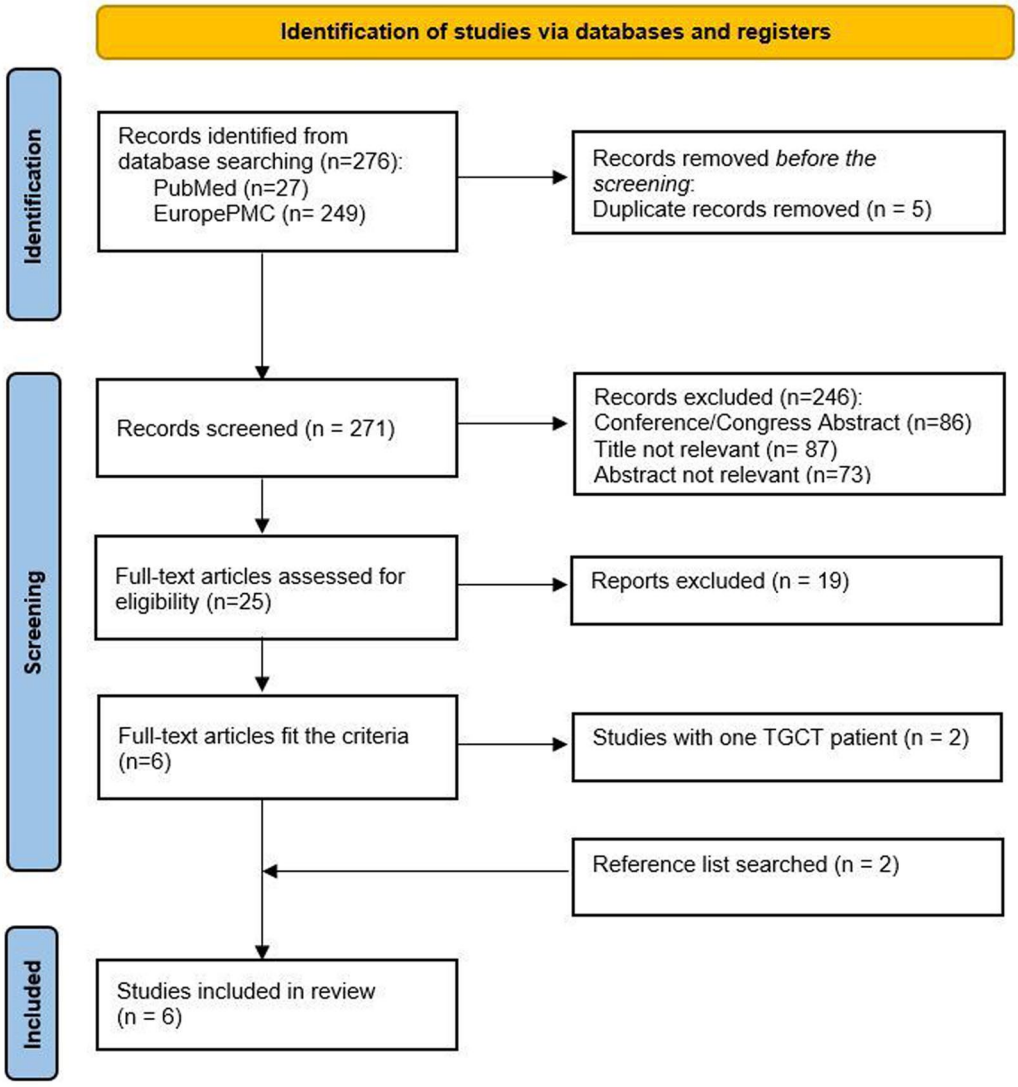


Figure 2. PRISMA flowchart.

patients which served as the control group (14 renal and 10 bladder carcinoma patients).[37] Ten of the 14 SE and six of the 11 NSE patients had unmethylated XIST fragments detectable, while none of the non-TGCT patients had. This gives unmethylated *XIST* a 64% sensitivity for TGCT, 71% sensitivity for SE, and 55% for NSE in cfDNA, with 100% specificity in all cases. The second study investigated cfDNA methylation of six genes in blood serum of 73 TGCT patients (36 SE and 37 NSE patients) and 35 healthy controls (HT).[38] When making a diagnostic panel using cfDNA methylation status of *RASSF1A*, *PTGS2*, and *P14*(*ARF*), 22 of 36 SE, 27 of 37 NSE patients, and 0 of 35 HT were detected. This made the sensitivity of the panel 67% for TGCT, 61% for SE, and 73% for NSE, with 100% specificity in all cases. The final study investigated *RASSF1A* methylation in blood serum of 98 TGCT patients (21 SEs and 77 NSEs) and 29 HT.[15] Nineteen of 21 SE patients, 66 of 77 NSE patients, and 0 of 29 HT had detectable *RASSF1A* hypermethylation. This meant a sensitivity of 87% for TGCT, 91% for SE, and 86% for NSE, with 100% specificity in all cases.

The study investigating mutations in cfDNA had 10 recruited patients with TGCT and 10 healthy controls.[32] Of the 10, NGS analysis of cfDNA and tumor genomic DNA has managed to identify mutations in six patients, while the remaining

four have not had any mutations detected. Of the six, four patients had mutations present in the tumor, which were also detected in the cfDNA of three patients, and undetected in one. Finally, two patients had mutations detected in cfDNA which were not present in the tumor itself. This would give NGS molecular profiling a 50% sensitivity and 100% specificity.

Regarding cfDNA concentrations, the study investigating mitochondrial cfDNA in blood serum of 74 TGCT patients (39 SEs and 35 NSEs) and 35 healthy controls found the greatest diagnostic precision by quantifying a 79 bp long mitochondrial fragment, giving a median concentration of 2.04×10^6 copies/mL in TGCT patients versus 0.47×10^6 copies/mL in healthy controls.[39] This resulted in 60% sensitivity and 94% specificity in TGCT (49% sensitivity and 97% specificity for SE and 69% sensitivity and 94% specificity for NSE), which was further improved when combining mitochondrial cfDNA with STM, with sensitivity being 85% and specificity 91%. The second study quantified the total cfDNA in blood plasma of 74 TGCT patients (39 SEs and 35 NSEs) versus 35 healthy controls by quantifying three fragments of the *ACTB* gene (106 bp, 193 bp, and 384 bp long fragments), with the strongest diagnostic effect obtained by quantifying the 193 bp long fragment.[40] In TGCT patients the average concentration was 9.03 ng/mL of cfDNA (8.08 ng/mL in SE and 10.08 ng/mL in NSE) vs 1.01 ng/mL in HT, this gave the 193bp long fragment an 88% sensitivity and 97% specificity (85% sensitivity and 97% specificity for SE and 91% sensitivity and 94% specificity for NSE), further improved when combined with STM to 93% sensitivity. cfDNA integrity 384/106 bp has shown a sensitivity of 57% and specificity of 89% in TGCT patients (60% sensitivity and 87% specificity for SE and 60% sensitivity and 89% specificity for NSE).

Discussion

Context of the results

Interest in cfDNA as a biomarker has been rapidly increasing every year.[23] While in comparison with other cancer types, research on cfDNA in TGCT is orders of magnitude behind.

Of the 244 search results, our search has managed to retrieve eight publications investigating the potential of cfDNA as a biomarker in TGCT, with six making the final cut. Of the six, four were published by 2009, with the remaining two being published in 2021. Effectively, this means there was an 11-year gap with no research on cfDNA in TGCT being published. Of the six, two have investigated cfDNA in blood plasma, while four have investigated cfDNA in blood serum.

To give the results of cfDNA studies, the necessary context, we have compared them with studies investigating miR-371a-3p and STM (Table 1). STMs are used in TGCT clinical staging, the TNM system,[41] while miR-371a-3p has been the most investigated LB TGCT biomarker of the last 10 years and with the most clinical promise.[42] Its use has been demonstrated in large patient groups, by various research groups and in comprehensive metanalyses.[15] We have selected prominent articles on STM and miR-371a-3p found within the results of our systematic review results and the reference list search. Only those studies on STM that had data on the combined sensitivity and specificity of all three biomarkers were used for the comparison. Since some of the studies on STM are missing specificity, we have decided to use the lowest specificity from the studies that included it, as a conservative placeholder.

While the sensitivity of cfDNA (50–88%) as a biomarker for TGCT detection is higher than in classical serological markers (38–71%), it is outperformed by miR-371a-3p (67–96%) (Figure 3(a)). According to the studies identified, cfDNA stands between the two in raw accuracy. As for specificity, cfDNA has shown a very high specificity (89–100%), comparable with STM (90–100%), and greater than miR-371a-3p (61–100%). In relation to SE, cfDNA has shown a sensitivity (49–91%) greater than STM (23–61%) and comparable with miR-371a-3p (53–91%) (Figure 3(b)). The specificity of cfDNA for SE (87–100%) is comparable with STM (90–100%) and miR-371a-3p (90–100%). In relation to NSE, the sensitivity of cfDNA (55%–91%) has outperformed STM (55%–83%) and is comparable to miR-371a-3p (45%–94%) (Figure 3(c)). The specificity of cfDNA in NSE (89–100%) is again comparable with STM (90–100%) and miR-371a-3p (90–100%).

Challenges in cfDNA application in TGCT patient management

As to why cfDNA did not find a larger interest as a biomarker for TGCT, we postulate a few possible challenges.

Table 1. Studies on cfDNA in TGCT identified by systemic review, as well as studies identified that assessed STM and miR-371a-3p sensitivity and specificity.

ctDNA–TGCT						AFP or bHCG or LDH–TGCT					miR-371a-3p–TGCT				
Study		Sensitivity		Specificity		Study	Sensitivity		Specificity		Study	Sensitivity		Specificity	
		TGCT	SE NSE	TGCT	SE NSE		TGCT	SE NSE	TGCT	SE NSE		TGCT	SE NSE	TGCT	SE NSE
Kawakami[37]	Methylation	**64%**	71%	**100%**	100%	Cremerius[43]	**67%**		**100%**		Dieckmann[44]	**67%**	85%	**100%**	100%
			55%		100%								45%		100%
Ellinger[38]	Methylation	**67%**	61%	**100%**	100%	Dieckmann)[44]	**38%**	23%	**100%**	100%	Gillis[45]	**89%**	85%	**61%**	
			73%		100%			55%		100%			91%		
Ellinger[40]	Concentration	**88%**	85%	**97%**	97%	Syring[46]	**46%**	36%	**92%**	92%	Syring[46]	**85%**	83%	**99%**	99%
			91%		94%			67%		92%			89%		99%
	Integrity	**57%**	60%	**89%**	87%	Dieckmann[71]	**50%**	46%	**100%**	100%	Agthoven[47]	**89%**	89%	**90%**	90%
			60%		89%			58%		100%			89%		90%
Ellinger[39]	Mitochondrial conc.	**60%**	49%	**94%**	97%	Lobo[48]	**71%**	61%	**100%**	100%	Dieckmann[71]	**88%**	86%	**93%**	93%
			69%		94%			83%		100%			90%		93%
Lobo[15]	Methylation	**87%**	91%	**100%**	100%	Dieckmann[49]	**60%**	45%	**90%**	90%	Dieckmann[50]	**90%**	87%	**94%**	94%
			86%		100%			82%		90%			94%		94%
Tsui (2021)[32]	Mutation	**50%**		**100%**		Dieckmann[50]	**60%**				Nappi[68]	**96%**		**100%**	
						Mørup (2020)[51]	**62%**	44%			Mørup[51]	**68%**	53%	**100%**	100%
								74%					78%		100%
						Badia (2021)[52]	**58%**		**100%**		Badia[52]	**93%**		**100%**	
											Lobo[15]	**86%**	91%	**100%**	100%
													84%		100%
											Piao[64]	**90%**		**85%**	

cfDNA, cell-free DNA; NSE, non-seminoma; SE, seminoma; AFP, alpha-feto-protein; bHCG, beta human chorionic gonadotropin; LDH, lactate dehydrogenase; TGCT, testicular germ cell tumors.

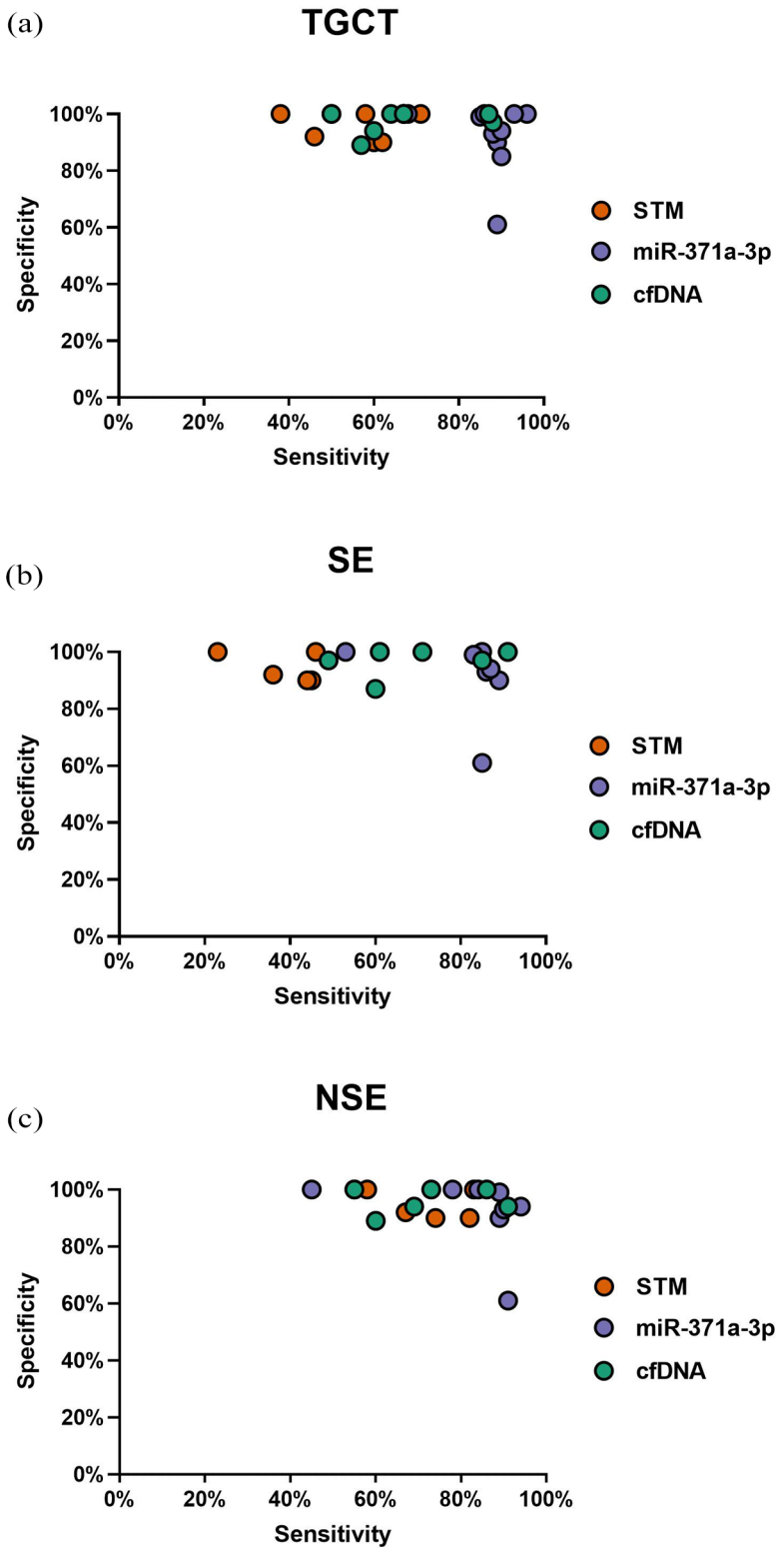


Figure 3. The specificity and sensitivity of identified cfDNA studies in TGCT in relation to studies on STM and miR-371a-3p. (a) Comparison of sensitivities and specificities for TGCT overall, (b) comparison between SE components, and (c) comparison between NSE components.

The localization of TGCT itself could be the first possible challenge. As mentioned before, the amount of ctDNA present in the plasma is variable, depending on factors, such as stage of the disease and tumor type. Colon, breast, and skin cancer are associated with releasing large amounts of ctDNA into the blood, while gliomas and prostate cancers are associated with the smallest amounts of ctDNA released.[25,28] This could in part be due to the brain and prostate being isolated by the blood–brain barrier and the blood–prostate barrier, limiting the diffusion of cfDNA into the blood.[25,31,53] The testis with its blood–testis barrier, which is a strong physiochemical barrier consisting of continuous cell layers[54] might have the same effect. The barrier is strong enough to even limit the effect of chemotherapy, with most childhood leukemia relapses being in the testis.[55] The average concentration of cfDNA in patients with TGCT was measured at 10 ng/mL while healthy controls had 1 ng/mL, which showed discriminatory power.[40] However, multiple studies have shown that the concentration of cfDNA in the blood of healthy controls is highly variable and ranges from 0 to 100 ng/mL with the average value of 30 ng/mL, while in patients with cancer the range is 5–1000 ng/mL.[18,19,23] The concentration of 10 ng/mL leaves a lot of space for possible overlap and would need to be validated in a much larger patient and control cohorts.

Low amounts of ctDNA are a major factor in limiting the sensitivity and application of ctDNA as a biomarker.[56] Novel methodologies and approaches are increasing the efficacy of biomarker detection, low ctDNA concentration is still the biggest challenge in this niche.[13] While it seems that a realistic goal for the early detection of cancer would be the detection of a tumor with a diameter of 5 mm, current approaches are only able to detect tumors greater than 1 cm.[27] Although these are still early stage tumors, they already show clinical signs and symptoms of cancer in patients, and can be identified through imaging,[27] which limits the usefulness of ctDNA in early detection of tumors.

The biology of TGCT itself could be another potential issue, with molecular profiling by NGS being among the main approaches of cfDNA applications in patient management.[27,29] This results in two challenges. First, an adequate

amount of cfDNA has to be extracted from the LB for the analysis to be reliable.[57] As mentioned before, in certain cancer types there isn't a large enough increase in ctDNA quantity[25,28] which makes NGS analysis problematic, with this being the case in TGCT as well. Second, a characteristic of TGCTs is its low mutational burden[58] and no high-penetrance susceptibility genes existing.[59,60] This would make most commercial cfDNA assays, which are highly focused, not well suited for TGCT, and more broader sequencing assays should be employed which remain cost-prohibitive for near-term clinical implementation.[32,49] This is only additionally complicated by ultra-deep sequencing picking up inconsequential mutations in other high turnover components (such as bone marrow) when needing to interpret rare alleles.[19] As research has shown,[32] while 6 of the 10 TGCTs have been detected by NGS, only 3 of them have had the same mutations identified in cfDNA and DNA from the solid tumor, 2 have had mutations detected only in cfDNA (which could be the before-mentioned inconsequential mutations), and 1 had tumor mutations not detected in cfDNA.

From a technical standpoint cfDNA research in general is hindered by issues in pre-analytical and analytical method standardization.[61–63] While multiple studies have examined the impact of variations in the cfDNA research process on the final result, there are still no commonly accepted standards in laboratory practice.[23,61] Everything from the time of day when sampling, the satiety and exertion level of the patient, the type of needle in blood drawing, the selection of blood tubes, sample transportation, the temperature, and time that takes from the blood to be processed, choice between blood serum and plasma, centrifugation protocols, storage of plasma or serum before cfDNA isolation, freeze-thawing of plasma or serum, HIL (hemolysis, icterus and lipemia) presence, choice of cfDNA isolation and quantification methods as well as cfDNA storage methods and duration has been shown to create variability in the final analysis results, especially with regards to cfDNA concentration and fragmentation.[23,62–66] This is both due to the effects of these variables on cfDNA degradation and isolation, but even more importantly, variations in sample processing can lead to genomic DNA contamination.[23,62,67] Even when certain procedures have become widely accepted or suggested, such as double-step centrifugation in blood plasma or serum preparation, minimizing the number of freeze-thawing events, usage of blood plasma and use of automated methods for cfDNA isolation the variations persist.[23,68] Finally, the issue is further exacerbated by no commonly accepted standards even in the way results are reported, adding more difficulties in data comparability.[69] Minimizing variability is the key in making cfDNA research reproducible and inter-lab comparable, which is what is hampering clinical acceptance of cfDNA.[63]

Selection of matrix for cfDNA research, between blood serum and plasma has been a widely investigated subject.[63] This is important to stress since blood plasma and serum differ as LB sources.[70] While serum has a higher amount of cfDNA, it also has a higher proportion of longer fragments potentially derived from leukocyte lysis. Despite the larger amount of cfDNA present, detection of cancer-specific mutated alleles was lower in serum than in blood plasma, meaning the overall ctDNA fraction was lower.[70,71] Due to this, there is a high chance of contamination by genomic DNA, and 'drowning-out' ctDNA.[70,71] However, despite blood plasma being strongly recommended studies have confirmed the utility of serum in cfDNA research, as long as its limitations are kept in mind and the results are not compared with those obtained on plasma.[61,70,72] Of the identified studies on TGCT cfDNA, four were done using blood serum. Since no specific reasoning was provided for its use, we can only assume it was either due to in-house lab practices or because blood serum is commonly used in micro-RNA (miRNA) research that is very prevalent now for TGCT. Replicating the studies on blood plasma could possibly provide higher resolution and more accurate results.[70,71]

Finally, a lack of interest in cfDNA as a biomarker for TGCT could very well be due to the shift in clinical interest to microRNAs that have exhibited a very high sensitivity and specificity for TGCT.[17,73] Studies have shown that quantification of miR-371a-3p (the M371 test) alone is sufficient for the detection of TGCT,[4,16,73–75] its accuracy is impacted very little by inter-lab heterogeneity and therefore holds the greatest potential for real-world clinical application,[4,17] with prospective trials under way.[42]

We have also confirmed that studies investigating cfDNA in TGCT have been focusing on type II TGCT.[13] Types I and III TGCT require detailed investigation of their own into the potential of cfDNA and LBs as potential biomarkers.

Possible approaches

This, however, does not mean that there is no application for cfDNA in TGCT management but instead highlights what and where its potential role may be, elucidating the potential approach that would result in clinical use. The before-mentioned aspects of TGCT management in need of non-invasive biomarkers are better follow-up methods, relapse prediction, and prognostic biomarkers. These are needed to avoid possible overtreatment with cisplatin-based chemotherapy, as well as better ways of post chemotherapy assessment of TE and necrosis/fibrosis for assessing residual disease.[2,13] As ctDNA has shown itself as perspective in detection of advanced cancers due to the before-mentioned specifics of ctDNA biology,[18,27] efforts could be focused toward therapy selection (detection of aggressiveness), treatment response or detection of relapse.[18] So far, a commercially available cfDNA NGS assay has correctly determined poly ADP ribose polymerase (PARP) inhibitor efficacy in a single TGCT patient,[36] signifying potential use in therapy selection for TGCT.

While miRNA-371a-3p will most certainly be introduced to the clinic, an aspect of TGCT remains unsolved, namely, TE detection and GCNIS detection.[2,15,73,75] So far, results indicate that miR-371a-3p cannot be used to screen preinvasive TGCT, making testicular biopsy and immunohistochemistry the only GCNIS detection method.[75] While eight miRNAs have been found specific for TGCT, none of them has been able to identify TE.[2,73] TE are characterized by chemoresistance and malignant potential, being commonly found in metastatic masses after chemotherapy as well as being independently associated with cancer specific mortality.[2,15,42] Elevated levels of miR-371a-3p have been found in the majority of patients with recurrent disease, detecting tumors less than 0.5 cm in diameter,[17] the sensitivity is lower than for detection of primary TGCT, owning to the larger percentage of TE in recurrent disease being found in 25–40% of resected lymph nodes, while 40–50% exhibit no signs of residual disease.[75–77] No combination of miRNAs adds to the diagnostic accuracy of miR-371a-3p and cannot reliably detect TE.[74,78] While mir-375 has been suggested to detect TE and be used in combination with miR-371a-3p, subsequent studies have found it to be non-informative.[72,77,79,80] Mir-885-5p and miR-448 were then suggested for TE detection, however, this could not be validated in further studies too.[17,72,77,79] The last small RNA sequencing study that was performed has not managed to verify any small RNA for TE detection.[77] With it clearly shown that there is no expression of STM in TE,[15,81] cfDNA analysis is currently the only proven method of TE detection.[15]

As said before, many of the challenges with ctDNA detection are the result of preanalytical and analytical process variation, genomic DNA contamination and analytical methods lacking power. Their resolution should improve with novel approaches, advances in technology (especially sequencing), and greater standardization.[19,32,56,82] Governing bodies imposing standardizes procedures on cfDNA clinical studies and pressure on producing more detailed protocols and metadata documentation of the pre-analytical process and patients involved in the research could serve to increase cfDNA research reproducibility.[61,63] NGS and polymerase chain reaction (PCR) methods are continuously refined in light of new biomarkers and to accommodate low target frequencies.[61,65] The advent of digital droplet PCR has already sparked a renewed interest in various aspects of ctDNA research.[15,29,83] However, if sequencing is to be employed, perhaps efforts focused on amplification of chromosome 12 (i12p), as a near-universal TGCT marker, should be investigated.[3,9]

The accuracy and resolution of diagnostic methods could also be improved by finding ways to increase the amount of ctDNA in body fluids. Research has shown that multiple factors, such as exercise, satiety, fasting, and time of day, impact cfDNA levels.[25,63,84] Exercise-induced cfDNA increase originates from a single cell source and is therefore not applicable in cancer biology, ideally, patients would have a rest period prior to blood draw to minimize genomic DNA contamination.[63,84] However, certain procedures, such as punctures, during tissue biopsy retrieval increase the amount of cfDNA found in the blood for a time window.[25] By analyzing cancer-specific mutations, it has been shown that the increased levels of cfDNA are due to a larger fraction of ctDNA.[25] It is possible that a similar induced time window could be found for TGCT. While testicular biopsies, as mentioned before, are highly problematic, a similar effect could perhaps be produced post-orchiectomy, post-testis sparring surgery or perhaps even after testicular self-inspection. In fasting subjects and when blood was drawn up to midday, the highest amount of

cfDNA was obtained, introducing a routine of taking samples in the morning while fasting could be beneficial.[63] Similarly, improvements in cfDNA yield could be obtained using plasmapheresis, using cfDNA capture devices and by greater standardization and optimization of preanalytical techniques.[27]

While detection of tumors with a diameter of 1 cm is less than ideal in other types of cancers,[27] in TGCT all tumors below 3 cm in diameter are considered less malignant.[85] Other types of cancers are first detected by imaging with the size of 1 cm in diameter,[27] TGCT is diagnosed at the average size of 3 cm for both SE and NSE.[86,87] Similarly, 34% of recurrences escape early detection and present with retroperitoneal lymphadenopathy of $>$ 2 cm.[75] Reliable diagnostic methods for either first detection or detection of recurrence of TGCT at the size of 1 cm diameter would be a great improvement in patient management.

Instead of focusing on incremental technical improvements, a different approach would be to take into account the specific biology of TGCT, focusing on biologically more significant non-genetic markers, such as cfDNA methylation or fragmentation.[18,27,29] Methylation of cfDNA is a robust pathology-specific modification that is not impacted by preanalytical methods,[23] has already found clinical application and is FDA approved in colorectal cancer.[27] What makes it especially valuable is that research has shown that cfDNA methylation can be detected even when ctDNA makes up less than 10% of the total cfDNA fraction.[38] For this reason, cfDNA methylation has been investigated in prostate cancer,[25] with prostate cancer and TGCT having similar challenges relating to blood as LB source. The importance of epigenetics in TGCT is well known, with its key drivers being epigenetic.[3] Of the three studies that investigated cfDNA methylation in TGCT,[37,38] two from 2004 and 2009 have had comparable sensitivity (64% and 67%) and 100% specificity for TGCT even against other cancers. Methylation of cfDNA is more specific than STM in distinguishing from other pathologies (i.e. inflammation or hemorrhage). One study has included bisulfite conversion, while one has used methylation-specific restriction enzymes. Due to the significant amount of DNA degradation during bisulfite conversion novel kits and protocols can serve to mitigate this loss.[61] However, development and refinement of analytical methods that avoid bisulfite conversion can only improve the efficiency its cost-effectiveness of methylation analysis.[38,61,88] The third study on cfDNA methylation has confirmed this, analyzing *RASSF1A* methylation using methylation-specific restriction enzymes on digital droplet PCR achieving a sensitivity of 87% and specificity of 100%, as well as a high accuracy in TE detection. Such an increase in specificity (47% vs 87% specificity when comparing methylated *RASSF1A* alone) was in part due to the higher sensitivity enabled by digital droplet PCR.[15] As for cfDNA fragmentation, research has shown sensitivity and specificity of over 90% across seven cancer types.[18] While the cfDNA study in TGCT has found a sensitivity of 57% and 89% specificity for cfDNA integrity as a biomarker,[40] which shows the same power as STM. However, the usefulness of cfDNA fragmentation in TE detection or relapse detection has yet to be investigated.

Selection of smaller fragments for PCR amplification increases detection of ctDNA fragments,[27,89] while selection of longer fragments does the same immediately post trauma-induced cfDNA enrichment step.[25] This process can also be used to bypass the issue of potential genomic DNA contamination, due to it being or larger fragment size.[69]

While most of the research on cfDNA has been done on blood as LB source, different body liquids can serve this role, including cerebrospinal fluid, urine and semen.[24,28] It has been argued that since sometimes they are situated closer to the tumor they could carry more tumor DNA.[63] The concentration of cfDNA from seminal plasma was higher than from other biological fluids.[24,28] This increase was noted in prostate cancer patients, with cfDNA levels being 100 times larger in semen than in blood.[18,31] Detected cfDNA quantity was able to distinguish between prostate cancer patients and healthy individuals,[18,24,28] as was cfDNA fragment analysis in semen with longer DNA fragments being present in cancer patients.[18,31] The larger amount of cfDNA present means that robust cost-effective methods, such as fluorimetry may be used.[28] This is of special interest for cancers with low levels of blood cfDNA that are in direct contact with other body fluids,[28] such as TGCT and semen. Semen is being investigated as LB source in small RNA analysis already.[90] However, most of the research on cfDNA in TGCT has been done on blood serum, and not blood plasma, despite the recommendations for potential clinical use to be

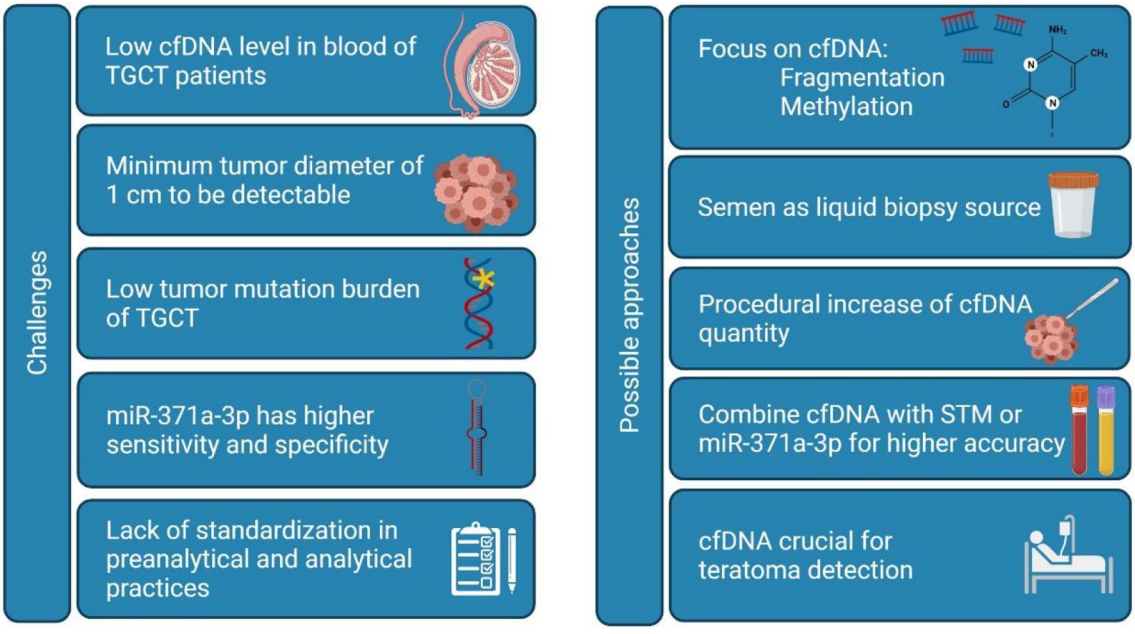
investigated in blood plasma as LB source.[70,71] Investigating the same parameters in blood plasma would alone perhaps yield results unobscured by genomic DNA contamination and potentially show a more accurate result.

A simple way of increasing accuracy in evaluation and diagnostics is with a multiparameter approach, STM when combined with abdominal and chest imaging most accurately stages early TGCT.[62,65,91] The research on cfDNA biomarkers confirms this, cfDNA concentration had a similar sensitivity and specificity in both STM-positive and -negative patients, combining the two biomarkers has resulted in an increase in accuracy, with total cfDNA amount being increased from 88% sensitivity to 97% and mitochondrial cfDNA from 60% to 85%.[39,40] Classical serum tumor markers are measured both on the start of TGCT management and on following routine check-ups to detect possible recurrence.[13] Detection of SE and TE components is problematic using STM,[15] so that a complementary analysis is required for accurate TGCT detection. Detection of miR-371a-3p has a similar issue, while it manages to detect SE and complements STM in that regard, the combining of the two methods leaves undetected TE components. However, combining miR-371a-3p and *RASSF1A* cfDNA methylation has resulted in 100% specificity and 100% sensitivity, detecting all TE in the patient cohort.[15] Calls have been made for an investigation of miR-371a-3p- and i12p-specific mutations.[42] Whatever it may be, the way forward in TGCT diagnostics seems to include cfDNA analysis as a companion parameter.

Conclusion

While advances in technology only serve to increase interest in cfDNA as a biomarker, its possible exact application in TGCT is underinvestigated. Challenges in cfDNA research relating to TGCT biology and analytic methodology have diverted attention to miR-371a-3p, which promises higher accuracy and easier detection. However, TE and relapse detection have shown to be undetectable by either microRNA panels or combinations of STM and microRNA. cfDNA has shown to be complementary to both STM and miR-371a-3p, detecting patients otherwise negative, including TE patients, which are chemotherapy insensitive. Further steps should be the validation of results obtained in serum in blood plasma, as well as exploration of other body liquids, such as semen, as LB sources.

Graphical depiction of the key points, created with BioRender.com



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Supplemental material

Supplemental material for this article is available online.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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