Improving outcomes in germ cell cancers using miRNA

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***Abstract*:** Owing to advances in treatment paradigms across the last five decades, testicular cancer is now eminently curable. However, current serum tumour and imaging biomarkers lack adequate sensitivity, specificity, and predictive value. Subsequently, their utility in detecting active malignancy and informing treatment decisions is minimal in a large proportion of men with testicular cancer. Micro-ribonucleic acids (miRNA), pertinently miR-371a-3p, offer a new tool, which based on early data, appears to fill many of the gaps that existing biomarkers leave. This paper reviews the evolution of the technology, potential limitations, and discusses the clinical relevance of miRNA as it moves towards the clinic.

Keywords: blood-based biomarker, germ cell tumour, molecular oncology, predictive biomarker, prognostic biomarker

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Introduction

Testicular germ cell tumors (TGCT) are the most common malignancy diagnosed in young men in Western countries. Fortunately, these malignancies are considered highly curable.[1] However, treatment may result in significant, long-term morbidities in men with an otherwise excellent prognosis and there is room to improve the care of this population.

Almost two thirds of men diagnosed with TGCT will have disease confined to the testis at diagnosis (clinical stage 1, CS1), with the remainder having either regional nodal involvement (clinical stage 2, CS2) or distant spread (clinical stage 3, CS3). Following formal staging, subsequent treatment depends on histological subtype, disease stage, and prognostic group [as defined by the International Germ Cell Cancer Collaborative Group, (IGCCCG)], as well as patient factors and is guided by local and international guidelines.[1–4] While chemotherapy, radiotherapy, and surgery are undoubtedly very effective treatments, there is a growing panoply of evidence demonstrating the potential long-term morbidities associated with these therapies. In a study of survivors, high rates of obesity, sensory neuropathy, hypogonadism, erectile dysfunction, and cardiovascular disease[5,6] were reported. In addition, there is accumulating evidence showing a deleterious impact that these treatments have on health-related quality of life.[7–10] Surgical techniques are also not without their problems, with many men reporting problems with anejaculation and infertility; however, these may be lessened by modern, nerve-sparing techniques.[11–13] Therefore, it is integral that clinicians accurately select men who are most likely to benefit from treatment.

Despite widespread endorsement in surveillance guidelines,[1–4] current serum tumour and imaging biomarkers in TGCT offer limited sensitivity, specificity, and predictive value in detecting active disease,[14] adding little value to treatment algorithms. Ultimately, there remains a number of important clinical questions that current tools fail to enlighten.

An ideal biomarker would help diagnose TGCT and accurately detect features of early relapse. It would reduce the issues associated with false positives seen with existing serum and imaging biomarkers, preventing unnecessary and potentially morbid treatments. An ideal biomarker would also detect minimal residual disease (MRD) post-orchidectomy and help select men who will most

benefit from adjuvant treatment, rather than applying an all-or-nothing approach for this common clinical scenario. It would also help nuance the management of non-specific radiological changes and post-chemotherapy residual masses, differentiating fibrosis and necrosis from active germ cell tumors (GCT), including teratoma. In turn, it would eliminate pN0 retroperitoneal lymph node dissections (RPLND), sparing a proportion of men from this invasive procedure and directing this therapy to men with active GCTs who will derive benefit. An ideal biomarker would also help define treatment algorithms, including monitoring, and choice between treatment modalities in advanced disease, as well as inform prognosis and risk of relapse for men following treatment for TGCT. Clearly, there is room for improvement in a multitude of clinical scenarios.

There has been increasing interest in micro-ribonucleic acids (miRNA) as predictive biomarkers in various cancer types, with certain clusters of miRNAs present almost invariably in the blood of men with active TGCT[15–17] opening the door for further development. Early signs point towards miRNA being valuable biomarkers in the care of men with TGCT, filling some of the gaps that current biomarkers leave and offering accurate predictive information, improving the focus on the long-term health of this population. The evolution of knowledge regarding miRNA has been rapid with the majority of work undertaken by a few key research groups.

This paper will review the growing evidence base for miRNAs in the routine management of men with TGCT, discussing current limitations and applications in clinical practice.

Current serum tumour biomarkers and their deficiencies

Alpha-fetoprotein (AFP), beta-human chorionic gonadotropin (hCG), and lactate dehydrogenase (LDH) are variably expressed between histologic subtypes of TGCT and are the only existing serum biomarkers endorsed in the diagnosis and surveillance of men with this malignancy.[18] The detection of these biomarkers in the serum of men with TGCT is explained by their embryological origins from gonocytes in normal development.[19]

Spermatogonia (and oogonia) arise from primordial germ cells contained within the extraembryonic mesoderm; these later become identifiable in the endoderm of the yolk sac.[20] AFP is produced by the embryological yolk sac in normal fetal development and by the liver later in gestation. It is, therefore, commonly measurable in the serum of men with yolk sac tumors (YST) and embryonal carcinoma (EC).[21] In men with significant AFP elevations, YST must be suspected, even if it was not apparent in the original orchidectomy specimen.[1] Importantly, AFP may also be detectable in the serum of patients with hepatic disease, including hepatocellular carcinoma[21,22] and chronic liver disease secondary to alcohol misuse, viral hepatitis, and biliary tract disorders[18,21],as well as other gastrointestinal cancers,[23] inherited conditions such as hereditary ataxia-telangiectasia syndrome,[24] and, occasionally, specific drugs.[18] It is rarely detected in seminoma or choriocarcinoma (CHC). The half-life of AFP is 5–7 days, which is important when interpreting elevated levels post-orchidectomy.[21]

The half-life of hCG is shorter and normally reduces within 12–36 h following orchidectomy. hCG is produced by syncytiotrophoblastic components in tumors, arising from trophoblasts associated with the placenta in embryological development. For this reason, elevation in hCG is commonly seen in CHC, and occasionally in pure seminoma, as well as other non-TGCT malignancies including neuroendocrine, bladder, renal, and lung carcinomas and non-malignant conditions including hypogonadism and tetrahydrocannabinol use.[21]

Of the existing serum tumor biomarkers, LDH is least specific for TGCT and is ubiquitously expressed by non-malignant cells, purely reflecting cell turnover. In malignancy, LDH is often measured at diagnosis as a surrogate for tumour bulk and risk of tumor lysis, and may be elevated in TGCTs, as well as non-malignant conditions including shock, liver disease, and muscle damage.[25–27]

Ultimately, AFP, hCG, and LDH are only elevated in 26–34%, 38–47%, and 33–44% of men at diagnosis with any TGCT, respectively,[28] making their role in surveillance of men without elevation fraught. In seminoma, the value of serum tumor biomarkers is particularly low, with elevation of AFP, hCG, and LDH seen in $<3\%$,[26,28–30] 18–31%,[26,31] and ~30%[32,33] at diagnosis, respectively, and similarly low rates are also seen at the time of relapse are also seen.[21] In fact, in the event of a significant elevation of serum tumor biomarkers in tumors otherwise thought to represent

pure seminoma histologically, the diagnosis is revised from pure seminoma to NSGCT.[1,4] For this reason, serum tumor biomarkers are no longer strongly recommended as part of routine surveillance in some guidelines for men with seminoma,[21] leaving clinicians relying on physical examination, modern imaging [including computerised tomography (CT) or magnetic resonance imaging, (MRI)] and symptoms as the only surveillance tools to detect relapse.

In NSGCT, serum tumor biomarkers are more commonly elevated. The degree of elevation often reflects clinical staging, with 10–20%, 20–40%, and 40–60% of men with NSGCT having elevation of AFP at diagnosis in CS1, CS2, and CS3, respectively. Elevation of hCG is slightly less common, seen in 10–20%, 20–30%, and 40%, respectively. LDH elevation is seen in 40–60% of patients across the disease spectrum.[32] Despite this, <50% of men who relapse will have elevated serum tumour biomarkers,[1] with elevation more common if there was lymphovascular invasion in the original orchidectomy specimen.[34–36] Given the greater reliability in this population, however, serum tumor biomarkers continue to be used to determine IGCCCG prognostic risk classification and may influence treatment options.[4] Despite this, these remain imperfect and subject to the issues outlined earlier.

Current imaging (bio)markers and their deficiencies

CT has long-formed part of the routine care in men with TGCT. It provides important structural information in the initial staging of malignancy and has a reasonable sensitivity in assessing advanced, untreated TGCT.[37] However, CT does have significant limitations. One of the major factors impacting the sensitivity of CT is its inability to detect malignancy in lymph nodes <1 cm, resulting in a missed opportunity to detect early relapse.[4] Another key limitation is the failure of CT to differentiate between necrosis and fibrosis from active malignancy in post-chemotherapy residual masses in advanced disease. This is important, as men with persisting tumor masses at CT may go onto have post-chemotherapy RPLND (pcRPLND), only to find no viable malignancy or teratoma in the specimen (pN0), resulting in an unnecessary procedure.[38,39] 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET) may add additional value in this scenario.

In seminoma, FDG-PET offers improved sensitivity (80% *versus* 70%), specificity (100% *versus* 74%), and positive (PPV; 100% *versus* 37%) and negative predictive values (NPV; 96% *versus* 92%) for detecting viable malignancy in post-chemotherapy residual masses compared to CT and is routinely recommended in men with a residual mass >3 cm at least 6 weeks following chemotherapy.[40,41] There may also be a place for FDG-PET in the evaluation of men with seminoma experiencing rising serum tumour biomarkers following chemotherapy. In this area, FDG-PET offered a PPV of 92%, however the NPV is low, at just 50%.[42] Unfortunately, FDG-PET has been shown to not be very useful in the evaluation of NSGCT,[43] nor in evaluation of small, non-specific lymph nodes across TGCT histologic subtypes.

The optimal frequency of CT surveillance is uncertain; however, it is important issue, given concerns regarding cumulative radiation exposure in this young population. A recent study[44] investigating an alternate surveillance schedule for CT in men with CS1 seminoma demonstrated that a reduced frequency of imaging was non-inferior to conventional surveillance. In their study, the risk of relapse beyond 36 months was <1%, providing reassurance that radiological surveillance may be safely reduced beyond this time point. In addition, MRI was non-inferior to CT, offering an attractive alternative approach and avoiding cumulative radiation exposure in well-resourced settings.[44]

While imaging (bio)markers add useful clinical information for some men with TGCT, there remain a number of clinical scenarios where better biomarkers could revolutionize and personalize the care of these men. miRNAs are a promising biomarker in this space.

The evolution of microRNAs

miRNAs are small, non-coding ribonucleic acid molecules involved in the regulation of post-transcriptional gene expression. They contribute to important embryological functions, including organogenesis in normal development,[45–47] and have also been implicated in oncogenesis of various different solid organ and haematological malignancies. They interact closely with messenger RNA, affecting normal protein translation, and, when involved in oncogenesis, may act as either an oncogene or tumour suppressor gene.[48–51] Multiple

adjacent miRNA genes considered 'clusters,' are recognised, and hold collective functions in normal development and oncogenesis; however, individual single miRNA molecules may, alone, hold the key to tumour growth, proliferation, and survival in some cancers.[52]

In TGCT, miRNA-371 (miR-371) to -373 clusters are over-expressed by most histologic subtypes.[17] Other miRNA types commonly seen in TGCT include miR-199a-3p, -302a-d, -214, -223-3, -367-3p, -383, -449, and -514a-3p. Adding weight to the integral role that miRNA may have in oncogenesis, specific miRNA recognised ubiquitously in TGCT are also present in tissue samples of germ cell neoplasia *in situ*, suggesting that over-expression may be an early step in oncogenesis.[53] Furthermore, miRNA clusters, for example miR-372-373, interfere with normal p53 function by interacting with the large tumor suppressor kinase 2 (*LATS2*) gene, inhibiting associated cyclin-dependent kinase (CDK) function, and leading to activation of the oncogenic Wnt/ß-catenin signaling pathway and uncontrolled cellular growth in TGCT development.[49,53–56]

Other pathways to oncogenesis in TGCT include tumor suppressor miR-26a, and Let-7a, a miRNA precursor, which inhibit proliferation, migration, and invasive capacity in seminoma.[57] Importantly, these miRNA and miRNA precursors are downregulated in seminoma, leading to cell proliferation and growth.[58] miR-449 may also act as a tumour suppressor in TGCT, with its normal regulatory function embedded closely with CDK6 and the cell cycle. Concomitant retinoblastoma mutations in TGCT, however, lead to downregulation of miR-449, resulting in cell cycle progression and cell proliferation.[59] Furthermore, in EC, over-expression of miR-383 interferes with normal cell cycle regulation.[60–62] Given the increasingly clear role that miRNAs have in TGCT oncogenesis, these miRNAs and their oncogenic pathways will continue to attract attention and may become targets for drug development in the future.

However, there is some variability in tissue expression amongst TGCTs: teratoma demonstrates little-to-no expression of miR-371-373, miR-302, and miR-367, while seminomas are characterized by 'average' expression, and EC has extremely high expression. Importantly, these miRNAs are detectable in most histologic TGCT subtypes, regardless of primary site (gonadal *versus* extragonadal) and including ovarian primaries and across both pediatric and adult cases.[17] While miRNAs are present in normal tissue, the expression profile of malignant tissue samples demonstrates relative dysregulation compared to their cell of origin, and importantly, the specific miRNAs that typify TGCT are not detectable in the serum in other cancers or disease states, which differs from our existing serum biomarkers.[63]

Enveloped in an exosome, miRNAs resist breakdown by ribonucleases,[64] making them eminently measurable in serum, plasma, and other bodily fluids.[29,55,65,66] miRNAs are released into the bloodstream by malignant cells. This explains why higher concentrations of miRNA, specifically miR-371, are observed in testicular vein blood samples compared to the peripheral blood of men with TGCTs,[53] notwithstanding the fact that peripheral blood sampling is most practical in the clinical environment. Of the miRNAs detected in TGCT, miR-371a-3p, a member of the miR-371 cluster, has been most extensively studied, and appears to have the highest sensitivity and specificity of the biomarkers.[67,68] To-date, there has been no consensus as to the optimal way of measuring miRNA in peripheral blood, with practice varying significantly between the main research groups, including fundamentally which miRNA to routinely evaluate, use of serum or plasma analysis, assay choice, and cut-off values to define positivity.

When evaluating serum, peripheral blood is collected into serum separator tubes, and then centrifuged, aliquoted and frozen at −80°C while awaiting RNA extraction.[68] Instead, plasma is collected into cell-free deoxyribonucleic acid (DNA) tubes, containing an anticoagulant and cell preservative. Following collection, provided appropriate storage, samples may safely last several days, before being frozen as plasma aliquots and later undergoing RNA extraction. Plasma sampling offers additional advantages over serum, including the fact that circulating tumour DNA (ctDNA) may also be collected. This may be of increasing relevance as mutation profiles in TGCTs could have implications on diagnosis and treatment algorithms in the future.[69] In addition, isochromosome 12 (i12p), another evolving biomarker with high sensitivity and specificity for TGCT,[70–74] can be detected in ctDNA *via in situ* hybridization or next generation sequencing, which makes plasma an attractive medium. Importantly, hemolysis may vary between the

analysis of miRNA in serum *versus* plasma, leading to unreliable results; as a consequence, this needs to be considered in sample processing and the interpretation of results.[68]

The issue of cut-off values to define results is also an important consideration as miRNAs move towards the clinics. In the main studies, the relative quantity (RQ) of RNA defining positivity following quantitative polymerase chain reaction (PCR) has varied, with RQs of miR-371 between 2- and 5-times control, defined as positive. Dieckmann *et al.*[75] evaluated healthy blood donors and men with non-malignant testicular pathology and demonstrated equivalent, low RQs following PCR amplification in this population, whereas men with active TGCT had RQs of miR-371a-3p of >5. This cut-off was determined using a receive operator characteristic (ROC) analysis and Youden's index. A consensus approach to measurement, analysis, and interpretation must be reached before this becomes a routine component of patient care.

Of the known miRNA, miR-371a-3p has been most comprehensively investigated in TGCT. Following orchidectomy for CS1 TGCT, serum miR-371a-3p reliably falls to <5% of pre-operative levels within 24 h,[55,76] with normalization within 6 days.[53,55,56] Its reported half-life is just <7 h.[76] As a diagnostic tool, miR-371a-3p also successfully discriminates active TGCTs from controls with a sensitivity of 91.8% and a specificity of 96.1%.[75] The sensitivity and specificity of miR-371a-3p does vary in different clinical scenarios; however, these high levels are generally seen across the board. As such, early data has determined the superiority of the assay when compared to conventional serum tumour biomarkers in detecting active TGCT.[75,77,78] From a health economics standpoint, early data supports the use of routine miRNA as a surveillance strategy.[79]

Other studies have described the utility of miR-371 at diagnosis,[14,53,56,76,80–82] in surveillance,[14,78,80,83] during treatment,[81,83,84] and in refining prognosis of TGCTs.[85] Unfortunately, many of these early studies lacked sufficient clinical follow-up to determine the full utility of these test in a real-world setting, providing impetus to embed this technology into randomised controlled trials to demonstrate its clinical utility.[86–88]

Potential clinical application of miR-371

Given the shortfalls of our current biomarkers and early results with miR-371, there is clear appetite for miR-371 to enter routine practice. Pivotal work relating to the potential clinical utility of miR-371 has been conducted since 2011 and has been discussed extensively in the literature since. [57,67,68,71,89,90] The remainder of this paper will focus on the potential clinical applications of miRNA, specifically miR-371, and whether this tool is likely to become a routine component of care of men with TGCT.

Diagnosing TGCT

The most common presentation of TGCT is the development of a clinically apparent testicular mass. Thereafter, men go onto have further investigation, including testicular ultrasound, serum tumour biomarker evaluation, and CT to exclude metastatic disease.[1] Ultimately, the diagnosis is clinched on histopathological analysis of the orchidectomy specimen confirming TGCT.

As a diagnostic tool, conventional serum biomarkers are complementary to other tests, but lack adequate sensitivity and specificity.[25–27] In a variety of studies, miR-371 has been shown to have superior diagnostic value when compared to these biomarkers.[14,75,77,91] In a large study by Dieckmann *et al.*[75] serum miR-371a-3p offered a sensitivity, specificity, PPV, and NPV of 92%, 96%, 97%m and 83%, respectively, regardless of histologic subtype. Similarly high sensitivity and specificity was seen in seminoma ($n = 323$, 90% and 96% respectively) and NSGCT ($n = 199$, 95% and 96% respectively) making miR-371a-3p an attractive additional tool for the initial evaluation of men with TGCTs and offering higher diagnostic precision than older biomarkers. In addition, Dieckmann *et al.*[75] demonstrated a correlation between tumour size (pT1 *versus* pT2-4, $p < 0.001$), disease extent, (CS1 *versus* CS2-3 $p < 0.001$) and miR-371a-3p, such that the contemporary biomarker may also help accurately stage TGCTs and inform treatment recommendations. Given that up to 35% of men with CS2 TGCT may be down-staged to pathologic stage 1 at RPLND,[38,39,92] accurate staging is key, as it may significantly alter treatment recommendations, i.e., definitive chemotherapy, radiotherapy, or surgery *versus* active surveillance if deemed CS1.

A minority of patients diagnosed with GCTs will have an extragonadal primary, typically within the retroperitoneum or mediastinum, and rarely, primary intracranial GCTs. Despite anatomical variation, these tumours share similar embryologic origins with TGCTs,[93] and it is therefore reasonable to assume similar miRNA expression patterns to TGCTs. The accurate diagnosis of GCT is crucially important when evaluating men with extragonadal primaries, as, unlike most other non-GCT malignant histopathologies involving the retroperitoneal nodes, extragonadal GCTs may be offered curative-intent therapy. miRNAs may help to clarify this scenario when there is diagnostic uncertainty and avoid invasive biopsies.[29,94] In a small study, which evaluated serum miR-371, -372, -373, and -367 clusters in extracranial pediatric GCTs, including extragonadal and ovarian primaries, serum levels of these miRNA were significantly higher in patients with malignant GCTs when compared to patients with benign teratoma, other malignancies, and no known malignancy.[29] In particular, miR-371 offered an area under the curve (AUC) for diagnosis in a ROC analysis of 0.97 ($p = 0.002$), with other miRNA also offering a high level of diagnostic accuracy.

Intracranial GCTs pose a particularly unique clinical dilemma, given the significant risks associated with surgical biopsies required to refine the diagnosis. A small case series evaluating cerebrospinal fluid miR-371a-3p levels as a diagnostic tool for this group of patients showed that this tool may accurately diagnose GCT, sparing these patients from invasive biopsy.[95] These observations need to be further evaluated in a larger group of patients, ideally in a prospective manner, to inform clinical practice.

Recommending adjuvant treatment

One of the ongoing controversies in the care of men with CS1 TGCT is the role for adjuvant therapy, with a move away from routinely offering this treatment to all men with CS1 TGCT as was done historically. Following orchidectomy alone, most men with will be cured; however, a small proportion ultimately relapse and go onto require further treatment.[1] Adjuvant chemotherapy or radiotherapy may reduce the risk of relapse from ~20% to ~4% in seminoma, or ~30% to ~3% in NSGCT;[32,96] however, this comes with the perils of over-treating 70–80% of men who were destined to never relapse. While pathological features from the orchidectomy specimen may offer some insights into the risk of relapse,[34–36,97–106] existing serum tumour biomarkers cannot identify men who will most benefit from adjuvant treatment. There has been a wide array of research into the role of miR-371 in detecting MRD following orchidectomy.[14,56,75,107,108]

The largest study in this area was undertaken by Dieckmann *et al.*[75] They conducted a prospective, multicentric study including 256 men with CS1 seminoma and 112 men with CS1 NSGCT (74 mixed GCT, 29 EC, 3 YST, 6 teratoma), to evaluate the sensitivity and specificity of serum miR-371a-3p in this space. They observed a marked fall in measurable serum miR-371a-3p following orchidectomy ($p < 0.001$) in men with CS1 TGCT in 91.8% of patients. Unfortunately, this study lacked sufficient clinical follow-up to report upon the outcomes of the remaining ~8% of men who did not experience biochemical resolution of miR-371a-3p following orchidectomy. However, it is plausible that the ongoing elevation of the biomarker in these men may have represented MRD, making them more likely to relapse and also more likely to benefit from adjuvant therapy.

In contrast to these results, however, Lobo *et al.*[108] recently conducted a retrospective analysis of stored serum samples of 151 men with CS1 TGCT, including 101 with seminoma and 50 with NSGCT. In their cohort, which included relapses in 23% of men, post-orchidectomy serum miR-371a-3p levels or relative decline in serum levels post-orchidectomy did not accurately predict risk of relapse, suggesting that it may not be useful to guide decisions around adjuvant therapy. However, they did demonstrate the utility of miR-371a-3p in diagnosing relapse, with 94% of men experiencing elevation of the contemporary biomarker at relapse, compared to just 38% of men with elevation in AFP or hCG.

Clearly, miR-371 offers unique insights into the possible presence of active GCT, unlike existing biomarkers. However, prospective clinical trials are needed to further elucidate the potential role of the biomarker in refining recommendations for adjuvant therapy and detecting MRD.

Diagnosing relapse

While uncommon, the most frequent pattern of relapse in men originally diagnosed with CS1 TGCT is within retroperitoneal lymph nodes and

for those with CS2+ disease at diagnosis, including extragonadal primary TGCTs, the first site of recurrence is commonly other nodal chains or visceral sites.[1] Reassuringly, the majority of men who do relapse can be offered curative-intent treatment with chemotherapy, radiotherapy, or surgery, depending on clinical characteristics and prior treatment. However, it is important to accurately diagnose relapse to ensure all available treatment options can be considered, and also, to reduce the risk of over-treatment and its associated toxicities.

In a multitude of studies, miR-371 has been shown to outperform existing biomarkers at the time of relapse.[14,75,78,83,108] In one of the largest studies evaluating plasma miR-371a-3p in men with *confirmed* active TGCT,[14] 44/46 evaluable patients had detectable plasma miR-371a-3p at relapse. The test offered a sensitivity of 96%, specificity of 100%, and NPV of 98%. It should be noted that the two patients who did not have detectable plasma miR-371a-3p had levels measured below the defined cut-off for 'positivity'; the presence of active TGCT was later determined by histopathology ($n = 1$) or progressive conventional serum tumour biomarker elevation ($n = 1$), which speaks to the potential limitations of the new biomarker. Another study performed utilizing serum miR-371a-3p in this context also offered high sensitivity and specificity of 83% and 96%, respectively.[75] Furthermore, in a group of 28 men where the probability of recurrence was deemed to be 'high-risk' (90–100%) due to untreated, definitive regional, or distant TGCT, defined by conventional serum biomarkers or imaging, plasma miR-371a-3p diagnosed relapse with a sensitivity of 96%, specificity, and PPV of 100%; however, NPV in this analysis was lower at 66%.[14]

Serum and plasma miR-371a-3p appears to have reasonably high sensitivity, specificity, and predictive value in detecting relapse; however, larger, prospective trials are needed. Niche areas where miRNAs may add value includes the management of non-specific imaging changes, the evaluation of post-chemotherapy residual masses, and the identification of teratoma, where current tools leave significant gaps.

The management of non-specific radiological changes

Not infrequently, men will present with a surveillance-detected nodal mass with negative serum tumour biomarkers, leaving some doubt about the presence of active TGCT and resulting management approach. Depending on the extent of disease at the time of relapse, men may be committed to intensified surveillance, or alternatively one or more of chemotherapy, radiotherapy, or surgery.

Nappi *et al.*[14] conducted an analysis of men with a prior diagnosis of CS1 TGCT, who had suspicious imaging findings, defined as 10–30 mm of nodal enlargement ± minor serum tumour biomarker elevation. They estimated that these men had a 'moderate risk' (25–50%) of harbouring active TGCT based on their clinical characteristics. Evaluating 34 men, they showed that plasma miR-371a-3p offered a sensitivity of 91%, specificity and PPV of 100%, and NPV of 96%. There was no apparent difference in the precision of plasma miR-371a-3p between seminoma and NSGCT in this analysis. Importantly, plasma miR-371a-3p also outperformed existing tools in these men, and those with CS1b NSGCT or post-chemotherapy residual masses with minor AFP elevation.[14] In a ROC analysis for this whole population, the AUC for plasma miR-371a-3p was 0.89 [95% confidence interval (CI) 0.76–1.02] compared to 0.66 (95% CI 0.50–0.82) for CT, 0.65 (95% CI 0.48–0.84) for AFP, 0.61 (95% CI 0.43–0.81) for hCG, and 0.70 (95% CI 0.52–0.90) for LDH. While this data is compelling, the analysis may have been impacted by a short period of follow-up; hence, prospective evaluation with longer follow-up is required.

If miR-371 can accurately identify men with relapsed disease, particularly small-volume retroperitoneal nodal recurrences where existing tools leave considerable uncertainty, this will have a significant impact on the shape of care. While combination chemotherapy is an effective treatment for these men, there has been a move towards offering RPLND to men with small-volume retroperitoneal nodal recurrences of NSGCT[4] and increasingly, seminoma,[109,110] due to the risk of long-term morbidity associated with systemic treatment.[5,6] Therefore, early diagnosis is key to avail all possible treatment options to men who do relapse.

The management of post-chemotherapy residual mass

Chemotherapy often results in substantial radiological reduction in tumour bulk; however, not

uncommonly, a residual mass remains. With current tools, there continues to be uncertainty regarding the presence of active TGCT in residual masses. In seminoma, FDG-PET may help differentiate between residual tumour and fibrosis or necrosis in men with residual masses >3 cm.[40,41] In NSGCT, where FDG-PET is not helpful, these men will routinely be subjected to RPLND if they have persistent masses >1 cm, despite normalization of serum tumour biomarkers. In men undergoing this procedure, 40–45% will have residual teratoma and 10–15% will have viable, non-teratoma NSGCT. Despite this, the procedure still subjects up to 50% of men to major surgery from which they will derive no benefit.[111–113]

In the largest study assessing the role of serum miRNA in detecting active NSGCT in post-chemotherapy residual masses, 82 men undergoing pcRPLND for this indication in a single, tertiary institution were evaluated.[81] While conventional tumour biomarkers, specifically AFP and hCG, correlated well with disease stage and treatment response following chemotherapy, they were inadequate at detecting active disease in men who had a residual mass. In a group of 39 men with serial, serum miRNA samples pre- and post-chemotherapy, and following pcRPLND, miR-371a-3p, miR-373-3p, and miR-367-3p correlated well with residual active TGCT. Serum miR-371a-3p and -367-3p levels reliably fell during chemotherapy. In men whose pcRPLND specimen ultimately demonstrated fibrosis, necrosis, or teratoma only, no further reduction in miRNA levels was seen following surgery; however, men with active TGCT contained within the residual mass had a significant fall in measurable miRNA following pcRPLND. Notably, men with active TGCT in their pcRPLND specimen also had higher levels of miR-371, -373, and -367 post-chemotherapy than men who went onto have fibrosis, necrosis, or teratoma only. miR-371 had the highest discriminatory capability of the miRNA evaluated, with an AUC of the ROC 0.87 (95% CI 0.77–0.97, $p < 0.0001$). In a subgroup analysis of men with a residual mass up to 3 cm in largest axial diameter and without extra-retroperitoneal disease, miR-371a-3p accurately predicted men with active TGCT in their pcRPLND specimen with 100% sensitivity, 54% specificity, and 100% NPV ($p = 0.02$).[81]

These findings were corroborated in a smaller group by Nappi *et al*,[14] who demonstrated a 100% sensitivity, specificity, and NPV in men with post-chemotherapy residual masses ± serum tumor biomarker elevation.

With such high sensitivity and NPV for detection of active NSGCT in post-chemotherapy residual masses, miR-371 clearly has tremendous potential to transform treatment paradigms for these men in the future. Less is known about the role for miR-371 in seminoma in this specific clinical context.

Using miRNA to evaluate teratoma. Given the risk of malignant transformation of benign teratoma, a classically chemotherapy- and radiotherapy-resistant pathology associated with a poor prognosis,[1] it is important to distinguish between teratoma, other active TGCT, and post-treatment fibrosis and necrosis. While miR-371 may help distinguish between fibrosis/necrosis and active non-teratoma NSGCT, it cannot adequately distinguish between fibrosis/necrosis and teratoma.[81] Existing biomarkers are also unhelpful.[98] Given that miR-371 is not highly expressed by teratoma in serum or tissue, it is perhaps not surprising that miR-371 lacks sufficient sensitivity in the evaluation of this histologic subtype.[17] Alternatively, there is preliminary evidence that an alternative miRNA cluster, miR-375, is expressed by teratoma,[19] which may guide treatment decisions if clinically validated.

Nappi *et al.*[114] conducted an exploratory study evaluating plasma miR-375 alone or integrated with miR-371a-3p in men with teratoma. In their initial analysis, plasma miR-375 was significantly higher in men with active teratoma, when compared to either CS1 GCT post orchidectomy during active surveillance ($p = 0.01$), or advanced seminoma ($p = 0.04$); and plasma miR-371a-3p was undetectable in both active teratoma and CS1 GCT post-orchidectomy during active surveillance ($p < 0.0001$). The resulting sensitivity, specificity, PPV, and NPV of plasma miR-375 for identifying teratoma were 0.90 (95% CI 0.69–0.97), 0.81 (95% CI 0.66–0.90), 0.69 (95% CI 0.50–0.83), and 0.94 (95% CI 0.81–0.98), respectively. However, in a validation cohort, miR-375 performed less well. When integrated with miR-371a-3p, the AUC of the ROC for miR-375 was 0.95 (95% CI 0.90–0.99), which was higher than either plasma miRNA alone.

In contrast, Lafin *et al.*[115] were unable to demonstrate that miR-375, specifically miR-375-3p and -5p, was an effective biomarker in this space.

They prospectively evaluated 40 pre-operative/post-chemotherapy serum samples of men undergoing pcRPLND for residual masses >1 cm. Histopathological review of pcRPLND samples was undertaken, confirming 19 teratomas, two mixed GCTs comprising teratoma, and either YST or EC, and 21 cases of fibrosis, necrosis, or benign lymph nodes. In their analysis, pre-operative serum miR-375-3p did not accurately predict the presence of teratoma in pcRPLND specimen, offering 86% sensitivity, 32% specificity, 58% PPV, and 67% NPV. Serum miR-375-5p at the same time point also lacked sensitivity (55%) and specificity (67%) in 20 men evaluated. Similarly, two other groups also found miR-375 had insufficient diagnostic value for teratoma,[116,117] with poor performance of miR-375-3p in a ROC analysis.[116]

Given the clinical relevance of this question, further prospective trials evaluating the role of miRNA clusters will be integral to answer this question for patients and the clinicians who care for them.

Treatment monitoring during chemotherapy

In men with serum tumour biomarker elevation at the time of relapse, the time to AFP and hCG normalization during chemotherapy has been shown to have prognostic value.[118–120] In a study which evaluated 653 men with advanced NSGCT from a collection of eight prospective trials, hCG normalization by week 3 favored an improvement in 4-year progression-free (PFS, $p < 0.001$) and overall survival (OS, $p < 0.001$). Normalization of AFP by week 3 similarly yielded an improvement in OS ($p = 0.039$), but not 4-year PFS ($p = 0.054$).[119] As a result, there are ongoing studies relating to treatment intensification for men whose serum tumour biomarkers do not normalize by this time point.

In a similar fashion, a variety of studies have evaluated the natural history of miR-371 during chemotherapy, demonstrating a significant reduction in miR-371 levels during treatment, particularly after the first cycle of chemotherapy.[75,81,83] In an analysis of 70 men undergoing chemotherapy for CS2a and CS2b disease,[75] a significant fall in serum miR-371a-3p was seen following cycle 1, with a relatively smaller decline following cycle 2 and then plateau at normal levels. Clearly, serum miR-371a-3p is sufficiently sensitive to demonstrate treatment benefit; however, there is no correlative data which offers prognostic information in the same way that existing serum tumour biomarkers may in NSGCT. This too, is being evaluated in prospective trials.[88]

Importantly, miRNA may also aid clinical decision-making where there is persistence of conventional serum tumour biomarkers and concern about chemo-resistance during treatment. In a single case report that reported a persistent rise in AFP during chemotherapy, serum miR-371a-3p and miR-367-3p were undetectable, and elevation of the conventional serum tumour biomarker was later explained by concomitant hepatic injury,[121] providing clinicians further reassurance regarding the specificity of the contemporary biomarker in this context.

Choosing treatment modality in relapsed or advanced disease

Over the last five decades, there have been significant advances in the chemotherapy regimens, as well as surgical and radiotherapy techniques for men with advanced TGCT. In general, where there are multiple sites of disease identified on CT or solitary serum tumour biomarker elevation in NSGCT (usually S1), combination chemotherapy is the treatment of choice. However, where disease occurs in a single radiotherapy or surgical field, or serum tumour biomarkers are non-contributory, there remains some uncertainty around the 'best' treatment option, which may include any of the three modalities.[83] Presently, there is insufficient clinical data relating to the role that miRNA may have in this space; however, some studies have shed light on this issue.

Given that miR-371a-3p elevation has been correlated with tumour size and clinical stage,[75] it is possible that the biomarker may be utilized as a surrogate for disease extent. In the future, men with elevated miR-371a-3p without recurrence defined by existing serum tumour and imaging biomarkers may be recommended chemotherapy in the same way that men with CS1S NSGCT are currently treated. By the same rationale, chemotherapy may be preferred in men with disease traditionally defined as resectable in the event of significant miRNA elevation. These hypotheses need to be evaluated in prospective trials.

Plaza *et al.*[85] has shown that higher pre-chemotherapy serum miR-371a-3p, miR-373-3p, and miR-367-3p levels in men with advanced TGCT predicts a higher risk of relapse compared to men

Text box 1. Current Serum Tumour and Imaging Biomarkers

The detection of serum tumour biomarkers reflects the embryologic origins of TGCT
Serum AFP, hCG, and LDH have poor sensitivity, specificity, and predictive value to diagnose TGCT, particularly seminoma
Serum tumour biomarker evaluation is no longer routinely recommended as part of surveillance in some guidelines for men with seminoma
Modern imaging, including CT and MRI, are unable to detect active malignancy in small lymph nodes; nor are they able to accurately differentiate between necrosis, fibrosis, and active malignancy
There is an established role for FDG-PET in evaluating post-chemotherapy residual masses >3 cm in seminoma

Text box 2. MicroRNA

Small, non-coding ribonucleic acid molecules involved in post-transcriptional gene expression, interacting with messenger RNA, and may act as either an oncogene or tumour suppressor gene
Certain clusters of miRNAs typify TGCT, with miR-371-372 over-expressed by most histologic subtypes; others include miR-302a-d and miR-367-3p
Measurable in serum, plasma, and other bodily fluids; resist breakdown by ribonucleases
Significant variation in practice around sample collection methods, including the use of serum or plasma; with focus required to refine definitions of 'positive' and 'negative' miRNA results

who had lower levels before chemotherapy, despite normalization of these miRNA during treatment and complete radiological response. It is plausible that men with significant miRNA elevation may benefit from treatment intensification with consolidative treatment or alternative chemotherapy regimens, however the cut-off value to define 'high' levels needs to be refined. In contrast, those with lower levels may benefit from de-intensification of treatment. This observation was replicated in another study, whereby men with 'positive' pre-chemotherapy plasma miR-371a-3p in the setting of advanced TGCT experienced an inferior PFS and OS than men with 'negative' miR-371a-3p and correlated these results with IGCCCG risk groups.[80] Clearly, there is room for improvement in this space.

Conclusions

miRNAs, particularly miR-371a-3p, are promising new biomarkers in the care of men with TGCT. The evolution of science underpinning miRNA in this space has been rapid, with significant advances in the technology and knowledge base across the last 10 years. In multiple studies, miR-371a-3p has been shown to be superior to existing serum tumour and imaging biomarkers. It has robust characteristics, with an apparent role in diagnosis and surveillance of TGCT, as well as offering prognostic information following orchidectomy and chemotherapy. Early evidence suggests that miR-371a-3p may fill many of the important gaps left by current diagnostic and surveillance tools. In teratoma, miR-375 may offer similarly helpful information; however, results have been conflicting to-date.

In addition to refining the above clinical scenarios, there is space to embed miRNA evaluation into routine surveillance, reducing the frequency of CT, as well as a possible place in treatment given the integral role miRNA play in the oncogenesis of TGCTs.[49,53–62,122–125] Prospective trials evaluating the role of miRNA in CS1 TGCT, such as SWOG1823 and AGCT1531, are currently ongoing, however additional trials across the TGCT disease spectrum are required to

Text box 3. Potential clinical applications of miRNA

Diagnosis: Serum miR-371a-3p offers sensitivity, specificity, PPV, and NPV of 91.8%, 96.1%, 97.2%, and 82.7%, respectively, in diagnosing TGCT regardless of histologic subtype and is superior to existing serum tumour biomarkers
Relapse: miR-371a-3p accurately detected relapsed disease in men with a confirmed relapse, with a sensitivity, specificity, and NPV of 96%, 100%, and 98% respectively
Adjuvant treatment: The role of miRNA in guiding use of adjuvant therapy is less clear. Some datasets have demonstrated that a minority of men with CS1 TGCT will not experience biochemical resolution of miR-371a-3p following orchidectomy, suggesting that they may harbor residual microscopic disease; however, Lobo *et al.* since demonstrated that post-orchidectomy miR-371a-3p levels did not accurately predict risk of relapse
Management of non-specific radiological changes: There appears to be a possible role for miRNA to finesse the management of non-specific radiological changes, with plasma miR-371a-3p accurately diagnosing active malignancy in men deemed at 'moderate risk' of harbouring TGCT with sensitivity, specificity, PPV, and NPV >90%
Post-chemotherapy residual masses: In men with post-chemotherapy residual masses >3 cm, miR-371a-3p accurately detected active TGCT with sensitivity and NPV of 100%; however, specificity was lower at just 54%
Teratoma: miR-371 seems less useful in the evaluation of teratoma, however a composite of miR-371a-3p and miR-375 may be helpful in diagnosing this condition
Treatment decisions and prognosis: miR-371a-3p falls reliably during chemotherapy, particularly after C1, however there is no correlative data relating the relationship between this observation and survival outcomes. There is also limited data pertaining to the way in which miRNA may guide treatment decisions, particularly around treatment intensification or consolidation and de-intensification. Early data has shown that higher levels of specific miRNA prior to chemotherapy may confer a worse prognosis than those with lower, albeit detectable levels at the same timepoint.

validate existing datasets and further elucidate the role of this technology in the clinics.[86–88] If early data are able to be replicated in these prospective trials, serum miR-371a-3p has the ability to alter routine surveillance for many men, offering an inexpensive, safe, and precise tool that will personalize care and prevent over-treatment in a group of men with an otherwise excellent prognosis.

Conflict of interest statement

BT reports grants and personal fees from Amgen, grants and personal fees from Astra Zeneca, grants from Astellas, grants and personal fees from BMS, grants and personal fees from Janssen, grants and personal fees from Pfizer, grants and personal fees from MSD, grants and personal fees from Ipsen, personal fees from IQVIA, personal fees from Sanofi, personal fees from Tolmar, personal fees from Novartis, grants and personal fees from Bayer, and personal fees from Roche, outside the submitted work.

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