

## The present and future of serum diagnostic tests for testicular germ cell tumours

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**Abstract** | Testicular germ cell tumours (GCTs) are the most common malignancy occurring in young adult men and the incidence of these tumours is increasing. Current research priorities in this field include improving overall survival for patients classified as being 'poor-risk' and reducing late effects of treatment for patients classified as 'good-risk'. Testicular GCTs are broadly classified into seminomas and nonseminomatous GCTs (NSGCTs). The conventional serum protein tumour markers  $\alpha$ -fetoprotein (AFP), human chorionic gonadotrophin (hCG) and lactate dehydrogenase (LDH) show some utility in the management of testicular malignant GCT. However, AFP and hCG display limited sensitivity and specificity, being indicative of yolk sac tumour (AFP) and choriocarcinoma or syncytiotrophoblast (hCG) subtypes. Furthermore, LDH is a very nonspecific biomarker. Consequently, seminomas and NSGCTs comprising a pure embryonal carcinoma subtype are generally negative for these conventional markers. As a result, novel universal biomarkers for testicular malignant GCTs are required. MicroRNAs are short, non-protein-coding RNAs that show much general promise as biomarkers. MicroRNAs from two 'clusters', miR-371–373 and miR-302–367, are overexpressed in all malignant GCTs, regardless of age (adult or paediatric), site (gonadal or extragonadal) and subtype (seminomas, yolk sac tumours or embryonal carcinomas). A panel of four circulating microRNAs from these two clusters (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) is highly sensitive and specific for the diagnosis of malignant GCT, including seminoma and embryonal carcinoma. In the future, circulating microRNAs might be useful in diagnosis, disease monitoring and prognostication of malignant testicular GCTs, which might also reduce reliance on serial CT scanning. For translation into clinical practice, important practical considerations now need addressing.

Testicular germ cell tumours (GCTs) are the most common malignancy occurring in young adult men and the incidence of these tumours is increasing over time<sup>1</sup>. Current research aims include increasing overall survival for patients deemed to have poor-risk disease and improving quality of survival for patients with low-stage and/or good-risk disease. Testicular GCTs are broadly classified into seminoma (a malignant subtype) and nonseminomatous GCTs (NSGCTs). The latter group include the malignant subtypes yolk sac tumour (YST), embryonal carcinoma and choriocarcinoma (CHC), as well as teratoma. Teratomas in pre-pubertal patients are generally considered benign, but postpubertal cases might display malignant potential<sup>2</sup>. GCTs that contain more than one subtype are known as mixed malignant GCTs; those with both seminomatous and nonseminomatous components are classified as combined NSGCTs<sup>3</sup>.

The histological classification and clinical staging of testicular GCTs guide subsequent treatment decisions. Stage I testicular GCTs (that is, those confined to the testes) are initially treated with orchidectomy, although management after orchidectomy varies and can be dictated by clinician and/or national preference<sup>4</sup> and/or known risk factors<sup>5–10</sup>. Chemotherapy is routinely administered for patients with stage II–IV testicular GCTs according to International Germ Cell Consensus Classification (IGCCC) prognostic criteria<sup>11</sup>.

The conventional serum protein biomarkers  $\alpha$ -fetoprotein (AFP) and human chorionic gonadotropin (hCG) are used to assist in the diagnosis and follow-up assessment of GCTs, but their use is generally restricted to tumours containing the relevant malignant subtypes YST and CHC, respectively<sup>12</sup> (TABLE 1). Consequently, only ~60% of patients with testicular malignant GCTs test positive for these markers at diagnosis<sup>13</sup>. Serum

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## Key points

- The conventional serum protein tumour markers  $\alpha$ -fetoprotein (AFP), human chorionic gonadotrophin (hCG) and lactate dehydrogenase (LDH) show utility in the management of testicular malignant germ cell tumours (GCTs)
- AFP and hCG show limited sensitivity and specificity for all malignant GCTs, being representative of yolk sac tumour and choriocarcinoma or syncytiotrophoblast subtypes, respectively; LDH is a very nonspecific biomarker
- Novel universal biomarkers for testicular malignant GCTs are required, particularly for seminoma and embryonal carcinoma subtypes that are typically negative for conventional markers
- MicroRNAs are short, non-protein-coding RNAs that show much promise as universal markers in malignant GCTs
- Individual microRNAs from two microRNA clusters, miR-371–373 and miR-302–367, are overexpressed in all malignant GCTs, regardless of patient age, tumour site and subtype
- A panel of four circulating microRNAs from these two clusters (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) is highly sensitive and specific for the diagnosis of malignant GCT, including seminoma and embryonal carcinoma subtypes
- Practical considerations need to be addressed to standardize the translation of circulating microRNA studies from a research tool to a routine clinical test

lactate dehydrogenase (LDH) levels are also used at diagnosis to assist with treatment decisions in patients with metastatic NSGCTs but this marker lacks sufficient specificity to be useful in diagnosis or monitoring<sup>3</sup> (TABLE 2). Current priorities in GCT research include the identification of universal circulating biomarkers of malignant disease, which might assist timely diagnosis and reduce the need for repeated cross-sectional CT imaging in follow-up monitoring, along with the associated radiation burden and risk of second cancer development<sup>14</sup>. One 2015 study, for example, showed that only 3% of patients relapsing following a diagnosis of stage I seminoma were identified using conventional serum tumour markers (CTMs), but that figure was 87% for CT imaging<sup>9</sup>. Other important issues that remain to be fully addressed include the most appropriate management of stage I testicular GCTs, particularly NSGCTs (active surveillance versus adjuvant chemotherapy), and the early identification of patients who are destined to have poor clinical outcomes within the overall IGCCC poor-risk disease cohort<sup>11</sup>. These issues are important, as many concerns exist regarding the substantial long-term sequelae of conventional chemotherapy treatment<sup>15–21</sup>, which include pulmonary fibrosis (with bleomycin), nephrotoxicity and/or ototoxicity (with cisplatin) and neuropathy (with cisplatin and taxol agents)<sup>20</sup>. Particular concerns also exist about the increased incidence of cardiovascular disease<sup>17,18</sup> and second cancer<sup>15,21</sup> associated

with conventional chemotherapy. Minimizing these risks through improved patient stratification is important; novel circulating biomarkers might assist with this challenge.

This Review provides a comprehensive overview of the current clinical value of serum diagnostic testing for testicular GCTs and discusses how the issues highlighted above could be addressed. It covers the utility of the traditional markers AFP, hCG and LDH in the diagnostic and prognostic management of this disease. In addition, it provides insights into the rapidly developing area of quantification of specific circulating short non-protein-coding RNAs termed microRNAs in GCTs, and discusses potential clinical applications of these markers. Practical aspects that must be considered before such microRNA testing can be incorporated into future routine clinical practice are also highlighted. The ultimate aims of developing circulating microRNA testing alongside conventional tumour marker testing are threefold. Firstly, to reduce or omit the use of CT scans in follow-up monitoring for patients whose disease is usually negative for conventional markers (predominantly seminoma subtype) at diagnosis. Secondly, to improve quality of patient survival through the rational reduction and/or omission of chemotherapy, along with its concomitant late effects, for patients with stage I disease, who are predicted to have excellent outcomes. Thirdly, to improve overall survival for those patients with poor-risk disease, through identification of a robust prognostic signature.

## AFP, hCG and LDH in testicular GCT

The serum protein markers AFP, hCG and LDH have all shown utility for the management of testicular GCTs and are currently widely used in clinical practice<sup>3</sup>.

## Physiology, background and limitations

AFP is a glycoprotein comprising a carbohydrate moiety attached to an ~600 amino acid  $\alpha$ -globulin molecule<sup>22</sup>. In fetal development, AFP is produced by the yolk sac and then predominantly the liver, with a minor contribution from the gastrointestinal tract in later gestation<sup>23</sup>. Postnatally, AFP is gradually replaced as the major circulating protein by albumin, which is exclusively derived from the liver<sup>22</sup>. In clinical practice, AFP levels are determined using a quantitative automated chemiluminescent sandwich enzyme immunoassay<sup>24</sup>, and measured against known AFP standards according to WHO International Standard 72/225 (REF 22). The half-life of AFP in the bloodstream is usually ~5–7 days<sup>3,22,25</sup> and levels are measured as ng/ml or kU/l, with normal values being <12 ng/ml (<10 kU/l; conversion 1 ng/ml = 0.84 kU/l)<sup>22</sup>. The features of AFP production during development are pertinent to the elevations observed in the serum of some patients with GCTs. Raised serum AFP levels are predominantly observed in GCTs containing YST components, although moderately elevated values can also occur in some embryonal carcinoma and immature teratoma lesions<sup>22</sup> (TABLE 1). In pure immature teratoma, serum AFP can be raised owing to the presence of immature liver and/or gastrointestinal tissues within the lesion<sup>22</sup>.

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AFP values are also typically elevated in patients with hepatocellular carcinoma<sup>3</sup>, in patients with other uncomplicated chronic liver disease<sup>26</sup>, those who have undergone gastrointestinal tract and/or hepatic surgery and in patients with certain conditions such as hereditary ataxia telangiectasia<sup>27</sup>. Infrequent reports of elevated AFP levels have also been described in gastrointestinal malignancies<sup>3,22</sup> (including gastric, pancreatic and colon carcinoma), which have the potential for AFP production to be de-repressed, as well as in carcinoma of the lung<sup>3</sup>. Interestingly, patients with GCTs have been reported to have false elevations of AFP, secondary to liver damage caused by chemotherapy, anaesthetic agents or other drugs<sup>28</sup>. Without awareness of such possibilities, patient management could potentially be altered based on such results. Thus, interpretation of AFP levels should only be undertaken with full knowledge of the patient's history and examination findings, including age, symptoms, past medical and family history and any relevant drug history<sup>22</sup>. In patients in whom potential liver disease is a concern, a full serum liver function test should be performed alongside AFP estimation<sup>22</sup>.

hCG measurements can be of the free  $\beta$ -subunit alone (monomer), the  $\alpha$ - $\beta$  dimer ('intact hCG'), or both ('total hCG', often referred to as ' $\beta$ -hCG')<sup>3</sup>. hCG is measured with a double antibody immunometric assay<sup>3</sup>, typically using lanthanide fluorescence. Normal hCG levels are <2 IU/l, although levels can be measured in ng/ml (where 5 IU/l = 1 ng/ml). The half-life of hCG is much shorter than AFP, usually ~12–36 h (REFS 3,29), and, therefore, levels should decay much more rapidly. hCG is produced primarily by CHC components, but low levels of hCG production can be observed in patients with pure seminoma containing syncytiotrophoblast (placental-like) cells (TABLE 1). However, even patients with advanced-stage pure seminoma only show raised hCG values in 15–20% of cases<sup>3</sup>. hCG, like AFP, also shows limitations in sensitivity and specificity. Levels can also be elevated in other malignancies, including neuroendocrine, bladder, renal and lung carcinomas<sup>3</sup>. The treating clinician needs to be aware of the limited sensitivity of both hCG and AFP to ensure appropriate patient management.

LDH is an enzyme that catalyses the conversion of lactate to pyruvate<sup>3</sup> and is ubiquitously expressed in cells of the body. Elevations in LDH are therefore very non-specific, but can indicate increased cell turnover, such as in malignancy, including testicular GCTs. The half-life of LDH in the bloodstream is not well reported as serial measurements are not usually taken, but is measured in days rather than hours. An enzymatic assay is used to measure circulating levels, but assays used between laboratories are very variable. LDH is often routinely measured at the time of cancer diagnosis, providing a surrogate measure for tumour bulk, and high levels are associated with an increased risk of tumour lysis syndrome, which can occur in patients with malignant GCTs<sup>30</sup>. Increased levels are also observed in lymphoma, lung cancer and bone tumours such as osteosarcoma and Ewing sarcoma, as well as in any nonmalignant condition

Table 1 | Serum AFP and hCG levels in GCTs<sup>22</sup>

GCT histological subtype	AFP	hCG
Yolk sac tumour	++	-
Seminoma	-	±
Embryonal carcinoma	±	±
Choriocarcinoma	-	++
Teratoma	±	-

AFP,  $\alpha$ -fetoprotein; GCT, germ cell tumour; hCG, human chorionic gonadotrophin. ++, strongly positive levels; ±, levels may be negative or moderately positive; -, negative levels.

that results in cellular lysis or injury, such as myocardial infarction or liver or muscle damage or disease<sup>3</sup>. These variations limit the use of LDH as a biomarker.

### Diagnosis and early follow-up monitoring

**Preorchidectomy.** Measurement of CTM levels before orchidectomy is advised in all cases, as a substantially elevated preoperative AFP level precludes a diagnosis of pure seminoma regardless of histological findings, and, in addition, this approach assists the interpretation of postorchidectomy estimations<sup>3</sup>. Furthermore, in rare cases, such as testicular GCTs with considerable tumour burden where urgent treatment is required, or extragonadal cases (such as retroperitoneal or mediastinal primary), in which the risks of surgical biopsy are deemed excessive, a substantially raised AFP and/or hCG might be sufficient for diagnosis<sup>3</sup>.

**Stage I disease.** Stage I testicular GCT is defined by the absence of demonstrable disease elsewhere on radiological imaging (that is, the disease is confined to the testis) and either normal levels of AFP and hCG, or if these levels were raised preoperatively, normalization after surgery. However, a proportion of patients with stage I malignant GCTs will develop clinical relapse during follow-up monitoring, and, therefore, for some patients, postorchidectomy risk stratification is performed.

Patients with NSGCTs are one such group for whom risk stratification is usually undertaken; those at low risk of subsequent relapse are monitored with surveillance and patients with high-risk disease might be offered adjuvant therapy instead. Effective adjuvant treatments that substantially reduce relapse risk in this setting include single-dose carboplatin or radiotherapy for stage I seminoma and one course of bleomycin, etoposide and cisplatin (BEP) chemotherapy for stage I NSGCT<sup>4</sup>. The most important and consistently reported risk factor for recurrence in patients with stage I NSGCT has been the presence of lymphovascular invasion (LVI)<sup>5–7,9,10</sup>. Patients without LVI have a predicted 15–20% risk of recurrence; in those with LVI, this risk is 40–50%. The percentage embryonal carcinoma in the resection specimen (%EC)<sup>6,7,10</sup> and rete testis invasion (RTI)<sup>6</sup> have also been reported to affect recurrence rates. In one large study of >1,000 patients, the overall relapse risk at 5 years after orchidectomy was 12% with none of these risk factors, compared with 50% with all three of these risk factors (31% for the cohort as a whole)<sup>6</sup>. A

Table 2 | IGCCC classification of metastatic GCTs<sup>11</sup>

Clinical variable	Seminoma	NSGCT
<b>Good-prognosis group</b>		
Primary site	Any	Testis or retroperitoneal
Metastases	No NPVM	No NPVM
AFP (ng/ml)	Normal	<1,000
hCG (IU/L)	Any	<5,000
LDH (×ULN)	Any	<1.5 × ULN
<b>Intermediate-prognosis group</b>		
Primary site	Any	Testis or retroperitoneal
Metastases	NPVM	No NPVM
AFP (ng/ml)	Normal	≥1,000 and ≤10,000*
hCG (IU/L)	Any	≥5,000 and ≤50,000*
LDH (×ULN)	Any	≥1.5 and ≤10 × ULN*
<b>Poor-prognosis group</b>		
Primary site	None <sup>†</sup>	Mediastinal <sup>§</sup> ; or testis or retroperitoneal with any of the risk factors below
Metastases	None <sup>†</sup>	NPVM <sup>§</sup>
AFP (ng/ml)	None <sup>†</sup>	>10,000 <sup>§</sup>
hCG (IU/L)	None <sup>†</sup>	>50,000 <sup>§</sup>
LDH (×ULN)	None <sup>†</sup>	>10 × ULN <sup>§</sup>

AFP, α-fetoprotein; GCT, germ cell tumour; hCG, human chorionic gonadotrophin. IGCCC, International Germ Cell Consensus Classification; LDH, lactate dehydrogenase; NSGCT, non-seminomatous GCT; NPVM, non-pulmonary visceral metastases; ULN, upper limit of normal. \*Any one of these risk factors will classify a patient with NSGCT as having an intermediate prognosis; <sup>†</sup>No patients with seminoma classified as having poor prognosis; <sup>§</sup>Any one of these risk-factors will classify a patient with NSGCT as having poor prognosis.

further study demonstrated that immunohistochemical expression of CXCL12 (also known as stromal cell-derived factor 1 (SDF1)), in stage I NSGCTs was associated with reduced relapse risk<sup>31</sup>, a finding that has now been independently validated and shown to offer additional risk stratification benefit over LVI alone<sup>7</sup>. The authors propose a new prognostic index using CXCL12 expression in addition to LVI and %EC, with three proposed risk groups (low, with a ~10% relapse risk; moderate, with a ~30–40% relapse risk; and high, with a ~70% relapse risk)<sup>7</sup>, although this index remains to be prospectively validated in a trial setting.

Risk stratification of patients with seminoma has been more controversial than for those with NSGCTs. A review of 638 patients identified tumour size, RTI and LVI as being risk factors for relapse on univariate analysis with tumour size and RTI remaining as risk factors on multivariate analysis<sup>32</sup>. A risk of relapse of 12%, 15% and 31% was identified if zero, one or two risk factors were present, respectively<sup>32</sup>. This prognostic index has never been fully validated and indeed, in an analysis of data from 685 patients with stage I seminoma, only tumour size was a significant risk factor for relapse<sup>33</sup>. Risk of relapse increased from 9% for a 1 cm diameter

tumour to 26% for an 8 cm diameter tumour<sup>33</sup>. Accurate prediction of relapse risk in this patient group remains elusive.

Novel circulating biomarkers could offer considerable additional value and refinement to such stratification systems in patients with stage I disease. For instance, a marker that shows increased sensitivity for residual microscopic disease might substantially reduce the overall need for adjuvant therapy.

**Metastatic disease.** Chemotherapy is routinely administered to patients with stage II–IV testicular GCTs according to IGCCC prognostic criteria<sup>11</sup> (TABLE 2). Current standard-of-care treatment is three courses of BEP chemotherapy or four cycles of etoposide and cisplatin (EP) for good-risk disease (60% of patients; 91% 5-year overall survival) and four BEP cycles for intermediate-risk disease (26% of patients; 79% 5-year overall survival) and high-risk disease (14% of patients; 48% 5-year overall survival) patients<sup>11</sup>. By IGCCC definition, patients with pure seminoma have normal levels of AFP. However, in the pooled multivariate analysis, LDH levels of more than double the institutional upper limit of normal (ULN) in the metastatic seminoma cohort were predictive of shorter progression-free survival (PFS) and overall survival, but the IGCCC classification successfully stratified patients into intermediate-risk (10% of patients) and good-risk groups (90% of patients) solely by the presence or absence of nonpulmonary visceral metastases, respectively<sup>11</sup>. Thus, in patients with metastatic seminoma, the level of LDH and hCG does not affect their prognosis or treatment<sup>11</sup>. However, for those with NSGCT AFP, hCG and LDH all have a role in determining IGCCC risk group<sup>11</sup> (TABLE 2). The good-risk NSGCT group comprises patients with diagnostic serum levels of AFP <1,000 ng/ml, hCG <5,000 IU/l and LDH <1.5 times the ULN<sup>11</sup>. The intermediate-group values are AFP >1,000 but ≤10,000 ng/ml, hCG >5,000 but ≤50,000 IU/l or LDH >1.5 times but ≤10 times the ULN. Finally, the poor-risk group comprises those patients with any CTM values above the upper limit of the intermediate-risk group range, plus patients with a mediastinal primary and/or nonpulmonary visceral metastases<sup>11</sup> (TABLE 2). For patients who are AFP-positive and/or hCG-positive at diagnosis, measurement of AFP and/or hCG levels is recommended at the start of each chemotherapy cycle, in order to monitor the effectiveness of treatment. AFP and hCG should be measured at the end of treatment in all patients, as ~50% of patients who relapse after metastatic seminoma have an elevated hCG<sup>34,35</sup> and some relapses manifest as NSGCTs<sup>3</sup>. End-of-treatment AFP and hCG levels generally do not assist in the identification of patients who could avoid retroperitoneal lymph node dissection or resection of residual tumour masses — surgery should go ahead unless CTMs are increasing, in which case a change to alternative chemotherapy is likely to be indicated<sup>3</sup>.

Measurement of serum AFP levels and/or decline might in the future help to further segregate IGCCC cohorts. One study of patients with intermediate-risk disease used AFP levels to further separate this population



into two groups, with the small percentage of patients with values >6,200 ng/ml having an overall survival similar to the IGCCC poor-risk group, whereas those with values less than this threshold had outcomes that approximated to those in the IGCCC good-risk group<sup>36</sup>. This suggestion remains to be validated prospectively. Clinical outcomes remain suboptimal for the ~15% of patients with IGCCC poor-risk disease<sup>11</sup>. A previous attempt to further segregate the poor-risk group using the statistical technique of tree modelling was unsuccessful<sup>37</sup>. Furthermore, despite more than three decades of randomized controlled trials of treatment escalation (such as the addition of multiagent chemotherapy<sup>38</sup>, taxols<sup>39</sup>, or high-dose therapy<sup>40</sup>) very few studies have demonstrated any clear additional survival benefit for such approaches. However, the early rate of decline of serum AFP and hCG levels has been shown to be of additional prognostic value in patients with poor-risk disease<sup>41</sup>. Tumour markers were measured just before initiation of chemotherapy and again 3 weeks later (between days 18–21 inclusive), and rate of decline was expressed as predicted time to normalization (TTN)<sup>41</sup>. The 4-year PFS rates were 44% for the poor-risk group as a whole, but 64% and 38% in patients who had a favourable and an unfavourable TTN, respectively, independent of initial marker levels, disease site and the presence of nonpulmonary visceral metastases<sup>41</sup>. Thus, early TTN was identified as an independent prognostic factor in patients with poor-risk disease<sup>41</sup>. The prospective multicentre study GETUG13, which reported results in 2014, based treatment intensification on this early TTN and showed improved 3-year PFS in a randomized comparison of a dose-dense schedule versus standard BEP in patients with unfavourable marker decline (59% versus 48%, respectively)<sup>42</sup>. The 3-year PFS in the small number of patients remaining in the favourable marker decline group was 70%<sup>42</sup>. Thus, the GETUG13 study has been suggested to be a practice-changing step in the management of these NSGCTs<sup>43,44</sup>. However, some concerns exist with this approach. Firstly, the timing of the reassessment of AFP level is strict and the calculation of the rate of decline in clinical practice can be challenging. For example, an AFP 'surge' or 'flare' on day 8 (compared with levels on day 1) following initiation of chemotherapy in patients with NSGCTs has been shown to be of adverse prognostic significance<sup>45</sup>, and such a phenomenon does not, therefore, simply represent a marker of tumour lysis<sup>45</sup>. As a result, interpretation of AFP levels at the beginning of the second course of chemotherapy in patients displaying an AFP flare should be undertaken with appropriate caution<sup>3</sup>. The effect of early AFP flares in the GETUG13 results is not clear. Secondly, the majority of patients (80%) on the GETUG13 trial displayed unfavourable TTN of tumour markers<sup>41</sup>, and if these results of dose-dense schedules are adopted and replicated prospectively in other studies, most patients will receive treatment intensification with the potential for only modest benefit in PFS. Thirdly, even the identified 'good-risk' group within this poor-risk study had only a 70% 3-year PFS, suggesting that, even in this cohort, outcomes are suboptimal. Finally, some patients in the poor-risk group were successfully treated

by BEP alone, suggesting that improved prognostication would be valuable.

AFP and/or hCG values that show an increase during therapy (aside from an initial surge or flare), usually indicate (YST-containing and/or CHC-containing) resistant or refractory disease and warrant a change in management<sup>3</sup>. Novel circulating biomarkers to improve identification of all truly cisplatin-refractory cases upfront, either at the time of diagnosis or during early treatment (in both IGCCC intermediate-risk and poor-risk cohorts) would, therefore, be welcomed<sup>20</sup>.

### Detection of testicular GCT recurrence

In patients with stage I testicular GCT disease, active surveillance after orchidectomy has been shown to lead to excellent outcomes (5-year disease-specific survival of 99.7%)<sup>9</sup>. Close monitoring of such patients not receiving adjuvant chemotherapy is needed, as a proportion harbour occult metastases, leading to subsequent relapse<sup>7</sup>. Approximately 20–30% of patients with NSGCT and 10–20% of those with seminoma, respectively, relapse in this way<sup>6,7,9</sup>. The vast majority (92%) of NSGCT relapses have occurred by 2 years of follow-up duration<sup>9</sup>. Such relapse patterns and frequency guide the intervals for CTM estimation during follow-up monitoring. The American Society of Clinical Oncology Clinical Practice Guideline recommends that follow-up assessment includes measurement of AFP and hCG levels in patients with stage I, chemotherapy-naïve NSGCTs<sup>3</sup>. Markers are most commonly measured monthly during the first year of follow-up monitoring and every 2 months in the second year, with frequency reduced further thereafter<sup>3</sup>. Following a diagnosis of stage I pure seminoma, of those patients who will ultimately experience a relapse, 75% will have done so by 2 years and 92% by 3 years<sup>9</sup>. However, owing to the lack of sensitive markers, evidence for the clinical benefit of CTM estimation during follow-up visits is lacking in this population, and, therefore, not recommended<sup>3</sup>. For example, a 2015 study showed that only 3% of stage I seminoma recurrences were detected by CTM evaluation and, even for stage I NSGCT cohorts, more than half of all relapses were negative by CTM estimation<sup>9</sup>. In the latter groups, CTM evaluation was only positive at relapse in 60% and 41% of patients with stage I NSGCT with or without LVI, respectively<sup>9</sup>, explaining the reliance on 3D imaging in follow-up assessment and highlighting the clinical need for markers of increased sensitivity and specificity for malignant GCT disease.

For patients with metastatic NSGCT after chemotherapy, follow-up measurement of both AFP and hCG is recommended, with the same interval and duration of follow-up analysis as for stage I disease. For patients with advanced stage seminomas, 15–20% of whom have elevated hCG at diagnosis, follow-up measurement of CTMs is also recommended<sup>3</sup>. Such follow-up monitoring is recommended as rising CTM levels can represent the earliest sign of malignant GCT recurrence or relapse, before clinical or radiological evidence is present<sup>46</sup>, although most clinicians would wait to see evidence of disease on imaging findings before instigating

appropriate management changes. Owing to the lack of direct evidence for their clinical benefit (for example, improved overall survival), recommended intervals for CTM estimation following treatment of advanced seminoma are generally less frequent than for NSGCT, typically every 2 months during the first year of follow-up monitoring, every 4 months during the second year, and less frequently thereafter<sup>3</sup>. In addition, no clear evidence indicates that LDH measurement is useful for the detection of recurrence during follow-up monitoring of patients with testicular GCT (including those with low-stage or advanced-stage, NSGCT or seminoma), owing to its lack of specificity<sup>47,48</sup>, and, therefore, such evaluation is not advised<sup>3</sup>.

### Other potential markers and investigations

As AFP, hCG and LDH are not universal markers of malignant GCTs, researchers have attempted to identify other potential candidate blood-based biomarkers and investigations for GCTs.

### Circulating *XIST* transcripts

Demethylated promoter regions of the long non-coding RNA X inactive specific transcript (*XIST*) gene at Xq13.2 have been reported in the plasma of men with testicular GCTs<sup>49</sup>. However, the 2004 study showing this finding was small, it only demonstrated an overall sensitivity of 64% for malignant testicular GCTs<sup>49</sup> and the finding has not been confirmed by other reports. Thus, *XIST* promoter demethylation currently lacks the sensitivity and specificity required for a clinical test. Consequently, an urgent need remains to identify highly sensitive and specific serum markers for all malignant testicular GCTs, particularly for the detection of seminoma and embryonal carcinoma subtypes, which are predominantly marker-negative by CTM estimation.

### Circulating mRNA transcripts

No reports have described circulating protein-coding mRNA transcripts as potential biomarkers in any malignant GCTs. Translation of any such candidate mRNA biomarker into the clinic would also be difficult, as mRNAs display relative instability at room temperature and in samples stored suboptimally<sup>50,51</sup>.

### PET scans

<sup>18</sup>F-fluorodeoxyglucose (FDG)–PET scans show better sensitivity and specificity for detecting active disease in seminoma masses after chemotherapy than does anatomical CT imaging, and consequently has become the standard of care<sup>52,53</sup>. FDG–PET might also have a role in detecting sites of relapse in patients with rising CTM levels after chemotherapy<sup>54</sup>. However, despite encouraging pilot data, FDG–PET was insufficiently sensitive in patients with stage I high-risk LVI-positive NSGCT to identify a substantial proportion of patients who subsequently relapsed<sup>55</sup>. Likewise, this imaging modality has not been useful in predicting the histology of residual masses in patients with NSGCT treated with surgery. A full description of the role of PET scans in testicular GCT is outside the scope of this Review.

### MicroRNAs in diagnosis and monitoring

The discovery of biological abnormalities that are common to all malignant GCTs is clinically relevant. Such abnormalities are likely to be critically important in disease pathogenesis, offer potential as biomarkers and might assist the identification of novel therapeutic targets in this disease<sup>20</sup>. MicroRNAs are short, non-protein-coding RNAs that regulate the expression of protein-coding genes. MicroRNAs are dysregulated in cancer, but expression profiles retain the characteristics of the cell of origin<sup>20</sup>. The first report of microRNA expression in malignant GCTs showed that the miR-371–373 microRNA cluster (at chromosomal location 19q13) was highly expressed in adult testicular disease, and might function as a potential oncogene through inhibition of *LATS2* (REF. 56). Subsequently, the specific overexpression of the miR-371–373 cluster in testicular GCTs was confirmed in a study that also included some adult ovarian malignant GCT cases<sup>57</sup>. These findings were then extended in a report demonstrating that the miR-371–373 cluster and, in addition, the miR-302–367 cluster (miR-302a-d plus miR-367; at 4q25) were both overexpressed in all malignant GCTs, independent of patient age (paediatric or adult), tumour histological subtype (YST, seminoma or embryonal carcinoma) or anatomical site (gonadal or extragonadal)<sup>58</sup>, representing the first universal molecular abnormality identified in this disease<sup>20</sup>. Across >100 clinical cases, the expression levels of the eight main microRNA members from the miR-371–373 and miR-302–367 clusters accurately separated malignant GCTs from nonmalignant samples, comprising normal gonadal control samples and teratomas<sup>58</sup> (FIG. 1). These findings suggest that microRNAs from the miR-371–373 and miR-302–367 clusters are potential highly sensitive and specific universal biomarkers of all malignant GCTs<sup>20</sup>. These observations have now been independently confirmed<sup>59</sup>, including the observation that these microRNA changes occur in germ cell neoplasia *in situ*<sup>60</sup>, the precursor lesion to testicular GCTs, implying that overexpression of these microRNAs represents an early molecular change that is likely to have a fundamental role in GCT pathogenesis. Importantly, these microRNAs have not been shown to be coordinately overexpressed in any other cancer or disease state, adding to their biomarker potential<sup>58</sup>.

MicroRNAs are released into the bloodstream from cancer cells, often contained within membrane-bound particles, termed exosomes, and are, therefore, protected from degradation<sup>61,62</sup>. Consequently, the quantification of circulating microRNAs offers substantial promise for cancer diagnosis and monitoring<sup>63</sup>. For malignant GCTs, a multiplexed quantitative reverse transcription (qRT)–PCR methodology approach was used, which included a preamplification step, to demonstrate that circulating levels of all eight main members of the miR-371–373 and miR-302–367 clusters were elevated in the serum of an index patient with extragonadal disease, compared with levels in pooled normal serum<sup>63</sup>. Levels of miR-372-3p fell to normal levels during treatment and during uneventful clinical follow-up monitoring<sup>63</sup>. This proof-of-principle investigation was followed by a small study across the clinical

spectra of age (paediatric and adult), anatomical site (gonadal (including testicular) and extragonadal) and histological subtype (YST, seminoma and embryonal carcinoma), confirming universal elevation at malignant GCT diagnosis of serum levels of miR-372-3p and miR-367-3p<sup>64</sup>. Importantly, most of the patients described in this study were marker-negative by CTM estimation (specifically the patients with seminoma and embryonal carcinoma), demonstrating potential clinical utility in these cohorts.

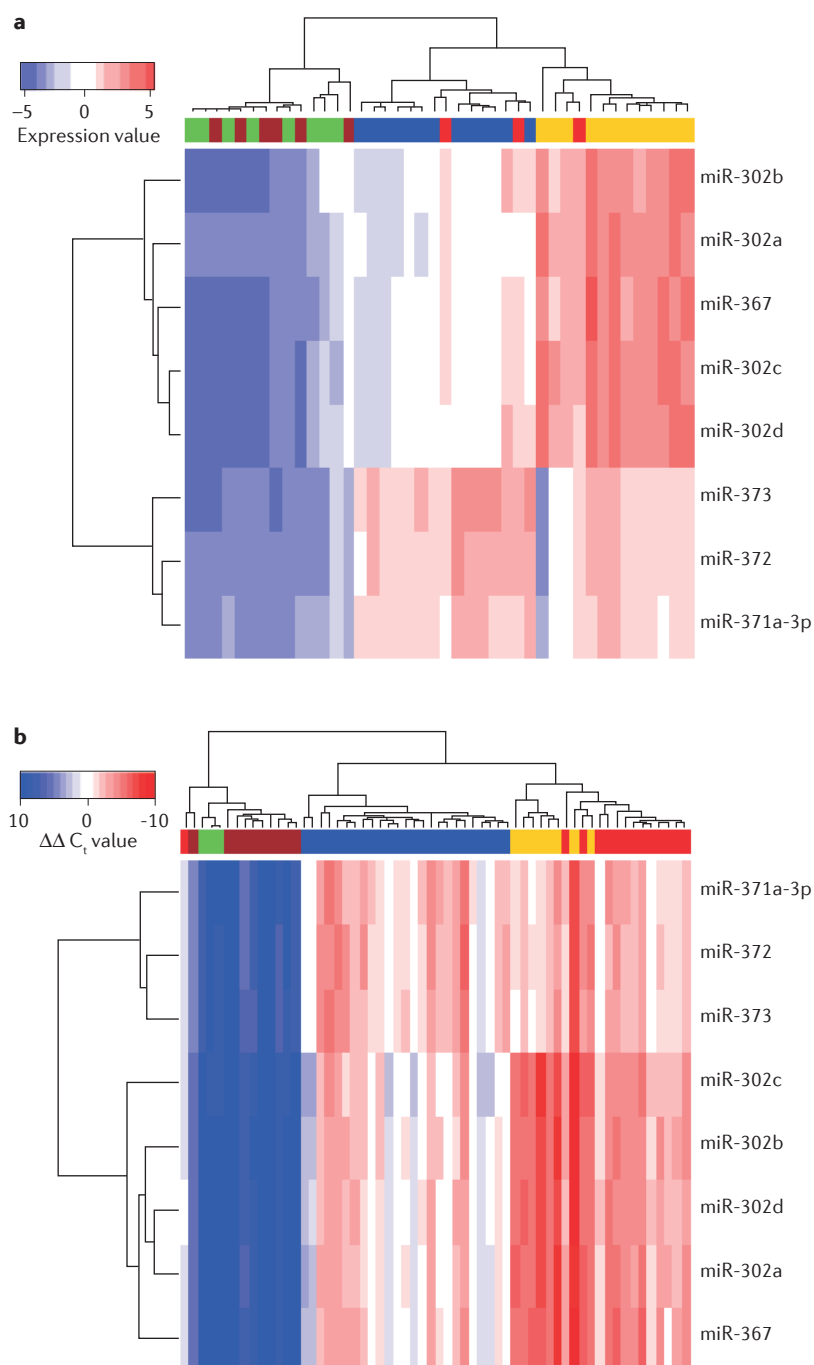
These initial findings have since been replicated and extended, based on the qRT-PCR methodology

described<sup>12,13,64–70</sup>, predominantly in malignant testicular GCTs. Indeed, a panel of just four circulating microRNAs (namely miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) is highly sensitive and specific for diagnosis of malignant GCT<sup>12,66,70</sup>. Circulating microRNA levels from the panel fall after definitive surgery and/or chemotherapy treatment<sup>12,13,63,66,70</sup>, and are also highly sensitive for detecting relapse<sup>12</sup>.

### Practical considerations

Circulating microRNAs are now starting to be studied in prospective clinical trials in patients with GCTs, with the ultimate aim of embedding microRNA quantification in routine clinical practice. However, at present no agreed protocol exists for sample collection, RNA extraction, quality control assessment or actual PCR quantification, and indeed, some groups are not assessing the full panel of four microRNAs that might offer the greatest sensitivity and specificity for detecting all malignant testicular GCTs (TABLE 3; studies are listed in chronological order). These issues are all critical and need to be overcome to ensure optimal reliability and reproducibility and to drive these research technologies towards future clinical use in testicular GCTs.

**Sample collection.** To date, all studies specifically reporting circulating microRNA levels from the miR-371–373 and miR-302–367 clusters in malignant GCTs have used serum<sup>12,13,63–70</sup>. To ensure consistent results, the whole blood collected in these serum separator tubes should be centrifuged in a standard fashion within a few hours following receipt, and the resultant serum aliquoted into separate tubes for processing, with subsequent storage at  $-80^{\circ}\text{C}$ . The starting serum volumes used for testing have reduced in recent years. Initially, 400  $\mu\text{l}$  was used<sup>63,64</sup>; 200  $\mu\text{l}$  is now the most common starting volume, but as little as 50  $\mu\text{l}$  can be sufficient<sup>66,67</sup> (TABLE 3). Plasma might also be suitable for testing, as serum and plasma microRNA levels are believed to correlate well<sup>71</sup>, although such a correlation remains to be formally demonstrated for the panel of four circulating microRNAs at the time of malignant GCT diagnosis. If proven to be suitable, the use of plasma would offer the additional benefit of enabling any circulating tumour



**Figure 1 | Differential expression of the microRNAs (miR)-371–373 and miR-302–367 clusters in malignant germ cell tumours (GCT).** Hierarchical clustering analysis based on the eight main microRNAs from the miR-371–373 and miR-302–367 clusters (rows) segregates **a** | paediatric and **b** | adult malignant GCT samples from nonmalignant controls (comprising benign teratomas and normal gonadal controls) (columns). In the heatmap, red represents relative microRNA overexpression and blue represents underexpression. Green columns = normal gonadal controls; brown columns = teratoma; blue columns = seminoma; yellow columns = yolk sac tumour; red columns = embryonal carcinoma. Modified with permission from AACR publications, Palmer, R. D. *et al. Cancer Res.* <http://dx.doi.org/10.1158/0008-5472.CAN-09-3301> (2010).

DNA (ctDNA) that is present to be extracted, which is important as mutational profiles in testicular GCTs using whole-exome sequencing have now been reported<sup>72</sup>. Mutations occurred in 43% of the tumours interrogated, with aberrations in the *KIT* gene (occurring in 14.3% of tumours) being the most common. Of particular interest, two treatment-refractory cases were identified, both of which harboured mutations of *XRCC2*, a gene implicated in cisplatin resistance<sup>72</sup>. Collection of

plasma in prospective studies might, therefore, offer further opportunities for the molecular diagnosis and risk stratification of malignant GCTs.

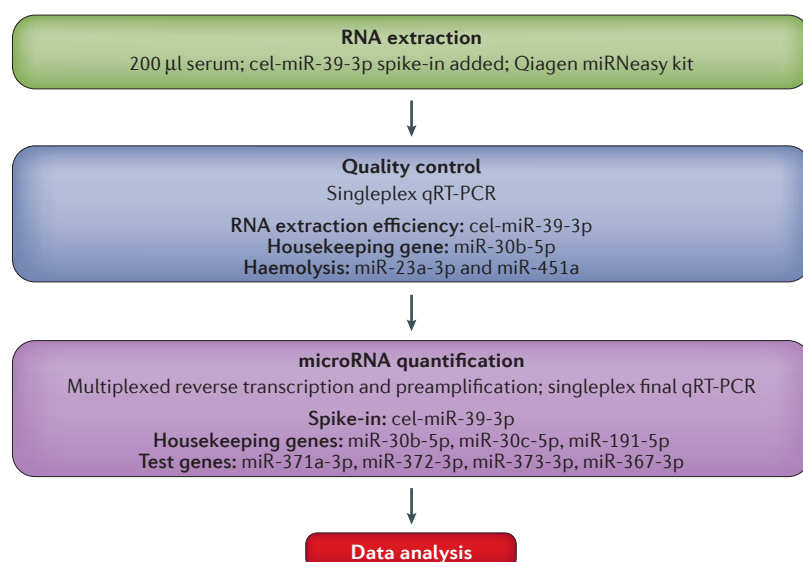
**RNA extraction.** RNA is extracted from serum using commercially available proprietary kits (TABLE 3). The subsequent PCR reactions are also performed using standard TaqMan assay kits and platforms (TABLE 3). To avoid false-positive and false-negative results,

Table 3 | Serum microRNA studies in malignant germ cell tumours (GCTs) and important practical considerations

Author (year)	Serum volume used (µl)	RNA extraction method	Test microRNAs or microRNA clusters quantified	Formal haemolysis assessment	Nonhuman exogenous spike-in RNA added	Normalization approach	Comments
Murray <i>et al.</i> (2011) <sup>63</sup>	400	miRVana PARIS kit (Ambion)	• miR-371–373 ( <i>n</i> = 3) • miR-302–367 ( <i>n</i> = 5)	No	No	Small RNA concentration	First report of utility of serum microRNAs in GCTs
Belge <i>et al.</i> (2012) <sup>65</sup>	Not described	Not described	miR-371–373 ( <i>n</i> = 3)	No	No	18S RNA	Letter, with sparse technical details
Murray and Coleman (2012) <sup>64</sup>	400	miRVana PARIS kit (Ambion)	• miR-372–3p ( <i>n</i> = 1) • miR-367–3p ( <i>n</i> = 1)	No	No	Small RNA concentration	Suggested additional specificity of using miR-367–3p
Dieckmann <i>et al.</i> (2012) <sup>13</sup>	200	miRNeasy mini kit (Qiagen)	miR-371–373 ( <i>n</i> = 3)	No	No	18S RNA	Different reverse transcription step for 18S versus microRNAs, risking technical bias
Gillis <i>et al.</i> (2013) <sup>66</sup>	50	TaqMan miRNA ABC purification kit; Panel A (Life Technologies)	• miR-371–373 ( <i>n</i> = 3) • miR-302/367 ( <i>n</i> = 4)	No	Yes; cel-miR-39-3p ath-miR-159a	miR-20a-5p miR-93-5p	Four serum microRNA panel* identified as most sensitive and specific
Syring <i>et al.</i> (2015) <sup>70</sup>	400	miRVana PARIS kit (Ambion)	• miR-371–373 ( <i>n</i> = 3) • miR-302/367 ( <i>n</i> = 4)	No	Yes; cel-miR-39-3p	None	Four serum microRNA panel* confirmed as most sensitive and specific
Spiekermann <i>et al.</i> (2015) <sup>68</sup>	200	miRNeasy mini kit (Qiagen)	miR-371a-3p ( <i>n</i> = 1)	No	No	miR-20a-5p	Only single microRNA tested
Rijlaarsdam <i>et al.</i> (2015) <sup>67</sup>	50	TaqMan miRNA ABC purification kit; Panels A&B (Life Technologies)	Global profiling study ( <i>n</i> ~750)	No	Yes; ath-miR-159a	Global normalisation (PMID 19531210)	• Appropriate normalization approach for a global profiling study used • Confirmation of the relevance of miR-371–373 microRNAs
Spiekermann <i>et al.</i> (2015) <sup>69</sup>	200	miRNeasy mini kit (Qiagen)	miR-371–373 ( <i>n</i> = 3)	No	No	18S RNA, but suggested none required	• Different reverse transcription step for 18S. • Suggestion that normalization (relative quantification) not required
Murray <i>et al.</i> (2016) <sup>12</sup>	200	miRNeasy serum/plasma kit (Qiagen)	• miR-371–373 ( <i>n</i> = 3) • miR-302–367 ( <i>n</i> = 5)	Yes: ΔCt (miR-23a-3p–miR-451a)	Yes; cel-miR-39-3p	miR-30b-5p	• Four serum microRNA panel* confirmed. • First demonstration of relapse detection
van Agthoven & Looijenga (2016) <sup>74</sup>	50	TaqMan miRNA ABC purification kit; Panel A (Life Technologies)	• miR-371–373 ( <i>n</i> = 2) • miR-367–3p ( <i>n</i> = 1)	No	Yes; ath-miR-159a	miR-30b-5p	• Confirmed utility of serum microRNA panel at diagnosis. • Showed miR-93-5p suboptimal as housekeeping gene
Dieckmann <i>et al.</i> (2016) <sup>75</sup>	200	miRNeasy mini kit (Qiagen)	• miR-371–373 ( <i>n</i> = 3) • miR-367–3p ( <i>n</i> = 1)	No	No	miR-93-5p	Confirmed utility of serum microRNA panel at diagnosis and miR-371a-3p in follow-up, including for relapse detection

\*Four serum microRNA panel comprising miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p.





**Figure 2 | A proposed pipeline for quantification of circulating microRNAs (miRs) in malignant germ cell tumours (GCTs)<sup>12</sup>.** At the RNA extraction stage (green box), a fixed quantity of the nonhuman spike-in RNA cel-miR-39-3p is added to enable subsequent exogenous normalisation. An initial quality control quantitative reverse transcription (qRT)-PCR step is then performed (blue box) to check for satisfactory levels of exogenous cel-miR-39-3p (indicating satisfactory RNA extraction), endogenous housekeeping microRNA (miR-30b-5p) and for formal haemolysis assessment. Once a serum sample has passed this step, formal microRNA quantification is then performed, using a multiplexed reverse transcription and preamplification step (purple box), which includes the panel of four test microRNAs (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p). Data analysis is then undertaken (red box).

no-template controls should be run for each assay (to exclude nonspecific amplification) and any differences in RNA extraction efficiency between samples measured, so that these differences can be accounted for in subsequent data analysis<sup>73</sup>. The latter is performed by adding a fixed quantity of exogenous nonhuman spike-in microRNA(s) (for example, *C. elegans* (cel)-miR-39-3p or *A. thaliana* (ath)-miR-159a), that does not occur naturally in human serum, which is then quantified and adjusted for. Not all reported studies use such a quality control step (TABLE 3), despite it providing additional stringency and being considered best practice<sup>12</sup>, as per Minimal Information in Q-PCR Experiment (MIQE) guidelines<sup>73</sup>. MIQE guidelines assist both the transparency of result reporting and the comparison of results between different studies, which could facilitate the subsequent standardization of protocols<sup>73</sup>. The selection of endogenous circulating microRNA housekeeping genes for data normalization (relative quantification) is also of critical importance, as this step minimizes technical bias and maximizes detection of true biological variation between serum samples<sup>12</sup>. Early studies did not include such normalization steps<sup>63,64</sup>, as no stable housekeeping microRNAs had been established. In many subsequent reports, no clear rationale is given for the selection of different housekeeping genes for this purpose (TABLE 3). However, one MIQE-compliant 2016 report has identified miR-30b-5p as being the most stable microRNA in serum, based on a global microRNA study ( $n = 741$ ) and subsequent validation in serum from patients with malignant GCTs<sup>12</sup>. Some researchers

suggest that such relative quantification might be unnecessary for clinical testing and that raw PCR results provide adequate diagnostic discrimination<sup>69</sup>. However, such an approach might not be optimal when using current qRT-PCR methodology to quantify the full panel of four circulating microRNAs that offer the greatest accuracy for malignant GCT diagnosis<sup>12</sup>. The dynamic range for circulating miR-372-3p, miR-373-3p and miR-367-3p is lower than for circulating miR-371a-3p<sup>12</sup>. Consequently, differences in extraction efficiency will have a relatively high impact on overall PCR measurements and, therefore, careful control via relative quantification approaches is still recommended<sup>12</sup>.

**Haemolysis.** Haemolysis might also be an important practical consideration in adopting serum microRNA tests for routine clinical detection of testicular malignant GCTs, as variations in red blood cell lysis between serum and plasma samples might result in altered circulating microRNA expression levels for technical, rather than biological, reasons. Consequently, a 2016 study only used samples without macroscopic evidence of haemolysis, and calculated  $\Delta Ct$  (miR-23a-3p minus miR-451a) values to formally assess differences in microscopic haemolysis between samples<sup>12</sup> (TABLE 3). MiR-23a-3p quantification is used as a housekeeping gene to enable a  $\Delta Ct$  value to be estimated, and miR-451a levels directly reflect the degree of haemolysis of the sample being interrogated, as this microRNA is one of the most abundant in red blood cells<sup>12</sup>. Other formal methods, such as spectrophotometry for quantification of free haemoglobin in the serum, can also quantify the degree of haemolysis within a sample. The value of such assessments in circulating microRNA testing remains to be established for the diagnosis of malignant GCT. However, we recommend that a formal method of haemolysis assessment should continue to be performed at present, to inform how much contribution haemolysis makes to any potential false-positive or false-negative results in future trials of circulating microRNAs in malignant GCTs, and to enable appropriate adjustments to be made.

**Using the full panel of four microRNAs.** For maximal sensitivity and specificity, using the full panel of four microRNAs from the miR-371–373 and miR-302–367 clusters is advisable<sup>12,66,70</sup>. The sensitivity of miR-371a-3p alone is ~90% for the diagnosis of malignant GCT<sup>68</sup>, but that value can be increased further by using the panel<sup>12,66,70</sup>. For example, the particularly rapid reduction in miR-371a-3p levels following orchidectomy in stage I disease<sup>68</sup> can result in negative results in samples taken more than 1 day after surgery, whereas the other microRNAs in the panel have longer half-lives<sup>12</sup>. Accordingly, in cases in which the timing of the ‘diagnostic’ serum sample is suboptimal (for example, taken following surgery), assessment of miR-371a-3p alone might result in false-negative results. Furthermore, assessment of circulating levels of miR-367-3p increases specificity for the identification of malignant GCTs, as it is transcribed from a separate chromosome locus (4q25) to the other three microRNAs in the panel (19q13)<sup>12</sup>. Such

assessment is important, as although individual microRNAs from either the miR-371–373 or the miR-302–367 cluster have been shown to be increased in certain malignancies, coordinate overexpression of microRNAs from both clusters have not been demonstrated in any tumour other than malignant GCTs, or in any other disease state<sup>57</sup>. We have proposed a pipeline for the quantification of circulating microRNAs in malignant GCTs that addresses these issues (FIG. 2).

In mid 2016, two further serum microRNA studies in testicular GCTs were published<sup>74,75</sup>, both of which showed that the panel of miR-371–373 and miR-367–3p microRNAs was significantly elevated in patients with malignant testicular GCTs at the time of diagnosis, using the originally described preamplification step<sup>63</sup>, which maximizes the sensitivity of the test. MiR-371a-3p was the most sensitive and specific individual microRNA from the serum panel in these analyses, with area under the curve values of 0.95 and 0.94, respectively<sup>74,75</sup>. One study did not use an exogenous nonhuman spike-in microRNA and used miR-93-5p for normalization<sup>75</sup>. The other used a nonhuman spike-in and selected miR-30b-5p as the housekeeping gene<sup>74</sup>, as previously described<sup>12</sup>. Interestingly, use of this microRNA was because miR-93-5p was found to be unsuitable for normalization owing to a significant difference in levels between the malignant GCT group and the control patients<sup>74</sup>. Neither study used a formal haemolysis method to assess the samples (TABLE 3).

**Cost and the future.** Currently, as a relatively low-throughput research method using the described pipeline<sup>12</sup> (FIG. 2), circulating microRNA analysis costs approximately UK£40 per sample. This figure compares favourably with the cost of a single CT scan of ~£200. In due course, when such a method is in routine clinical use with higher throughput, economies of scale are likely to reduce these costs further. In the future, robust absolute quantification PCR methods might overcome the need for relative quantification when measuring

circulating microRNA levels, although the requirement to use nonhuman spike-ins or other appropriate standards will remain, to ensure the accuracy of RNA extraction steps and subsequent PCR quantification within and between samples. Such measures should improve sensitivity and specificity overall and provide greater confidence in borderline cases, ensuring that clinical decisions are not undermined by subtle technical effects<sup>12</sup>.

## Conclusions

The conventional tumour markers AFP, hCG and LDH have demonstrated value in the clinical management of testicular malignant GCTs, particularly for early risk-stratification of metastatic NSGCTs and detection of relapse in patients with NSGCT. However, their limitations in sensitivity and specificity prevent more universal application, especially in patients with seminoma. Circulating microRNAs show exciting promise as a universal marker of testicular malignant GCTs. Such biomarkers could considerably contribute to improving the quality of survival for patients in low-stage and/or IGCCC good-risk groups, through rational reductions in adjuvant chemotherapy, improved targeted imaging and detection of relapse at an earlier stage. Their use might also enable new approaches to increase overall survival among IGCCC poor-risk and relapsed disease cohorts by modifying treatment and identifying patients who should receive intensified treatment.

As these novel biomarkers are now being studied in prospective clinical trials, the practical considerations discussed in this Review should be addressed and incorporated into standardized protocols. We suggest that haemolysis assessment, alongside normalization to both a nonhuman exogenous spike-in RNA (for example, cel-miR-39-3p) and carefully selected endogenous housekeeping microRNAs (for example, miR-30b-5p), are optimal approaches for the relative quantification PCR methods currently in use. Such robust protocols will ensure the optimal reliability and reproducibility of these tests in clinical settings.

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# Author contributions

All authors researched data for the article, made a substantial contribution to discussions of content, wrote and edited the manuscript before submission.

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