Supplementary Materials and Methods

Data normalisation and analysis. Following qRT-PCR, the serum sample with the lowest raw mean cel-miR-39-3p Cq value (i.e. the sample with the highest abundance and therefore greatest RNA recovery) was used to calculate individual correction factors for all other samples, using the equation:

\[
\text{cel-miR-39-3p correction-factor} = (\text{sample raw mean cel-miR-39-3p Cq}) - (\text{lowest raw mean cel-miR-39-3p Cq})
\]

Individual correction-factors were then applied to the respective raw mean housekeeping miRNA (miR-30b-5p, miR-30c-5p, miR-191-5p) Cq values, using the equation:

\[
\text{cel-miR-39-3p-adjusted housekeeping miRNA Cq} = (\text{sample raw mean housekeeping miRNA Cq}) - (\text{sample cel-miR-39-3p correction-factor})
\]

As the standard deviation of the serum Cq values across the whole cohort of 41 samples was lowest for miR-30b-5p, this miRNA was selected for subsequent normalisation purposes. Thus, for each target miRNA under consideration, a delta-Cq value was obtained, using the equation:

\[
\text{sample target miRNA delta-Cq} = (\text{sample mean target miRNA Cq}) - (\text{sample mean cel-miR-39-3p-corrected miR-30b-5p Cq})
\]

Target miRNA delta-delta-Cq values were then calculated, using the equation:
sample mean target miRNA delta-delta-Cq = (non-tumour control group mean target miRNA delta-Cq value) - (sample mean target miRNA delta-Cq value)

Finally, relative expression was quantified using the equation:

sample relative expression = $2^{\text{sample mean target miRNA delta-delta-Cq}}$
Supplementary Results

Clinical case 1: Longitudinal follow-up of a patient with a testicular malignant GCT.

A 14-year-old male presented with a short history of left testicular swelling (patient MGCT_ExC#3). Serum AFP and HCG levels at diagnosis were elevated (Figure 4A/B). Following ultrasound, a left orchidectomy was performed, with histopathology showing a mixed malignant GCT that contained yolk sac tumour (YST), embryonal carcinoma (EC) and choriocarcinoma (CHC) as well as immature teratoma (IT) components. CT scan of the chest and abdomen/pelvis confirmed stage 1 disease (Figure 4E-1). After discussion with the patient and family regarding subsequent management, namely adjuvant chemotherapy versus a surveillance approach, the latter strategy was chosen for follow-up.

The patient was followed with serum AFP/HCG markers that initially fell at day (d) 14. A further CT scan at d28 showed left retroperitoneal lymphadenopathy (Figure 4E-2). At this time, AFP levels had not increased, while HCG levels showed a modest increase from their previous values at d14 (Figure 4A/B). Repeat markers at d33 showed a modest increase in AFP, and a further increase in HCG, compared with their respective d28 values (Figure 4A/B). Together, the clinical, radiological and biochemical findings were consistent with a malignant GCT relapse.

The patient was assigned to the good-risk group as per the International Germ Cell Cancer Collaborative Classification (IGCCC), and treated with three cycles of standard ‘BEP’ chemotherapy (cisplatin, etoposide and bleomycin), commencing on d33. After an initial flare on d42, serum AFP and HCG markers rapidly normalised and remained low (Figure 4A/B). An end-of-treatment CT scan revealed a further increase in size of the left retroperitoneal
lymph node mass (Figure 4E-3). Clinically, the differential diagnosis was between growing teratoma syndrome and refractory ‘marker-negative’ EC. In the absence of a suitable serum marker to rule out EC, the patient underwent a template retroperitoneal lymph node dissection (RPLND). Histopathology showed growing teratoma syndrome, with no evidence of malignant components. A further CT scan post-RPLND confirmed radiological remission (Figure 4E-4). The patient made an uncomplicated clinical recovery and remains well more than two years following initial presentation, with normal serum AFP/HCG, miR-371a-3p and miR-372-3p levels (Supplementary Figure S3A).

**Clinical cases 2: Intracranial tumours.**

The first patient (MGCT_IC#1), a 12-year-old male, presented with a two month history of headaches and vomiting and a two week history of diplopia. An MRI head scan revealed a solid pineal tumour. Serum and CSF AFP and HCG markers were negative. He underwent uneventful neurosurgical biopsy which showed a germinoma, before successful combined chemoradiotherapy, as per the current European SIOP-CNS-GCT-II protocol.

The second patient (MGCT_IC#2), a 12-year-old female, presented with a three month history of headache and nausea, as well as polydipsia and polyuria suggestive of diabetes insipidus. An MRI head scan revealed a solid suprasellar tumour (Figure 5A, left panel). Serum markers revealed a normal AFP but an elevated HCG (92.4 IU/L), consistent with a diagnosis of a secreting non-germinoma, and she therefore avoided neurosurgical biopsy. She underwent successful intensified combined chemotherapy as per the SIOP-CNS-GCT-II protocol.
The third patient, an 11-year-old female (MGCT_IC#3), presented with a five month history of headache and growth failure, a one week history of vomiting and development of status epilepticus on the day of admission. She was ventilated and transferred to the tertiary paediatric intensive care unit. An MRI head scan revealed a large suprasellar tumour with solid and cystic components (Figure 5A, middle panel). An emergency extraventricular drain was inserted. Serum and CSF AFP and HCG markers were negative. She underwent neurosurgical biopsy but post-operatively developed diabetes insipidus, causing disturbed sodium homeostasis and further seizures which required re-admission to intensive care. Histopathology showed a germinoma. Following stabilisation, she underwent successful treatment with combined chemoradiotherapy as per the current European SIOP-CNS-GCT-II protocol.

The fourth patient (B-non-GCT_IC#1), a 15-year-old female, presented with a three year history of headaches, growth failure and delayed puberty. An MRI head scan revealed a solid suprasellar tumour (Figure 5A, right panel). The most likely diagnosis radiologically was a malignant GCT. Serum and CSF AFP and HCG markers were negative. She underwent neurosurgical biopsy and histopathology showed a ganglioglioma, WHO grade 1. Subsequent follow-up MRI scans show stable appearances and she is currently being monitored with a surveillance approach.

The fifth patient (M-non-GCT_IC#1), was a 2-year-old male with MYCN-amplified neuroblastoma (Murray et al, 2015a), who developed intracranial metastatic disease during treatment and subsequently rapidly died of disease.
Supplementary Discussion

Our study also confirms that despite being expressed at elevated levels in all malignant GCT tissues (Palmer et al, 2010), miR-302a-d miRNAs are not universally elevated in the serum of affected patients, compared with the other tumour and non-tumour control groups (Gillis et al, 2013; Syring et al, 2015). We previously showed that miR-302a-d miRNAs were elevated at diagnosis in the serum of a patient with bulky malignant GCT disease (Murray et al, 2011). In the present study, we observed elevated miR-302a-c levels in the serum of a patient with an extracranial malignant GCT and the CSF of a patient with an intracranial malignant GCT, again both presenting with a relatively heavy disease burden. However, across the range of malignant GCTs encountered (which were well represented in our sample cohort), miR-302a-d miRNAs did not provide any additional diagnostic benefit to the four serum miRNA panel identified.

A recent international consensus document regarding aspects of the management of intracranial GCTs has highlighted the critical importance of biospecimen collection, including CSF and serum, in order to facilitate the identification of new biomarkers (Murray et al, 2015b). On this note, we have also demonstrated that members of the four miRNA panel are elevated in the CSF of patients at the time of diagnosis of intracranial malignant GCTs. Interestingly, in two of three such cases examined, the miRNAs were also elevated in the serum. The panel therefore offers a potential non-invasive diagnostic method for segregating intracranial malignant GCT from other intracranial tumours, such as gliomas. For treatment purposes, it is necessary not only to identify the presence of malignant intracranial GCT disease, but also to specify accurately the most aggressive malignant GCT subtype present, as patients with tumours containing YST, CHC or EC components are treated more
intensively than those with pure germinoma, due to inferior outcomes (Murray et al, 2015b). It will therefore also be important to identify miRNA or other biomarkers of malignant GCT histological subtypes, as these could also potentially be measured in serum and/or CSF samples. For intracranial malignant GCTs, it will be particularly valuable to segregate germinoma from EC cases, as both are typically AFP and HCG negative and currently require invasive neurosurgical biopsy for full diagnosis.
Supplementary Table and Figure Legends.

Supplementary Table S1. The miRNAs quantified in the serum and CSF samples. Assays marked by an asterisk represent those used in the miRNA quantification step (Figure 1, Box C). Raw mean NTC Cq values from the miRNA quantification step are listed. N/A = not applicable.

Supplementary Figure S1. Mean Cq values in each serum sample assessed during initial quality control (QC) steps (Figure 1, Box B1) for: A) the exogenous non-human spike-in control cel-miR-39-3p, B) the housekeeping miRNA miR-30b-5p and C) the haemolysis miRNA miR-451a. D) Delta Cq (miR-23a-3p minus miR-451a) levels in the serum samples. Those samples with a delta Cq of <8 were designated as not substantially haemolysed. In panels A-D, control samples are shown in green, benign GCT samples in brown, non-GCT samples in yellow and malignant GCT samples in blue. E) Correlations for cel-miR-39-3p and miR-30b-5p between the Cq values obtained in the initial QC (y-axis) and miRNA quantification (x-axis) steps (i.e. Figure 1, Box B1 and Figure 1, Box C, respectively).

Supplementary Figure S2. Relative serum levels of: A) miR-371a-3p, B) miR-372-3p, C) miR-373-3p and D) miR-367-3p for individual patients at diagnosis. Patients are grouped into extracranial malignant GCTs, benign GCT/non-GCT pathology and the non-tumour control group. The broken horizontal line in each graph represents the cut-offs derived from the serum samples, i.e. 2.0 times the highest level seen in the non-tumour control group. Error bars = SEM. E) Correlations in serum samples between Cq and delta Cq values for i) miR-371a-3p, ii) miR-372-3p, iii) miR-373-3p and iv) miR-367-3p. Each point represents the
mean value of technical triplicates, as determined by qRT-PCR. Delta Cq values were calculated relative to the housekeeping miRNA miR-30b-5p.

**Supplementary Figure S3.** Full follow-up of the patient with a testicular mixed malignant GCT (MGCT_ExC#3). A) Levels of the serum protein biomarkers AFP (top left panel) and HCG (top right), and relative serum levels of miR-371a-3p (bottom left) and miR-372-3p (bottom right), from d0 (diagnosis) to d782 of follow-up. Asterisks denote the clinical stages of: diagnosis (1), malignant relapse (2), end-of-treatment (3) and retroperitoneal lymph node dissection (RPNLD) (4). The arrows show the timing of each chemotherapy cycle. B) Scatter plots showing the correlations between: AFP and HCG (top left panel); miR-371a-3p and miR-372-3p (top right); AFP and miR-371a-3p (centre left); AFP and miR-372-3p (centre right); HCG and miR-371a-3p (bottom left) and HCG and miR-372-3p (bottom right), for all 18 longitudinal serum samples from patient MGCT_ExC#3. Outlier points are caused by the more sensitive early detection of malignant relapse using serum miRNAs (red arrows) and, in contrast to AFP/HCG, the lack of a flare in miRNA levels following initiation of chemotherapy (green arrows).

**Supplementary Figure S4.** A) Comparison of Cq values obtained in the miRNA quantification step (Figure 1, Box C) across the four cerebrospinal fluid (CSF) samples for four putative housekeeping miRNAs (miR-124-3p, miR-30b-5p, miR-30c-5p and miR-191-5p) plus the exogenous non-human spike-in cel-miR-39-3p. B) Raw Cq values for miR-23a-3p and miR-451a in the haemolysis quality control steps (Figure 1, Box B3). The delta Cq values shown were determined from miR-23a-3p minus miR-451a levels. C) Comparison of the raw Cq values in serum and CSF samples classified as positive or negative using our pipeline for: i) miR-371a-3p, ii) miR-372-3p, iii) miR-373-3p and iv) miR-367-3p.
Supplementary References


### Supplementary Table S1

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Role</th>
<th>miRBase accession number</th>
<th>Nucleotide sequence</th>
<th>Assay number</th>
<th>Raw mean no-template control Cq</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-371a-3p</td>
<td>Target</td>
<td>MIMAT0000723</td>
<td>AAGUGCCGCAUCUUUUGAGUGU</td>
<td>002124*</td>
<td>32.86 ± 0.22</td>
</tr>
<tr>
<td>hsa-miR-372-3p</td>
<td>Target</td>
<td>MIMAT0000724</td>
<td>AAAGUGCGCGACAUUUUGACGCU</td>
<td>000560*</td>
<td>32.51 ± 0.55</td>
</tr>
<tr>
<td>hsa-miR-373-3p</td>
<td>Target</td>
<td>MIMAT0000726</td>
<td>GAAGUGCUUCCAUUUUGGGGUGU</td>
<td>000561*</td>
<td>45.00</td>
</tr>
<tr>
<td>hsa-miR-302a-3p</td>
<td>Target</td>
<td>MIMAT0000684</td>
<td>UAAGUGCUUCAUGUUUUGGUGA</td>
<td>000529*</td>
<td>33.45 ± 0.17</td>
</tr>
<tr>
<td>hsa-miR-302b-3p</td>
<td>Target</td>
<td>MIMAT0000715</td>
<td>UAAGUGCUUCAUGUUUAGUAG</td>
<td>000531*</td>
<td>29.48 ± 0.09</td>
</tr>
<tr>
<td>hsa-miR-302c-3p</td>
<td>Target</td>
<td>MIMAT0000717</td>
<td>UAAGUGCUUCAUGUUUUCAGUGG</td>
<td>000533*</td>
<td>33.20 ± 0.59</td>
</tr>
<tr>
<td>hsa-miR-302d-3p</td>
<td>Target</td>
<td>MIMAT0000718</td>
<td>UAAGUGCUUCAUGUUUGAGUG</td>
<td>000535*</td>
<td>29.82 ± 0.86</td>
</tr>
<tr>
<td>hsa-miR-367-3p</td>
<td>Target</td>
<td>MIMAT0000719</td>
<td>AAUUGACUUUAGCAUGUGA</td>
<td>000555*</td>
<td>31.19 ± 0.65</td>
</tr>
<tr>
<td>ccel-miR-39-3p</td>
<td>Non-human spike-in</td>
<td>MIMAT0000010</td>
<td>UCACCGGGUGUAAUACAGCUUG</td>
<td>000200*</td>
<td>45.00</td>
</tr>
<tr>
<td>hsa-miR-30b-5p</td>
<td>Housekeeping gene</td>
<td>MIMAT0000420</td>
<td>UGUAACAUCCUACUCAGCU</td>
<td>000602*</td>
<td>30.04 ± 0.11</td>
</tr>
<tr>
<td>hsa-miR-30c-5p</td>
<td>Housekeeping gene</td>
<td>MIMAT0000244</td>
<td>UGUAACAUCCUACUCACUCAGC</td>
<td>000419*</td>
<td>32.51 ± 0.23</td>
</tr>
<tr>
<td>hsa-miR-191-5p</td>
<td>Housekeeping gene</td>
<td>MIMAT0000440</td>
<td>CAACGGAAUCCAAAAGCAGCUG</td>
<td>002299*</td>
<td>42.59 ± 1.50</td>
</tr>
<tr>
<td>hsa-miR-23a-3p</td>
<td>Haemolysis assessment</td>
<td>MIMAT0000078</td>
<td>AUCACAUUGCACGGGAUUCC</td>
<td>000399</td>
<td>N/A</td>
</tr>
<tr>
<td>hsa-miR-451a</td>
<td>Haemolysis assessment</td>
<td>MIMAT0001631</td>
<td>AAACCGUUACCAGUACUGU</td>
<td>001141</td>
<td>N/A</td>
</tr>
</tbody>
</table>