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Circulating microRNAs as biomarkers to assist the management of the malignant germ-cell-tumour subtype choriocarcinoma

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ABSTRACT

Germ-cell-tumours (GCTs) are heterogeneous and management is complex. The current conventional biomarkers, alpha-fetoprotein and human-chorionic-gonadotropin (HCG), have limited utility for diagnosis/follow-up as secretion is restricted to specific malignant-GCT subtypes and long half-life can make interpretation and clinical decision-making challenging. We sought to identify circulating microRNAs that reflected choriocarcinoma disease activity more accurately than HCG in a metastatic primary mediastinal nonseminomatous-GCT (PMNSGCT) case with elevated diagnostic serum HCG (>250,000 U/L), consistent with pure choriocarcinoma. We undertook comprehensive microRNA profiling ($n = 754$ microRNAs) using two 384-well TaqMan Low-Density-Array cards in 16 serum samples; 10 from PMNSGCT diagnosis/follow-up and six controls. Key findings underwent confirmatory qRT-PCR. We identified a serum panel of choriocarcinoma-specific 'chromosome-19-microRNA-cluster' (C19MC) microRNAs that were highly elevated at diagnosis but fell rapidly on treatment and normalised before the second full chemotherapy course. We also re-confirmed serum elevation of the previously identified malignant-GCT marker miR-371a-3p at diagnosis. These circulating microRNA markers reflected choriocarcinoma disease activity more accurately than serum HCG and real-time knowledge would have assisted clinical decision-making. With further study, these microRNA markers will facilitate future management of such patients and are likely to result in improved outcomes.

Abbreviations:

AFP	alpha-fetoprotein
C19MC	chromosome-19-microRNA-cluster
CHC	choriocarcinoma
HCG	human-chorionic-gonadotropin
GCT	germ-cell-tumour
IGCCC	International Germ Cell Consensus Classification
miRNA	microRNA
NPVM	non-pulmonary visceral metastases
PMNSGCT	primary mediastinal nonseminomatous-GCT

Introduction

Germ-cell-tumours (GCTs) are classified into seminoma and non-seminomatous tumours (NSGCTs), the latter comprising the malignant subtypes yolk-sac-tumour, embryonal carcinoma and choriocarcinoma (CHC), in addition to teratomas, which show extensive somatic differentiation [1]. The conventional biomarkers alpha-fetoprotein (AFP), human-chorionic-gonadotropin (HCG) and lactate dehydrogenase (LDH) have limited utility for the diagnosis and follow-up of malignant-GCTs [2]. LDH is very non-specific and secretion and detection of AFP and HCG in body fluids is restricted to specific subtypes, with levels raised predominantly in tumours containing yolk-sac-tumour and CHC, respectively [2]. Even where levels of AFP and/or HCG are elevated, clinical issues remain. These include the long half-lives of both AFP (~5–7 days) and HCG (~12–36 h) and their limited specificity, which may make clinical interpretation complicated [3,4]. Therefore, even in patients where these biomarkers are elevated at diagnosis, making optimal management decisions may be challenging.

Patients with primary mediastinal NSGCTs (PMNSGCTs) are classified into the International Germ Cell Consensus Classification (IGCCC) poor-risk group, irrespective of any other additional risk factors, due to their inferior prognosis [5]. Overall, this described IGCCC cohort had 48% five-year survival [5], although current survival is marginally

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better. Poor outcomes for PMNSGCT patients have been confirmed in subsequent independent series [6,7]. Management is complex, particularly for PMNSGCT patients with very high diagnostic serum HCG levels, consistent with a pure CHC. Issues include delayed diagnosis, with GCT not considered in a timely fashion, and thus AFP/HCG levels only requested after pathological confirmation [8]. In addition, there is a high risk of sudden respiratory decompensation [9] and/or catastrophic pulmonary [10,11] or cerebral [12,13] haemorrhage in such patients on initial instigation of treatment, known as 'choriocarcinoma syndrome' [9]. In such patients, close assessment in a tertiary centre intensive care setting is warranted [9]. Furthermore, following induction chemotherapy, primary mediastinal surgery is important in patients with PMNSGCT in order to guide subsequent management [14]. However, the long half-lives of AFP/HCG make assessment of when to perform such surgery difficult [14]. Optimising management and timing of these interventions may well improve outcomes for PMNSGCT patients [14], including those with CHC [9].

Improved biomarkers, which are highly sensitive and specific at malignant-GCT diagnosis and which accurately reflect disease status during treatment and in follow-up, would therefore address such unmet clinical needs. Over the last decade, microRNAs (miRNAs) have emerged as a new generation of biomarkers for malignant-GCTs [15]. MiRNAs are short, non-protein coding RNAs that are highly stable and suitable for diagnosis and disease-monitoring [16]. MiRNAs from the miR-371~373 (chromosomal locus 19q13.41) and miR-302/367 (4q25) clusters are universally over-expressed in malignant GCT tissue samples [1]. Levels of miRNAs from these clusters are elevated in the serum at malignant-GCT diagnosis, decrease with treatment and increase at malignant relapse [17–24], and their role has recently been subject to a comprehensive review [15].

Other miRNA expression changes reflective of different malignant-GCT subtypes also occur in the tissues, such as overexpression of miRNAs from the 'chromosome-19-microRNA-cluster' (C19MC, or miR-515~526 [1]) in embryonal carcinoma [1]. C19MC is the largest human miRNA gene cluster, containing >40 miRNAs. Intriguingly, C19MC is co-located within 100 kb of the miR-371~373 cluster and occurs uniquely in primates [25]. C19MC is expressed at high levels in placental tissues [26] and miRNAs from this cluster have also been found to be at high levels in the bloodstream in pregnancy, both in physiological [27] and pathological states, e.g. pre-eclampsia [28] and gestational-trophoblastic-neoplasia (GTN) [29]. Given the similar pathological trophoblastic (placental-like) appearances between GTN and CHC, we hypothesised that in addition to miR-371~373 miRNAs, C19MC miRNAs may also offer value as circulating biomarkers for this particular malignant-GCT subtype. Previously, three C19MC miRNAs were quantified in testicular GCT and control tissues and just one (miR-517a-3p) selected for serum assessment from affected patients [30]. Of note, only two patients had a modest quantifiable CHC component (mixed malignant-GCTs containing 30% and 10% CHC) and no patients in the cohort had pure CHC [30], so the direct contribution to the circulating miRNA profile from pure CHC alone could not be assessed.

Here, using unbiased comprehensive miRNA profiling, we sought to identify circulating miRNAs that reflected CHC disease activity more accurately than HCG in a widely metastatic primary mediastinal non-seminomatous GCT (PMNSGCT) case with elevated diagnostic serum HCG level (>250,000 U/L), consistent with pure CHC. In future, with further study and validation, real-time knowledge of miRNA markers may facilitate clinical management of such patients.

Case report

A 13-year-old male presented with a short history of cough and haemoptysis. He was well on clinical examination, with normal respiratory findings. Chest X-ray revealed a large mediastinal mass and multiple pulmonary metastases up to 50 mm in diameter (Fig. 1A). CT confirmed the above findings (Fig. 1B), with the mediastinal mass 144 mm in maximal coronal diameter, and revealed additional non-pulmonary visceral metastases (NPVM) in the spleen and kidneys. Bloods revealed normal full blood count (FBC), urea and electrolytes (U&E) and liver function tests (LFTs). Lactate dehydrogenase (LDH) estimation (350 U/L) was within the reference range. Serum HCG was elevated at 260,760 U/L (range 0.0–4.0) (Fig. 2A). Serum AFP was normal (<2; range 0–10 kU/L). Coagulation studies revealed a prolonged prothrombin time (16.7 s; range 10.4–13.3), which was aggressively corrected with intravenous vitamin K and fresh frozen plasma. Further staging revealed disease in the L3 vertebral body and multiple cerebral metastases (Fig. 1C). The diagnosis was made of a widely metastatic mediastinal CHC which fell into the IGCCC poor-risk group [5] due to three independent risk factors: mediastinal primary, HCG >50,000 U/L, and NPVM.

Due to the high decompensation and/or life-threatening haemorrhage risk, central venous access was obtained and emergency 'Em-EP' chemotherapy commenced on intensive care on the day of presentation, with two days of cisplatin (20 mg/m^2/day) and etoposide (100 mg/m^2/day) [31] delivered. Early chest X-ray performed on day 3 revealed an apparent interval increase in size of the bilateral multiple pulmonary masses (Fig. 1D) although the patient remained clinically well. The standard treatment for IGCCC poor-risk patients is four courses of 'BEP' (Bleomycin, Etoposide and cisPlatin) chemotherapy. However, bleomycin was substituted for ifosfamide and four full courses of 'PEI' (or 'VIP') chemotherapy [cisPlatin, Etoposide (VP-16) and Ifosfamide] [32,33] delivered for multiple reasons. Firstly, the large mediastinal primary and multiple pulmonary lesions would have made assessment for bleomycin toxicity challenging, by significantly influencing baseline pulmonary function test results [34] if performed, obscuring possible chest X-ray changes, and in view of the pre-existing cough. Secondly, given patients with PMNSGCTs have a high rate of postoperative pulmonary failure and mortality from major thoracic surgical procedures following the use of BEP, some clinicians prefer to use PEI/VIP [35]. Thirdly, multiple metastatic CNS lesions were present, and PEI is standard treatment for CNS NSGCTs as ifosfamide is known to have good CNS penetration [36].

Four PEI/VIP courses were therefore delivered with GCSF support from day (d) 10 at 18–20 day intervals, upon count recovery. The patient remained clinically well throughout, with resolution of cough and haemoptysis within two weeks of commencing treatment. HCG estimation fell to 966.1 U/L on d28 (d1 of the second course of PEI chemotherapy), 369.3 U/L (d48; d1 of cycle 3) and 111.6 U/L (d66; d1 of cycle 4). However, the HCG level was still elevated at 56.1 U/L following the last chemotherapy course on d82 (Fig. 2A). Furthermore, radiological imaging revealed only modest reduction in mediastinal primary size and persistence of multiple pulmonary metastases (Fig. 3A). MRI head revealed only small foci of blood degradation products in the region of previous metastases, consistent with treatment response. Multidisciplinary discussion of these biochemical and chest findings at an outside institution (where surgery was planned) led to suggestions that surgery to the mediastinal primary might be cancelled and second-line chemotherapy for refractory disease instituted instead. However, in view of evidence that surgery in such cases should proceed regardless of conventional tumour marker levels, unless they are genuinely rising [14], surgery was undertaken on d94 following further discussion between the two institutions. Complete resection of the primary mediastinal mass was achieved (Fig. 3B). Of note, pathological examination of the mediastinal resection specimen revealed complete necrosis only, with no evidence of viable disease. Serum HCG levels continued to fall slowly following surgery and finally normalised by d138 (Fig. 2A). Consequently, an expectant 'watch-and-wait' surveillance approach was adopted. The patient's HCG levels remained within the reference range and chest radiological findings gradually resolved (Fig. 3C/D); pulmonary metastases were no longer visible on chest X-ray performed over two years after the end-of-treatment (Fig. 3D). The patient remains well, 45 months from

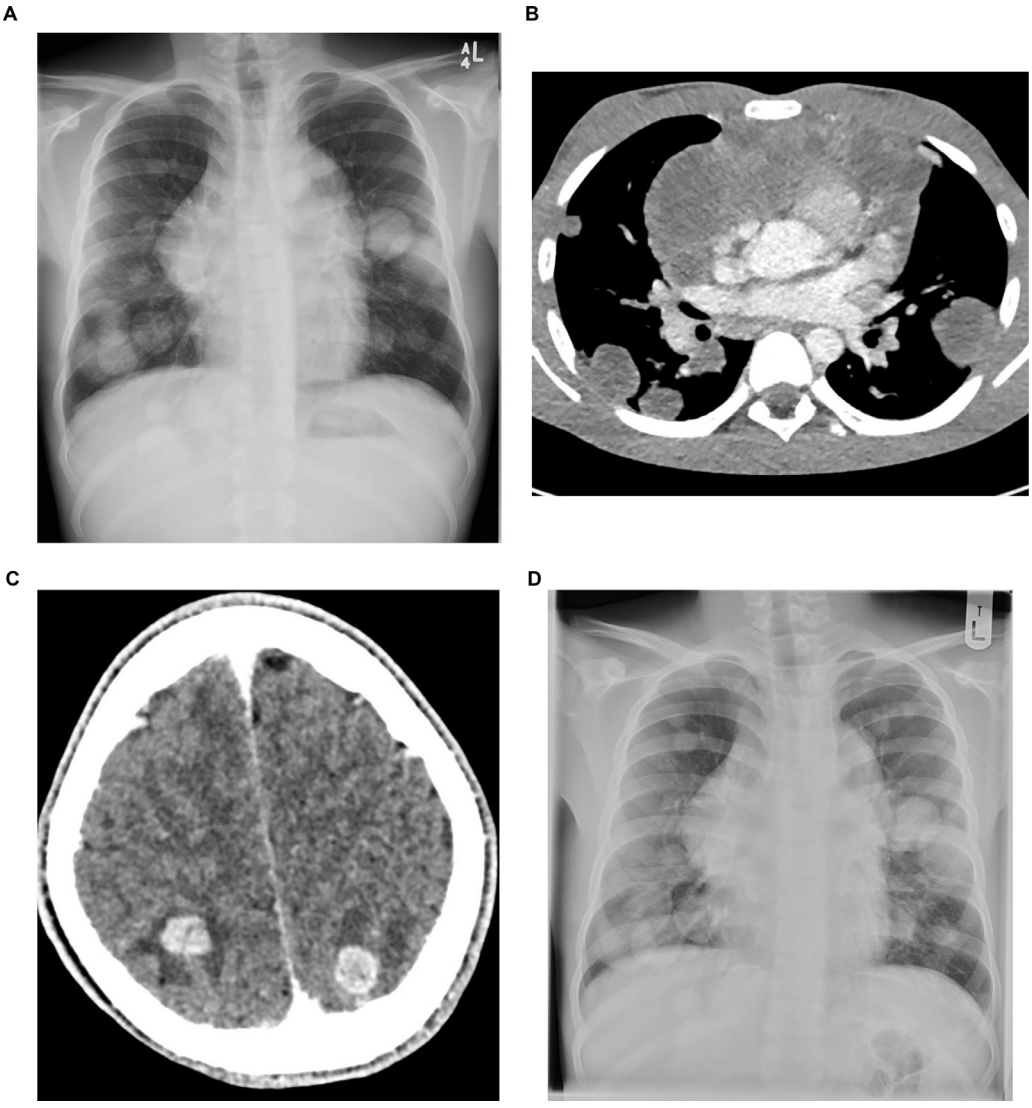

Fig. 1. Radiological imaging at the time of presentation of the patient with a metastatic primary mediastinal nonseminomatous germ-cell-tumour (PMNSGCT). A) Plain chest X-ray on the day of presentation showing mediastinal mass and multiple pulmonary metastases; B) Representative axial view of the CT chest scan confirming findings in A); C) Representative axial view of the CT head scan revealing cerebral metastases with surrounding vasogenic oedema; D) Plain chest X-ray on d3 of treatment showing an apparent increase in size of the pulmonary metastases.

initial presentation and 41 months following end of treatment, with an expected survival now in excess of 93% [37].

Materials and methods

Study rationale and serum samples analysed

Given the relatively long half-life of HCG, generally ~12–36 h [3,4], and the lack of correlation between radiological appearances and pathological response to chemotherapy in PMNSGCTs [14], we sought to identify circulating miRNAs that may offer a diagnostic and longitudinal profile more accurately reflecting disease activity, particularly at d82 when assessing the patient for primary mediastinal surgery, when the HCG was still elevated (Fig. 2A) and radiology showed persistent bilateral pulmonary metastases (Fig. 3A).

To this end, we undertook initial screening using comprehensive miRNA profiling of clinical serum samples in the index case at diagnosis and key subsequent time-points, leftover after routine clinical use, and compared results with serum samples from non-cancer control patients. Key findings were validated by targeted Taqman qRT-PCR in triplicate. The study received approval from the Cambridge Local-Research-Ethics-Committee (reference 01/128) and was performed with full informed parental consent. All experimental steps were MIQE compliant [38].

In total, we analysed 16 serum samples, namely 10 test samples from the index case and six controls. The comprehensive circulating miRNA profiling study (initial screening phase) comprised eight test samples; three separate individual samples (biological replicates) at original diagnosis (d0) and then the five time-points d28, d45, d66, d101 and d341. In addition, serum samples from a group of age-matched control subjects without tumours ($n = 4$) were utilised for comparison. For the targeted profiling study (validation phase), as well as the time-points above, two additional available serum samples from d17 and d181 time-points were analysed. Two of the three diagnostic (d0) test samples, and three of the four control samples, used in the comprehensive profiling study had sufficient serum available for the subsequent targeted profiling. These three existing controls were therefore supplemented by an additional two age-matched control samples for this validation phase.

Sample processing

As part of standard clinical care, blood was sampled in serum-separator tubes, before processing and centrifugation within four hours

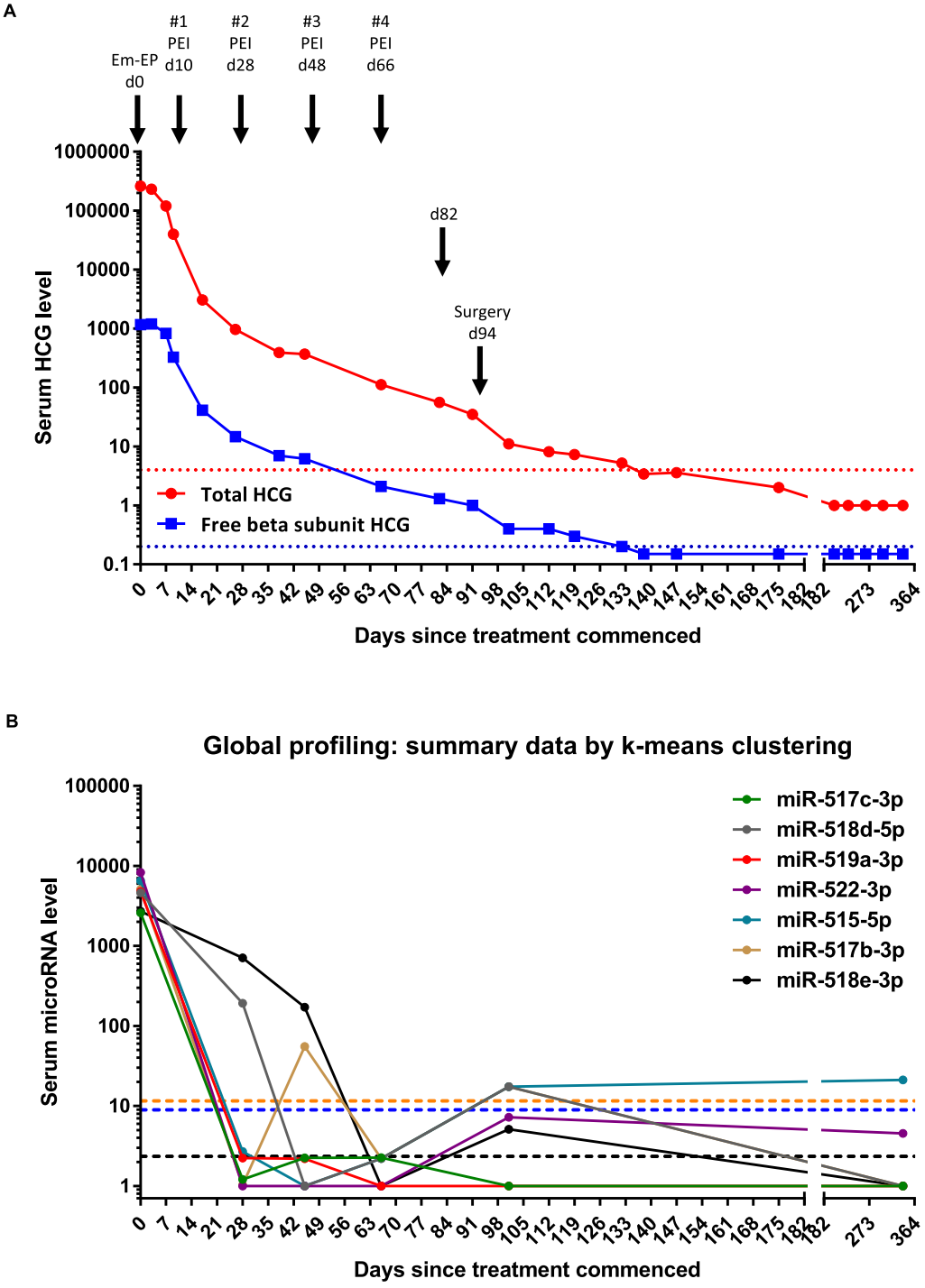

Fig. 2. Longitudinal circulating biomarker levels from diagnosis, through treatment and into follow-up (d341) for the patient with a metastatic PMNSGCT. A) Serial serum total and free beta-subunit human-chorionic-gonadotropin (HCG) levels shown. Horizontal dotted lines represent upper limit of reference range for total HCG (red) and free beta subunit HCG (blue) of HCG. For HCG values below the detection range (<2.0 for total and <0.2 for free beta subunit), arbitrary values of 1 and 0.15 were assigned, respectively. Key: Em-EP = emergency cisplatin-etoposide chemotherapy; PEI = cisplatin, etoposide and ifosfamide chemotherapy. B) Serial miRNA levels at time-points from the comprehensive profiling (screening phase) study. Data for the seven clustered C19MC miRNAs are shown. The first values represent the mean miRNA level obtained from analysis of the three independent serum samples taken at the time of original diagnosis (d0), assessed in singlicate. Subsequent levels are from time-points d28, d45, d66, d101 and d341, assessed in singlicate. All levels were normalised using the geometric mean approach. For miRNA values <1, an arbitrary value of 1 was assigned. Horizontal dotted lines represent maximum level (orange), mean+2SDs (blue), and mean level (black) of the control samples, across all seven C19MC miRNAs.

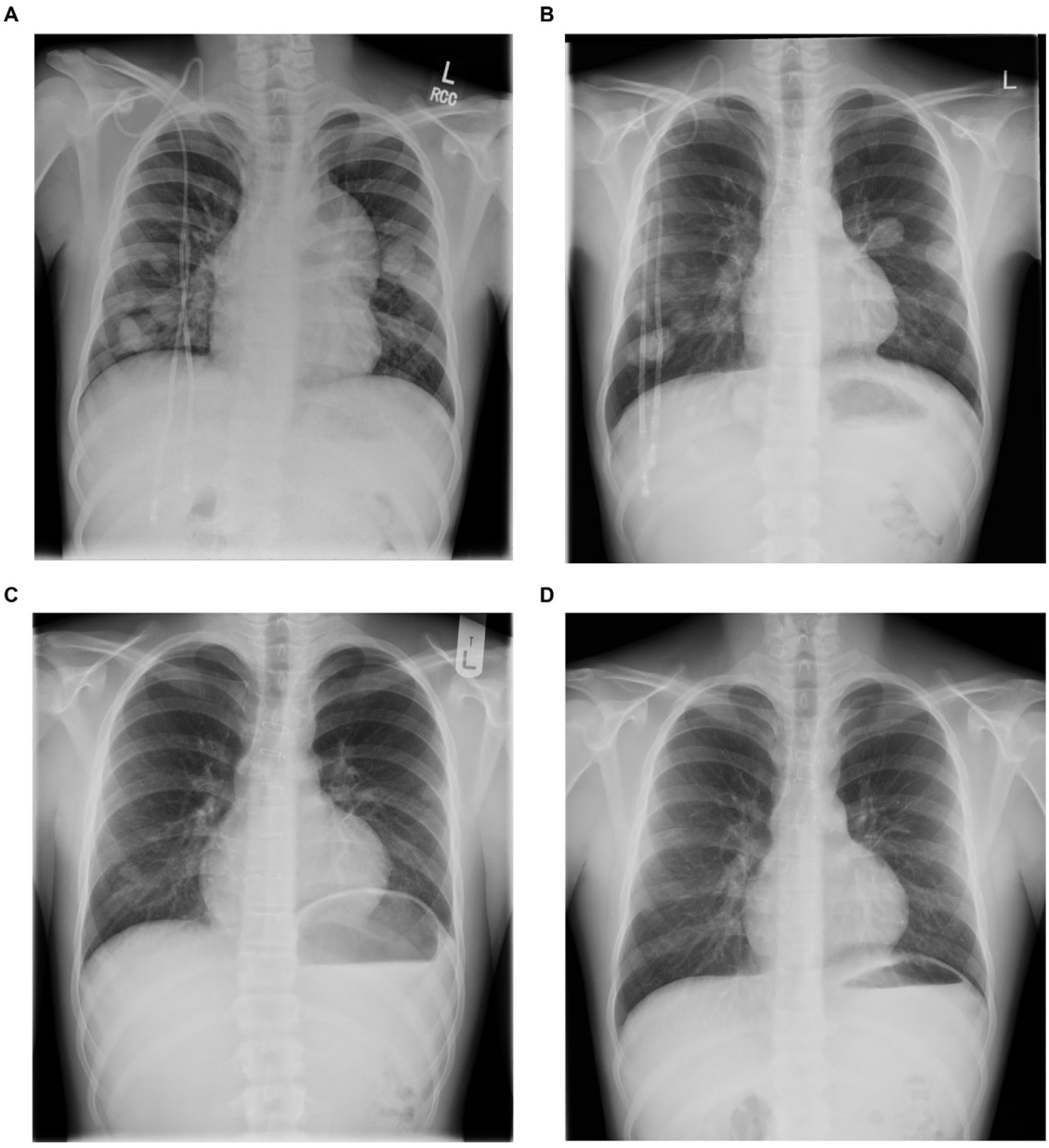

Fig. 3. Radiological imaging with plain chest X-rays in follow-up for the patient with a metastatic PMNSGCT. A) At the end of the four full courses of PEI/VIP chemotherapy (d82) revealing only modest reduction in the size of the mediastinal primary and the persistence of multiple pulmonary metastases; B) After surgery to the mediastinal primary on d94; C) Twelve months following end of treatment, showing gradual resolution of the pulmonary metastases, consistent with pathology of the mediastinal primary showing complete necrosis; D) 30 months from initial presentation and more than 27 months following end of treatment, finally showing complete resolution of the pulmonary metastases.

of receipt, as described [21]. After routine clinical measurements, samples were kept at 4 °C before residual serum was frozen and stored at −80 °C [21]. Only samples without macroscopic evidence of haemolysis were used [21,39], to avoid technical variations due to intracellular miRNA release from red-blood-cells [40].

RNA isolation and initial sample quality control

RNA from 200 µl of thawed serum was isolated using the Qiagen miRNeasy serum/plasma kit (catalogue number 217184), with addition of Sigma MS2-carrier-RNA to improve RNA yield [21,41]. In addition to a fixed quantity of exogenous non-human spike-in cel-miR-39–3p (Qiagen, catalogue number 219610), ath-miR-159a (Thermo-FisherScientific, catalogue number 4464066) was also added, as per best practice [4,38], to allow subsequent measurement of RNA extraction efficiency [21,41], as RNA concentration measurements are unreliable in RNA samples extracted from serum due to low abundance [42,43]. Total RNA was eluted from columns with either 15 µl or 100 µl [21] of nuclease-free-water, for comprehensive screening and targeted validation phases, respectively. Total RNA was stored at −80 °C until use. For samples eluted in 100 µl, quality control checks were performed by singleplex qRT-PCR for the exogenous (cel-miR-39–3p and ath-miR-159a) and endogenous (miR-30b-5p, miR-23a-3p and miR-451a) miRNAs, to exclude any technical issues or samples with substantial haemolysis, as described [21,41]. For samples eluted in 15 µl, 1 µl of RNA eluate was diluted to a final volume of 26 µl and then 5 µl of this mix was used as template for each individual reverse-transcription reaction.

Comprehensive circulating miRNA profiling – reverse-transcription step

Reverse-transcription (RT) was undertaken with 3 µl of total RNA for each sample using the Life-Technologies TaqMan microRNA Reverse-

Transcription Kit (catalogue number 4366596) using Life-Technologies Megaplex™-RT-Primers-Human-Pool-A (v2.1) or Pool-B (v3.0) (catalogue number 4444750, comprising both pools, overall comprising 754 unique miRNAs plus four control assays). The final volume was 7.5 μl for each reaction. For no-template-control (NTC) reactions, 3 μl of nuclease-free-water was used instead of RNA. Following a 5 min incubation on ice at 4 °C, samples underwent RT for 40 cycles on an MJ-Research-Peltier-Thermal-Cycler PTC-225 machine, comprising 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 second, and then 85 °C for 5 min to complete the reaction prior to a final hold at 4 °C. Complementary-DNA (cDNA) was then stored at −20 °C until further use.

Comprehensive circulating miRNA profiling – pre-amplification PCR step

Pre-amplification was undertaken with 5 μl of cDNA for each sample, as per the manufacturer's instructions. Pre-amplification primers for Human-Pool-A (v2.1) or Human-Pool-B (v3.0) were used (2.5 μl) with Life-Technologies TaqMan PreAmp-Master-Mix (catalogue number 4391128) (12.5 μl) and nuclease-free-water (5 μl), to make a final reaction volume of 25 μl. Following 5 min incubation on ice at 4 °C, samples underwent pre-amplification PCR on an MJ-Research-Peltier-Thermal-Cycler PTC-225 machine, comprising initial hold steps of 95 °C for 10 min, 55 °C for 2 min and 72 °C for 2 min prior to 15 cycles of 95 °C for 15 s and 60 °C for 4 min, before 100 °C hold for 10 min to complete the reaction prior to a final hold at 4 °C. The 25 μl of neat pre-amplification product was then stored at −20 °C until further use.

Comprehensive circulating miRNA profiling – final amplification PCR step

This was undertaken on two 384-well TaqMan Low-Density-Array A&B cards (catalogue number 4444913) with reaction volumes as per the manufacturer's instructions. In short, 9 μl of neat pre-amplified product (to maximise assay sensitivity) for each sample was added to 425 μl of TaqMan Universal-PCR-Master-Mix, No-AmpErase-UNG (Applied-Biosystems) and 416 μl of nuclease-free-water to make a final volume of 850 μl, and the cards were then loaded as per the manufacturer's instructions. Each card contained 377 unique assays (corresponding to 754 unique miRNAs across both cards, assessed in singlicate) and four controls, namely three candidate endogenous (U6 snRNA, RNU44, and RNU48) assays and one exogenous (ath-miR-159a) assay. PCR was carried out on an Applied-Biosystems 7900HT-Fast-Real-Time-PCR-System using a TaqMan®-Array-Micro-Fluidic-Card Thermal-Cycling-Block and associated standard PCR protocol.

Comprehensive circulating miRNA profiling – data analysis, normalisation and identification of biomarkers

All data analysis was conducted with the *R* statistical computing language using the *HTPCR, ggplot2* and *tidyr* packages. The data were first filtered to exclude any samples which failed quality control (none failed this step) and miRNAs which were undetectable or invariant (225, 29.8%). After comparing normalisation strategies, a geometric mean approach was employed for all downstream analysis. To find groups of co-expressed miRNAs using unbiased methodology, we employed k-means clustering with silhouette analysis [44]. Silhouette analysis for k between 2 and 40 yielded a maximum score at $k=7$; this value was used in the k-means clustering. We examined any clusters in detail to find those whose expression profile most closely matched the clinical course of the patient in order to identify new candidate biomarkers. This was achieved by using the mean expression value for each cluster to calculate the greatest decrease between diagnosis and subsequent time-points after treatment commenced. Cluster means were then selected which showed no significant increase after the initial decrease in expression. Next, raw Ct values were analysed to assess overall biomarker abundance; miRNAs within clusters with raw Ct values >30 at diagnosis were excluded to ensure maximal assay sensitivity for remaining candidates. Mean diagnostic expression levels (taken from the three biological replicates) were compared with mean expression levels from the control group to derive fold-changes. For the control group, mean, maximum, and mean plus two standard deviation (mean+2SD) levels were determined. The reference range was considered to be values up to the mean+2SD threshold.

Confirmatory targeted miRNA profiling – reverse-transcription step

We next undertook targeted assessment of selected miRNAs identified in the comprehensive discovery screening phase of the study in the full longitudinal panel of serum samples from the index case, along with miR-517a-3p [30]. These were assessed along with the endogenous housekeeping miRNA miR-30b-5p [17,21,24,39,45], and the exogenous spike-in miRNAs cel-miR-39-3p and ath-miR-159a using targeted qRT-PCR. Taqman assay-IDs for these primer/probes were as follows: miR-517a-3p (ID 002402), miR-517c-3p (001153), miR-519a-3p (002415), miR-515–5p (001112), miR-518d-5p (002389), miR-522-3p (002413), miR-30b-5p (000602), cel-miR-39–3p (000200) and ath-miR-159a (000338). In addition, miR-371a-3p (ID 002124) was also assessed in targeted profiling in diagnostic samples. We first performed a multiplexed RT step using the relevant TaqMan miRNA-specific stem-loop RT primers each diluted 1:50 with 1x Tris-EDTA buffer (pH 8.0) in an equal mixture of each relevant RT primer, adapted from [22], and using 5 μl of RNA eluate. The final volume of 15 μl for each reaction underwent RT, as described above for the comprehensive miRNA profiling phase above.

Confirmatory targeted miRNA profiling – pre-amplification PCR step

For the pre-amplification step, reactions were multiplexed using the relevant individual TaqMan assay probes, allowing greater assay sensitivity and increasing the number of target miRNAs measured from the small starting volume, as described [22]. Briefly, an equal mix of all 20x TaqMan assay probes was prepared for each reaction and diluted to 0.2x with 1x Tris-EDTA buffer (pH 8.0), as described [22]. The pre-amplification reaction contained 25 μl of 2x TaqMan PreAmp-Master-Mix, 12.5 μl of the diluted TaqMan assay probe mix and 12.5 μl of multiplexed cDNA product [22]. After heating to 95 °C for 10 min, 14 cycles of 95 °C for 15 s and 60 °C for 4 min were run on a MJ-Research-Peltier-Thermal-Cycler PTC-225 machine [22]. The resulting reaction products were diluted 1:10 with nuclease-free-water to make a final volume of 500μl, sufficient to perform all subsequent singleplex final miRNA PCR reactions in triplicate.

Confirmatory targeted miRNA profiling – final amplification PCR step

Nine μl of the diluted pre-amplified cDNA product was added to 10 μl of TaqMan 2x Universal-PCR-Master-Mix, No-AmpErase-UNG and 1 μl of each individual 20x TaqMan assay probe, thereby maintaining ratios identical to those previously described [22]. All reactions were performed in triplicate. PCR was performed using the following conditions: TaqMan activation (hold) 95 °C for 10 min, followed by 45 cycles of denaturation (95 °C 15 s) and annealing/extension (60 °C for 1 min) on a Realplex-Mastercycler. For both the targeted miRNA quantification steps, the Ct threshold was set manually to 2000 fluorescence units across all PCR plates, as described [21]. MiRNAs with results within two Ct values of the NTC sample were classed as non-expressing, as described [21]. Samples which gave no Ct value, or a Ct value of >40, were arbitrarily assigned a Ct value of 40. Mean diagnostic expression levels (taken from the two available biological replicates) were compared with mean expression levels from the control group to derive fold-changes. As for the comprehensive profiling study, mean, maximum, and mean+2SD levels were determined and the reference range was considered to be values up to the mean+2SD threshold.

Table 1
Candidate circulating miRNA biomarkers for the germ-cell-tumour subtype choriocarcinoma (CHC). The seven k-means clustered C19MC miRNA candidate biomarkers are shown, plus miR-517a-3p identified from literature review. Data shown include formal miRBase accession number, Taqman probe ID, mean raw Ct value from the comprehensive miRNA profiling study (mean of three biological replicates from diagnosis, each measured in singlicate), and fold-change relative to the mean expression levels of the control group, and those miRNAs selected for subsequent targeted qRT-PCR with confirmatory diagnostic fold-change (mean of two biological replicates from diagnosis, each measured in technical triplicate).

microRNA	Taqman probe ID	MIMAT accession number at miRBase (http://www.mirbase.org/)	Mean raw Ct value (global profiling)	Mean normalised fold-change vs. controls (global profiling)	Selected for targeted qRT-PCR profiling	Mean normalised fold-change vs. controls (targeted profiling)
miR-517c-3p	001153	MIMAT0002866	22.7	2588	Yes	1401
miR-519a-3p	002415	MIMAT0002869	22.4	4787	Yes	1569
miR-518d-5p	002389	MIMAT0002864	26.8	4603	Yes	1578
miR-515-5p	001112	MIMAT0002827	26.3	6527	Yes	1547
miR-522-3p	002413	MIMAT0002868	24.7	8289	Yes	1016
miR-517a-3p	002402	MIMAT0002852	22.8	4456	Yes	3540
miR-517b-3p	001152	MIMAT0002857	26.7	5112	No	N/A
miR-518e-3p	002395	MIMAT0002861	25.8	2700	No	N/A
miR-371a-3p	002124	MIMAT0000723	29.9	71.1	Diagnosis only	994

Results

In initial screening work on the comprehensive serum miRNA profiling dataset, all samples passed quality control. After exclusion of undetectable/invariant miRNAs (225 from 754; 29.8%) and normalisation strategy comparison, the geometric mean approach, using the remaining 529 miRNAs (70.2%) from the two 384-well TaqMan Low-Density-Array cards, was employed for all downstream analysis, as this was optimal. This number was very similar to the 568/741 miRNAs (76.7%) used for normalisation in our previous discovery microRNA profiling study [39]. In addition, normalisation using the established single endogenous miRNA miR-30b-5p [17,21,24,39,45] also gave very similar results, indicating its utility for subsequent targeted miRNA profiling.

To find groups of co-expressed miRNAs using unbiased methodology, we employed k-means clustering ($k = 7$ clusters after silhouette analysis) [44]. A single cluster most closely matched the clinical course of the patient. This cluster contained an initial 10 miRNAs; three were excluded as they had mean raw Ct values >30 (namely miR-302d-3p, probe ID 000535, mean raw Ct 37.7; miR-200a-5p, 001011, 36.7; and miR-630, 001563, 32.6). The remaining seven miRNAs in this single cluster were all derived from the C19MC cluster at chromosomal locus 19q13.41, namely miR-515–3p, miR-517b-3p, miR-517c-3p, miR-518d-3p, miR-518e-3p, miR-519a-3p and miR-522-3p (Table 1). These seven candidate miRNAs were highly elevated at the time of CHC diagnosis (Table 1, Fig. 2B and Supplementary-Fig.-S1). Following treatment, levels of five of these candidates, namely miR-517c-3p, miR-519a-3p, miR-518d-5p, miR-515-5p and miR-522-3p had fallen to, and/or remained within, the reference range by d28 and d45 of treatment, i.e. before/during the second full course of chemotherapy (Fig. 2B, Supplementary-Fig.-S1). In addition, miR-517a-3p, selected from literature review [30], also met this criterion (Supplementary-Fig.-S1). These six miRNAs were therefore selected for subsequent targeted profiling, in triplicate, following this comprehensive screening in singlicate. In addition, miR-371a-3p, an established universal serum miRNA biomarker in malignant-GCTs [17–24], had an elevated circulating level at diagnosis, although more modest than the fold-changes seen for the C19MC candidates (Table 1), and was consequently only selected for confirmation in diagnostic samples. We noted that two of the C19MC miRNAs selected for validation did show marginally elevated levels at subsequent time-points in this comprehensive screening phase, performed in singlicate (namely miR-518d-5p at d101 and miR-515-5p at d101/d341) (Fig. 2B, Supplementary Fig.-S1), hence the need for subsequent validation by triplicate qRT-PCR.

Further confirmation by targeted qRT-PCR across all available time-points was performed for the six selected C19MC miRNA candidates. This was an important next step, as targeted qRT-PCR was performed in technical triplicate for each miRNA, whereas on the profiling cards, each miRNA was only represented once, and hence more prone to variation in levels. Circulating levels of all six of these C19MC miRNAs were confirmed to be highly elevated at diagnosis compared with the mean of the control group (Table 1, Fig. 4). With the additional time-points, we could confirm that levels of these miRNAs had normalised prior to the start of the second course of chemotherapy (d17 and d28) and as a panel remained low subsequently (Fig. 4). Importantly, levels remained low at d82 (Fig. 4) when the patient was being assessed for primary mediastinal surgery, when the serum HCG was still elevated (Fig. 2A) and radiology showed persistent bilateral pulmonary metastases (Fig. 3A). In addition, we confirmed that miR-371a-3p showed elevated serum levels at diagnosis (Table 1).

Discussion

The clinical management of germ-cell-tumours (GCT) is complex due to their heterogeneity [1]. This includes both gonadal and extragonadal (often relatively inaccessible) disease, different histological subtypes, and risk-groups with differing outcomes [4]. A major current issue is the limited sensitivity and specificity of the current serum markers alpha-fetoprotein (AFP), human-chorionic-gonadotrophin (HCG) and lactate dehydrogenase (LDH), which are only positive at diagnosis in ~50% of cases [4]. Even where positive at diagnosis, a further limitation includes their long half-lives, making optimal management decisions for patients difficult.

Importantly, in this patient with a widely metastatic primary mediastinal nonseminomatous GCT (PMNSGCT) and diagnostic serum HCG level consistent with pure CHC, we identified a panel of C19MC miRNAs that were highly elevated at diagnosis. We also confirmed elevation of the established miRNA marker miR-371a-3p [17–24]. Levels of these C19MC miRNAs fell rapidly on treatment, normalising before the second full course of chemotherapy and which, if available in real-time, would have facilitated clinical management. Here, we show that circulating C19MC miRNA levels provided a much more accurate readout of a good early response to treatment than conventional markers/imaging. Consistent with the very short circulating half-life (<<12 h) [46] and excellent correlation with malignant-GCT disease activity reported for miR-371a-3p e.g. [17,21,22,24,47], we speculate that the successful and aggressive early treatment approach pursued here reduced the viable CHC tumour cells to such low numbers that circulating C19MC levels were within the reference range prior to the second full course of chemotherapy. Of note, the decay of C19MC miRNAs observed here appears even more rapid than for miR-371a-3p, which still showed a significant decrease between the second and third chemotherapy courses in malignant-GCT patients with stage 3 disease [17]. Furthermore, following completion of induction chemotherapy with four full courses of PEI/VIP, surgery to remove the mediastinal primary is recommended for

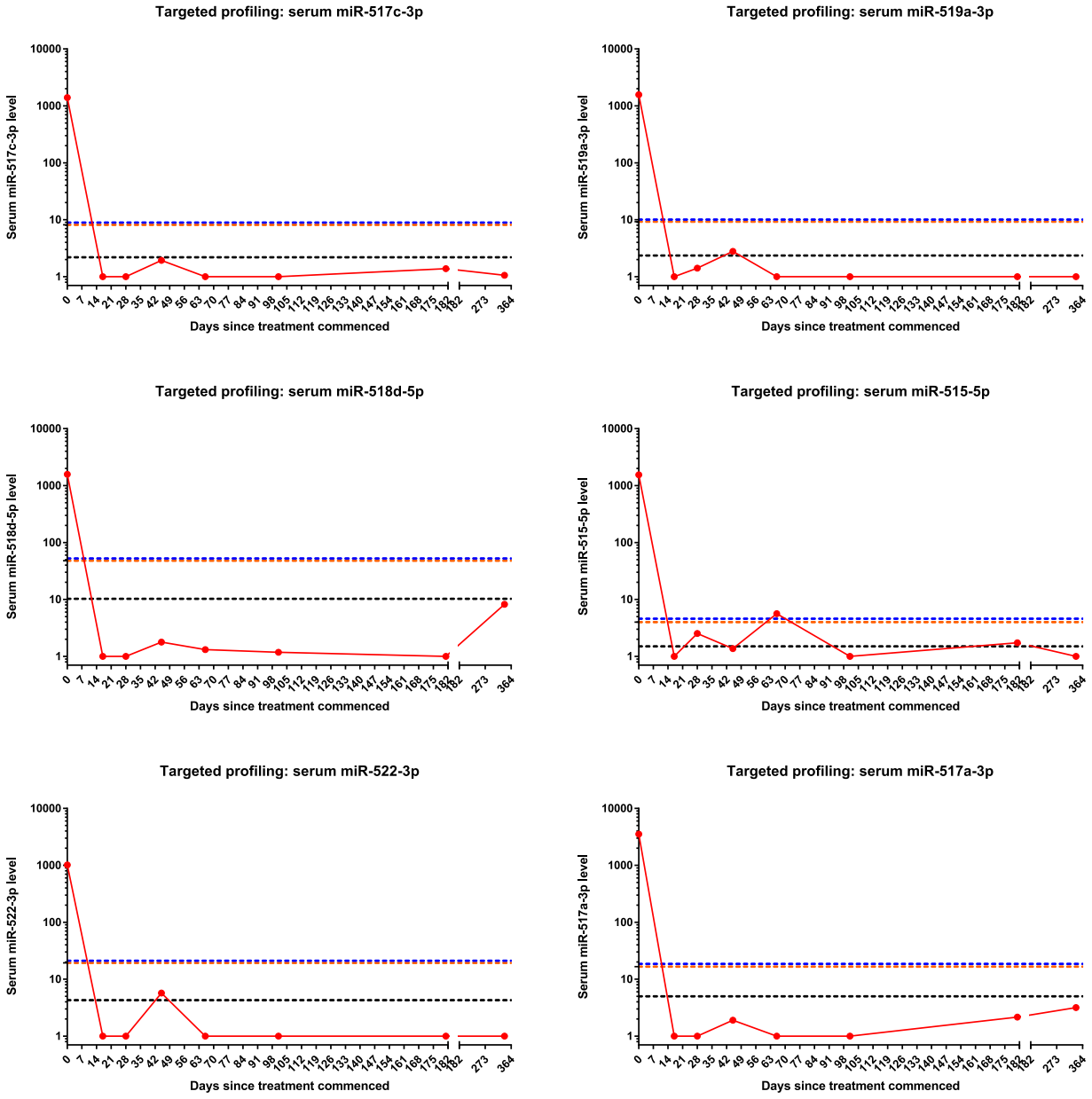

Fig. 4. Confirmation of targeted longitudinal circulating miRNA biomarker levels from diagnosis, through treatment and into follow-up for the patient with a metastatic PMNSGCT. Data shown for the six C19MC miRNAs identified in the comprehensive profiling study, namely miR-518d-5p, miR-519a-3p, miR-517c-3p, miR-515-5p and miR-522-3p and from literature review (miR-517a-3p). All levels were performed in technical triplicate and normalised to the established endogenous housekeeping miRNA miR-30b-5p. For miRNA values <1, an arbitrary value of 1 was assigned. Horizontal dotted lines represent mean+2SDs (blue), maximum level (orange) and mean level (black) of the control samples, for each individual miRNA.

patients with PMNSGCT to assist subsequent management decisions, but the prolonged half-life of AFP and HCG can make establishing the optimal timing of surgery difficult [14]. Here, when surgery was being considered at d82, the serum HCG level was still raised (Fig. 2A) and chest X-ray showed minimal changes from diagnosis (Fig. 3A). As a result, the outside surgical institution recommended that second-line chemotherapy for refractory disease be instituted. With our knowledge that surgery should proceed unless conventional markers were genuinely rising [14], our institution advocated strongly for surgery, which was performed on d94. This management is also consistent with a report which showed that <10% of patients who presented with HCG levels >50,000 kU/L (i.e. IGCCC poor-risk) had a normal HCG at the time of the fourth and final course of chemotherapy [48]. However, ~50% of those patients remained event-free despite no further chemotherapy, confirming that salvage therapy should only be initiated if and when there is HCG progression [48]. Such clinical decision-making would therefore have been facilitated by knowledge of the early normalisation of serum C19MC miRNAs. Consistent with these observations, pathology of the resected mediastinal mass showed complete necrosis. No further treatment has been necessary, and the patient now has an expected survival of >93% [37].

Previous study of circulating miRNAs for patients with the CHC malignant-GCT subtype has been exceptionally limited. No previous studies have assessed patients with pure CHC. One study quantified serum levels of the single C19MC miRNA miR-517a-3p, but only two patients in the cohort had a quantifiable CHC component, both with ≤30% involvement within a mixed malignant-GCT [30]. As exemplified here, our identified panel of C19MC miRNAs, plus miR-517a-3p

[30] and along with miR-371a-3p, offer potential advantages for monitoring patients with CHC, due to limitations of the accompanying elevated serum HCG levels, as well as for patients with embryonal carcinoma [30], who may be AFP/HCG marker negative. Future use of serum miRNA markers to optimise management for malignant-GCT patients [15], including those with PMNSGCTs [14] and/or pure CHC [9], will likely improve patient outcomes. Importantly, serum miRNAs are moving from research promise towards clinical reality for the management of patients with malignant-GCTs [15]. Of note, despite extensive searches, there were no suitable publicly available datasets to extend the observations described here at this stage. Hence, future work will be needed to confirm the utility of our observations.

In addition to the biomarker potential of these miRNAs [15], they have a functional role in malignant GCT development and progression. Selective activation and expression of C19MC miRNAs in the nonseminomatous malignant-GCT subtypes embryonal carcinoma and CHC is likely to confer additional clinical aggressiveness to these tumours when compared with their seminomatous counterparts, over and above that provided by universal miR-371~373 and miR-302/367 overexpression [1]. MiRNAs regulate protein-coding gene expression through binding to the 3′ untranslated region (3′UTR) of messenger RNAs (mRNAs), determined primarily by the 'seed' region at nucleotide positions 2–7 (2–7 nt). Intriguingly, many C19MC miRNAs contain the same 2–7 nt seed region, namely 'AAGUGC', as miRNAs from the miR-371~373 and miR-302/367 clusters, and therefore have overlapping mRNA targets. 'AAGUGC' seed-containing miRNAs allow cells to continue unchecked through the cell cycle, resulting in rapid cellular proliferation and a selective growth advantage [49]. Altered C19MC expression has been described in other tumours to date [25] and C19MC miRNAs may also act through negative regulation of apoptosis [50]. Of note, expression levels of miR-371~373 and C19MC miRNAs in malignant-GCT cells have been shown to substantially increase upon development of cisplatin resistance, further demonstrating a key functional role for these short non-coding RNAs [51]. The dysregulated expression of miRNAs observed in malignant-GCTs could therefore potentially be targeted therapeutically, in order to ultimately improve patient outcomes with poor-risk disease [52].

In summary, we describe a patient with a widely metastatic PMNSGCT and findings consistent with a pure CHC, for whom associated clinical challenges are described. We identify a panel of C19MC miRNAs, as well as miR-371a-3p, which were elevated at diagnosis. We show that levels of the C19MC miRNAs fell rapidly on treatment and normalised early during chemotherapy treatment, reflecting CHC disease activity more accurately than HCG. Following further study, real-time knowledge of these miRNA markers will facilitate future management of such patients and are likely to result in improved clinical outcomes.

CRediT authorship contribution statement

Matthew J. Murray: Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing, Funding acquisition. **Stephen Smith:** Data curation, Writing - original draft, Writing - review & editing, Funding acquisition. **Dawn Ward:** Data curation, Writing - review & editing. **Lorena Verduci:** Data curation, Writing - review & editing. **James C. Nicholson:** Conceptualization, Writing - original draft, Writing - review & editing. **Cinzia G. Scarpini:** Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Nicholas Coleman:** Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2020.100904.

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