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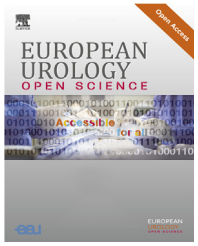

European Association of Urology

Testis Cancer

Perioperative Serum MicroRNA 371a-3p and 372-3p Levels in Patients with Clinically Localized Testicular Masses

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Abstract

Background: MicroRNAs (miRNAs) show promise as blood-based tumor markers for germ cell tumors (GCTs), with miRNA-371-3p being the most studied. The marginal benefit of including other candidate miRNAs to aid with the management of testicular GCTs remains unclear.
Objective: To assess the performance of our combined miRNA assay (371a-3p and 372-3p) in patients with clinically localized testicular masses.
Design, setting, and participants: This was a retrospective review of patients prospectively enrolled in an ongoing protocol collecting serum miR-371a-3p and miR-372-3p levels (together, Memorial Sloan Kettering Cancer Center [MSK] miRNA assay [MMA]) in patients with a suspected or diagnosed testicular GCT.
Outcome measurements and statistical analysis: The coprimary outcomes of interest were sensitivity and specificity of miR-371a-3p and 372-3p, individually and together, to detect nonteratomatous GCTs in the orchiectomy specimen. Secondary outcomes included additional assay diagnostic parameters, the relationship of patient and disease factors with variations in miRNA levels, and temporal patterns of miRNA normalization after orchiectomy.
Results and limitations: Sixty-two patients were included, 52 had a viable GCT at orchiectomy, and ten had no cancer or a non-GCT. Forty-six patients with a GCT had positive preorchiectomy MMA (sensitivity 88.5% [95% confidence interval {CI}: 79.8, 97.2]), and one patient had positive preorchiectomy MMA but no GCT (specificity 90.0% [95% CI: 71.4, 100]). The diagnostic performance of miR-371a-3-p and miR-372-3p was similar. The time for miRNA to decrease to undetectable levels varied, with some patients having positive levels up to 3 wk after orchiectomy.

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Conclusions: The biomarkers miR-371a-3p and miR-372-3p demonstrated high sensitivity and specificity for localized testicular GCTs, but causes of variation in relative miRNA levels and time to normalization for individual patients remain unclear.
Patient summary: We studied the ability of the blood-based biomarkers miR-371a-3p and miR-372-3p to detect testicular cancer (germ cell tumors) in patients with small testicular masses. We found that together and individually these were sensitive and specific for testicular cancer.
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1. Introduction

MicroRNAs (miRNAs) are short noncoding RNAs involved in the regulation of gene expression [1–3]. MicroRNAs can play a role in carcinogenesis by modulating gene expression, resulting in cell proliferation and resistance to apoptosis [4]. Overexpression of several miRNA clusters, most notably miR-367 and miR-371–373, has been identified in germ cell tumors (GCTs), and expanding evidence has demonstrated that circulating miRNAs are both sensitive and specific in detecting GCTs. Traditional serum tumor markers (STMs), β-human chorionic gonadotropin (β-HCG), α-fetoprotein (AFP), and lactate dehydrogenase (LDH) are essential to the management of GCTs [5,6]. However, elevation in these is not universal among patients with disease, with only 50% of GCTs producing elevated STMs [7–10]. Owing to their high sensitivity and specificity, circulating miRNAs are poised to overcome these shortcomings.

Prior studies have largely focused on miR-371a-3p and its ability to detect GCTs across stages of disease with high sensitivity and specificity [11–14]. Other candidate miRNAs are less studied, and the diagnostic potential of these as independent or combination assays is not well known. Additionally, the widespread adoption of miRNAs in testicular cancer management will require consensus regarding interpretation and standardized thresholds for abnormal results. Understanding how test characteristics are affected by disease and patient characteristics is necessary to determine how miRNA values vary between patients and how miRNA perform in discrete clinical situations.

Our previously described combination assay (miR-371a-3p and miR-372-3p, collectively Memorial Sloan Kettering Cancer Center [MSK] miRNA assay [MMA]) was created based on the prevailing performance characteristics of miR-371a-3p and the potential benefit of miR-372-3p. The extent literature suggests that miR-371a-3p alone demonstrates lower sensitivity in the detection of relapse; miR-372-3p has been shown to be markedly elevated in pediatric yolk sac tumors and has a longer half-life, and therefore was selected for inclusion in our assay [15]. We sought to study diagnostic test characteristics of our combined assay in patients with localized testicular masses to determine possible marginal benefit of this approach, to determine what patient and tumor factors may influence levels of these markers, and to explore postorchiectomy kinetics.

2. Patients and methods

2.1. Data sources and patients

All patients with suspected or known GCTs treated at MSK are eligible to participate in an institutional review board–approved ongoing prospective diagnostic protocol assessing MMA levels throughout diagnosis and treatment. Recruitment and enrollment for this cohort of patients occurred between May 28, 2021 and February 1, 2024. We retrospectively identified 62 patients with testicular masses concerning for GCTs who were planned for radical or partial orchiectomy and had, at minimum, one presurgical MMA drawn. All patients had clinically localized disease. Patients were staged with a computed tomography (CT) scan of the chest, abdomen, and pelvis with contrast prior to or immediately after radical orchiectomy, and STMs are trended after orchiectomy until normalization.

2.2. Measures and outcomes

Patient demographics included age and race/ethnicity. Patient comorbidity measures included body mass index (BMI), estimated glomerular filtration rate (eGFR), liver function (aspartate aminotransferase and alanine aminotransferase levels), and male hormonal function (morning free/total testosterone, follicle-stimulating hormone, luteinizing hormone, estradiol, and sex hormone binding globulin). Tumor size, histologic components, pT stage, lymphovascular invasion (LVI), rete testis invasion (RT+), and hilar soft tissue invasion were abstracted from the synoptic pathology report. All patients underwent surgery at MSK, and pathology was reviewed by our genitourinary pathologists.

Our coprimary outcomes of interest were sensitivity and specificity of each miRNA separately and together compared with the gold standard, which was the presence of viable nonteratomatous GCTs in the orchiectomy specimen. Additional secondary outcomes included the correlation between individual miRNA levels (both relative and C_T values) and patient and histology factors. We also assessed

temporal trends in miRNA value normalization after orchiectomy as an exploratory outcome.

2.3. Specimen processing, RNA extraction, and stem-loop quantitative reverse transcription polymerase chain reaction (TaqMan assay)

Specific miRNA levels were determined using stem-loop quantitative reverse transcription polymerase chain reaction, as described previously [15]. Briefly, extracted total RNA was reverse transcribed into cDNA using the TaqMan microRNA Reverse Transcription Kit (Cat #4355696; Thermo Fisher Scientific, Waltham, MA, USA) in singleplex. TaqMan assays were used for preamplification and quantification of miRNAs. Relative quantification of miRNAs was calculated using the comparative C_T method ($2^{\Delta\Delta C}{}_T$), normalizing with cel-miR-39-3p and miR-30b-5p acting, respectively, as exogenous and endogenous controls, and additionally normalizing with a previously described cohort of healthy volunteers used as the reference value [15]. Indeterminate ranges were created by applying the mean CV% for intra-assay variability to the established relative expression threshold for a positive result.

2.4. MicroRNA result Interpretation

The MMA result was determined using miR-371a-3p (<0.5 negative, ≥0.5–<0.75 indeterminate, and ≥0.75 positive) and miR-372-3p (<1.0 negative, ≥1.0–<1.5 indeterminate, and ≥1.5 positive) relative values according to the methods published previously [15]. In the case of an indeterminate relative value for either miRNA, C_T values were used to adjudicate MMA positivity (for miR-371a-3p [≥34 negative and <34 positive] and miR-372-3p [≥30 negative and <30 positive]). The overall MMA result was designated as positive if either miR-371a-3p or miR-372-3p was positive.

2.5. Statistical analysis

Baseline patient characteristics were summarized using descriptive statistics. GCT disease characteristics were summarized for patients with viable disease. Sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values were calculated with 95% exact confidence intervals (CIs) for preorchiectomy MMA relative to the presence of viable nonteratomatous GCTs. Receiver operating characteristic curves were generated, and the areas under the curve (AUCs) were calculated for miR-371a-3p and miR-372-3p

Table 1 – Baseline patient and disease characteristics

	Viable NT-GCT (*n* = 52)	No viable NT-GCT[c] (*n* = 10)
Baseline characteristics		
Age (yr), median (range)	35 (25, 57)	30 (18, 73)
Body mass index, median (range)	27.3 (20.1, 45.3)	23.2 (19.3, 36.3)
Preorchiectomy serum tumor markers		
AFP [a] (ng/ml), median (range)	3.4 (1.2, 2703)	3.6 (2.0, 7.5)
AFP [a] elevated (>15), *n* (%)	6 (12)	0
HCG (U/l), median (range)	1.4 (<0.5, 836)	<0.5 (<0.5, 5.3)
HCG elevated (>2.2), *n* (%)	18 (35)	1 (10)
LDH (U/l), median (range)	178 (132, 653)	153 (132, 235)
LDH elevated (>246), *n* (%)	9 (17)	0
Any serum tumor marker elevated, *n* (%)	24 (46)	1 (10)
Preorchiectomy laboratory values, median (range), *N*		
Estimated glomerular filtration rate (ml/min/1.73 m^2)	102 (66, 125), 50	121 (79, 129), 8
Alanine transaminase (U/l; ≤55)	25 (<0.1, 88), 45	19.0 (<0.1, 39), 7
Aspartate aminotransferase (U/l; ≤37)	22 (13, 92), 45	17 (15, 58), 7
Testosterone (ng/dl; 221–716)	524 (264, 2160), 26	573 (283, 660), 8
Free testosterone (ng/d; 4.7–24.4)	14.6 (7.2, 52.8), 20	16.3 (8.1, 140.0), 8
Follicle-stimulating hormone (mU/ml; 0.9–12.0)	5.2 (0.0, 77.2), 21	4.0 (0.8, 15.8), 7
Luteinizing hormone (mU/ml; 2.0–9.0)	3.1 (0.0, 16.3), 21	2.6 (1.6, 4.6), 7
Estradiol (pg/ml; 10–40)	18 (12. 52), 19	19 (10, 81), 7
Sex hormone binding globulin (nM/l; 13.3–89.5)	32.1 (12.4, 83.8), 18	31.1 (14.2, 70.4), 8
GCT disease characteristics		
Histologic type, *n* (%)		
Pure seminoma	37 (71)	–
Mixed or nonseminoma [b]	15 (29)	–
Seminoma present	12 (80)	–
Embryonal carcinoma present	11 (73)	–
Yolk sac present	9 (60)	–
Teratoma present	6 (40)	–
T stage, *n* (%)		
pT1	41 (79)	–
pT2	11 (21)	–
Lymphovascular invasion, *n* (%)	11 (21)	–
Rete testis invasion, *n* (%)	34 (65)	–
Hilar soft tissue invasion, *n* (%)	7 (13)	–
Tumor size (cm), median (range)	3.7 (0.2, 10.3)	–

AFP = α-fetoprotein; GCT = germ cell tumor; HCG = human chorionic gonadotropin; LDH = lactate dehydrogenase; NT-GCT = nonteratomatous germ cell tumor.
[a] Pre-orchiectomy value was not available for one patient.
[b] Nonseminoma includes two pure embryonal carcinoma, one pure yolk sac, and others with mixed histology.
[c] Non-GCT histology included two patients with epidermoid cysts, four patients with Leydig or sex cord stromal tumors, two patients with regressed/burnt out tumors, one patient with an intratesticular hematoma, and one patient with a dedifferentiated liposarcoma.

Table 2 – Preorchiectomy MMA result and STM elevation for the detection of viable GCT at orchiectomy [a]

MSK miRNA assay				Serum tumor markers			
MMA	Viable GCT			STM	Viable GCT		
	Positive	Negative	Total		Positive	Negative	Total
Positive	46	1	47	Elevated	24	1	25
Negative	6	9	15	Not elevated	28	9	37
Total	52	10	62	Total	52	10	62
Sensitivity	88.5% (95% CI: 76.6, 95.7)			Sensitivity	46.2% (95% CI: 32.2, 60.5)		
Specificity	90.0% (95% CI: 55.5, 99.8)			Specificity	90.0% (95% CI: 55.5, 99.8)		
PPV	97.9% (95% CI: 88.7, 100)			PPV	96.0% (95% CI: 79.7, 99.9)		
NPV	60.0% (95% CI: 32.3, 83.7)			NPV	24.3% (95% CI: 11.8, 41.2)		
AUC (95% CI)				AUC (95% CI)			
miR-371a-3p relative	0.94 (95% CI: 0.88, 1.00)			AFP	0.50 (95% CI: 0.33, 0.67)		
miR-371a-3p C_T	0.94 (95% CI: 0.88, 0.99)			HCG	0.71 (95% CI: 0.57, 0.85)		
miR-372-3p relative	0.90 (95% CI: 0.82, 0.98)			LDH	0.70 (95% CI: 0.49, 0.91)		
miR-372-3p C_T	0.90 (95% CI: 0.82, 0.98)						

AFP = α-fetoprotein; AUC = area under the curve; CI = confidence interval; GCT = germ cell tumor; HCG = human chorionic gonadotropin; LDH = lactate dehydrogenase; miRNA = microRNA; MMA = MSK miRNA assay; MSK = Memorial Sloan Kettering Cancer Center; NPV = negative predictive value; PPV = positive predictive value; STM = serum tumor marker.

[a] Preoperative MMA results and preoperative STMs were compared with final pathology from orchiectomy. Sensitivity, specificity, PPV, and NPV are shown for the combined MMA assay and for all STMs together. Individual AUCs are shown for each individual miRNA and for each individual STM.

relative expression and C_T values, and for preorchiectomy STMs. Undetectable results were assigned the maximum Ct value (40) and the corresponding relative values were assigned a value of zero. For visualization of relative values on the log scale, zero was substituted with a relative value 1 order of magnitude smaller than any of those observed (0.001). Associations between preorchiectomy miR-371a-3p and miR-372-3p relative expression and C_T values with patient and GCT disease characteristics were assessed using the Spearman correlation coefficient and Wilcoxon rank-sum test. Pre- and postorchiectomy miRNA levels were plotted over time in discrete weekly intervals after the procedure. All tests were evaluated for statistical significance at an alpha level of 0.05. A statistical analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC, USA). Study results were reported according to the Standards for Reporting Diagnostic accuracy studies (STARD) guidelines [16].

3. Results

3.1. Cohort description

Sixty-two patients met the criteria and were included in this study. Sixty patients underwent radical orchiectomy and two patients underwent partial orchiectomy. Of these patients, 52 had viable nonteratomatous GCTs and ten had benign or non-GCT pathology. Both patients who underwent partial orchiectomy had benign disease and no germ cell neoplasia in situ (GCNIS) on peripheral testis biopsy. Thirty-seven patients (71%) had pure seminoma, while 15 patients (29%) had nonseminoma or mixed GCT. Most tumors (79%) were pT1; the remainder were pT2 (21%). The median tumor size was 3.7 cm. Baseline patient and tumor characteristics are shown in Table 1. Non-GCT pathology included dedifferentiated liposarcoma (n = 1), regressed/burnt out tumor(s) without GCNIS (n = 2), hematoma (n = 1), Leydig cell tumor (n = 4), and epidermoid cyst (n = 2).

3.2. Combination miR-371a-3p and miR-372-3p results

Forty-six patients with viable nonteratomatous GCTs had positive preorchiectomy MMA results (sensitivity 88.5%, 95% CI: 79.8, 97.2; Table 2). Six patients had false negative MMA results (five had pure seminoma measuring 0.2–2.8 cm, the sixth had a 1-cm tumor comprising >90% teratoma and remainder seminoma). One of ten patients without a GCT had positive preorchiectomy MMA (specificity 90.0%, 95% CI: 71.4, 100). This patient had a 0.6-cm Leydig cell tumor without peripheral GCNIS. The PPV of MMA was 97.9% (95% CI: 93.8, 100) and NPV was 60% (95% CI: 35.2, 84.8; Table 2).

3.3. Individual performance of miR-371a-3p and miR-372-3p

Individually, miR-371a-3p demonstrated sensitivity of 86.5% (95% CI: 77.3, 95.8), specificity of 100% (95% CI: NA, NA), PPV of 100% (95% CI: NA, NA), and NPV of 58.9% (95% CI: 35.4, 82.2) while miR-372-3p demonstrated sensitivity of 76.5% (95% CI: 64.8, 88.1), specificity of 90.0% (95% CI: 71.4, 100), PPV of 97.5% (95% CI: 92.7, 100), and NPV of 42.9% (95% CI: 21.7, 64.0; Supplementary Table 1). The AUCs of miR-371a-3p and miR-372-3p were, respectively, 0.94 (95% CI: 0.88, 1.00) and 0.90 (95% CI: 0.82, 0.98) for the presence of viable nonteratomatous GCTs at orchiectomy (Fig. 1). No patients had an indeterminate miR-371a-3p value. One patient found to have a 6-mm Leydig cell tumor had an indeterminate miR-372-3p relative value of 1.11. This was adjudicated by the miR-372-3p C_T value of 29.27, resulting in the study's lone false positive.

3.4. Variation in miRNA levels before orchiectomy

There was a wide range of preorchiectomy miRNA-371a-3p and miR-372-3p relative levels (Fig. 2) despite all patients having localized disease. Relative miR-371a-3p expression among patients with positive results in our cohort ranged

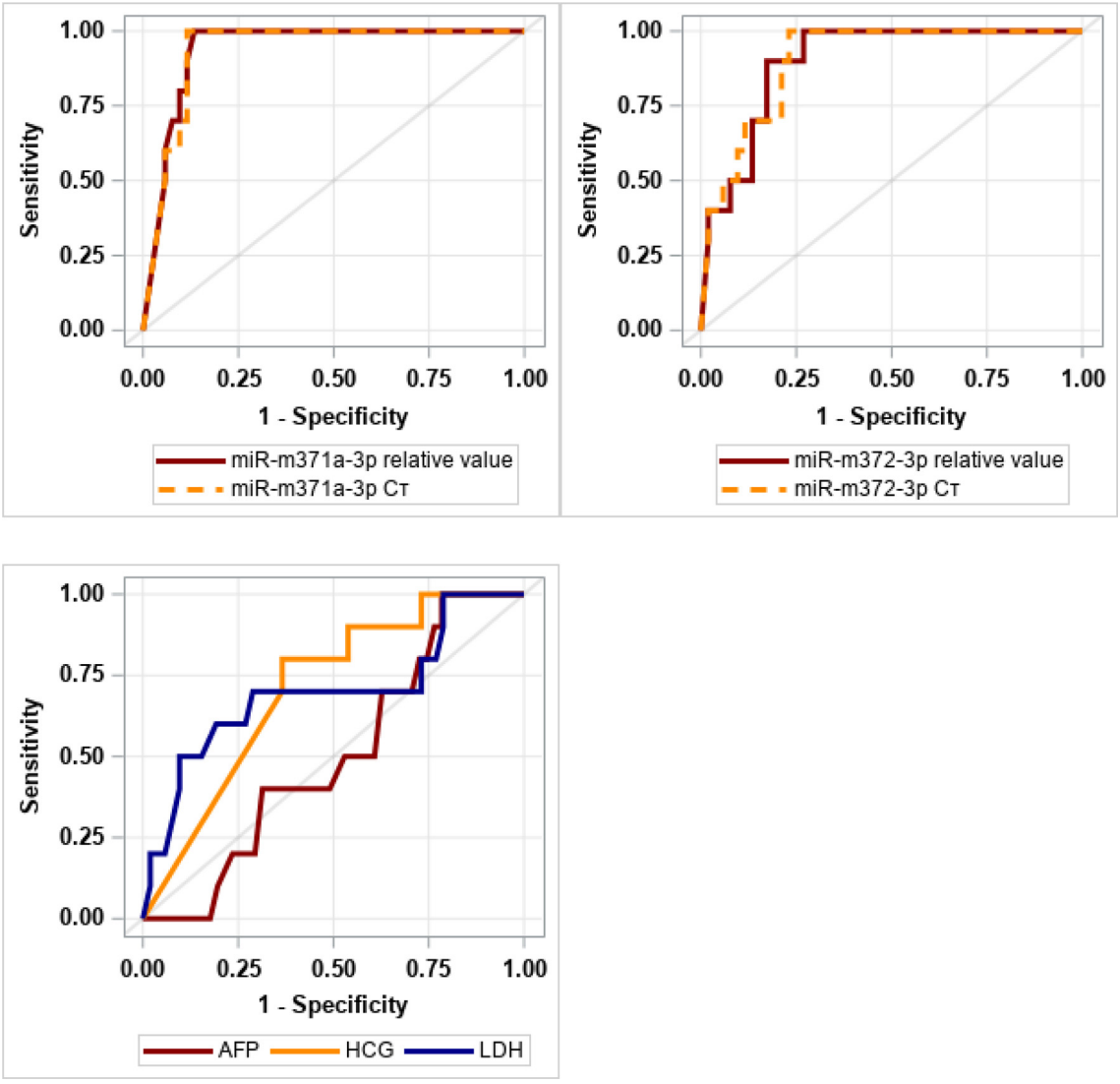

Fig. 1 – Receiver operating curves for miR-371a-3p, miR-372-3p, and traditional serum tumors markers (AFP, HCG, and LDH) to detect viable GCT. AFP = α-fetoprotein; GCT = germ cell tumor; HCG = human chorionic gonadotropin; LDH = lactate dehydrogenase.

from 0.49 to 16 337.62. Preorchiectomy levels of both miR-371a-3p and miR-372-3p were positively correlated with tumor size (Spearman coefficient ρ = 0.64 and 0.68, respectively; $p < 0.001$), but levels were not statistically significantly different by tumor histology (seminoma vs nonseminoma), pT stage, or presence of LVI, rete testis, or hilar soft tissue invasion (Supplementary Table 2). Preorchiectomy levels of both miR-371a-3p and miR-372-3p were correlated with preorchiectomy β-HCG (ρ = 0.53 and 0.58, respectively; $p < 0.001$) and LDH levels (ρ = 0.53 and 0.56, respectively; $p < 0.001$). Neither was correlated with AFP levels in patients with nonseminoma histology (Supplementary Table 3). No examined patient characteristics, including age, BMI, or preorchiectomy laboratory values (eGFR, liver function tests, and endocrine function panel) were correlated with preorchiectomy miR-371a-3p or miR-372-3p levels (Supplementary Table 3).

3.5. *Postorchiectomy kinetics*

Thirty-seven patients had at least one postorchiectomy MMA within 3 wk of surgery (Fig. 3). Among the 19 patients with normalized postorchiectomy values, none subsequently relapsed with a median of 7.2 mo of follow-up (range: 1.9, 18.8). Of the 18 patients with at least one positive 3-wk postorchiectomy MMA value, seven (39%) experienced a median time to relapse of 2.5 (range: 1.0, 8.3) mo, and the median follow-up for those who did not relapse was 10.0 (range: 0.9, 17.2) mo.

4. Discussion

In this study of 62 patients with a suspected localized testicular GCT, miR-371a-3p and miR-372-3p levels reliably identified viable nonteratomatous GCTs from non-GCT tes-

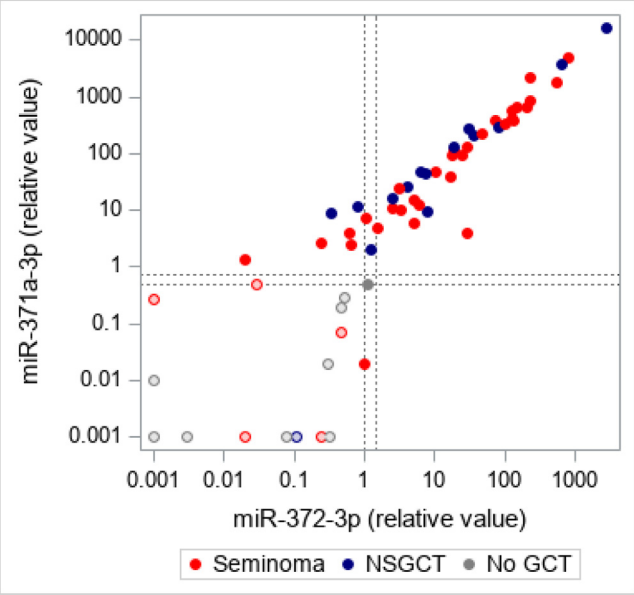

Fig. 2 – Preorchiectomy m371a versus m372 relative values by orchiectomy histology type. Reference (dashed) lines for the determination of result for m371a (<0.5 negative, 0.5–0.75 indeterminate, >0.75 positive) and m372 (<1.0 negative, 1.0–1.5 indeterminate, >1.5 positive) relative values. Relative values are shown on the log scale. Solid points were determined to be MMA positive and transparent points MMA negative. Spearman correlation between preorchiectomy m371a and m372 relative values is 0.995 (p < 0.001). GCT = germ cell tumor; MMA = MSK microRNA assay; MSK = Memorial Sloan Kettering Cancer Center; NSGCT = nonseminomatous GCT.

ticular masses with high sensitivity and specificity, both as a combined assay (MMA) and separately. Aside from tumor size, serum miR-371a-3p and miR-372-3p levels did not correlate with studied patient or disease factors such as BMI or hepatic, renal, or endocrine function. In addition, we observed a wide range of pretreatment miRNA levels despite all patients having clinically localized GCTs. Finally, we also demonstrate the potential for a longer than expected time to normalization in patients after orchiectomy, which challenges the concept of short half-lives for all miRNA clusters.

The ideal management of small (<2 cm) or incidentally detected nonpalpable testicular masses remains controversial. Some have advocated for surveillance or organ-sparing surgery in patients with small masses or those with a history of hormone disorders, since the proportion of patients found to have testicular GCTs is lower than in those with larger masses [17,18]. Our combined assay resulted in six false negatives; five occurred in patients with small pure seminoma (0.2–2.8 cm). The sixth occurred in a patient with a 1-cm mixed GCT comprising mostly (>90%) teratoma and seminoma. Patients with these masses stand to gain the most from sensitive biomarkers as they often present with equivocal physical examination, imaging, and STMs. While this limitation may potentially be addressed by lowering the threshold value necessary for a positive result, any potential benefit in sensitivity may be offset by a higher false positive rate.

Similarly, the use of a combined assay such as MMA may increase sensitivity over that of miR-371a-3p alone. The addition of miR-372-3p identified an additional patient with a 2.6-cm seminoma, which also resulted in the assay's single false positive—a patient with a 6-mm Leydig cell tumor. In previous studies, Leydig cell tumors have not resulted in elevated miR-371a-3p [11,13,19]. Given their unclear malignant potential and metabolic activity, the treatment for Leydig cell tumors is often excision; therefore, the clinical implications of a false positive result are debatable. While it appears that the marginal benefit conferred by the addition of mi-372-3p in localized disease is minimal, it remains possible that a combination assay may have a potential diagnostic benefit in the nonlocalized setting, which should be studied. These should be weighed against the costs, time, and possible decreased interpretability of a combined assay.

Interpretation of STM levels are sometimes limited by non-GCT causes including hepatic dysfunction and endocrine or metabolic causes [7–9,20,21]. We sought to assess whether patient factors such as age, BMI, and hepatic, renal, and endocrine function were correlated with preoperative miRNA levels. In our cohort, serum miR-371a-3p and miR-372-3p expression levels were not correlated with age, BMI, or preoperative hepatic, renal, or endocrine function, suggesting that normal serum miRNA levels may be generalizable across patients. Our assessment of these relationships is limited by a nonexhaustive approach to all potential patient factors. Further attention should be given to evaluating other factors, which may influence miRNA levels (circadian timing, laboratory variation, etc.) in patients with comorbid disease states.

Our study demonstrated wide variation in preorchiectomy miR-371a-3p and miR-372-3p expression. We confirm the previously described correlation between miRNA levels and tumor size, suggesting that increased disease burden is associated with increased miRNA production, an important feature of ideal tumor markers [12,22,23]. However, no other pathologic factors (LVI, histologic subtypes, and tumor stage) were correlated with preorchiectomy miRNA levels. While prior publications have reported higher miR-371a-3p expression in patients with LVI [24], there was no significant correlation in our study. Dieckmann and colleagues [12] observed lower levels of miR-371a-3p expression in patients with seminoma. However, other studies have found no difference in expression levels between seminoma and nonseminoma [11,13]. Similarly, we found no significant difference in either miR-371a-3p or miR-372-3p levels between seminoma and nonseminoma. In the absence of any correlation with these patient and tumor factors, it is unclear what causes such wide variation in presenting miR-371a-3p relative expression levels or whether the degree of elevation will demonstrate prognostic significance.

In an exploratory analysis, we assessed the kinetics of MMA normalization following orchiectomy in a subset of patients who had a short-term (within 3 wk) postorchiectomy MMA draw. MicroRNA levels did not normalize within 3 wk in roughly half of patients with clinical stage (CS) I disease following radical orchiectomy. However, all patients who were normalized were relapse free with 7+ mo of median follow-up, indicating the potential prognostic value of

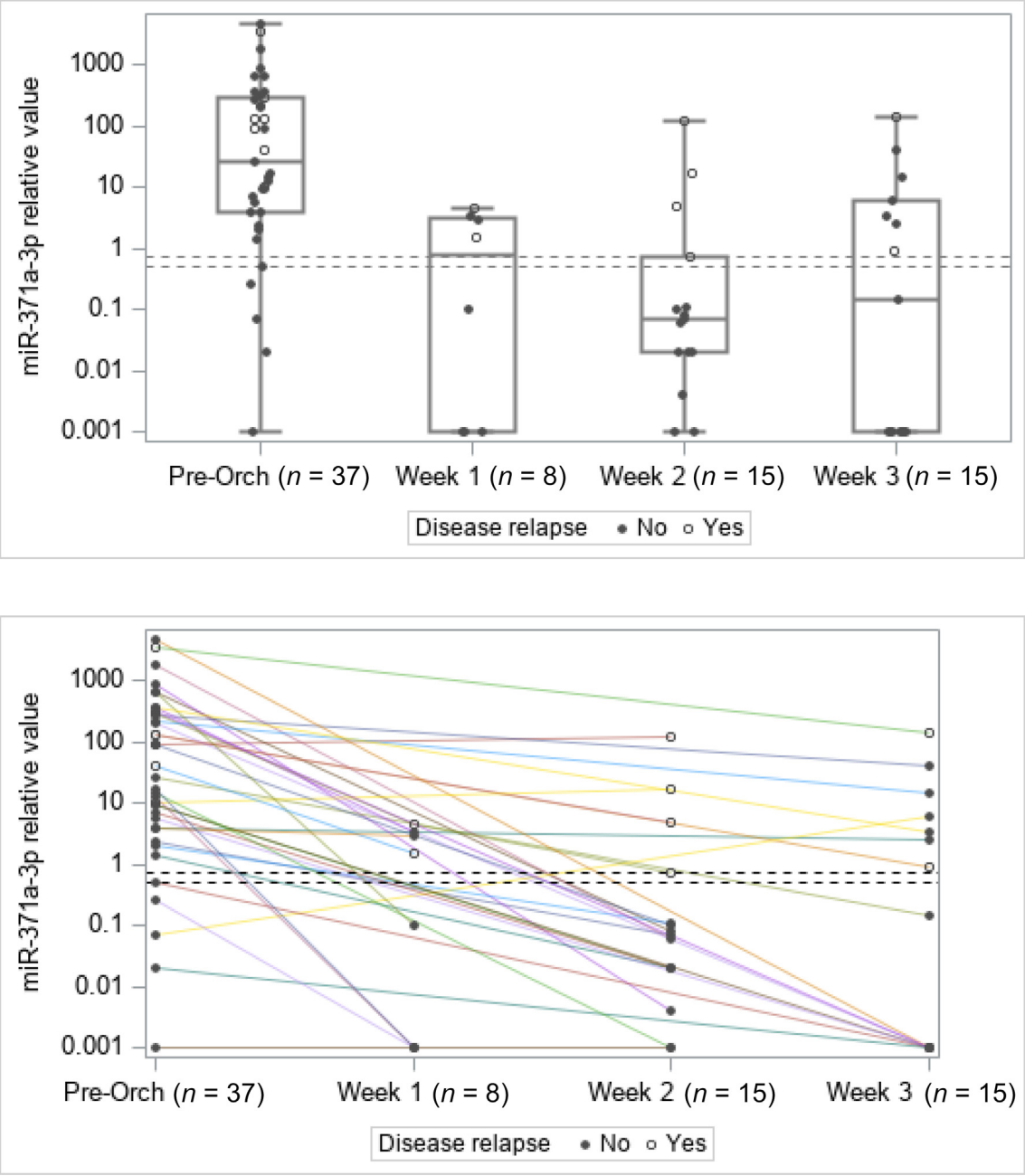

Fig. 3 – Pre- and postorchiectomy miR-371a-3p and miR-372-3p values for patients found to have a GCT on pathology. Box plots and line graphs demonstrate pre- and postoperative miR-371a-3p and miR-372-3p levels for each patient. At least one postorchiectomy MMA was measured within 3 wk of surgery for 37 patients. Seven patients known to experience subsequent relapse are depicted by white circles in each graph. Each line is representative of an individual patient. GCT = germ cell tumor; MMA = MSK microRNA assay; MSK = Memorial Sloan Kettering Cancer Center; Pre-Orch = preorchiectomy.

normalization. Conversely, of those who were not normalized, 39% relapsed and the remainder were relapse free at a median follow-up of 10 mo. Prior studies have reported rapidly normalized serum miRNA levels following radical orchiectomy in most patients with localized disease, and a half-life of 3.7–7 h for miR-371a-3p has been reported [12,13,25,26]. Although our study lacked repeated short-interval postoperative draws necessary to calculate a half-life, we found that not all patients were normalized in a time consistent with a <24-h half-life. While nadir levels and percentage decline of miR-371a-3p following orchiectomy have not been shown to predict relapse, miR-371a-3p levels have been shown to rise prior to or at the time of relapse [27]. Belge et al [28] reported on their experience using miR-371a-3p in a cohort of 371 men with a CS I GCT on surveillance following orchiectomy. While their study demonstrated that miR-371a-3p had high sensitivity and specificity for the detection of relapse, they found that postorchiectomy levels, in aggregate, were not higher in patients who relapsed than in those who did not, although this analysis was based on five relapse events. Since the median time to relapse for patients with CS I GCT varies

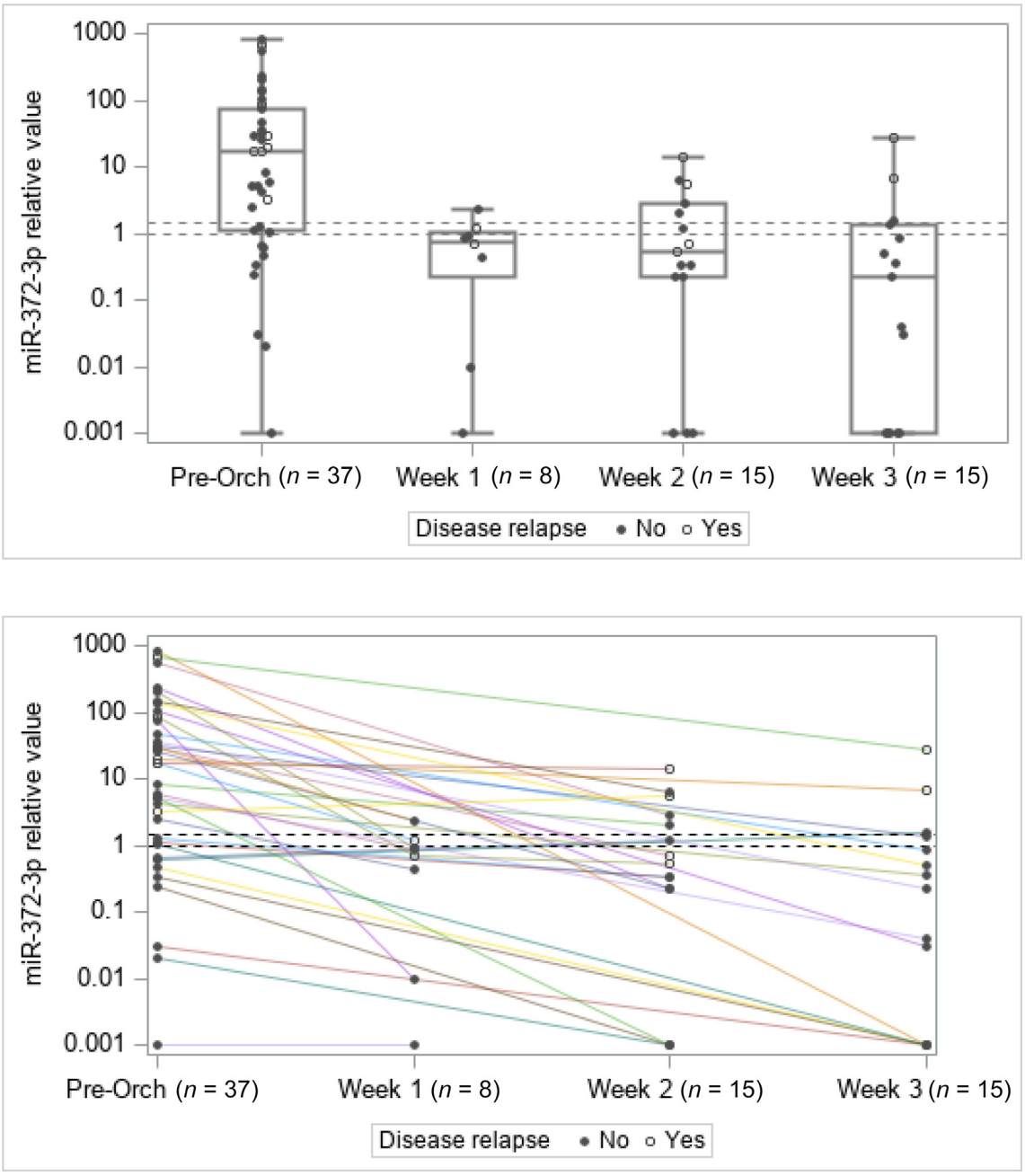

Fig. 3 (*continued*)

between 6 and 14 mo, it is possible that some patients in our study who have not normalized their miRNA values but remain free of disease (median follow-up time 10 mo) may relapse in the future with more complete follow-up, a limitation of this exploratory analysis that warrants further study.

This study is limited by a modest sample size and a nonexhaustive approach to assessing potential patient factors that may explain variation in pretreatment miRNA levels. Notably, our study focuses only on miR-372-3p as an additional biomarker, and we acknowledge the potential for other candidate miRNA clusters to perform differently, if tested. We also found a longer than expected time to normalization for some patients with apparent CS I disease without relapse, calling into question the reported <24-h half-life of miRNA. This finding has implications for ongoing miRNA studies as it remains to be seen whether postorchiectomy miRNA levels can be used safely to guide adjuvant therapy.

5. Conclusions

A combined assay of miR-371a-3p and miR-372-3p demonstrated high sensitivity and specificity for testicular GCTs in men with testicular masses and suspected localized cancer. We were not able to identify any patient demographic or comorbidity characteristics that were associated with pre-

orchiectomy miRNA values. Finally, while normalization of serum miRNA did not occur universally within 3 wk, no patient who were normalized within 3 wk experienced relapse at a median follow-up of 7.2 mo.

***Author contributions*:** Richard S. Matulewicz had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Administrative, technical, or material support: Williams, Kantor, Hossain, Bromberg, Valentino, So.
Supervision: Matulewicz, Sheinfeld, Funt, Ye, Feldman.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euros.2024.08.003.

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