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Isabella Syring, Joanna Bartels, Stefan Holdenrieder, Glen Kristiansen, Stefan C. Müller, Jörg Ellinger

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Circulating serum microRNA (miR-367-3p, miR-371a-3p, miR-372-3p, miR-373-3p) as biomarkers for patients with testicular germ cell cancers

Authors: Isabella Syring [(1)]; Joanna Bartels [(1)]; Stefan Holdenrieder [(2)]; Glen Kristiansen [(3)]; Stefan C. Müller [(1)]; Jörg Ellinger [(1)]

Affiliations: [(1)] Universitätsklinikum Bonn, Klinik und Poliklinik für Urologie und Kinderurologie, Bonn, Germany; [(2)] Universitätsklinikum Bonn, Institut für Klinische Chemie und Klinische Pharmakologie, Bonn, Germany; [(3)] Universitätsklinikum Bonn, Institut für Pathologie, Bonn, Germany

Corresponding author: Dr. med. Isabella Syring, Universitätsklinikum Bonn, Klinik und Poliklinik für Urologie und Kinderurologie, Bonn, Germany; Sigmund-Freud-Strasse 25, 53105 Bonn; Phone: ++49 228-287-15109; Fax: ++49 228-287-14185; Mail: isabella.syring@ukb.uni-bonn.de

Runninghead: microRNAs as biomarker for testicular cancer patients

Keywords: testicular cancer; biomarker; serum; microRNA; miR-371a-3p

ABSTRACT

Purpose: Classic serum tumor markers (HCG, AFP, LDH) play an important role in management of testicular germ cell tumor (TGCT). As only 60% of all TGCT patients have elevations of these, there is need for new biomarkers which offer greater sensitivity/specificity.

MicroRNAs are deregulated in cancer and could serve as non-invasive serum biomarkers; the present study explores the role of serum microRNAs as novel biomarkers in patients with **TGCT**.

Materials and Methods: Total RNA was isolated from serum, and microRNA levels were quantified using quantitative real-time PCR. MicroRNAs (miR-302a-3p, miR-302b-3p, miR-302c-3p, miR-367-3p, miR-371a-3p, miR-372-3p, miR-373-3p) were assessed in a sub-cohort of 30 **TGCT** and 18 healthy subjects. Validation was performed with 76 patients undergoing inguinal exploration suspicious for **TGCT** (cancer n=59; benign disease n=17) and 84 healthy male subjects.

Results: Serum levels of miR-367-3p, miR-371a-3p, miR-372-3p and miR-373-3p were significantly increased in **TGCT** compared to healthy individuals and non-malignant testis diseases. In particular, miR-371a-3p allowed sensitive (**84.7**%) and specific (99%) identification of patients with **TGCT** and thus outperformed human chorionic gonadotropin (HCG) or alpha-1 Fetoprotein (AFP) testing. Furthermore, miR-367-3p was increased in non-seminoma compared to seminoma patients. Serum microRNA levels were elevated in patients with advanced local stage and metastasis. In nine patients with localized (clinical stage 1A) **TGCT**, serum microRNAs levels (miR-371a-3p) decreased after surgery indicating the tumor specific release.

Conclusions: miR-371a-3p allows better identification of **TGCT** than AFP and HCG, and could be helpful for clinical management of **TGCT**, **especially monitoring surveillance therapy and residual disease after chemotherapy**.

INTRODUCTION

MicroRNAs are single-stranded, small RNA molecules. They regulate the expression of approximately a third of human genes, and thereby influence important cellular functions like cell cycle, development, proliferation, apoptosis and differentiation.[1] MicroRNA expression profiles are altered in many diseases including urological cancer.[2, 3] Importantly, microRNA expression is not only different in malignant and benign tissue; also, various tumor entities show specific microRNA expression profiles.[4] MicroRNAs are detectable in a variety of body fluids including serum/plasma.[5] MicroRNAs are protected in serum against degradation by RNases;[6] many external influences (i.e. pH-alteration, storage, freezing and thawing) do not affect microRNA stability.[7] Thus, circulating microRNAs have a great potential as diagnostic/prognostic biomarkers (e.g. bladder cancer,[8] breast cancer,[9] gastric cancer,[10] lung cancer,[11] prostate cancer,[12] renal cell carcinoma[13]).

MicroRNAs also play a relevant role during testis carcinogenesis: Previous studies highlighted a prominent role of the microRNA cluster miR-371a-373-3p: Overexpression of the miR371a-373-3p, miR-302 and miR-367-3p clusters was reported in malignant **testicular** germ cell cancer **(TGCT)** tissue.[14-16] Both, miR-372-3p and miR-373-3p are involved in regulation of proliferation and cancer cell invasion via neutralization of p53-mediated CDK inhibition[17] and activation of the Wnt/β-catenin signaling pathway.[18]

Circulating microRNAs may also be of importance in **TGCT**: Serum levels of microRNAs from miR-371a-373-3p and miR-302/367-3p clusters were elevated in a child with yolk sac tumor at diagnosis, with levels of miR-372-3p (from the miR-371 – 373 cluster) falling during treatment.[19] The increase of miR-371a-373-3p levels in testicular vein blood[20] and the normalization of increased microRNA levels in patients with organ-confined testicular cancer

following surgery[20, 21] indicate the tumoral origin of circulating microRNAs. Notably, the informative value of the circulating microRNAs was improved compared to the classical tumor markers (AFP, HCG) **(Supplementary Table 3)**, but the small sample size did not allow drawing meaningful conclusions.[20, 21] We therefore studied circulating microRNAs in a large cohort of patients with **TGCT (Supplementary Table 3)**.

MATERIAL AND METHODS

2.1 Sample collection

We investigated first serum microRNA expression in a screening cohort which included 30 **TGCT** patients (seminoma, n=15; non-seminoma, n=15) and 18 healthy subjects. Validation of the screening results was performed with 76 patients undergoing inguinal exploration suspicious for testicular cancer; among these 59 patients had malignant testicular cancer (seminoma n=40, non-seminoma n=19) and 17 had non-malignant diseases (histological diagnosis: epidermoid cyst n=7; leydig cell tumor n=4; mature teratoma n=1; adenomatoid tumor n=1; testicular infarction n=1; stromal spindle cell tumor n=1; scar tissue n=1; fibroma n=1). The non-seminoma cohort (n=19) included the following subtypes: 1 pure embryonal carcinoma, and 18 mixed malignant tumors (components: embryonal carcinoma n=13, teratoma n=11, yolk sac carcinoma n=7, choriocarcinoma n=2, seminoma n=10). In addition, we investigated an age-matched cohort of 84 healthy male individuals as control subjects. Detailed clinicopathological parameters are provided in Supplementary Table 1 and 2.

All samples were collected according local standard operating procedures within the framework of the Biobank initiative at the CIO Köln/Bonn. In brief, venous blood was taken in Serum S-Monovette Gel tubes with clotting activator (Sarstedt, Nümbrecht, Germany)

prior to surgery. For the comparison of pre- and postoperative microRNA levels, we also obtained serum samples on day 5 following surgery. After 60-180 minutes the clotted serum was centrifuged (10 min, 2500 g), separated and stored in cryotubes at -80°C until use. Sample collection was performed between 1997 and 2012. Written informed consent was obtained from each individual and the study was approved by the local ethic committee (approval number: 169/12).

2.2 RNA Isolation, Reverse Transcription and quantitative Real-Time PCR

Total RNA was isolated from 400 µl serum using the mirVana PARIS Kit (Ambion, Foster City, CA, USA) after addition of 25 fmol of the synthetic miRNA cel-miR-39 (Qiagen, Hilden, Germany); RNA was eluted in 50 µl elution solution. In humans, a cel-miR-39-sequence does not exist and thus, its determination allows quantification of a target miRNA with normalization to the RNA isolation and reverse transcription efficiency. Furthermore, the use of cel-miR-39 as reference gene was supported by Sanders et al.[22] Reverse Transcription was performed with the TaqMan MicroRNA Reverse Transcription Kit; cDNA synthesis differed in screening and validation experiments somewhat: the Megaplex PreAmp Primer Pool A v2.1 was used in the screening experiments, whereas a pool with specific primers (miR-367-3p, miR-371a-3p, miR-372-3p, miR-373-3p, cel-miR-39; see catalog numbers below) was created as reported earlier.[13] In order to increase the sensitivity of the assay, we performed a preamplification with the TaqMan PreAmp Master Mix with the Human Pool A MegaPlex PreAmp Primer set according the manufacturer's recommendations. Preliminary experiments (data not shown) demonstrated that serum microRNA levels were too low for quantification without preamplification, and was also necessary in former studies.[20, 23]

All PCR experiments were carried out in triplicate on an ABIPrism 7900HT in 384 well plates using the TaqMan Universal Master Mix II with UNG kit; PCR primers were purchased from Applied Biosystems (assay-ID: cel-miR-39 #000200; miR-302a-3p #000529; miR-302b-3p #000531; miR-302c-3p #000533; miR-367-3p #000555; miR-371a-3p #002124; miR-372-3p #000560; miR-373-3p #000561). Each run included water blanks and genomic DNA as negative controls, as well as the germ cell tumor line Tera1 as positive control;[14] serial dilutions of Tera1 RNA were used to assure PCR efficiency (92-100%). Each run consisted of 35 cycles; microRNA levels in samples without amplification signal (undetermined) were considered as zero. PCR data were analyzed with the SDS Relative Quantification Software v2.4 and the RQ Manager v2.1.1; relative quantification (2d^dCt) was performed using Data Assist v3.0. All reagents not otherwise specified and all software packages were obtained from Applied Biosystems (Foster City, CA, USA). Detailed information is provided in the MIQE protocol in supplement.

2.3 Statistical analysis

The Mann-Whitney-U test and the Kruskal-Wallis-test were used to determine differences between cancer patients and healthy controls, and to associate microRNA expression with clinical-pathological parameters, as appropriate.[12] The Pearson test was used to correlate AFP/HCG with microRNA levels. Receiver Operator Curve (ROC) analyses were performed to determine the sensitivity and specificity of microRNAs as diagnostic biomarkers;[12] the Youden index was used to identify the optimal threshold. Statistical significance was concluded at $p<0.05$. Correction for multiple hypothesis testing was not performed in order not to refuse a true hypothesis; however, the use of a validation cohort overcomes this limitation. Statistical analyses were performed using SPSS Statistics v21 (IBM, Chicago, Illinois, USA).

RESULTS

3.1 Screening Phase: analysis of seven circulating microRNAs

We investigated a sub-cohort of 30 **TGCT** patients (seminoma, n=15; non-seminoma, n=15) and 18 healthy subjects. The median recovery rates of cel-miR-39 were 22.1% in the cancer group and 60.1% in the healthy control group. **The mean s**erum miR-367-3p (p=0.001), miR-371a-3p (p<0.001) and miR-372-3p (p<0.001) levels were significantly increased in **TGCT** patients. The other microRNAs were not different in both cohorts (miR-302a-3p p=0.071; miR-302b-3p p=0.268; miR-302c-3p p=0.735; miR-373-3p p=0.055). See Figure 1.

3.2 Circulating microRNA levels are different in testicular cancer patients and control subjects

We next investigated the most interesting target microRNAs in a larger cohort which included 59 patients with testicular cancer (seminoma n=40, non-seminoma n=19), 84 healthy individuals and 17 patients with non-malignant testicular disease. The median recovery rate of the cel-mir-39 in the validation cohorts was 35.3% in the cancer patients, 44.3% in the healthy controls, and 34.2% in the patients with non-malignant testicular disease (Supplementary Figure 1).

The mean serum levels of miR-367-3p, miR-371a-3p, miR-372-3p and miR-373-3p were significantly (all p<0.001) increased in **TGCT** patients compared to healthy individuals, and importantly, also compared to patients with non-malignant testis diseases. Circulating microRNA levels were similar in patients with benign disease and healthy individuals (all p>0.3). MiR-367-3p levels were increased in non-seminoma compared to seminoma patients

(p=0.004), whereas the other microRNAs were circulating at similar levels in both cohorts (p=0.303-0.897). See Figure 2. **Individual data are provided in Supplementary Table 5.**

We next determined the diagnostic information of circulating microRNAs using receiver operator characteristic analyses: As shown in Figure 3, all four microRNAs were useful for the discrimination of cancer patients and control subjects: In particular, miR-371a-3p allowed highly sensitive (84.7%) and specific (99.0%) discrimination (area under curve 0.929, 95% confidence interval 0.876-0.982) of patients with testicular cancer and control subjects (healthy male and non-malignant testicular disease) at a threshold of serum miR-371a-3p level 0.004. The diagnostic characteristics were similar in seminoma and non-seminoma patients (see Table 1). Values for AFP and HCG were not determined in healthy controls; under the assumption of negative tumor markers in the subjects, we determined a significant improvement of diagnostic information for serum microRNAs compared to classical tumor markers (AFP: sensitivity 13.6%, AUC 0.568, 95% confidence interval 0.416-0.719; HCG: sensitivity 37.3%, AUC 0.686, 95% confidence interval 0.560-0.813).

3.3 Circulating microRNA levels are increased in patients with advanced testicular cancer

To investigate the association of serum microRNAs with advanced tumor stage, we combined the screening and validation cohort to increase the statistical power. Serum microRNA levels were increased in patients with advanced disease: patients with pT3 stage had significantly increased miR-367-3p, miR-371a-3p and miR-373-3p levels compared to pT1 and pT2 patients (all $p<0.05$). Patients with metastatic testicular cancer (i.e. clinical stage 2 and 3 vs. clinical stage 1) were associated with higher miR-367-3p ($p<0.001$), miR-371a-3p (p=0.001), miR-372-3p (0.032) and miR-373-3p levels ($p<0.001$) compared to organ-confined tumors. The IGCCCG prognosis classification system was not associated with serum

microRNA levels (all p>0.4), but it should be noticed that the case numbers were low, in particular for patients with poor prognostic disease. Furthermore, miR-367-3p (p=0.041, correlation coefficient 0.202) levels were weakly positively associated with AFP levels.

3.4 Circulating microRNA levels decrease after surgery in patients without metastasis

In order to prove the tumoral origin of specific microRNAs we determined circulating microRNA levels in serum obtained before and five days after orchiectomy in nine patients with clinical stage 1A testicular cancer. None of them suffered from relapse, however only three patients underwent surveillance, while two had retroperitoneal lymphadenectomy (pN0), three underwent radiotherapy and one received carboplatin chemotherapy; **all patients were free of recurrence during a follow-up period of at least two years.** Serum miR-371a-3p decreased in all patients after surgery; it decreased below the detection limit in 5 patients, thereby indicating the tumor-specific release of miR-371a-3p. MiR-367-3p also declined in 6 patients with measurable levels; the remaining three patients had undetectable miR-367-3p levels before surgery. MiR-372-3p and miR-373-3p were also lower in most patients after surgery, but two (miR-372-3p) respectively **three** (miR-373-3p) patients had a marker increase. See Figure 4 **and Supplementary Table 4.**

DISCUSSION

Serum tumor markers (HCG, AFP, LDH) play an important role in the management of men with a suspected **TGCT**: they are used for risk stratification, to assess the response to treatment and for surveillance following treatment. However, they are often within normal ranges, especially in patients with early stage disease. **However, as only 60% of all GCT patients have elevations of the classic tumor markers (HCG, AFP, LDH), there is an ongoing**

need for new biomarkers[20, 21] **which offer greater sensitivity and specificity for diagnosing or monitoring malignant GCTs.**[19]

Additional it is well known that the expression of AFP and HCG can change during the course of disease affected by different factors (e.g. malignant and non-malignant diseases, drugs, tumor lysis).[24] Thus, improved biomarkers could help to improve the treatment of **TGCT** patients.

Circulating microRNAs have been identified as potential novel diagnostic/prognostic biomarkers for a variety of human malignancies.[8-13] Recently, increased serum levels of the miR-371a-373-3p cluster have been detected in **TGCT** patients,[20, 21, 24] however these studies suffered from low patient numbers and only analyzed a very small number of patients with non-malignant testicular masses **(Supplementary Table 3)**. In the present study, we observed that the presence of miR-367-3p and miR-371a-3p in serum was highly specific for patients with **TGCT**; importantly, most patients with benign testicular disease had undetectable miR-367-3p (94%) and miR-371a-3p (81%) levels. Notably, serum levels of miR-367-3p, miR-371a-3p, miR-372-3p and miR-373-3p were significantly increased in cancer patients compared to healthy control and patients with benign testis disease. These results confirm the findings of Gillis et al.[24] Although our study included **59** patients with testicular cancer, **17** patients with benign testis disease and 84 age-matched healthy controls - a large number of cases for a study on **TGCT** - reliable normal-ranges have to be established in further studies. **Furthermore, our study distinguishes to the others in the use of high patient numbers with benign disease and a high amount of healthy patients (Supplementary Table 3).** Using ROC-analyses, we determined that serum miR-371a-3p levels provided a sensitivity of 8**4**.7% and a specificity of 99.0% for the identification of patients with **TGCT**. Thus, microRNAs perform better than the classical tumor markers AFP

and HCG which were elevated in 19.8% and 40.7% of patients with malignant testicular cancer **(Supplementary Table 3)**;[24] especially patients with seminoma - who are often marker-negative - could benefit from this new marker, which worked well in both seminoma and non-seminoma patients (miR-371a-3p: AUC 0.924 vs. 0.937, respectively). We could also demonstrate a decline of miR-371a-3p after surgery for localised malignant disease, as shown by others.[20, 21] The analysis of miR-371a-3p could be useful for monitoring of tumor burden during chemotherapy or in surveillance to detect recurrence. MicroRNAs may also be helpful to differentiate between non-seminoma and seminoma germ cell cancer: miR-367-3p levels were increased in non-seminoma patients, as would be expected given their relative increased levels in such tumor specimens tissue.[14]

In addition to the diagnostic potential, serum microRNA levels were positively associated with advanced local tumor stage and clinical stage: i.e. miR-367-3p, miR-371a-3p and miR-373-3p were increase in patients with pT3 stage, and miR-367-3p, miR-371a-3p and miR-373-3p levels were increased in patients with clinical stage III. Serum miR-367-3p, miR-371a-3p and miR-373-3p levels were also weakly correlated with AFP levels; this could also be related to increased microRNA levels in patients with advanced cancer; increased tumor markers are commonly observed in these patients. It should be kept in mind that the number of patients with advanced disease was small and the statistical power is therefore limited. However, this study indicates an association of serum microRNAs with advanced clinical and tumour stage, as has been suggested in smaller studies by others.[24] These findings clearly deserve future verification and **validation** in an independent cohort.

CONCLUSIONS

Serum miR-371a-3p levels are derived from malignant germ cell cancer cells, and allow distinguishing patients with cancer and non-malignant testicular disease. The high sensitivity and specificity of miR-371a-3p testing could help the clinical management of **TGCT** patients, **especially for monitoring surveillance therapy and residual disease after chemotherapy**. A prospective multicenter study to confirm the value of this assay is warranted.

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FIGURE LEGENDS

Figure 1

Detection of serum microRNA in testicular cancer patients and healthy individuals. Grey boxes indicate detectable serum microRNAs.

Figure 2

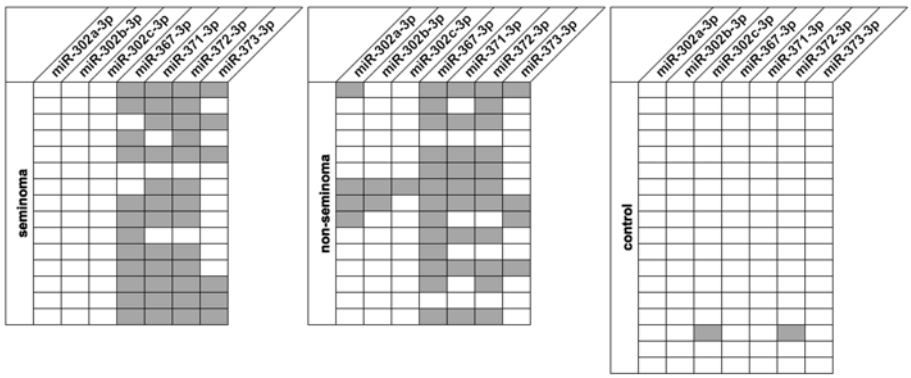
Serum microRNA (miR-367-3p, miR-371a-3p, miR-372-3p, miR-373-3p) levels are increased in testicular cancer patients compared to healthy individuals and patients with benign testicular disease.

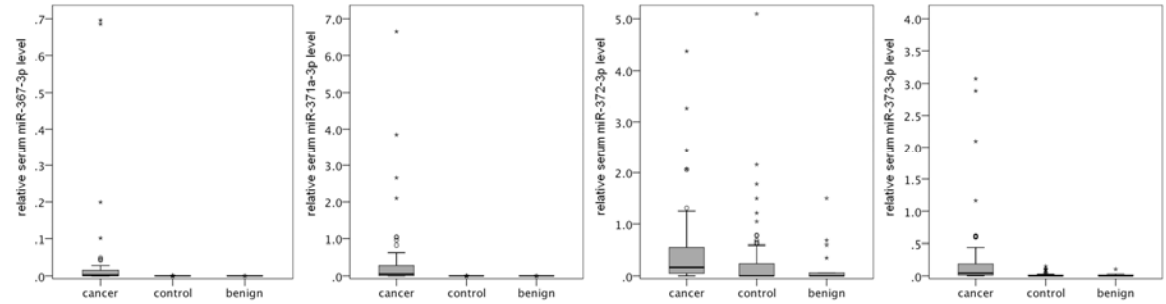
Figure 3

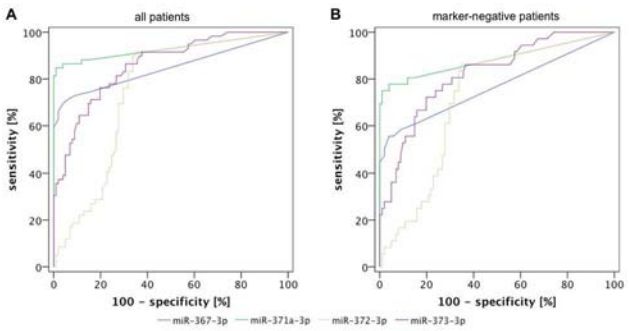
Receiver operator characteristic analysis demonstrate that serum microRNA (miR-367-3p, miR-371a-3p, miR-372-3p, miR-373-3p) levels allow sensitive and specific **discrimination** of testicular germ cell cancer **(TGCT) patients and healthy subjects/benign testicular diseases; (A) includes the whole TGCT study group (n=59), (B) includes only TGCT patients with negative tumor markers (n=36)**.

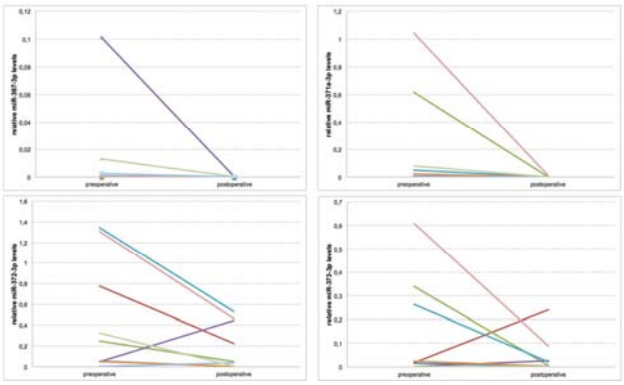
Figure 4

Comparison of serum microRNA levels in nine patients with testicular cancer in clinical stage 1A without recurrence during at least 2 years of follow-up before and five days after inguinal orchiectomy.









Abbreviations

AFP	alpha-1 Fetoprotein
AUC	area under curve
HCG	human chorionic gonadotropin
miRNA	microRNA
real-time PCR	real-time polymerase chain reaction

Supplementary Table 1

Clinical-pathological parameters of patients investigated in the screening experiments.

	TGCT (n=30)	seminoma (n=15)	non-seminoma (n=15)	healthy (n=18)
Age				
mean	34.5	37.1	31.9	28.7
min-max	21 - 51	24 - 51	21-46	19 - 49
pT-stage				
pT1	19 (63.3%)	9 (60.0%)	10 (66.7%)	n.a.
pT2	5 (16.7%)	3 (30.0%)	2 (13.3%)	n.a.
pT3	5 (16.7%)	2 (13.3%)	3 (20.0%)	n.a.
"burned out"	1 (3.3%)	1 (6.7%)	0 (0%)	n.a.
Clinical stage (Lugano)				
stage 1	12 (40.0%)	7 (22.6%)	5 (33.3%)	n.a.
stage 2	14 (46.7%)	7 (22.6%)	7 (46.7%)	n.a.
stage 3	4 (13.3%)	1 (6.7%)	3 (20.0%)	n.a.
IGCCCG classification				
good prognosis	12 (40.0%)	8 (53.3%)	4 (26.7%)	n.a.
intermediate prognosis	4 (13.3%)	0 (0%)	4 (26.7%)	n.a.
poor prognosis	2 (6.7%)	0 (0%)	2 (13.3%)	n.a.
not applicable	12 (40.0%)	7 (46.7%)	5 (33.3%)	n.a.
Tumor marker				
AFP	10 (33.3%)	1 (1.8%)	9 (60.0%)	n.d.
HCG	15 (50.0%)	5 (30.9%)	10 (66.7%)	n.d.
Missing	0 (0%)	0 (0%)	0 (0%)	18 (100%)

note: 1 seminoma patient had known increased AFP levels due to chronic liver disease; abbreviations: n.a., not applicable; n.d., not done

Supplementary Table 2

Clinical-pathological parameters of patients investigated in the validation experiments excluding the patients of the screening cohort.

	TGCT (n=59)	seminoma (n=40)	non-seminoma (n=19)	healthy (n=84)	benign (n=17)
Age					
mean	35.9	38.2	31.1	32.4	31.9
min-max	14-59	23-59	14-54	19-64	18-57
pT-stage					
pT1	45 (76.3%)	31 (77.5%)	14 (73.7%)	n.a.	n.a.
pT2	10 (16.9%)	5 (12.5%)	5 (26.3%)	n.a.	n.a.
pT3	2 (3.4%)	2 (5.0%)	0 (0%)	n.a.	n.a.
"burned out"	2 (3.4%)	2 (5.0%)	0 (0%)	n.a.	n.a.
Clinical stage (Lugano)					
stage 1	47 (79.7%)	33 (82.5%)	14 (73.7%)	n.a.	n.a.
stage 2	9 (15.3%)	6 (15.0%)	3 (15.8%)	n.a.	n.a.
stage 3	3 (5.1%)	1 (2.5%)	2 (10.5%)	n.a.	n.a.
IGCCCG classification					
good prognosis	8 (13.5%)	4 (10.0%)	4 (21.1%)	n.a.	n.a.
intermediate prognosis	4 (6.8%)	3 (7.5%)	1 (5.2%)	n.a.	n.a.
poor prognosis	0 (0%)	0 (0%)	0 (0%)	n.a.	n.a.
not applicable	48 (81.3%)	33 (82.5%)	15 (78.9%)	n.a.	n.a.
Tumor marker increase					
AFP	8 (13.6%)	0 (0%)	8 (42.1%)	n.d.	0 (0%)
HCG	22 (37.3%)	11 (27.5%)	11 (57.9%)	n.d.	0 (0%)
Missing	0 (0%)	0 (0%)	0 (0%)	84 (100%)	2 (11.8%)

abbreviations: n.a.. not applicable; n.d.. not done

Supplementary Table 3

Comparison of case numbers und diagnostic sensitivity in different studies on cell-free serum microRNA in testicular germ cell cancer patients.

	Case number			sensitivity		
	cancer	healthy	benign	AFP	HCG	miR-371a-3p
Syring	59	84	17	19.8%	40.7%	84.7%
Dieckmann [1]	24	17	0	16.7%	8.3%	70.8%
Gillis [2]	80	47	0	3% (S) 45% (NS)	62% (S) 66% (NS)	>90%
Murray [3]	3	3	0	n.r.	n.r.	n.r.
Belge [4]	11	12	0	n.r.	n.r.	n.r.

S=seminoma; NS=non-seminoma, n.r.=not referred

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Supplementary Table 4

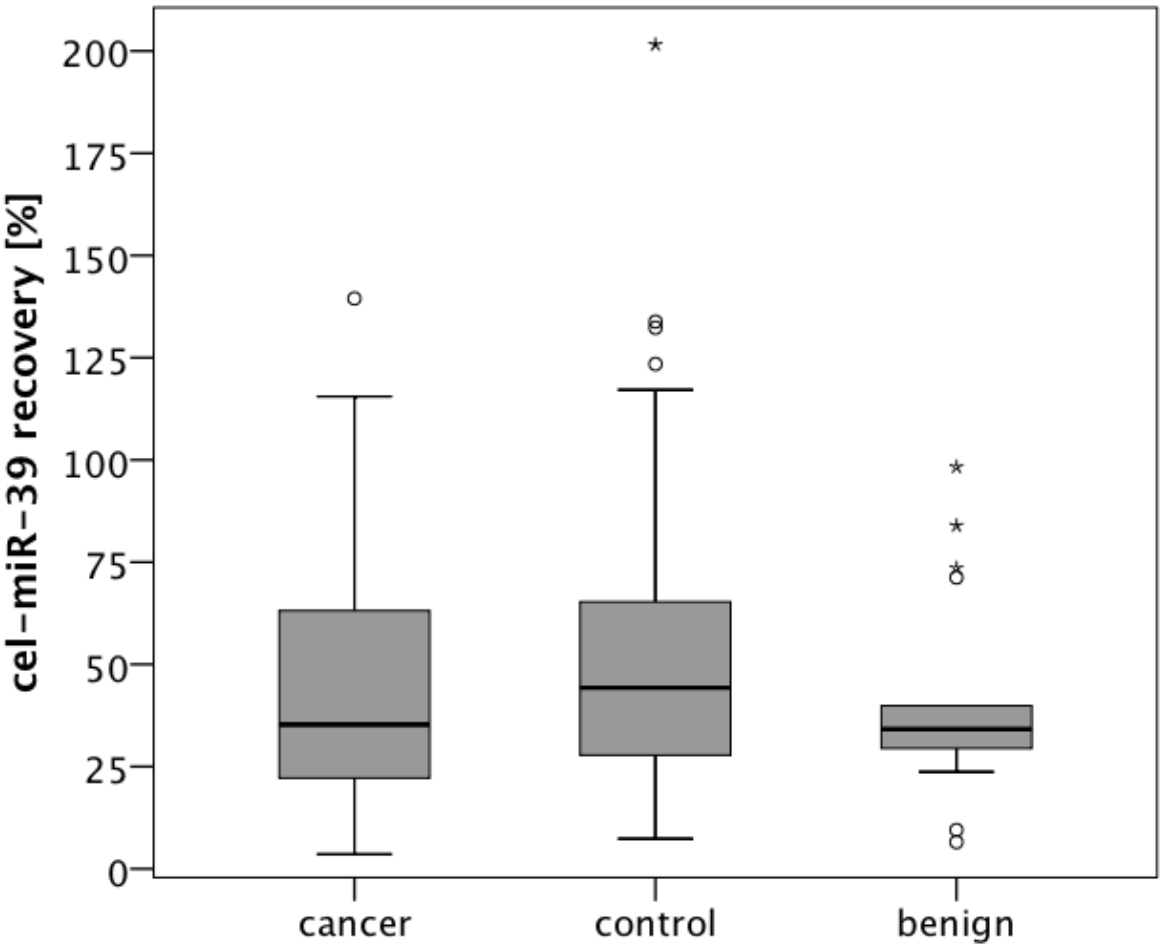
Comparison of pre- and postoperative (5 days after orchiectomy) serum miRNA levels for patients with Lugano stage 1A. Postoperative increases of circulating serum microRNA are highlighted bold.

Tumor	Therapy	miR-367-3p		miR-371a-3p		mir-372-3p		miR-373-3p	
		pre	post	pre	post	pre	post	pre	post
S	R	0.0000	0.0000	0.0054	0.0038	0.0529	0.0000	0.0163	0.0031
S	C	0.0000	0.0000	0.0075	0.0025	0.7756	0.2240	0.0174	***0.2413***
S	Sur	0.0003	0.0001	0.0502	0.0000	1.3453	0.5290	0.2629	0.0212
S	R	0.0131	0.0002	1.0449	0.0118	1.3091	0.4621	0.6094	0.0884
S	R	0.0027	0.0000	0.0799	0.0000	0.3258	0.0061	0.0012	0.0012
NS(EC,S)	RLA	0.1012	0.0001	0.6168	0.0000	0.2487	0.0487	0.3396	0.0060
NS(EC,T)	Sur	0.0008	0.0000	0.0075	0.0002	0.0480	***0.4440***	0.0020	***0.0258***
NS(EC,S)	RLA	0.0012	0.0000	0.0216	0.0000	0.0520	0.0000	0.0240	0.0029
NS(EC,YS)	Sur	0.0000	0.0000	0.0035	0.0000	0.0000	***0.0392***	0.0017	***0.0039***

abbreviations: pre=preoperative; post=postoperative; S=seminoma; NS=non-seminoma; EC=embryonal carcinoma; T=mature teratoma; YS= yolk sac carcinoma; R=radiotherapy; C=carboplatin chemotherapy; Sur=surveillance; RLA=retroperioneal lymphadenectomy

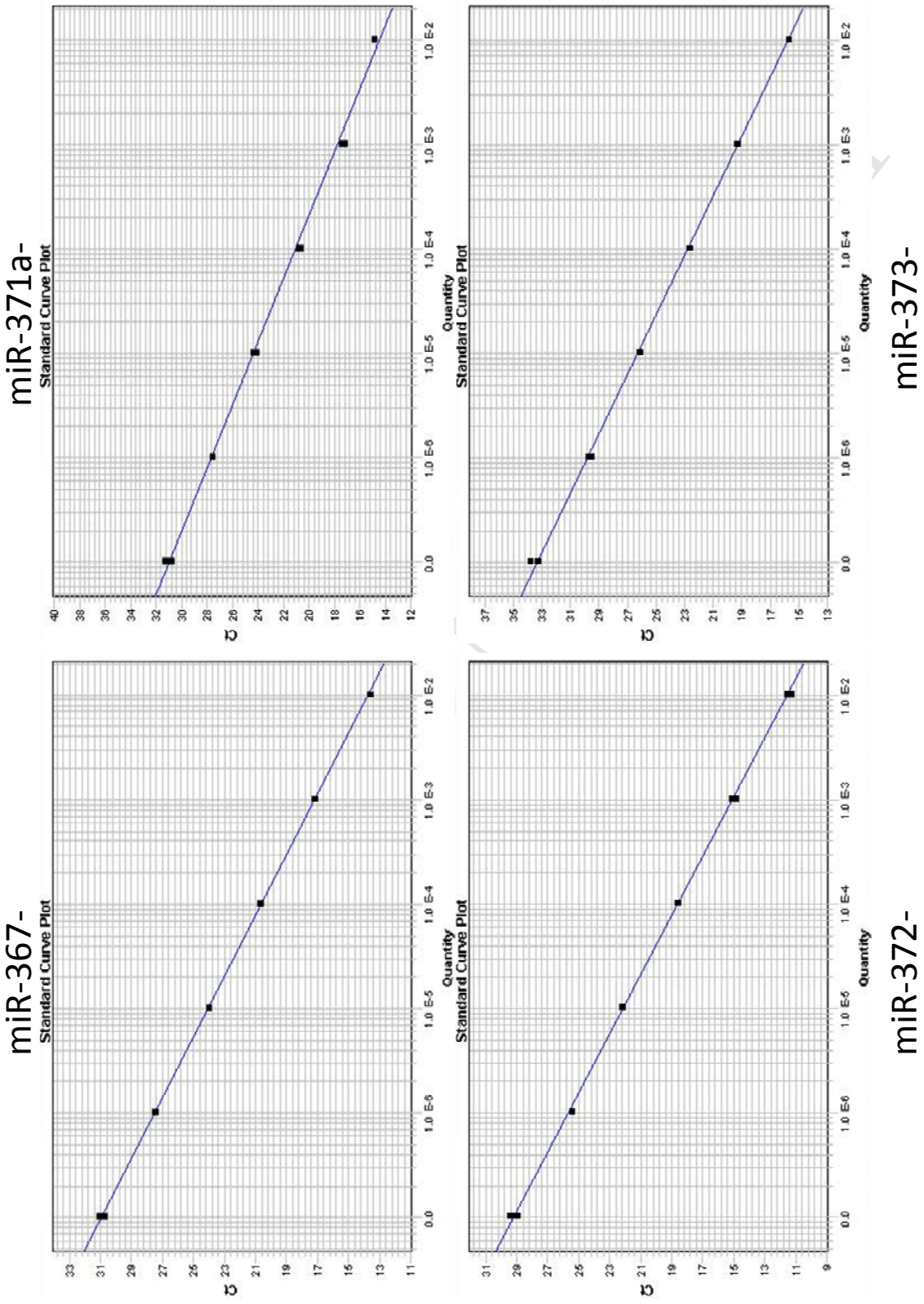
Supplementary Figure 1

Recovery rate (%) of cel-miR-39 in the validation cohort.



Supplementary Figure 2

Calibration curves of miR-367-3p, miRa-371-3p, miR-372-3p and miR-373-3p.



MIQE-COMPLIANT REPORT OF EXPERIMENTAL METHODS

EXPERIMENTAL DESIGN

Definition of experimental and control groups

We analyzed separate samples at each step of the study; serum samples were not pooled at any time.

Screening cohort: The experimental group consisted of patients undergoing inguinal exploration suspicious for testicular cancer with malignant and non-malignant testicular disease. We also investigated an age-matched cohort of healthy male individuals as control subjects Cancerous serum and serum from different healthy individuals were used.

Validation cohort: The experimental group consisted of patients undergoing inguinal exploration with suspicious for testicular cancer; thus, the study cohort consisted of patients with testicular cancer, benign testicular tumors (i.e. leydig cell tumor) and a control group consisting of healthy men. Only serum samples were used.

Number within each group

Phase-1: testis cancer n=**30** (seminoma, n=15; non-seminoma, n=**15**), healthy control n=18.

Phase-2: testis cancer n=**59**, non-malignant testicular disease n= **17**, healthy control n=84.

See Supplementary Table 1 and 2 for detailed clinical-pathological parameters.

Assay carried out by core lab or investigator's lab?

All serum samples used were collected and stored in at the laboratories of the Klinik und Poliklinik für Urologie und Kinderurologie at the Universitätsklinikum Bonn.

RNA purification and qPCR of all samples was done at the laboratories of the Klinik und Poliklinik für Urologie und Kinderurologie at the Universitätsklinikum Bonn.

Acknowledgement of authors' contributions

Study concept and design: Ellinger, Syring

Acquisition of data: Bartels

Analysis and interpretation: Ellinger, Syring, Bartels

Drafting the manuscript: Ellinger, Syring

Critical revision of the manuscript for important intellectual content:,Bartels, Holdenrieder, Kristiansen, Müller

Statistical analysis: Ellinger, Syring, Bartels

Obtaining funding: no external funding

Administrative, technical, or material support: Holdenrieder, Kristiansen, Müller

Supervision: Müller, Ellinger

SAMPLE

Description

Screening cohort: Serum samples, blood withdrawal prior to surgery.

Validation cohort: Serum samples, blood withdrawal prior to surgery and five days postoperatively.

Volume/mass of sample processed

A total of 400 µl serum was used for RNA purification.

Processing procedure
The collection of serum samples was performed according to the SOPs of the Biobank at the CIO Köln/Bonn: Serum samples were withdrawn in Serum-S-Monovette (Saerstedt), and after serum separation stored at -80°C in cryotubes until use.

If frozen - how and how quickly?
After clotting, serum was separated after centrifugation within 60-180 minutes and stored in cryotubes at -80°C until use.

Sample storage conditions and duration (especially for FFPE samples)
Serum samples, collected between 1997 and 2012, were stored in cryotubes at -80°C.

NUCLEIC ACID EXTRACTION
Procedure and/or instrumentation
RNA isolation was performed according to the manufacturer's recommendations using the mirVana PARIS Kit (catalog number AM-1556; Ambion, Foster City, CA, USA).

Name of kit and details of any modifications
The mirVana PARIS Kit (Ambion, Foster City, CA, USA) was used to purify total RNA from 400µl serum. RNA isolation was performed according to the manufacturer's recommendation (final elution volume 50 µl) with one exception: we added 25 fmol of a synthetic Caenorhabditis elegans microRNA, cel-miR-39 (Qiagen, Hilden, Germany; catalog number MSY0000010) to the serum at the beginning of the isolation procedure.

Source of additional reagents used
Synthetic Caenorhabditis elegans microRNA, cel-miR-39 (Qiagen, Hilden, Germany; catalog number MSY0000010). 2-Mercaptoethanol (Sigma, St. Louis, MO, USA). 100% Ethanol (Merck, Darmstadt, Germany; index number 603-002-00-5)

Contamination assessment (DNA or RNA)
Contamination assessment was not performed because the serum RNA levels were too low.

Purity (A260/A280)
Purity was not controlled because the serum RNA levels were too low.

RNA integrity method/instrument; RIN/RQI or Cq of 3' and 5' transcripts; Electrophoresis traces
RNA integrity was not checked because the amount of isolated RNA in serum was too small for RNA integrity analysis.

Inhibition testing (Cq dilutions, spike or other)
The addition cel-miR-39 (Qiagen, Hilden, Germany; catalog number MSY0000010) to the serum and its quantification allowed controlling technical variability.

REVERSE TRANSCRIPTION

Complete reaction conditions

Screening cohort: The TaqMan MicroRNA Reverse Transcription Kit (catalog number: 4366596; Applied Biosystems, Foster City, CA, USA) was used to synthesize single-stranded cDNA from total RNA samples. All reactions were performed as specified in the protocols of the manufacturer: 2,5 µl total RNA were added to 5 µl of the RT reaction mix (dNTPs with dTTP 100mM, MultiScribe Reverse Transcriptase 50U/µl, 10X RT Buffer, RNase Inhibitor 20 U/µl, specific RT primers [cel-miR-39 (Applied Biosystems Assay ID: 000200), hsa-miR-371a-3p (Applied Biosystems Assay ID: 002124), hsa-miR-372-3p (Applied Biosystems Assay ID: 000560), hsa-miR-373-3p (Applied Biosystems Assay ID: 000561), hsa-miR-367-3p (Applied Biosystems Assay ID: 000555), hsa-miR-302a-3p (Applied Biosystems Assay ID: 000529), miR-302b-3p (Applied Biosystems Assay ID: 000531) and has-miR-302c-3p (Applied Biosystems Assay ID: 000533)] and Nuclease-free water). After incubation on ice for 5 minutes reverse transcription was performed using a thermal cycler (UNO-Thermoblock, Biometra, Göttingen, Germany).

In order to increase the sensitivity of the TaqMan Low Density Arrays, we performed a pre-amplification after the reverse transcription using the TaqMan PreAmp Master Mix (catalog number: 4384267; Applied Biosystems, Foster City, CA, USA) as well as the MegaplexPreAmp Primers, Human Pool A. All reactions were performed as specified in the protocols of the manufacturer. After incubation on ice for 5 minutes, reverse transcription was performed on a thermal cycler (UNO-Thermoblock, Biometra, Göttingen, Germany).

Validation cohort: The TaqMan MicroRNA Reverse Transcription Kit and specific RT primers were used to synthesize single-stranded cDNA from total RNA samples. All reactions were performed as specified in the protocols of the manufacturer with one exception: Reverse Transcription was performed with a self-created primer pool. To obtain this RT primer pool (20X), we speed vacuumed a mixture of each 320 µl of cel-miR-39 (Applied Biosystems Assay ID: 000200), hsa-miR-371a-3p (Applied Biosystems Assay ID: 002124), hsa-miR-372-3p (Applied Biosystems Assay ID: 000560), hsa-miR-373-3p (Applied Biosystems Assay ID: 000561), hsa-miR-367-3p (Applied Biosystems Assay ID: 000555), miR-302a-3p (Applied Biosystems Assay ID: 000529), miR-302b-3p (Applied Biosystems Assay ID: 000531) and miR-302c-3p (Applied Biosystems Assay ID: 000533) at 45°C for 3 hours with the Concentrator 5301 (Eppendorf, Wesseling, Germany). The RT primer pool was re-suspended in 320µl nuclease-free water. Then 2,5 µl total RNA were added to 5 µl of the RT reaction mix (RT primer pool 20X, dNTPs with dTTP 100 mM, MultiScribe Reverse Transcriptase 50 U/µl, 10X Reverse Transcription Buffer, RNase Inhibitor 20 U/µl and Nuclease-free water). After incubation on ice for 5 minutes, reverse transcription was performed on a thermal cycler (UNO-Thermoblock, Biometra, Göttingen, Germany).

Amount of RNA and reaction volume

We used 5 µl of isolated RNA from serum for cDNA synthesis. The reaction volume was 12.5 µl.

Reverse transcriptase and concentration

MultiScribe Reverse Transcriptase (50 U/µl) (Applied Biosystems, Foster City, CA, USA), component of the TaqMan MicroRNA Reverse Transcription Kit (catalog number: 4366596; Applied Biosystems, Foster City, CA, USA).

Temperature and time
30 minutes at 16°C followed by 30 minutes at 42°C and 5 minutes at 85°C; rest period at 4°C. The preamplification reaction was performed as specified in the manufacturer`s protocol: 10 minutes at 95°C, followed by 12 cycles with 15 seconds at 95°C and 4 minutes at 60°C.

Manufacturer of reagents and catalogue numbers
TaqMan MicroRNA Reverse Transcription Kit (catalog number: 4366596; Applied Biosystems, Foster City, CA, USA); TaqMan® Universal Master Mix II with UNG, TaqMan® Small RNA Assays.
TaqMan MicroRNA Reverse Transcription Kit (catalog number: 4366596; Applied Biosystems, Foster City, CA, USA), RT Primer: cel-miR-39 (Applied Biosystems Assay ID: 000200), hsa-miR-371a-3p (Applied Biosystems Assay ID: 002124), hsa-miR-372-3p (Applied Biosystems Assay ID: 000560), hsa-miR-373-3p (Applied Biosystems Assay ID: 000561), hsa-miR-367-3p (Applied Biosystems Assay ID: 000555), miR-302a-3p (Applied Biosystems Assay ID: 000529), miR-302b (Applied Biosystems Assay ID: 000531) and miR-302c-3p (Applied Biosystems Assay ID: 000533).
Preamplification reaction: TaqMan PreAmp Master Mix 2X (catalog number: 4391128; Applied Biosystems, Foster City, CA, USA); Megaplex PreAmp Primer Human Pool A v2.1 (catalog number: 4399233; Applied Biosystems, Foster City, CA, USA).

Cqs with and without RT
Not determined.

Storage conditions of cDNA
We stored cDNA at -20 °C for one week.

qPCR TARGET INFORMATION
If multiplex, efficiency and LOD (=limit of detection) of each assay.
The Low Density Arrays in Phase-1 were purchased pre-designed from Applied Biosystems; efficiency (90-110%) and limit of detection (10 copies) are guaranteed by the manufacturer. We did not perform multiplex PCR in Phase-2.

Location of amplicon; Amplicon length; In Silico specificity screen; Pseudogenes, retropseudogenes or other homologs; Sequence alignment; Secondary structure analysis of amplicon; Location of each primer by exon or intron; Splice variants
All primers were purchased pre-designed from Applied Biosystems, and the location/size of the amplicon is not provided by the manufacturer. According to the manufacturer, the primers do not target pseudogenes, retropseudogenes or other homologs according to the manufacturer. Sequence alignment for each primer is possible at the manufacturer's website:
(https://bioinfo.appliedbiosystems.com/genome-database/).

Gene symbol and Sequence accession number

miRNA Primer Assay	Assay ID	microRNA sequence 5'-3'
cel-mir-39	000200	UCACCGGGUGUAAAUCAGCUUG
hsa-miR-371a-3p	002124	AAGUGCCGCCAUCUUUUGAGUGU
hsa-miR-372-3p	000560	AAAGUGCUGCGACAUUUGAGCGU
hsa-miR-373-3p	000561	GAAGUGCUUCGAUUUUGGGGUGU
hsa-miR-367-3p	000555	AAUUGCACUUUAGCAAUGGUGA
hsa-miR-302a-3p	000529	UAAGUGCUUCCAUGUUUUGGUGA
hsa-miR-302b-3p	000531	UAAGUGCUUCCAUGUUUUAGUAG
hsa-miR-302c-3p	000533	UAAGUGCUUCCAUGUUUCAGUGG

qPCR OLIGONUCLEOTIDES

Primer sequences; RTPrimerDB Identification Number; Probe sequences

All primers were purchased pre-designed from Applied Biosystems, and primer/probe sequences are not supplied by the manufacturer. The primers are not listed in the RTPrimer database.

Location and identity of any modifications

The TaqMan MGB probe contains: a reporter dye (FAM dye), a minor groove binder MGB and a non-fluorescent quencher (NFQ).

Manufacturer of oligonucleotides

Applied Biosystems, Foster City, CA, USA.

Purification method

Information on purification method is not provided by the manufacturer.

qPCR PROTOCOL

Complete reaction conditions

Screening cohort: All PCR experiments were carried out in triplicate on an ABIPrism 7900HT in 384 well plates using the TaqMan Universal Master Mix II with UNG kit; PCR primers were purchased from Applied Biosystems (assay-ID: cel-miR-39 #000200; hsa-miR-302a-3p #000529; hsa-miR-302b-3p #000531; has-miR-302c-3p #000533; hsa-miR-367-3p #000555; has-miR-371-3p #002124; hsa-miR-372-3p #000560; has-miR-373-3p #000561).

Validation cohort: In the validation cohort these PCR primers from Applied Biosystems were used: cel-miR-39 #000200; hsa-miR-367-3p #000555; hsa-miR-371a-3p #002124; hsa-miR-372-3p #000560; hsa-miR-373-3p #000561)

All experiments were performed as specified in the manufacturer's protocols.

Reaction volume and amount of cDNA/DNA

Our aim was to identify microRNA levels in serum. As specified in the manufacturer`s protocol we used 2,5 µl of isolated RNA for cDNA synthesis. cDNA concentrations were not determined later since there is no sense in equalizing cDNA concentrations in this study.

Primer, (probe), Mg++ and dNTP concentrations
We used the TaqMan® Universal Master Mix II with UNG (catalog number: 4304437 ; Applied Biosystems, Foster City, CA, USA) according to the manufacturer`s protocol. The concentration of the primers, Mg++ and dNTPs is not provided by the manufacturer. The concentration of the primers used was 20X.

Polymerase identity and concentration
We used the TaqMan® Universal Master Mix II with UNG (catalog number: 4304437; Applied Biosystems, Foster City, CA, USA). It includes the AmpliTaq Gold DNA Polymerase. Its concentration is not provided by the manufacturer.

Buffer/kit identity and manufacturer; Exact chemical constitution of the buffer; Additives
We used the TaqMan® Universal Master Mix II with UNG (catalog number: 4304437; Applied Biosystems, Foster City, CA, USA). The exact constitution is not provided. We did not use any additives.

Manufacturer of plates/tubes and catalog number
For qPCR the following plates and seals were used: Thermo-Fast 384 (catalog number: TF-0384; ThermoScientific, Waltham; USA) and Absolute QPCR-Seal (catalog number: AB-1170; ThermoScientific, Waltham; USA).

Complete thermocycling parameters
The thermo cycling conditions were, as specified in the manufacturer's protocol: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles with 15 seconds at 95°C and 1 minute at 60°C.

Reaction setup (manual/robotic)
The qPCR was setup up manually using an 8-chanel pipette (Matrix 30 µl, ThermoScientific, Waltham, DE; USA).

Manufacturer of qPCR instrument
ABIPrism 7900HT (Applied Biosystems, Foster City, CA, USA).

qPCR VALIDATION
Evidence of optimisation (from gradients); Specificity (gel, sequence, melt, or digest); Standard curves with slope and y-intercept; PCR efficiency calculated from slope; Confidence interval for PCR efficiency or standard error; r^2 of standard curve; Linear dynamic range; Cq variation at LOD; Confidence intervals throughout range; Evidence for limit of detection (LOD)
All primers were purchased pre-designed from Applied Biosystems, and performance is guaranteed by the manufacturer.

If multiplex, efficiency and LOD of each assay.
We did not perform multiplex assays.

DATA ANALYSIS

qPCR analysis program (source, version)

The analysis of the real-time PCR data was done using the SDS software v2.4 (settings: automatic baseline, threshold 0.2); relative microRNA levels were calculated with the RQ Manager v1.2.1, and data was analyzed with DataAssist V 3.01 (all software packages: Applied Biosystems, Foster City, CA, USA).

Cq method determination

The relative quantification of microRNA expression was performed using the comparative CT (ΔΔCT) method using the DataAssist V 3.01 software.

Outlier identification and disposition

By analyzing the real-time PCR data using the SDS software v2.4 with the following settings *automatic baseline, threshold 0.2*, outliers were identified and erased.

Results of NTCs

NTCs gave no signal.

Justification of number and choice of reference genes

We normalized the amount of circulating microRNAs against cel-miR-39, which allows absolute quantification of circulating microRNAs with normalization to technical variability.

Description of normalisation method

Serum microRNA levels were normalized to cel-miR-39 (synthetic non-human-microRNA, spiked-in during RNA isolation) using the comparative CT (ΔΔCT) method; calculations were performed with DataAssist v2.0.

Number and concordance of biological replicates

Not tested.

Number and stage (RT or qPCR) of technical replicates

All PCR experiments were performed with three technical replicates.

Repeatability (intra-assay variation) and Reproducibility (inter-assay variation)

Not done.

Power analysis

Not done.

Statistical methods for result significance

Sensitivity, specificity and area under curve (AUC) for serum microRNA levels were determined using Receiver Operator Characteristic (ROC) analysis. Clinical-pathological parameters and microRNA levels were correlated using the Mann-Whitney-U or Kruskal-Wallis-test, as appropriate.

Software (source, version)

Statistical analyses were performed using SPSS V 21.0 (SPSS, Chicago, IL, USA).

Supplementary Table 5: Serum-levels of the microRNAs and the classical tumor markers and the clinical-pathological parameters of patients investigated in the validation experiments excluding the patients of the screening

group	age	pT-stage	N-stage	cM-stage	IGCCCG	Lugano	AFP	HCG	LDH	miR-39	miR-367-3p	miR-371a-3p	miR-372-3p	miR-373-3p
B	33	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	increased	31%	0.0000	0.0033	0.5988	0.0015
B	30	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	98%	0.0000	0.0000	0.0030	0.0006
B	47	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	missing	71%	0.0000	0.0000	0.0603	0.0015
B	45	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	34%	0.0000	0.0000	0.0000	0.0012
B	28	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	10%	0.0000	0.0000	0.3494	0.0146
B	34	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	38%	0.0002	0.0000	0.0000	0.0080
B	22	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	35%	0.0000	0.0000	0.0000	0.0319
B	18	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	26%	0.0000	0.0000	0.0000	0.0147
B	18	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	32%	0.0000	0.0006	0.0000	0.0005
B	36	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	74%	0.0000	0.0000	0.0086	0.0007
B	19	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	40%	0.0000	0.0000	0.6829	0.0014
B	25	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	29%	0.0000	0.0000	0.0000	0.0014
B	57	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	7%	0.0000	0.0000	0.0000	0.1055
B	28	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	39%	0.0000	0.0000	0.0000	0.0143
B	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	32%	0.0000	0.0000	0.0000	0.0079
B	29	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	negative	24%	0.0000	0.0002	1.4988	0.0042
B	40	n.a.	n.a.	n.a.	n.a.	n.a.	negative	negative	missing	84%	0.0001	0.0002	0.0000	0.0039
CTRL	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	7%	0.0007	0.0000	0.0000	0.0169
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	96%	0.0000	0.0000	0.0000	0.0003
CTRL	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	19%	0.0000	0.0013	0.0425	0.0027
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	15%	0.0000	0.0000	0.0000	0.0022
CTRL	32	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	10%	0.0000	0.0056	0.0000	0.0018
CTRL	32	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	28%	0.0000	0.0000	0.3165	0.0213
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	52%	0.0000	0.0000	0.0000	0.0068
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	75%	0.0000	0.0000	0.0000	0.0123
CTRL	30	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	36%	0.0000	0.0000	0.0000	0.0012
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	12%	0.0004	0.0000	0.0000	0.0016
CTRL	23	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	52%	0.0001	0.0000	0.0000	0.0009
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	45%	0.0002	0.0000	0.0000	0.0017
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	25%	0.0003	0.0000	0.0000	0.0009
CTRL	31	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	41%	0.0000	0.0000	1.4956	0.0003
CTRL	47	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	25%	0.0000	0.0000	0.0000	0.0059
CTRL	42	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	8%	0.0000	0.0000	0.0000	0.0153
CTRL	40	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	27%	0.0000	0.0000	0.6399	0.0000
CTRL	42	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	31%	0.0005	0.0000	0.5841	0.0031
CTRL	19	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	14%	0.0000	0.0000	0.0000	0.0024
CTRL	29	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	14%	0.0000	0.0000	0.0000	0.0030
CTRL	28	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	87%	0.0000	0.0000	0.0000	0.0037
CTRL	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	40%	0.0000	0.0000	0.0000	0.0075
CTRL	31	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	27%	0.0000	0.0000	1.7765	0.0010
CTRL	32	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	26%	0.0000	0.0000	0.0000	0.0123

CTRL	29	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	45%	0.0000	0.0010	0.0000	0.0016
CTRL	26	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	132%	0.0000	0.0000	0.2084	0.0010
CTRL	28	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	44%	0.0000	0.0000	0.0037	0.0006
CTRL	34	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	50%	0.0000	0.0000	0.7698	0.0028
CTRL	56	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	49%	0.0000	0.0000	0.0000	0.0120
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	59%	0.0000	0.0000	0.5945	0.0030
CTRL	30	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	123%	0.0000	0.0000	0.0000	0.0005
CTRL	48	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	65%	0.0000	0.0000	0.0072	0.0067
CTRL	64	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	73%	0.0000	0.0000	0.0000	0.0010
CTRL	26	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	67%	0.0000	0.0000	0.5263	0.1122
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	55%	0.0002	0.0000	0.0000	0.0128
CTRL	31	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	100%	0.0000	0.0000	0.0000	0.0010
CTRL	30	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	60%	0.0000	0.0031	0.0000	0.0011
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	78%	0.0000	0.0016	0.0000	0.0008
CTRL	23	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	103%	0.0003	0.0000	0.2996	0.0257
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	65%	0.0000	0.0000	0.0000	0.0024
CTRL	50	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	44%	0.0000	0.0000	0.0000	0.0026
CTRL	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	39%	0.0000	0.0000	0.0000	0.0012
CTRL	31	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	49%	0.0000	0.0000	0.0000	0.0007
CTRL	29	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	50%	0.0000	0.0000	0.6318	0.0008
CTRL	53	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	59%	0.0000	0.0006	0.4969	0.0025
CTRL	39	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	72%	0.0000	0.0000	0.0000	0.0036
CTRL	40	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	42%	0.0001	0.0015	0.5381	0.0913
CTRL	39	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	65%	0.0001	0.0000	0.6806	0.0028
CTRL	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	18%	0.0000	0.0000	0.0000	0.0030
CTRL	41	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	31%	0.0000	0.0000	0.1871	0.0019
CTRL	44	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	63%	0.0000	0.0000	1.2116	0.0117
CTRL	44	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	109%	0.0000	0.0001	0.0000	0.0045
CTRL	43	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	134%	0.0000	0.0000	0.1255	0.0004
CTRL	42	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	30%	0.0000	0.0037	0.0040	0.0017
CTRL	41	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	60%	0.0000	0.0000	0.0614	0.0010
CTRL	38	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	16%	0.0000	0.0000	5.0973	0.0977
CTRL	34	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	86%	0.0000	0.0000	0.0000	0.0064
CTRL	29	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	43%	0.0000	0.0000	0.0000	0.0512
CTRL	30	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	93%	0.0001	0.0000	0.0226	0.0119
CTRL	29	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	76%	0.0001	0.0000	0.0000	0.0022
CTRL	28	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	39%	0.0000	0.0000	0.0000	0.0026
CTRL	27	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	21%	0.0007	0.0009	2.1565	0.0200
CTRL	19	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	17%	0.0000	0.0000	0.0000	0.0018
CTRL	32	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	41%	0.0000	0.0000	0.0000	0.0471
CTRL	47	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	20%	0.0000	0.0000	0.0000	0.0067
CTRL	34	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	24%	0.0000	0.0000	0.0000	0.0203
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	25%	0.0000	0.0000	0.0000	0.0000
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	28%	0.0000	0.0000	0.0000	0.0006

CTRL	26	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	30%	0.0000	0.0000	0.0460	0.0040
CTRL	34	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	34%	0.0000	0.0000	0.0000	0.0126
CTRL	26	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	117%	0.0000	0.0000	0.0000	0.0010
CTRL	19	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	202%	0.0001	0.0010	0.0000	0.0037
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	28%	0.0000	0.0000	1.0462	0.0216
CTRL	29	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	56%	0.0000	0.0000	0.0000	0.0009
CTRL	43	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	45%	0.0000	0.0000	0.0000	0.0016
CTRL	27	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	114%	0.0000	0.0000	0.2691	0.0088
CTRL	31	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	28%	0.0000	0.0000	0.5198	0.0255
CTRL	21	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	37%	0.0000	0.0000	0.0000	0.0046
CTRL	41	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	47%	0.0000	0.0000	0.7828	0.1484
CTRL	38	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	39%	0.0000	0.0000	0.0126	0.0370
CTRL	30	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	34%	0.0000	0.0000	0.5159	0.0008
CTRL	23	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	44%	0.0000	0.0000	0.0000	0.0014
CTRL	28	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	33%	0.0000	0.0000	0.0259	0.0013
CTRL	28	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	99%	0.0000	0.0000	0.0000	0.0003
NS	35	pT1	N0	M0	n.a.	CS1	negative	increased	missing	6%	0.0185	0.0991	0.8208	0.0079
NS	28	pT1	N0	M0	n.a.	CS1	increased	increased	negative	11%	0.0454	0.0979	0.1527	0.0928
NS	37	pT1	N0	M0	n.a.	CS1	negative	negative	negative	32%	0.0002	0.0000	0.4856	0.0520
NS	50	pT1	N0	M0	n.a.	CS1	negative	increased	negative	86%	0.1012	0.6168	0.2487	0.3396
NS	23	pT2	N0	M0	n.a.	CS1	increased	increased	negative	4%	0.0165	0.0737	1.0158	0.0136
NS	39	pT1	N0	M0	n.a.	CS1	negative	increased	negative	82%	0.0012	0.0216	0.0520	0.0240
NS	20	pT1	N0	M0	n.a.	CS1	increased	increased	negative	9%	0.0098	0.1158	0.0691	0.0391
NS	30	pT1	N0	M0	n.a.	CS1	negative	increased	negative	38%	0.0067	0.1719	0.0729	0.1458
NS	36	pT1	N0	M0	n.a.	CS1	increased	increased	negative	48%	0.0119	0.2082	0.1878	0.1303
NS	23	pT1	N0	M0	n.a.	CS1	negative	negative	negative	139%	0.0008	0.0075	0.0480	0.0020
NS	54	pT1	N0	M0	n.a.	CS1	negative	negative	negative	34%	0.0000	0.0000	0.0144	0.0022
NS	34	pT1	N0	M0	n.a.	CS1	negative	negative	missing	24%	0.0182	0.8298	0.1217	0.5927
NS	32	pT1	N1	M0	n.a.	CS1	negative	negative	negative	11%	0.0495	0.0733	0.0995	0.1518
NS	29	pT2	N0	M0	n.a.	CS1	negative	negative	negative	86%	0.0077	0.0230	0.1655	0.0094
NS	30	pT1	N1	M0	good	CS2	negative	negative	negative	21%	0.0000	0.0084	0.0000	0.0342
NS	21	pT2	N3	M0	good	CS2	increased	increased	increased	6%	0.0283	0.6345	0.1670	0.6152
NS	14	pT2	N3	M0	good	CS2	increased	increased	increased	7%	0.6961	6.6533	3.2632	2.8836
NS	30	pT1	N2	M1	good	CS3	increased	negative	negative	6%	0.6859	3.8486	0.6298	3.0749
NS	25	pT2	N0	M1	intermediate	CS3	increased	increased	negative	95%	0.0012	0.0128	0.6461	0.0578
S	36	pT1	N0	M0	n.a.	CS1	negative	negative	negative	108%	0.0009	0.0578	0.0417	0.0442
S	42	pT1	N0	M0	n.a.	CS1	negative	negative	negative	75%	0.0000	0.0021	0.5496	0.0136
S	38	pT1	N0	M0	n.a.	CS1	negative	negative	negative	28%	0.0000	0.0000	0.0000	0.0018
S	23	pT1	N0	M0	n.a.	CS1	negative	negative	negative	54%	0.0006	0.0232	0.5262	0.0236
S	35	pT1	N0	M0	n.a.	CS1	negative	negative	negative	53%	0.0004	0.0490	0.0328	0.0445
S	28	pT1	N0	M0	n.a.	CS1	negative	negative	increased	72%	0.0064	0.3078	0.3141	0.1525
S	29	pT2	N0	M0	n.a.	CS1	negative	negative	negative	93%	0.0147	0.3501	0.3037	0.2819
S	31	pT1	N0	M0	n.a.	CS1	negative	negative	negative	4%	0.1990	2.0995	2.0585	2.0963
S	39	pT2	N0	M0	n.a.	CS1	negative	increased	missing	8%	0.0172	1.0352	2.0658	0.4361

S	31	pT1	N0	M0	n.a.	CS1	negative	negative	negative	24%	0.0000	0.0133	0.0158	0.0048
S	36	pT2	N0	M0	n.a.	CS1	negative	negative	negative	35%	0.0000	0.0052	0.0164	0.0257
S	31	pT1	N0	M0	n.a.	CS1	negative	negative	negative	20%	0.0025	0.2555	0.2171	0.2152
S	42	pT1	N0	M0	n.a.	CS1	negative	increased	negative	116%	0.0006	0.0223	0.0197	0.0073
S	54	pT1	N0	M0	n.a.	CS1	negative	negative	negative	25%	0.0000	0.0270	2.4428	0.0257
S	30	pT1	N0	M0	n.a.	CS1	negative	negative	negative	27%	0.0000	0.0003	0.0087	0.0178
S	56	pT1	N0	M0	n.a.	CS1	negative	increased	negative	20%	0.0040	0.4125	0.4359	0.3104
S	34	pT1	N0	M0	n.a.	CS1	negative	negative	negative	44%	0.0005	0.0111	0.0889	0.0303
S	30	pT1	N0	M0	n.a.	CS1	negative	increased	negative	81%	0.0003	0.0415	0.0319	0.0100
S	37	pT1	N0	M0	n.a.	CS1	negative	negative	increased	45%	0.0000	0.0000	0.0000	0.0134
S	46	pT1	N0	M0	n.a.	CS1	negative	negative	negative	63%	0.0014	0.0200	0.0759	0.0807
S	59	pT1	N0	M0	n.a.	CS1	negative	negative	negative	4%	0.0055	0.0947	4.3679	0.1083
S	33	pT1	N0	M0	n.a.	CS1	negative	negative	negative	28%	0.0000	0.0082	0.0000	0.0175
S	28	pT1	N0	M0	n.a.	CS1	negative	negative	missing	34%	0.0027	0.0799	0.3258	0.0012
S	45	pT1	N0	M0	n.a.	CS1	negative	negative	negative	34%	0.0000	0.0054	0.0529	0.0163
S	44	pT1	N0	M0	n.a.	CS1	negative	increased	missing	35%	0.0131	1.0449	1.3091	0.6094
S	34	pT1	N0	M0	n.a.	CS1	negative	negative	negative	40%	0.0000	0.0075	0.7756	0.0174
S	46	pT1	N0	M0	n.a.	CS1	negative	negative	negative	9%	0.0000	0.0000	0.0000	0.0046
S	48	pT1	N0	M0	n.a.	CS1	negative	negative	negative	31%	0.0000	0.0000	0.8189	0.0121
S	30	pT1	N0	M0	n.a.	CS1	negative	negative	negative	41%	0.0007	0.0505	0.6595	0.1223
S	33	pT1	N0	M0	n.a.	CS1	negative	negative	negative	29%	0.0020	0.0000	0.0649	0.0015
S	38	pT1	N0	M0	n.a.	CS1	negative	increased	negative	67%	0.0000	0.0070	0.3124	0.0043
S	34	pT3	N0	M0	n.a.	CS1	negative	negative	negative	28%	0.0021	0.0874	0.0567	0.0712
S	52	pT2	N0	M0	n.a.	CS1	negative	negative	negative	58%	0.0001	0.0227	0.5571	0.0070
S	51	pT3	N3	M0	good	CS2	negative	increased	increased	63%	0.0427	2.6565	0.2701	1.1628
S	25	burned out	N1	M0	good	CS2	negative	increased	negative	40%	0.0185	0.1542	0.1524	0.0730
S	33	pT1	N3	M0	good	CS2	negative	increased	increased	37%	0.0119	0.6023	0.4112	0.4421
S	34	burned out	N3	M0	good	CS2	negative	negative	increased	37%	0.0168	0.4093	1.2507	0.2603
S	43	pT1	N1	M0	intermediate	CS2	negative	increased	increased	102%	0.0007	0.0440	0.0341	0.0300
S	35	pT2	N3	M0	intermediate	CS2	negative	negative	negative	73%	0.0088	0.1798	0.1620	0.1596
S	53	pT1	N2	M1	intermediate	CS3	negative	increased	increased	40%	0.0246	0.9600	0.2353	0.6004

group	age	pT-stage	cN-stage	cM-stage	IGCCCG	Lugano	AFP	HCG	LDH	miR-39	miR-302a-3p	miR-302b-3p	miR-302c-3p	miR-367-3p	miR-371a-3p	miR-372-3p	miR-373-3p
CTRL	27	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	2%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	19	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	166%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	41	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	2%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	27	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	69%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	21	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	86%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	49	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	32%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	0%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	31	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	0%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.1400
CTRL	33	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	65%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	26	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	1%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	72%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	22	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	58%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	35	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	145%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	24	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	88%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	38%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	28	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	92%	0.0000	0.0000	0.0004	0.0000	0.0000	0.0002	0.0000
CTRL	25	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	36%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
CTRL	36	n.a.	n.a.	n.a.	n.a.	n.a.	missing	missing	missing	62%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NS	35	T1	N0	M0	n.a.	CS1	increased	increased	missing	2%	0.0000	0.0000	0.0000	0.0359	0.0000	0.0199	0.0000
NS	34	T1	N0	M0	n.a.	CS1	negative	increased	missing	16%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NS	22	T1	N0	M0	n.a.	CS1	increased	increased	increased	23%	0.1374	0.0022	0.0000	1.1048	2.9713	0.7978	2.1100
NS	35	T1	N0	M0	n.a.	CS1	increased	negative	negative	22%	0.0011	0.0000	0.0000	0.0875	0.0000	0.0025	0.1900
NS	33	T1	N0	M0	n.a.	CS1	increased	increased	negative	32%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
NS	46	T3	N2	M0	good	CS2	negative	negative	increased	18%	0.0005	0.0000	0.0000	0.0296	0.0259	0.0084	0.0000
NS	21	T2	N2	M0	good	CS2	negative	negative	negative	19%	0.0000	0.0000	0.0000	0.0282	0.0060	0.0031	0.0000
NS	42	T1	N2	M0	good	CS2	increased	negative	missing	19%	0.0000	0.0000	0.0000	0.0301	0.0000	0.0000	0.0000
NS	39	T2	N1	M0	intermediate	CS2	increased	increased	missing	30%	0.0000	0.0000	0.0000	0.0069	0.0015	0.0036	0.0000
NS	39	T1	N1	M0	intermediate	CS2	increased	increased	negative	1%	0.0000	0.0000	0.0000	4.6034	2.3477	0.7961	0.0000
NS	28	T1	N2	M0	intermediate	CS2	increased	increased	increased	1%	0.0000	0.0000	0.0000	0.2226	0.4948	0.1233	0.0000
NS	22	T1	N1	M0	intermediate	CS2	negative	increased	increased	18%	0.0000	0.0000	0.0000	0.0026	0.0000	0.0456	0.0000
NS	33	T3	N3	M1	good	CS3	negative	increased	increased	22%	0.0000	0.0000	0.0000	0.0437	0.2107	0.0188	0.1100
NS	29	T1	N3	M1	poor	CS3	negative	negative	increased	28%	0.0080	0.0000	0.0000	0.5003	0.3010	0.0754	0.0100
NS	21	T3	N3	M1	poor	CS3	increased	increased	increased	26%	0.0410	0.0058	0.0166	0.1748	0.2318	0.1684	0.1200
S	36	T1	N0	M0	n.a.	CS1	negative	negative	negative	26%	0.0000	0.0000	0.0000	0.0021	0.0132	0.0001	0.0000
S	32	T1	N0	M0	n.a.	CS1	negative	negative	negative	17%	0.0000	0.0000	0.0000	0.0003	0.0000	0.0015	0.0000
S	35	T1	N0	M0	n.a.	CS1	negative	negative	missing	40%	0.0000	0.0000	0.0000	0.0000	0.0071	0.0008	0.0000
S	30	T1	N0	M0	n.a.	CS1	negative	increased	missing	22%	0.0000	0.0000	0.0000	0.0003	0.0054	0.0042	0.0000
S	24	T1	N0	M0	n.a.	CS1	negative	negative	negative	29%	0.0000	0.0000	0.0000	0.0020	0.0475	0.0182	0.0000
S	36	T2	N0	M0	n.a.	CS1	negative	negative	negative	21%	0.0000	0.0000	0.0000	0.0474	0.5263	0.2228	1.0800
S	31	T2	N1	M0	n.a.	CS1	negative	negative	increased	38%	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
S	49	T1	N2	M0	good	CS2	negative	negative	negative	1%	0.0000	0.0000	0.0000	0.3447	0.0040	0.0044	0.0300
S	51	T3	N3	M0	good	CS2	negative	increased	increased	1%	0.0000	0.0000	0.0000	0.9242	11.6200	1.9081	51.2000
S	48	T3	N2	M0	good	CS2	negative	increased	missing	16%	0.0000	0.0000	0.0000	0.1041	0.2362	0.1347	0.9800
S	37	T2	N2	M0	good	CS2	increased	increased	negative	15%	0.0000	0.0000	0.0000	0.0000	0.0175	0.0022	0.1200
S	27	T1	N2	M0	good	CS2	negative	negative	negative	22%	0.0000	0.0000	0.0000	0.0016	0.0202	0.0058	0.0000
S	38	T1	N3	M0	good	CS2	negative	negative	negative	36%	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000
S	42	T1	N2	M0	good	CS2	negative	negative	missing	31%	0.0000	0.0000	0.0000	0.0067	0.0004	0.0011	0.0000
S	41	Tx	N3	M1	good	CS3	negative	increased	increased	45%	0.0000	0.0000	0.0000	0.0383	0.0834	0.0451	0.3000