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Original Article

Circulating miRNA landscape identifies miR-1246 as promising diagnostic biomarker in high-grade serous ovarian carcinoma: A validation across two independent cohorts

Paola Todeschini [a, b, 1], Elisa Salviato [c, 1], Lara Paracchini [d, 1], Manuela Ferracin [e], Marco Petrillo [f], Laura Zanotti [a], Germana Tognon [g], Angela Gambino [h], Enrica Calura [c], Giulia Caratti [d], Paolo Martini [c], Luca Beltrame [d], Lorenzo Maragoni [c], Daniela Gallo [f], Franco E. Odicino [h], Enrico Sartori [h], Giovanni Scambia [f], Massimo Negrini [i], Antonella Ravaggi [a], Maurizio D'Incalci [d, *], Sergio Marchini [d, 2], Eliana Bignotti [g, 2], Chiara Romualdi [c, 2]

[a] Department of Obstetrics and Gynecology, "Angelo Nocivelli" Institute of Molecular Medicine, University of Brescia, Brescia, Italy
[b] Doctorate School of Molecular and Translational Medicine, University of Milano, Milano, Italy
[c] Department of Biology, University of Padova, Padova, Italy
[d] Department of Oncology, IRCCS – "Mario Negri" Institute for Pharmacological Research, Milano, Italy
[e] Department of Experimental, Diagnostic and Specialty Medicine – DIMES, University of Bologna, Bologna, Italy
[f] Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Catholic University of the Sacred Heart, Rome, Italy
[g] Division of Obstetrics and Gynecology, ASST Spedali Civili di Brescia, Brescia, Italy
[h] Department of Obstetrics and Gynecology, University of Brescia, Brescia, Italy
[i] Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

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ABSTRACT

High-grade serous ovarian carcinoma (HGSOC) is the most lethal gynecologic neoplasm, with five-year survival rate below 30%. Early disease detection is of utmost importance to improve HGSOC cure rate.

Sera from 168 HGSOC patients and 65 healthy controls were gathered together from two independent collections and stratified into a training set, for miRNA marker identification, and a validation set, for data validation. An innovative statistical approach for microarray data normalization was developed to identify differentially expressed miRNAs. Signature validation in both the training and validation sets was performed by quantitative Real Time PCR (RT-qPCR).

In both the training and validation sets, *miR-1246*, *miR-595* and *miR-2278* emerged significantly over expressed in the sera of HGSOC patients compared to healthy controls. Receiver Operating Characteristic curve analysis revealed *miR-1246* as the best diagnostic biomarker, with a sensitivity of 87%, a specificity of 77% and an accuracy of 84%.

This study is the first step in the identification of circulating miRNAs with diagnostic relevance for HGSOC. According to its specificity and sensitivity, circulating *miR-1246* levels are worthy to be further investigated as potential diagnostic biomarker for HGSOC.

Introduction

The most common histological subtype of epithelial ovarian cancer (EOC), the high-grade serous ovarian carcinoma (HGSOC), is generally diagnosed late, when multiple synchronous lesions localised to the ovary, as well as in other anatomical sites within the peritoneal cavity. The five-year survival rate is less than 30%, as patients, despite an initial response to platinum agents, become progressively resistant and die from incurable disease. It is becoming evident that efforts to optimize patients' clinical outcome should be focused either on improving the therapeutic armamentarium or on enhancing early disease detection [1]. The

* Corresponding author. Department of Oncology, IRCCS Istituto di Ricerche Farmacologiche "Mario Negri", Via La Masa 19, 20156 Milano, Italy.
E-mail address: maurizio.dincalci@marionegri.it (M. D'Incalci).
[1] PT, ES and LP contributed equally to this work.
[2] SM, EB and CR are co-last authors.

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often asymptomatic nature of HGSOC has hampered till now any attempt to improve early diagnosis.

Results from a recently published UK collaborative trial on ovarian cancer (UKCTOCS), among more than 200,000 post-menopausal women, showed the limits of currently known predictors of disease, like pelvic examination, CA-125 levels and transvaginal ultrasound [2].

Given the complex molecular nature lagging behind the single definition of HGSOC, future screening strategies aimed to define an individual's risk of EOC should take into consideration this biologic heterogeneity [3]. One of the most promising field of research is based on the analysis of microRNA (miRNA) expression profile in liquid biopsies, as serum or plasma.

In the clinical practice, the identification of novel biomarkers in tumor tissues suffers of at least two main limitations. Firstly, tissue samples are difficult to acquire during patients' follow up, therefore assessing the prognostic role of tumor biomarkers in longitudinal analysis is limited. Secondly, since HGSOC is a systemic disease, molecular portraits obtained at the ovary not necessarily reflect those obtained from synchronous lesions in other anatomical sites [4]. Liquid biopsies are now becoming a new source to develop novel biomarkers with diagnostic and prognostic purposes. Previous studies have confirmed the potential use of miRNA profiling as a novel non invasive biomarker for diagnosis of ovarian cancer [5–7].

The focus of this study was the detection of the levels of circulating miRNAs in tissues and sera from patients with HGSOC as a first step in the evaluation process of their role as diagnostic biomarkers.

Materials and methods

Methods-patients

We retrospectively evaluated a cohort of 233 serum samples gathered together from two independent Italian serum collections. The first, collected at the Division of Obstetrics & Gynecology, ASST Spedali Civili, University of Brescia, Brescia, Italy, between 2003 and 2013 consists of serum samples from 110 patients with stage III-IV HGSOC and from 52 healthy individuals of comparable age. The second, collected at the Division of Gynecologic Oncology of Policlinico A. Gemelli, Catholic University, Rome, between 2005 and 2012, is made up of 58 serum specimens belonging to patients with HGSOC and 13 samples of comparable age healthy individuals (Table 1).

Matched flash-frozen tumor tissues were collected from 76 out of 110 HGSOC patients enrolled at the Division of Obstetrics & Gynecology, ASST Spedali Civili, University of Brescia. As controls, normal ovarian and fallopian tube epithelia were obtained from a total of 28 patients undergoing hysterectomy and bilateral salpingo-oophorectomy for benign pathologies at the same institution (Supplementary Table S1.1). This study was authorized by the institutional review board of ASST Spedali Civili of Brescia and Catholic University, Rome. The study was performed following the Declaration of Helsinki set of principles and approved by the Research Review Board -the Ethic Committee-of the Spedali Civili, Brescia, Italy (study reference number: NP1676). Written informed consent was obtained from all patients enrolled.

Total RNA extraction

Total RNA enriched in miRNA fraction was extracted from 200 μl of serum using miRNeasy Mini kit (Qiagen, Milan Italy). In particular, serum samples were thawed in ice, then 1 ml of QIAzol Lysis Reagent (Qiagen) was added to the samples and they were kept at room temperature for 5 min. Ten synthetic spike-in RNA oligos (12.5 fmol each in a total volume of 25 μl), without sequence homology to known human miRNAs, were added to samples to control for variations during the preparation of total RNA and subsequent steps. RNA oligo sequences are displayed in Supplementary Table S1.2. All the last steps of purification were performed following the manufacturer's instructions (Qiagen). RNA was eluted in 35 μl nuclease-free water.

Total RNA was extracted from tissue samples using TRIzol® Reagent, followed by a purification with RNeasy MinElute Cleanup® kit (Qiagen), with a modified protocol for co-purification of small RNAs according to the manufacturer's instructions. In details, the starting sample volume was adjusted to 50 μl with RNase-free water, instead of 100 μl. Then, after the addition of 350 μl of RLT buffer, 600 μl of 96–100% ethanol were mixed to the sample. Then, we followed the procedure steps reported on the RNeasy MinElute Cleanup Handbook (Qiagen). RNA concentration and 260/280 absorbance ratio ($A_{260/280}$) were measured with Infinite M200 spectrophotometer (TECAN). RNA integrity was assessed with RNA 6000 Nano LabChip kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity number (RIN), generated with Agilent 2100 Expert software, was superior to 8 for all RNA samples.

miRNA profiling by microarray

Two independent miRNA microarray profiling evaluations of serum and tissue samples were performed. In tissue miRNA arrays, 76 HGSOC tissues, 5 pools of HOSE obtained from 17 women, and 11 normal luminal fallopian tube epithelia were hybridized. In serum miRNA arrays, 110 sera from HGSOC patients and 19 sera from healthy controls were evaluated for miRNA expression profiling. Briefly, for tissue miRNA profiling, 100 ng of RNA, enriched in miRNA fraction, were Cyanine 3-pCp labeled and hybridized on the commercially available G4871A human miRNA Microarray kit, using a miRNA labeling and hybridization kit according to the manufacturer's instructions (Agilent Technologies). For the analysis of circulating miRNAs, we used the commercially available G4872A-046064 human miRNA Microarray Kit (Agilent Technologies), customized with probes for the detection of specific RNA oligo spike-in. For circulating miRNA profile, we hybridized fixed volume of eluted total RNA, derived from fixed serum volumes, for all samples tested. The arrays were washed and scanned with a laser confocal scanner (G2565BA, Agilent Technologies) according to the manufacturer's instructions. miRNA microarrays underwent standard post hybridization processing and the intensities of fluorescence were calculated by Feature Extraction software version 11 (Agilent Technologies). Raw data are available at ArrayExpress database (accession E-MTAB-4667).

Table 1
Clinico-pathological features of HGSOC patients and healthy controls enrolled in the study.

Clinico-pathological characteristics		Training set		Validation set	
		HGSOC patients (n = 110)	Healthy controls (n = 52)	HGSOC patients (n = 58)	Healthy controls (n = 13)
Median age (range), years		61 (36–85)	61 (38–73)	57 (34–76)	52 (26–67)
Menopausal status	Pre	24 (22%)	–	16 (27%)	–
	Post	85 (77%)	–	41 (71%)	–
	Missing	1 (1%)	–	1 (2%)	–
FIGO Stage	III	78 (71%)	–	55 (95%)	–
	IV	32 (29%)	–	3 (5%)	–
Level of CA-125 (median, UI/mL)	≥943	52 (47.5%)	–	29 (50%)	–
	<943	52 (47.5%)	–	28 (48%)	–
	Missing	6 (5%)	–	1 (2%)	–
Presence of ascites	Yes	90 (82%)	–	31 (54%)	–
	No	19 (17%)	–	3 (5%)	–
	Missing	1 (1%)	–	24 (41%)	–
Lymph node metastasis	Yes	43 (39%)	–	21 (36%)	–
	No	21 (19%)	–	36 (62%)	–
	Missing	46 (42%)	–	1 (2%)	–

cDNA synthesis and real-time quantitative PCR (RT-qPCR)

Signature validation by quantitative Real-Time-reverse transcription PCR (RT-qPCR) was performed starting from 5 μl of total RNA, purified as previously described [8,9], and reverse transcribed into cDNA, following manufacturer's instructions (miScript Reverse Transcription Kit, Qiagen). We used a fixed volume of eluted RNA sample as input for RT-qPCR, rather than using a fixed quantity of input RNA, as previously reported [10]. Two microliter of cDNA were used for RT-qPCR experiments in triplicate using Rotor-Gene Thermal Cycler (Qiagen). Experiments were run in triplicate and plates were prepared by automatic liquid handling station on a final volume of 10 μl (QiaAgility). As there are no established endogenous miRNAs acting as normalizers for serum miRNAs, RT-qPCR analysis was performed on raw cycle thresholds (Cq).

Droplet digital PCR workflow

Each EvaGreen amplification mixture (20 μl) was loaded into a disposable droplet generator cartridge (Bio-Rad) and mixed with 70 μl of droplet generator oil into the QX200 droplet generator (Bio-Rad), thus portioning each sample into 20,000 nL-sized droplets. Emulsified samples were then transferred into a 96-well PCR plate to perform PCR, using a conventional thermal cycle. The cycling steps were set as follow: 95 °C for 5 min, (95 °C for 30, 58 °C for 1 min) × 40 cycles, 4 °C for 5 min, 90 °C for 5 min and infinite 4 °C holding. The PCR plate was then loaded into the QX200 droplet reader (Bio-Rad) for sample automated analysis. A no template control (no cDNA in PCR) and a negative control for each reverse transcription reaction (RT-neg) were included in every assay run.

Statistical analysis

Expression data has been normalized using the cyclic lowess [11], in which the 10 spike-in oligos and a set of 10 invariant low expressed miRNAs were used as stabilizing factors. See Supplementary Material S2 for the details on data preprocessing. Empirical Bayes test (limma Bioconductor package) has been used to identify differentially expressed miRNAs with an FDR (False Discovery Rate) < 0.05. Hierarchical clustering has been performed with Euclidean distance and complete linkage. RT-qPCR data in the training and validation sets have been tested between patients and controls using t-test. Linear discriminant analysis and ROC curves were used to estimate sensitivity and specificity for each biomarker in the training and validation sets. Logistic multivariate model has been applied to test the prognostic performance of the integration of all biomarkers. This study was carried out following REMARK guidelines [12].

Results

Cohort description and study design

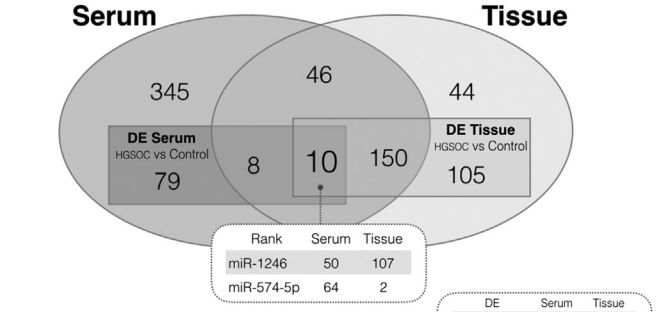
Table 1 shows clinico-pathological features of HGSOC patients and healthy controls from whom sera were selected for the study. Samples were gathered together from two independent Italian tumor tissue collections and subdivided into a training set and a validation set. Serum samples were withdrawn at diagnosis, before any treatment. The median age at diagnosis was 61 and 58 years for the training and the validation set, respectively. The vast majority of women were in postmenopausal status (77% and 71% for the training and the validation set, respectively). All patients were staged according to FIGO (Federation International of Gynecology and Obstetrics) guidelines as stage III-IV [13], with high-grade serous histological type. The median CA-125 value at diagnosis was 943 UI/ml. As shown in Table 1, the CA-125 levels were higher than the above level in the 47% of patients of the training set and in the 50% of patients of the validation set. Some patients showed presence of ascites (82% training set and 54% validation set, respectively) and lymph node metastasis (39% for the training set and 36% for the validation set). No patients received neo-adjuvant chemotherapy. The study was organised into two steps. Firstly, in the training set, array technology was used to identify miRNA species differentially expressed between HGSOC patients ($n = 110$) and healthy controls ($n = 52$). Secondly, the expression of the candidate circulating miRNAs was validated in an external and independent cohort of sera of HGSOC patients ($n = 58$) and healthy controls ($n = 13$).

Discovery of candidate diagnostic miRNAs in serum by microarrays

To identify the entire repertoire of known miRNA species expressed in patients with stage III-IV HGSOC, miRNA microarray experiments were performed in the training set. After a comparative analysis, we selected, as the best normalization strategy for our data, the cyclic lowess normalization with weights on spike-in oligos and low invariant miRNAs (hereafter called CLWsim, Supplementary Material S2). A total of 97 miRNAs were identified as differentially expressed between sera of HGSOC patients and healthy controls. The complete list of differentially expressed miRNA (from now on referred to as DEM) are reported in Supplementary Table S2.1. Of these, 92 miRNAs (95%) resulted up-regulated, and five (5%) resulted down-regulated in the sera of patients compared to healthy controls. Cluster analysis using DEM expression levels (Supplementary Fig. S2.3) identified three main clusters, C1, C2 and C3. With the exception of 7 healthy patients, cluster C2 and C3 are enriched in miRNAs from HGSOC patients, while cluster C1 is enriched in miRNA species from healthy controls. No differences in clinical characteristics have been observed between C2 and C3.

Comparison of miRNA expression between matched serum and tissue samples

To investigate the tissue of origin of selected DEM, we investigated miRNA expression profile in those patients ($n = 76$, Supplementary Table S1.1) for whom matched sera and tumor tissues were available. Analysis revealed 265 DEM (123 up-regulated and 142 down-regulated) in HGSOC biopsies compared to 28 normal tissues (Supplementary Material S3). As described in Fig. 1, only ten miRNAs resulted differentially expressed in both matched tissue and serum samples. Of these, eight miRNAs shared the same



	Total Detected	DE (up - down)
Serum	638	97 (92 - 5)
Tissue	363	265 (123 -142)
Intersection	214	10

Fig. 1. Venn diagram of the list of differentially expressed miRNAs between tissue and serum samples. After filtering and normalization steps, the total number of detectable microRNAs was 638 and 363 for serum and tissue samples respectively. The Venn diagram shows the partition of these miRNAs detected only in serum (345 + 79), or in tissue (105 + 44), or detected in both serum and tissue samples (46 + 150 + 8 + 10). Among the detected miRNAs, 97 and 265 were found to be differentially expressed (DE in the figure) in HGSOC samples compared to healthy controls as reported in the table on the left-bottom part of the figure (in brackets are reported the number of up and down regulated miRNAs). Of these, only ten miRNAs were found significantly differently expressed compared to healthy controls either in serum and in tissue samples. Eight out of ten miRNAs show a concordant trend (up/down regulated with respect to the healthy controls), and two show an opposite trend (as reported in the table on the bottom-right of the figure). Among these ten miRNAs, we selected *miR-1246* and *miR-574-5p* with their rank position in the list of differentially expressed reported in the table located in the center of the figure.

trend of regulation in both tumors and sera, while two miRNAs (*miR-1246* and *miR-1972*) displayed an opposite trend.

We further explored *miR-1246* expression trend in HGSOC versus separately ovarian and fallopian tube epithelia. Using our microarray data, we observed a significant downregulation of *miR-1246* with respect to ovarian epithelia ($p < 0.0001$), while the comparison between *miR-1246* levels in HGSOC tissues and normal fallopian tube epithelia was not significant ($p = 0.42$). These results were confirmed by RT-qPCR, using *SNORD48* as reference for proper data normalization, as previously published by our group [14].

Validation of candidate circulating miRNAs by RT-qPCR in the training set

Signature validation was performed by independent techniques, like RT-qPCR (quantitative Real Time PCR) or ddPCR (droplet digital PCR). Due to the limits of PCR-based approaches and the low abundance of miRNA species in the sera of HGSOC patients, validation experiments were performed on a selection of DEM according to the following criteria: *i*) we selected only miRNAs with at least 75% of good quality measures (not NA) across samples or within patients or within healthy controls, *ii*) highest log fold change, measured in patients compared to healthy controls, *iii*) lower adjusted p-value. Finally, only miRNA expression with $Ct < 36$ were considered reliable.

Supplementary Table S2.2 reports the list of nine DEM selected for independent validation. Of these, *miR-1246* and *miR-574-5p* resulted as DEM in matched tissue samples. To note, except for *miR-4281*, all DEM selected for further RT-qPCR validation lies in the intersection of the three different normalization approaches (Supplementary Figure S2.4).

Results of RT-qPCR are reported in Table 2. *miR-1246*, *miR-4290*, *miR-595*, and *miR-2278* ($P \leq 0.0002$, FC (Fold Change) = 7.78, FC = 1.97, FC = 2.08, FC = 7.01, respectively) are the most significantly up-regulated miRNAs in the serum of patients compared to healthy controls. *miR-574-5p* and *miR-483-3p* were not confirmed. RT-qPCR Ct values for *miR-32-3p, miR-4281* and *miR-3148* resulted above the selected cut-off (i.e., $Ct = 36$) and therefore were discarded from downstream validation.

Independent evaluation of candidate circulating miRNAs in HGSOC patients

Results were further validated in a second and independent cohort of sera (Table 1). Accordant with the results in the training set, the expression levels of *miR-1246, miR-595* and *miR-2278* displayed a significant over-expression (all $P \leq 0.03$, FC = 3.11, FC = 2.96, FC = 1.1, respectively) in the serum of HGSOC patients compared to healthy controls (Table 2 and Fig. 2). Conversely, *miR-4290* showed an opposite trend.

To further support the robustness of our findings, we normalised our RT-qPCR data against *miR-15b* expression. Also there is no consensus on the normalization strategy for circulating miRNA, *miR-15b* is reported as a reliable reference for circulating miRNA analysis [15], and from our microarray and RT-qPCR data (Supplementary Fig. S4.1), miR-15b resulted as one of the most invariant miRNA. Normalised data reported in Supplementary Table S4.1 confirmed *miR-1246* and *miR-595* as significantly up-regulated in HGSOC patients of both training and validation sets (Supplementary Table S4.1).

Collectively, these analyses suggest that circulating *miR-1246, miR-595* and *miR-2278* in serum may serve as candidate biomarkers for diagnosis of HGSOC.

Evaluation of the diagnostic potential of miRNAs for HGSOC

To assess the efficiency of above three miRNAs as diagnostic markers for HGSOC detection, we performed ROC curve analysis

Table 2
RT-qPCR expression analysis.

RT-qPCR		Training set		Validation set	
		Control	Case	Control	Case
miR-1246	median (CT) [IQR]	26.4 [2.13]	23.44 [2]	27.39 [0.49]	25.75 [1.3]
	mean (CT) [sd]	25.83 [1.33]	23.41 [1.6]	27.56 [0.62]	25.82 [1.15]
	p-value (2^{-CT})	**<0.00001**		**<0.00001**	
miR-574-5p	median (CT) [IQR]	28.12 [1.59]	28.24 [2.23]	30.35 [1.31]	29.58 [1.85]
	mean (CT) [sd]	28.16 [1.38]	28.32 [1.66]	30.67 [0.97]	29.6 [1.24]
	p-value (2^{-CT})	0.6637		<0.00001	
miR-483-3p	median (CT) [IQR]	32.41 [1.17]	32.77 [1.57]	31.87 [0.8]	32.12 [1.17]
	mean (CT) [sd]	32.44 [0.91]	32.72 [1.24]	31.84 [0.62]	32.28 [1.16]
	p-value (2^{-CT})	0.9799		0.4145	
miR-4290	median (CT) [IQR]	33.25 [1.95]	32.27 [2.33]	30.11 [0.75]	31.09 [1.46]
	mean (CT) [sd]	33.4 [1.58]	32.62 [1.86]	30.21 [0.8]	31.08 [1.19]
	p-value (2^{-CT})	0.0002		0.0344	
miR-595	median (CT) [IQR]	34.29 [2.01]	33.23 [2.55]	34.44 [2.03]	32.87 [2]
	mean (CT) [sd]	34.37 [1.76]	33.17 [2.39]	34.13 [1.28]	32.85 [1.76]
	p-value (2^{-CT})	**0.0002**		**<0.0001**	
miR-2278	median (CT) [IQR]	36.71 [2.99]	33.90 [1.56]	33,59 [0,32]	33,45 [0,74]
	mean (CT) [sd]	36.44 [2.21]	34.06 [1.61]	33.58 [0.36]	33.39 [0.52]
	p-value (2^{-CT})	**0.0000**		**0.0373**	
miR-32-3p	median (CT) [IQR]	35.87 [1.8]	37.12 [1.67]	38.23 [1.53]	36.84 [2]
	mean (CT) [sd]	36.16 [1.48]	37.16 [1.7]	38.34 [2.42]	37.29 [1.68]
	p-value (2^{-CT})	–		–	
miR-4281	median (CT) [IQR]	38.79 [3.15]	36.68 [1.67]	37.43 [2.4]	37.57 [1.81]
	mean (CT) [sd]	38.75 [1.98]	36.81 [1.68]	37.8 [1.48]	37.61 [1.65]
	p-value (2^{-CT})	–		–	
miR-3148	median (CT) [IQR]	39.43 [3.29]	37.75 [2.45]	38.31 [1.64]	38.2 [1.38]
	mean (CT) [sd]	39.83 [2.46]	37.83 [2.42]	37.55 [2.08]	38.35 [1.53]
	p-value (2^{-CT})	–		–	

For each of the nine selected miRNAs, expression values in the sera of both healthy controls (Control) and HGSOC patients (Case) are reported for the training and validation set. The median of the cycle threshold (CT) with inter-quantile range (IQR), the mean Ct with standard deviation and the p-value are shown.
The p-values of miR-1246, miR-595 and miR-2278, that showed a significant over-expression in the serum of HGSOC patients compared to healthy controls, both in the training and in the validation sets, are reported in bold.

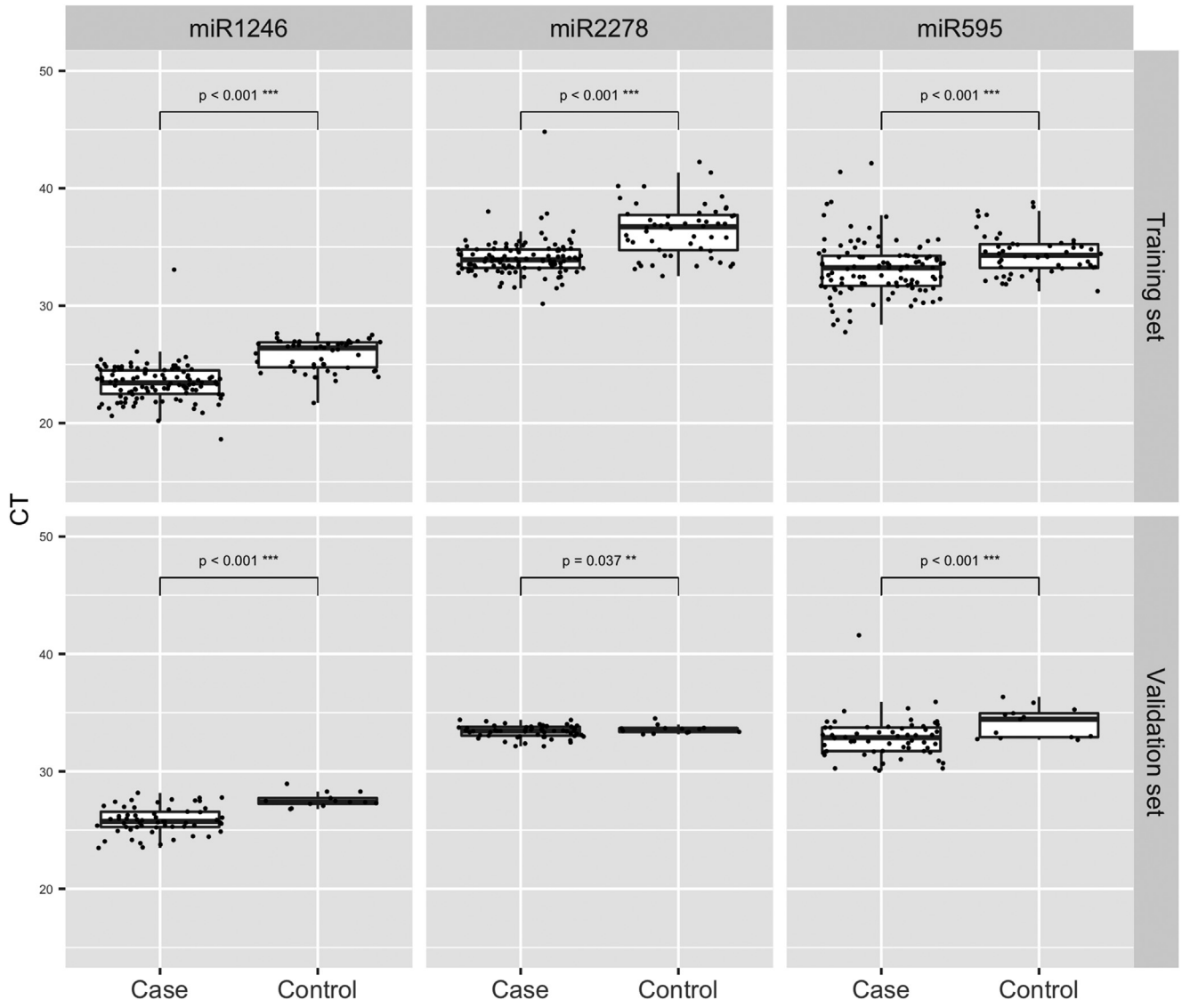


Fig. 2. Boxplots of the three selected miRNA expression values in patients divided into training and validation sets. Box plot diagrams showing the expression levels of *miR-1246*, *miR-595*, *miR-2278* measured by RT-qPCR in sera of training set (upper panel) and in sera of validation set (lower panel). The horizontal line within each box indicates the median. The top edge of the boxes represents the 75th percentile, the bottom edge the 25th percentile. The range is shown as a vertical line ending above and below the 75th and 25th percentile values, respectively. Control = healthy controls; Case = HGSOC patients.

separately on each miRNA to estimate sensitivity and specificity (Fig. 3 and Supplementary Table S5.1) For *miR-1246*, the sensitivity was 87%, the specificity was 77% and the accuracy was 84%, with an AUC (Area Under the Curve) of 0.89. For *miR-595*, the sensitivity was 47%, the specificity was 84% and the accuracy was 57%, with an AUC of 0.69. For *miR*-2278, the sensitivity was 81%, the specificity was 66% and the accuracy was 77%, with an AUC of 0.76. Then, we tested the diagnostic value of the integration of the three biomarkers. We found that *miR-1246* remains the strongest biomarker (P = 2.3e-09), while *miR-595* (P = 0.41) and *miR-2278* (P = 0.14) resulted to be not significant (Supplementary Table S5.2). Moreover, the combination of the three biomarkers resulted in a moderate increase of AUC only in the validation set (Fig. 3 and Supplementary Table S5.3).

As *miR-1246* resulted the most promising diagnostic serum biomarker, we decided to validate its expression levels with an additional and more sensitive technique, EvaGreen-based ddPCR technology. The quantification by ddPCR, expressed as copies/μl (Supplementary Fig. S6.1), confirmed the diagnostic potential of *miR-1246* (P < 0.0001) in discriminating HGSOC patients and healthy controls.

These data indicated that *miR-1246*, which showed the greatest ability in differentiating HGSOC patients from controls, could act as a suitable biomarker for detecting HGSOC patients.

Discussion

By exploiting different miRNA expression technologies and a dedicated computational approach, we have identified *miR-1246* as significantly up-regulated in the serum of patients with diagnosis of HGSOC, compared to healthy individuals. According to its specificity and sensitivity, our data suggest that *miR-1246* is worthy to be investigated in future studies as potential diagnostic biomarker for HGSOC.

Considering the lack of ovarian cancer screening tests able to significantly reduce the mortality of patients, the development of novel strategies for early diagnosis, such as the identification of novel biomarkers, is one of the possible strategies to pursue.

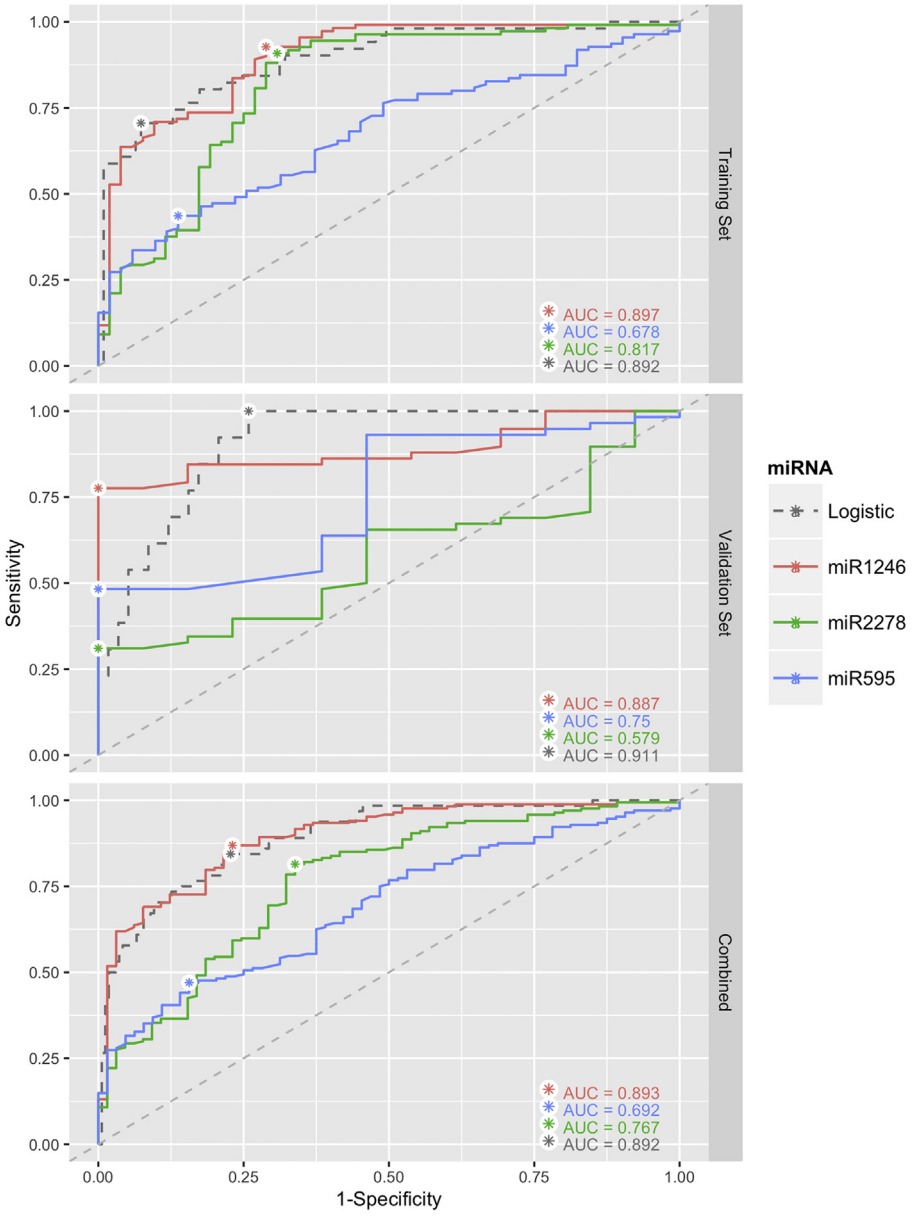


Fig. 3. ROC curves showing the diagnostic performance of each single miRNA markers in the training set, in the validation set and the combination of the two of them. Stars indicate the best combination of sensitivity and specificity with the highest AUC. In dashed black, the model integrating the three miRNAs.

Conceptually, along the path to patient bedside, there are different steps for a molecule to be selected as a biomarker. In the first preclinical exploratory phase, which is the main focus of the current work, tumor and non-tumor specimens are compared to identify biological molecules that characterize a normal or abnormal process and that could be used to generate hypothesis for clinical tests to detect cancer. Serum tumor biomarkers are currently considered one of the best tool to improve early diagnosis, aid to predict prognosis and eventually therapeutic response. This is particularly relevant for a neoplastic disease, like ovarian carcinoma, which is often asymptomatic at its onset and tissue samples are not always accessible during clinical follow-up. In clinical practice, patients at relapse are basically treated on the scanty molecular information obtained at diagnosis before treatment.

Over the last years, circulating miRNAs have been discovered and found highly stable in a variety of body fluids that can be obtained in a minimally invasive way. Although, the expression levels of circulating miRNAs reflect the cumulative effects of several underlying pathways, not fully elucidated yet, the levels and composition of miRNAs in blood, serum or plasma were found to mirror the presence of different malignant diseases, making them attractive tools for diagnostic and prognostic purposes. Many technical challenges in the analysis of circulating miRNAs (*i.e.,* samples storage and processing, profiling methods and data normalization) have complicated the comparison of independent datasets and delayed their entering into clinical settings. In the current study, we used microarray technology to achieve an efficient selection of the most promising miRNAs among the thousands of possible candidates sourced from the miRNome (miRBAse version 19). In addition, we developed a novel bioinformatic approach to identify specific circulating miRNAs characterizing HGSOC patients. The miRNA profile on the training set initially allowed us to identify 97 miRNAs with different expression levels between HGSOC patients and healthy controls. Among them, *miR-1246* and *miR-595*, were

further validated in a completely independent dataset. Receiver operating characteristic curve confirmed *miR-1246* as the most promising diagnostic biomarker, as it was able to accurately classify tumor patients compared to healthy controls, both in the training and in the validation cohorts.

Although a detailed biological analysis of *miR-1246* is far from the scope of this study, there are some data previously reported in the literature that are warranted to be discussed in detail. Its expression has been largely reported as upregulated in various cancer tissues [16–18], and as circulating marker, it has been proposed for the detection of several carcinomas [19,20]. Moreover, *miR-1246* has been associated with stemness in non-small cell lung cancer [16] and in pancreatic carcinoma [18]. To the best of our knowledge, *miR-1246* has not been previously associated to ovarian cancer, neither at the tissue nor at the serum level. This is not surprising, because, despite the abundance of published papers on circulating miRNAs in ovarian cancer diagnosis, a high level of inconsistency exists across studies. Pre-analytical and analytical challenges in circulating miRNA experiments, data analysis and normalization, statistical power and validation of results are the main causes of this poor overlap of results. Within this complex scenario, we believe that our study displays several improved features compared to previous studies, including: i) the focus on HGSOC, the most frequent and aggressive ovarian carcinoma subtype, ii) optimized protocols including collection, handling, storage and miRNAs extraction of serum samples, iii) haemolysis monitoring of serum samples, iv) the inclusion of two cohorts of HGSOC patients and controls, gathered from independent serum collections, v) the use of an innovative and effective statistical approach of microarray data normalization, combining synthetic spike-in RNA oligos and the most invariant endogenous miRNAs, vi) the use of two RT-qPCR techniques for miRNA validation and, in particular, of Exiqon primer sets with LNA technology, which maximizes sensitivity and specificity in detecting miRNA amplicons. This strict approach makes us confident in our results, reporting *miR-1246* as a novel diagnostic biomarker in HGSOC.

In line with previous studies, we found a modest overlap between miRNA expression pattern in serum and tissue [21–23], suggesting that circulating miRNAs could derive from a contribute of inflammation-related and tumor-specific miRNAs, selectively and actively secreted through microvesicles and exosomes as a novel mechanism of genetic exchange between cells.

In conclusion, *miR-1246* emerged as the most consistently upregulated miRNA in the serum of HGSOC patients compared to healthy controls, as assessed by three independent technologies (microarray, RT-qPCR and ddPCR) and validated in two independent cohorts of patients. To the best of our knowledge, this is the first report demonstrating *miR-1246* as a potential diagnostic serum biomarker in HGSOC.

Currently, serum CA-125 is the most frequently used biomarker for EOC detection, showing the best performance in advanced-stage HGSOC, while exhibiting both a low specificity and sensitivity to detect early-stage disease. Consequently, prospective studies on larger cohort of serum samples are needed either to test *miR-1246* potential clinical utility in late-stage HGSOC or to assess its value in early-stage diagnosis.

Conflict of interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.12.017.

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