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A signature of circulating microRNAs predicts the response to treatment with FOLFIRI plus aflibercept in metastatic colorectal cancer patients

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ABSTRACT

The benefit of adding the antiangiogenic drug aflibercept to FOLFIRI regime in metastatic colorectal cancer (CRC) patients resistant to or progressive on an oxaliplatin-based therapy has been previously demonstrated. However, the absence of validated biomarkers to predict greater outcomes is a major challenge encountered when using antiangiogenic therapies. In this study we investigated profiles of circulating microRNAs (miRNAs) to build predictive models of response to treatment and survival. Plasma was obtained from 98 metastatic CRC patients enrolled in a clinical phase II trial before receiving FOLFIRI plus aflibercept treatment, and the circulating levels of 754 individual miRNAs were quantified using real-time PCR. A distinct signature of circulating miRNAs differentiated responder from non-responder patients. Remarkably, most of these miRNAs were found to target genes that are involved in angiogenic processes. Accordingly, some of these miRNAs had predictive value and entered in predictive models of response to therapy, progression of disease, and survival of patients treated with FOLFIRI plus aflibercept. Among these miRNAs, circulating levels of hsa-miR-33b-5p efficiently discriminated between responder and non-responder patients and predicted the risk of disease progression. Moreover, the

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combination of circulating VEGF-A and miR-33b-5p levels improved clinical stratification of metastatic CRC patients who were to receive FOLFIRI plus aflibercept treatment. In conclusion, our study supports circulating miRNAs as valuable biomarkers for predicting better outcomes in metastatic CRC patients treated with FOLFIRI plus aflibercept.

1. Introduction

Colorectal cancer (CRC) is the third most incident cancer worldwide comprising 10 % of all diagnosed cancers and being the third most common cancer in men and the second most common cancer in women [1]. Moreover, CRC is the second tumour type responsible for cancer-related deaths [2,3]. Currently treatments for metastatic CRC (mCRC) include systemic chemotherapy, targeted therapy and immunotherapy. Election of first line of treatment will depend on several tumour and patients-related characteristics. Systemic chemotherapy includes fluoropyrimidines, such as 5-fluorouracil (5-FU) and capecitabine, the topoisomerase I inhibitor irinotecan and the platinum-based drug oxaliplatin [4]. The antitumor activity of 5-FU can be potentiated by using the folate analogue leucovorin, and the combination therapy FOLFIRI (5-FU/leucovorin and irinotecan), has become an efficacious treatment of mCRC [5]. On the other hand, targeted therapies for mCRC have been mainly developed against the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) signalling pathways [6]. Aflibercept is a novel antiangiogenic VEGF-targeted agent that blocks VEGF-A, VEGF-B, and placental growth factors [7].

The benefit of adding aflibercept to FOLFIRI regime in mCRC patients previously treated with oxaliplatin was demonstrated in the pivotal phase III VELOUR trial [8]. The benefits of aflibercept in combination with FOLFIRI were observed whatever age, gender, race, performance status, prior anti-VEGF treatment with bevacizumab and timing of first-line progression [9,10] and were the grounds for approving aflibercept for mCRC resistant to or progressive on an oxaliplatin-based therapy. However, the absence of validated biomarkers to predict greater outcomes is a major challenge encountered when using VEGF therapies [11]. In this regard, we have recently reported that circulating VEGF-A may constitute a potential biomarker to predict better outcomes following aflibercept plus FOLFIRI [12].

Aberrant microRNA (miRNA) expression is usually associated with molecular and cellular processes involved in human malignancies, including cell proliferation, apoptosis, metastasis, and drug response [13,14]. Moreover, several miRNAs have been reported to be involved in tumour angiogenesis regulation and they participate both in the promotion or the inhibition of angiogenic processes [15,16]. Different profiles of circulating miRNAs have been described for the diagnosis, prognosis, and prediction of response to therapy in CRC. Moreover, the combination of several miRNAs, rather than individual miRNAs profiles, has been shown to improve their diagnostic or predictive value [17,18]. Therefore, the present study was aimed to explore circulating miRNAs as predictors of better outcomes following aflibercept plus FOLFIRI treatment in mCRC patients.

2. Materials and methods

2.1. Patients

Ninety-eight patients from the open-label, single-arm, phase II trial POLAF (Clinicaltrials.gov number NCT02970916 and EudraCT number 2016–001508–45) were included in the study, that was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Córdoba (Córdoba, Spain; approval number/ID: 255). All subjects gave their informed consent for their inclusion in the study. Eligible patients were 18 years or older with mCRC resistant to or progressive on an oxaliplatin-containing regimen, who received aflibercept, followed by FOLFIRI [12].

2.2. Circulating microRNA extraction from plasma

Blood samples were drawn in EDTA tubes within seven days prior to first treatment administration and plasma was obtained by centrifuging at 1650×*g* during 10 min at 4ºC. Plasma samples were then aliquoted and stored at −80 °C until analysis. Circulating miRNAs were extracted from 0.2 mL of plasma using the miRNeasy Serum/Plasma Kit (Qiagen) following manufacturer instructions. During miRNA extraction 5 pM of phosphorylate ath-miR159a from *Arabidopsis thaliana* (Thermo) was added (spike-in) as exogenous control for miRNA levels normalization.

2.3. Circulating microRNA analysis in the screening cohort

First, 30 patients were selected as screening cohort. Circulating levels of 754 miRNAs in these 30 patients were determined using the TaqMan OpenArray MicroRNA Panel with advanced chemistry. Protocol was optimized by using 22 cycles of PCR in the pre-amplification reaction. Real-time PCR was carried out in a TaqMan OpenArray Human Advanced microRNA Panel (Applied Biosystems), which is a 3072-well microfluidic plate containing dried TaqMan primers and probes that enables quantification of the miRNA levels of up to 754 miRNAs and controls in three samples. The pre-amplified cDNA product was mixed with TaqMan OpenArray Real-Time PCR Master Mix (Applied Biosystems) and loaded into the array using the OpenArray Accufill System (Applied Biosystems). Real-time PCR was carried out in the QuanStudio 12 K Flex Real-Time PCR System (Applied Biosystems).

2.4. Circulating microRNA analysis in the validation cohort

The remaining sixty-eight patients were analysed for the validation assay using TaqMan Array MicroRNA Cards (Applied Biosystems) with advanced chemistry. Forty-seven miRNAs were selected based on the screening assay. Preparation of miRNAs for the analysis was performed using TaqMan Advanced miRNA cDNA Synthesis Kit as described above. In this case, protocol was optimized by increasing to 18 cycles for the pre-amplification reaction (miR-Amp reaction). Pre-amplification product was diluted 1:10 with 0.1X TE Buffer, mixed with TaqMan Fast Advanced Master Mix and loaded into the Custom TaqMan Array MicroRNA Cards (Format 48), which contain dry primers and probes for the forty-seven miRNAs selected plus the spike-in control and they can accommodate up to 8 individuals samples. Real-time PCR in the Cards was carried out in the ViiA7 Real-Time PCR System (Applied Biosystem).

2.5. Circulating microRNA normalization and transformation

Circulating miRNAs levels were normalized to the exogenous control and the mean levels of the three most stable miRNAs using the NormFinder algorithm [19]. Relative circulating levels were calculated with the $2^{-\Delta\Delta Ct}$ method [20]. Those miRNAs not detected in at least one third of the patients were excluded in further analyses. Data of circulating miRNA levels from 98 patients were $\log_2$ transformed for the construction of predictive models of response to treatment and survival analyses. In those patients in which a particular circulating miRNA was not detected, a non-zero value resulting of dividing by 10 the lowest circulating miRNA level detected was used.

Table 1
Baseline characteristics of metastatic CRC patients receiving FOLFIRI plus aflibercept treatment.

Patient Characteristics			n (%)
Age (median, range)			63, 36–83
Sex	Male		60 (61.2)
	Female		38 (38.8)
ECOG	0		46 (46.9)
	1–2		52 (53.1)
Primary Tumour Location	Colon	Yes	73 (74.5)
		No	23 (23.5)
	Rectum	Yes	30 (30.6)
		No	66 (67.3)
	Data Not Available		2 (2.0)
RAS status in tissue	RAS mutated		57 (58.2)
	RAS non-mutated		34 (34.7)
	Data Not Available		7 (7.1)
Stage at diagnosis	I+II+III		15 (15.3)
	IV		82 (83.7)
	Data Not Available		1 (1.0)
Histopathological Grade	Well differentiated		16 (16.3)
	Moderately or poorly differentiated		49 (50.0)
	Grade could not be determined		33 (33.7)
Metastatic Location	Liver	Yes	78 (79.6)
		No	20 (20.4)
	Lung	Yes	57 (58.2)
		No	41 (41.8)
	Distant lymph nodes	Yes	23 (23.5)
		No	75 (76.5)
	Peritoneum	Yes	18 (18.4)
		No	80 (81.6)
	Regional lymph nodes	Yes	11 (11.2)
		No	87 (88.8)
Primary tumour surgery prior to study inclusion		Yes	72 (73.5)
		No	26 (26.5)

2.6. Characterization of microRNAs target genes and biological functions

The Ingenuity Pathway Analysis (IPA) software (Qiagen) was used to perform a network analysis of miRNAs with their mRNA targets in the colorectal metastasis signalling pathway. The online miRNA set enrichment tool TAM 2.0 [21] was used to further analyse the relation between miRNAs and biological functions and diseases.

2.7. Statistical analysis

Statistical analyses were performed using SPSS Statistic 22.0.0, GraphPad Prism 6.0 Software and MetaboAnalyst web server [22]. Time-to-treatment-failure (TTF) was calculated from the start to finish of treatment. Progression-free survival (PFS) was calculated from the start of therapy date until disease progression or death. Overall survival (OS) was calculated from the enrolment to death from any cause. The survival rates were estimated using the Kaplan-Meier method and the Log-Rank test was used to identify the prognostic variables. In the screening cohort 15 patients with the longest TTF (responder patients) and 15 patients with the shortest TTF (non-responder patients) were included. On the other hand, for building predictive models the treatment response was defined according to RECIST (Response Evaluation Criteria In Solid Tumors), and patients were classified into reponders (patients with complete or partial response, and non-responders (patients with stable disease and progression). Non-parametric Mann-Whitney tests were used to compare two groups. Selection of variables to build the predictive models was performed using a univariate binary logistic regression. The predictive model was constructed by performing a stepwise regression with bidirectional elimination, which is a combination of forward selection and backward elimination. In each step, a variable is considered for addition to or subtraction from the model using p of F-to-enter ≤ 0.05 and p of F-to-remove > 0.10. Iteratively adding and removing predictor variables results in the best performing model, that is the model with the lower prediction error. Additionally, the relative risks were calculated.

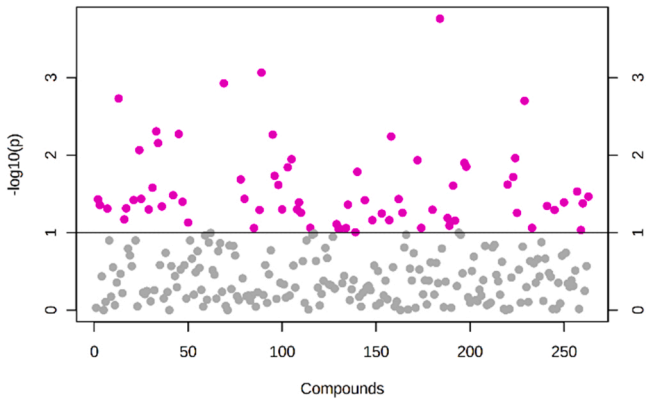

Fig. 1. miRNAs differentially expressed between patients responding or non-responding to treatment with FOLFIRI plus aflibercept. Seventy-one miRNAs (purple dots) were differentially expressed between responder and non-responder patients ($p < 0.1$).

3. Results

3.1. Clinicopathological characteristics of patients

Ninety-eight mCRC patients (60 men, 38 women) with 63 years median age (range 36–83 years) were included in the study from 2016 to 2017 (clinical characteristics are summarised in Table 1). More than 80 % of the patients were diagnosed with stage IV CRC and most patients had a primary tumour located in the colon (74.5 %) and metastases in the liver (79.6 %). RAS mutation was detected in tumor tissue in 57 patients (62.6 %).

3.2. Differential circulating miRNA levels between responder and non-responder patients to treatment with FOLFIRI plus aflibercept

Fifteen patients with the longest TTF (responder patients) and 15 with the shortest TTF (non-responder patients) were first included for screening. For each patient 754 circulating miRNAs were analysed and Ct values were obtained. Seventy-one miRNAs were found to be differentially expressed between responder and non-responder patients ($p < 0.1$) using the non-parametric Wilcoxon Rank test in the MetaboAnalyst

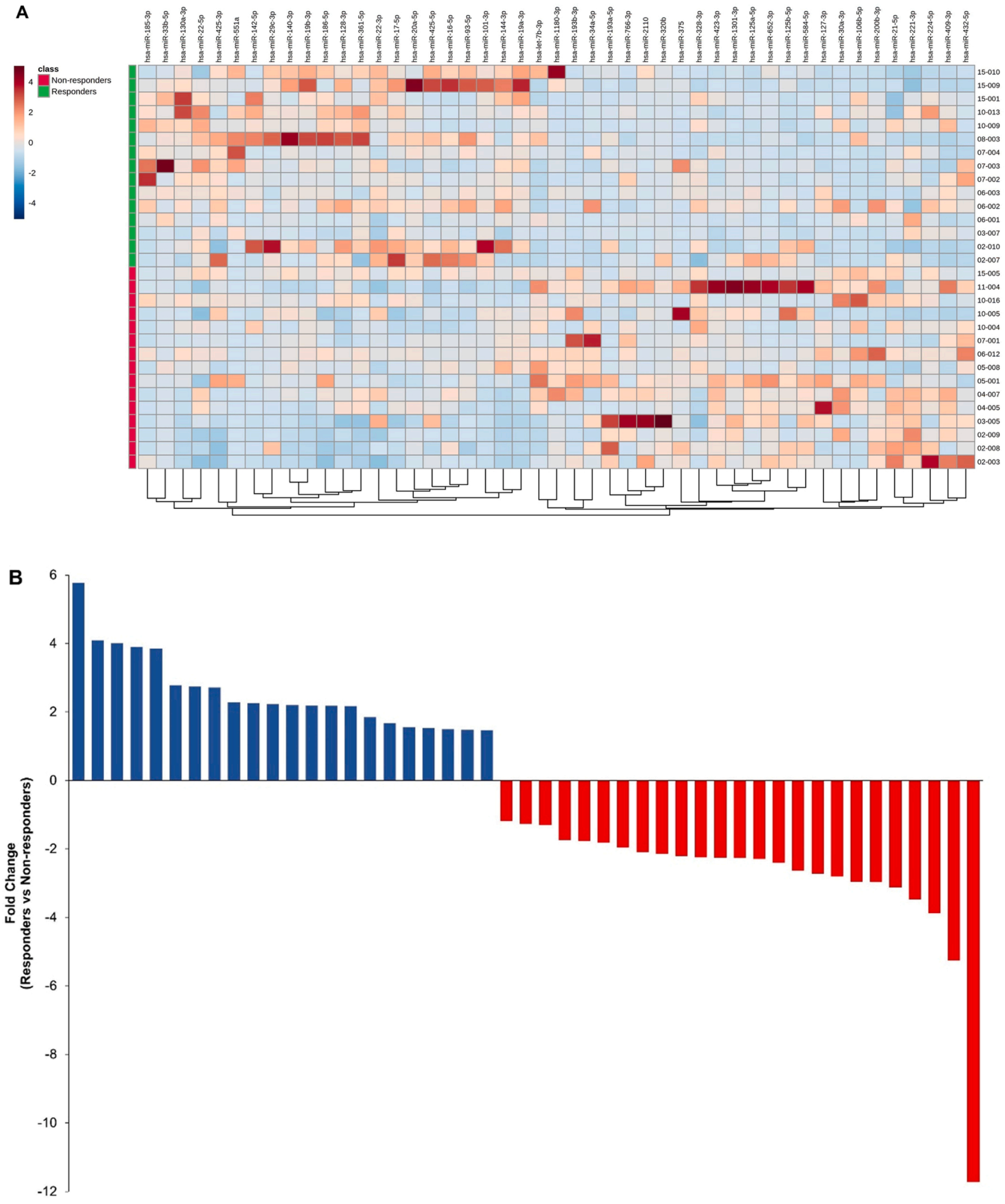

Fig. 2. Distinct circulating miRNA signature between patients responding or non-responding to treatment with FOLFIRI plus aflibercept. (**A**) Heatmap showing the distinct signature between responder and non-responder patients of the 47 circulating miRNAs selected. (**B**) Fold changes for the 47 miRNAs selected, with 22 miRNAs up-regulated (blue) and 25 miRNAs down-regulated (red) in responder patients.

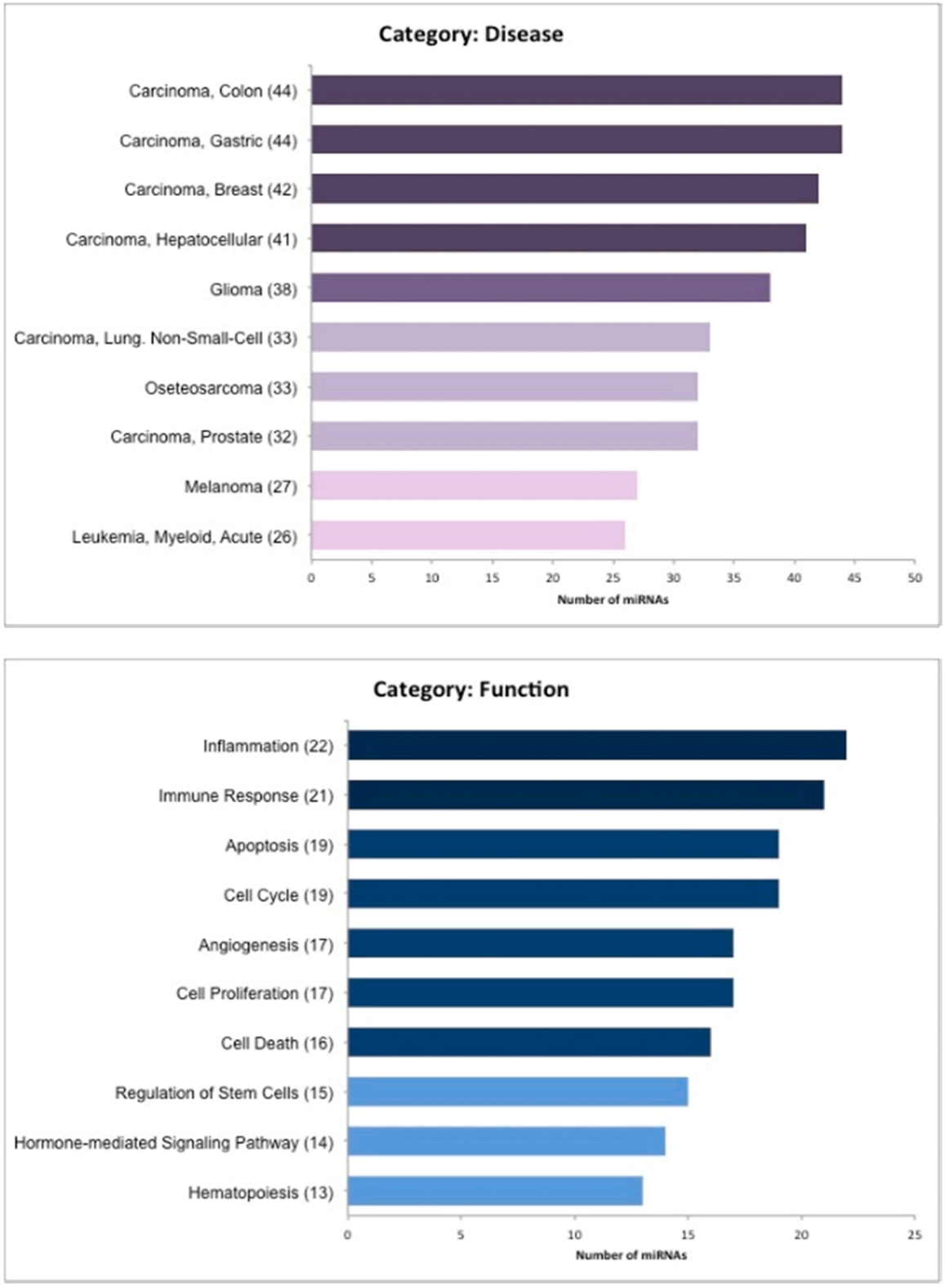

Fig. 3. Disease phenotypes and functions associated with the set of circulating miRNAs with distinct profile in patients responding or non-responding to treatment with FOLFIRI plus aflibercept. The top-ten diseases and functions most significantly associated with the set of 47 miRNAs are shown ranked by the number of miRNAs involved.

web server [22] and were selected for further analysis (Fig. 1).

Individual evaluation of these 71 miRNAs was then performed and based on this comparative analysis, 47 miRNAs that most clearly differentiated between responder and non-responder patients were finally selected for the validation step (Supplementary Table 1). As shown in Fig. 2**A**, a distinct miRNAs signature was found to differentiate responder from non-responder patients. Twenty-two and 25 miRNAs were up-regulated and down-regulated in responder patients,

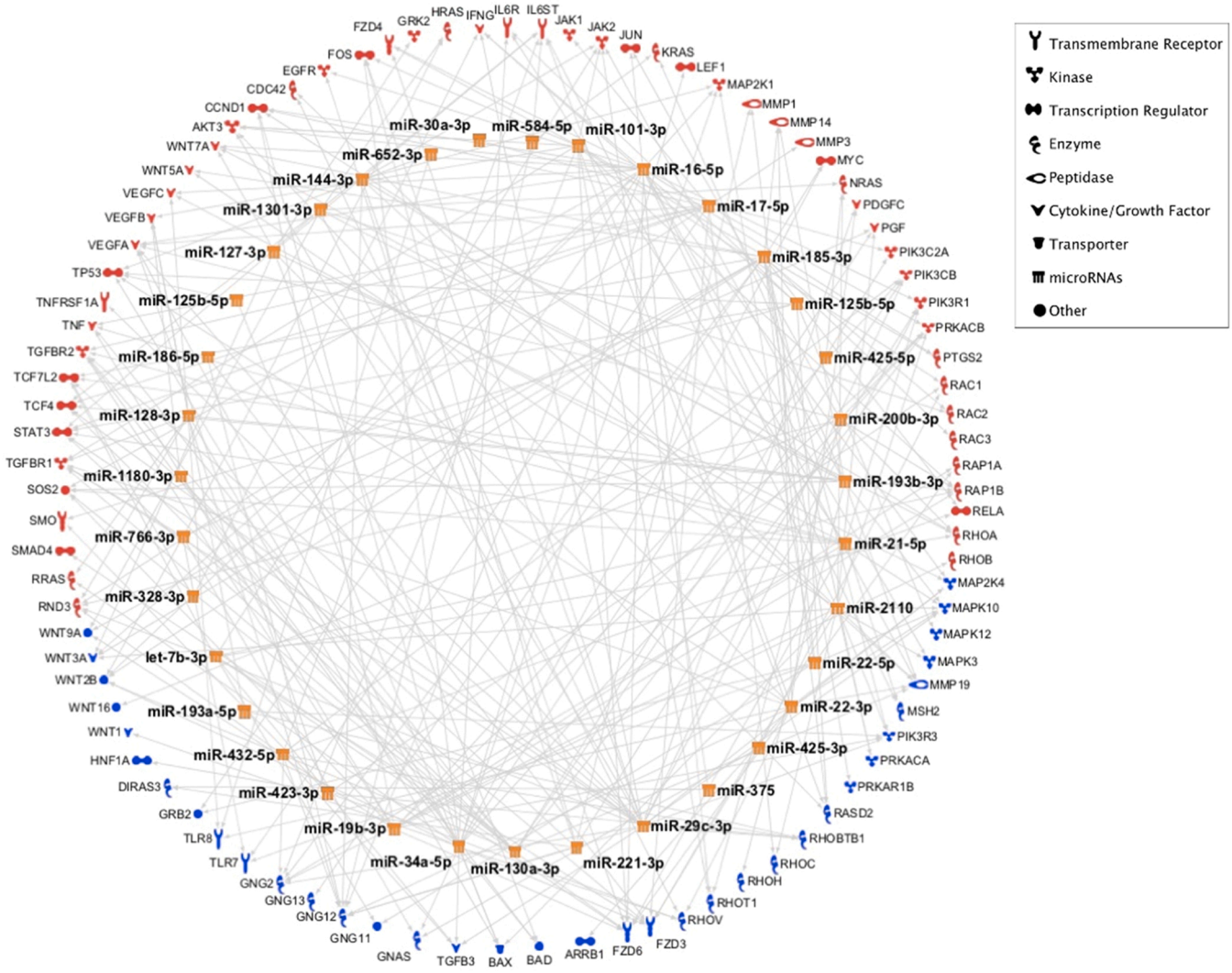

Fig. 4. Molecular network of miRNA targets. Interactions between differentially expressed miRNAs (orange) and corresponding target genes using the IPA (Ingenuity Pathway Analysis) software. Ninety-one targe genes (blue and red) were found involved in colorectal cancer signalling pathways and 55 of them were also found connected with angiogenesis (red).

respectively (Fig. 2**B**).

To identify those disease phenotypes and functions over-represented in this set of miRNAs, we next performed an enrichment analysis (Fig. 3**)** using the online miRNA set enrichment tool TAM 2.0 [21]. Notably, colon carcinoma was the most significant disease-association, with 44 miRNAs. Moreover, angiogenesis ranked among the most significant function-associations in this set of miRNAs, with 17 miRNAs associated with this vasculogenic process.

Interactions between differentially expressed miRNAs and target genes was next analysed using the IPA (Ingenuity Pathway Analysis) software (Qiagen). As shown in Fig. 4, the set of miRNAs was molecularly interconnected with 91 target genes involved in colorectal metastasis signalling. Moreover, 55 target genes (60.4 %) were also implicated in angiogenesis, supporting the connection between this set of miRNAs and angiogenic processes in colorectal tumours.

3.3. *Predictive model of response to treatment with FOLFIRI plus aflibercept*

A predictive model of response was constructed by combining the 47 miRNAs displaying differential profiles between responder and non-responder patients (Supplementary Table 1), with clinical variables (Table 1). The patients included in the study were classified as reponders (patients with complete or partial response, n = 19) and non-responders (patients with stable disease and progression, n = 79). Due to the high number of potential predictors included in the analysis, a univariate

Table 2
Predictive model of response to treatment with FOLFIRI plus aflibercept.

Variables	B	S.E.	Wald	df	Sig.	Exp (B)	95 % C.I. for Exp (B)
Primary tumour located in the colon (Yes versus No (ref.))	-1.258	0.631	3.970	1	0.046	0.284	0.083–0.980
hsa-miR-33b-5p	0.140	0.051	7.517	1	0.006	1.150	1.041–1.271
hsa-miR-30a-3p	-0.087	0.042	4.285	1	0.038	0.917	0.844–0.995

B, coefficient to calculate hazard ratio; S.E., Standard Error; Wald, Wald statistic; df: degrees of freedom; Sig., *p-value*; Exp (B), hazard ratio; C.I., confidence interval; ref., reference category.

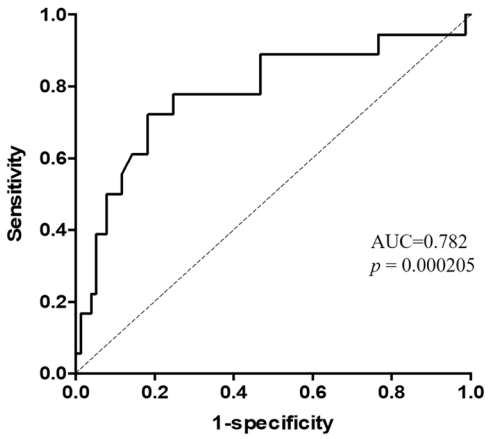

Fig. 5. ROC curve obtained from the predictive model of response to treatment with FOLFIRI plus aflibercept. ROC curve classifying responder and non-responder patients with AUC = 0.782, $p=p$ = 0.000205, 72.2 % sensitivity, 81.8 % specificity and 0.20 optimal cut-off.

Table 3
Relative risk table for the predictive model of response to treatment with FOLFIRI plus aflibercept.

Primary tumour location	hsa-miR-30a-3p	Hazard Ratio (Relative Risk)		
		hsa-miR-33b-5p		
		Minimum	Q3	Maximum
Rectum	Minimum	19.34	43.60	**50.29**
	Q3	6.87	24.52	33.68
	Maximum	3.38	14.40	22.01
Colon	Minimum	7.08	25.02	34.20
	Q3	2.12	9.73	15.69
	Maximum	1	4.91	8.40

binary logistic regression was first performed, and those variables with $p > 0.15$ for their association with response were excluded. Hence, thirteen variables with a likely higher weight in the model were finally included in the analysis. The predictive model was built by performing a stepwise regression with bidirectional elimination. Primary tumour located in the colon (not in rectum) and two miRNAs (hsa-miR-33b-5p and hsa-miR-30a-3p) were the predictor variables included in the final predictive model (Table 2). In brief, patients with primary tumour located in the colon presented a lower probability of response. Higher circulating levels of hsa-miR-33b-5p were also associated with higher probability of response, whereas higher circulating levels of hsa-miR-30a-3p were related with lower probability of response.

Using the predictive values, a ROC curve (AUC=0.782; $p = 0.000205$) was obtained (Fig. 5), showing that the model efficiently discriminates between responder and non-responder patients. Also, an optimal cut-off of 0.20 was determined by maximizing the sensitivity (72.2 %) and specificity (81.8 %).

Table 5
Relative risk table for the predictive model of progression on FOLFIRI plus aflibercept treatment.

Risk Pattern Variables	Minimum	Intermediate[a]	Maximum
Surgery of primary tumour prior to study inclusion	Yes	Yes	No
hsa-miR-142–5p	Maximum	Minimum	Minimum
hsa-miR-33b-5p	Maximum	Maximum	Minimum
hsa-miR-93–5p	Maximum	Median	Minimum
hsa-miR-193b-3p	Minimum	Maximum	Maximum
hsa-miR-29c-3p	Minimum	Maximum	Maximum
hsa-miR-328–3p	Minimum	Median	Maximum
hsa-miR-652–3p	Minimum	Maximum	Maximum
HR	1	3372.99	256435.4

[a] HR closest to the mean has been considered as intermediate pattern

Additionally, from the predictive model, the relative risks were calculated, with the qualitative variable (dichotomous) and the minimum, third quartile and maximum values for the quantitative variables, to establish the patterns. As shown in Table 3, the maximum chance of response to treatment with FOLFIRI plus aflibercept corresponded to a patient with primary tumour located in the rectum, minimum value of hsa-miR-30a-3p and maximum value of hsa-miR-33b-5p.

3.4. Predictive model of progression on FOLFIRI plus aflibercept treatment

Eighty patients progressed on FOLFIRI plus aflibercept treatment or died before the end of the study, whereas eighteen patients still had not progressed. All the clinical characteristics summarised in Table 1 and the 47 selected miRNAs (Supplementary Table 1) were included in the model. After exclusion of those variables with $p > 0.15$ (univariate binary logistic regression), twenty-three variables were finally included in the analysis. Stepwise regression with bidirectional elimination was performed, and primary tumour surgery prior to study inclusion and seven miRNAs, hsa-miR-142–5p, hsa-miR-193b-3p, hsa-miR-29c-3p, hsa-miR-328–3p, hsa-miR-33b-5p, hsa-miR-652–3p and hsa-miR-93–5p, were the variables finally included in the optimal model (Table 4). In brief, patients without surgery of primary tumour prior to study inclusion presented higher probability to progress or die on FOLFIRI plus aflibercept treatment. Similarly, higher circulating levels of hsa-miR-193b-3p, hsa-miR-29c-3p, hsa-miR-328–3p and hsa-miR-652–3p were also associated with higher probability of progression or death, whereas higher circulating levels of hsa-miR-142–5p, hsa-miR-33b-5p and hsa-miR-93–5p were related with lower probability to progress or die on FOLFIRI plus aflibercept treatment.

Additionally, from the predictive model, the relative risks were calculated, with the qualitative variable (dichotomous) and the minimum, median and maximum values for the quantitative variables, to establish the pattern. The maximum risk to progress or die on FOLFIRI plus aflibercept treatment corresponded to a patient without surgery of primary tumour prior to study inclusion, minimum value of hsa-miR-142–5p, hsa-miR-33b-5p and hsa-miR-93–5p and maximum value of

Table 4
Predictive model of progression on FOLFIRI plus aflibercept treatment.

Variables	B	S.E.	Wald	df	Sig.	Exp (B)	95 % C.I. for Exp (B)
Surgery of primary tumour prior to study inclusion (Yes (ref.) versus No)	0.760	0.270	7.920	1	0.005	2.138	1.259–3.630
hsa-miR-193b-3p	0.079	0.021	13.664	1	< 0.001	1.082	1.038–1.129
hsa-miR-29c-3p	0.075	0.037	4.166	1	0.041	1.078	1.003–1.158
hsa-miR-328–3p	0.547	0.182	9.006	1	0.003	1.729	1.209–2.472
hsa-miR-652–3p	0.285	0.132	4.648	1	0.031	1.330	1.026–1.724
hsa-miR-142–5p	-0.051	0.018	7.762	1	0.005	0.951	0.917–0.985
hsa-miR-33b-5p	-0.046	0.023	4.130	1	0.042	0.955	0.914–0.998
hsa-miR-93–5p	-0.566	0.164	11.966	1	< 0.001	0.568	0.412–0.782

B, coefficient to calculate hazard ratio; S.E., Standard Error; Wald, Wald statistic; df: degrees of freedom; Sig., *p-value*; Exp (B), hazard ratio; C.I., confidence interval; ref., reference category.

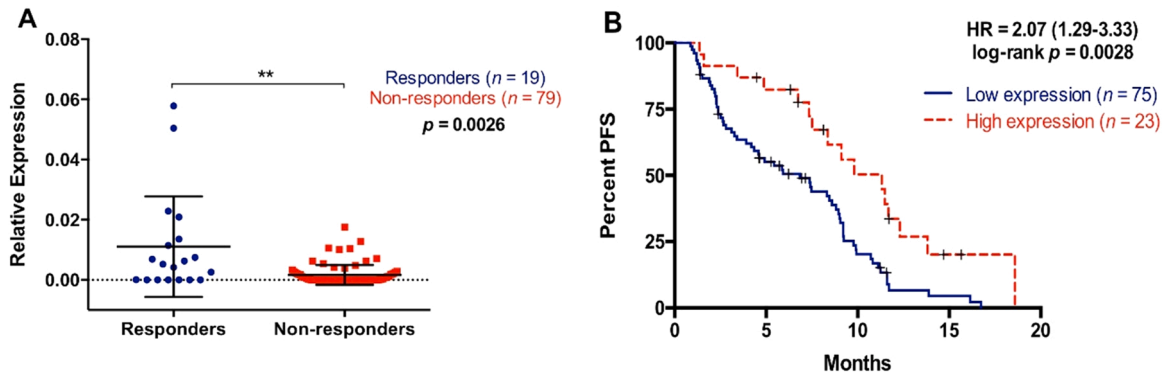

Fig. 6. Circulating levels of hsa-miR-33b-5p discriminate between responder and non-responder patients treated with FOLFIRI plus aflibercept and predict the risk of disease progression. (A) Circulating levels of hsa-miR-33b-5p according to response to treatment with FOLFIRI plus aflibercept; (**B**) PFS according to circulating levels of hsa-miR-33b-5p (mean value as cut-off). (** $p < 0.01$).

Table 6
Predictive model of survival of patients receiving FOLFIRI plus aflibercept treatment.

Variables	B	S.E.	Wald	df	Sig.	Exp (B)	95 % C.I. for Exp (B)
Sex (Men (ref.) versus Women)	-0.615	0.310	3.919	1	0.048	0.541	0.294–0.994
Primary tumour located in colon (Yes versus No (ref.))	0.770	0.346	4.964	1	0.026	2.160	1.097–4.251
RAS mutational status (WT (ref.) versus MUT)	0.712	0.307	5.378	1	0.020	2.039	1.117–3.722
Surgery of primary tumour prior to study inclusion (Yes (ref.) versus No)	0.645	0.306	4.448	1	0.035	1.906	1.047–3.472
hsa-miR-185–3p	-0.048	0.021	5.351	1	0.021	0.953	0.914–0.993
hsa-miR-19b-3p	-0.043	0.017	6.275	1	0.012	0.958	0.927–0.991
hsa-miR-425–5–5p	-0.964	0.240	16.193	1	< 0.0001	0.381	0.238–0.610
hsa-miR-432–5p	-0.051	0.024	4.745	1	0.029	0.950	0.907–0.995

B, coefficient to calculate hazard ratio; S.E., Standard Error; Wald, Wald statistic; df: degrees of freedom; Sig., *p-value*; Exp (B), hazard ratio; C.I., confidence interval; ref., reference category.

hsa-miR-193b-3p, hsa-miR-29c-3p, hsa-miR-328–3p and hsa-miR-652–3p (Table 5).

Remarkably, low levels of hsa-miR-33b-5p were associated with higher risk of disease progression in this model (Table 5), while higher levels of this miRNA predicted a higher probability of response to treatment with FOLFIRI plus aflibercept (Table 3). Therefore, we next evaluated the utility of hsa-miR-33b-5p as single biomarker to differentiate between responder and non-responder patients and also to predict the risk of disease progression. As shown in Fig. 6, patients responding to treatment with FOLFIRI plus aflibercept showed significantly higher hsa-miR-33b-5p circulating levels than non-responder patients ($p = 0.0026$; Fig. 6**A**). Furthermore, using the mean value level of hsa-miR-33b-5p as a cut-off, those patients with high circulating levels of this miRNA had a significant better PFS (9 versus 6 months; $p = 0.0028$; Fig. 6**B**).

3.5. *Predictive model of survival of patients receiving FOLFIRI plus aflibercept treatment*

Sixty-two patients died before the end of the study, whereas thirty-six patients still were alive. All the clinical characteristics summarised in Table 1 and the 47 selected miRNAs (Supplementary Table 1) were included in the model. After exclusion of those variables with $p > 0.15$ (univariate binary logistic regression) twenty-three variables were finally included in the analysis. Stepwise regression with bidirectional elimination was performed, and sex, primary tumour located in the colon, RAS mutation, primary tumour surgery prior to study inclusion, and four miRNAs (hsa-miR-185–3p, hsa-miR-19b-3p, hsa-miR-425–5p and hsa-miR-432–5p) were the variables finally included in the optimal model (Table 6). In brief, the characteristics associated with high risk of death on FOLFIRI plus aflibercept treatment were: male sex, tumour located in the colon, presence of tumour in the colon prior to study inclusion, RAS mutation, and lower circulating levels of hsa-miR-185–3p, hsa-miR-19b-3p, hsa-miR-425–5p and hsa-miR-432–5p.

Table 7
Relative risk table for the predictive model of survival of patients receiving FOLFIRI plus aflibercept treatment.

Risk Pattern Variables	Minimum	Intermediate[a]	Maximum
Surgery of primary tumour prior to study inclusion	No	No	Yes
Primary tumour located in colon	No	No	Yes
Sex	Male	Male	Female
RAS mutational status	Wild type	Mutant	Mutant
hsa-miR-185–3p	Maximum	Median	Minimum
hsa-miR-19b-3p	Maximum	Minimum	Minimum
hsa-miR-425–5–5p	Maximum	Minimum	Minimum
hsa-miR-432–5p	Maximum	Median	Minimum
HR	1	458.33	13886.80

[a] HR closest to the mean has been considered as intermediate pattern

Additionally, from the predictive model, the relative risks were calculated, with qualitative variables (dichotomous) and the minimum, median and maximum values for the quantitative variables, to establish the patterns. The maximum risk to die on FOLFIRI plus aflibercept treatment (Table 7) corresponded to a female patient with primary tumour located in the colon, with primary tumour surgery prior to study inclusion, RAS mutated and minimum value of all the miRNAs included in the final model (hsa-miR-185–3p, hsa-miR-19b-3p, hsa-miR-425–5p and hsa-miR-432–5p).

3.6. *The combination of VEGF-A and miR-33b-5p levels improves clinical stratification of metastatic CRC patients who were to receive FOLFIRI plus aflibercept treatment*

As mentioned above, we recently described circulating VEGF-A as a potential biomarker to predict better outcomes after aflibercept plus

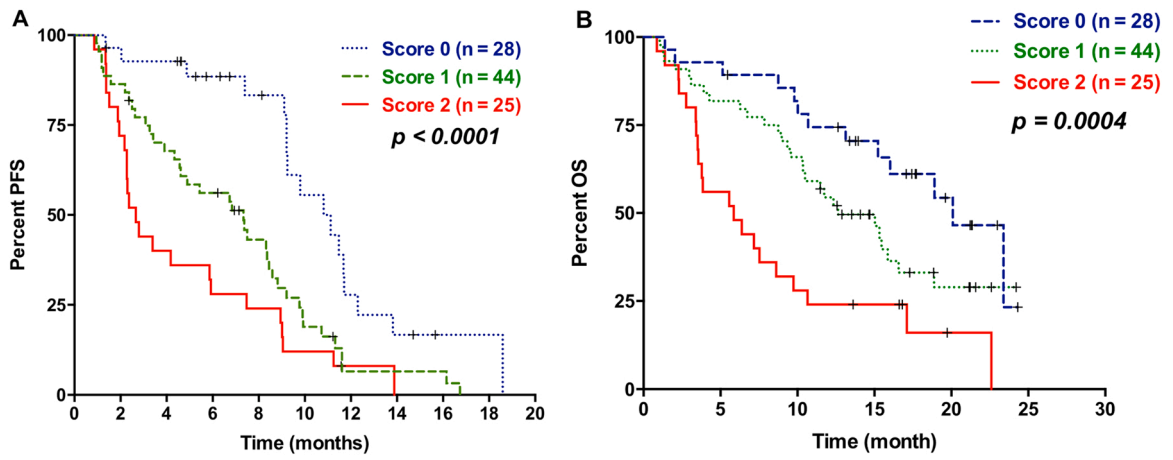

Fig. 7. The combination of circulating VEGF-A and miR-33b-5p levels improves prognostic stratification in patients who will receive FOLFIRI plus aflibercept treatment. (A) Progression-free survival (PFS) according to the VEGF-A and miR-33b-5p combination; (B) Overall survival (OS) according to the VEGF-A and miR-33b-5p combination. See text for details.

FOLFIRI [12]. Therefore we next stratified the patients in our study according to a scoring system combining VEGF-A and miR-33b-5p circulating levels. The cut-off value for miR-33b-5p was the mean (0.0035) and for VEGF-A was 1941 pg/mL as previously described [12]. Taking into account that high levels of miR-33b-5p were associated with better response and prognosis (Tables 4, 5, Fig. 6), whereas low levels of VEGF-A have been associated with a better outcome after FOLFIRI plus aflibercept treatment [12], a relative expression < 0.0035 was considered positive for miR-33b-5p biomarker and > 1941 pg/mL was considered positive for VEGF-A biomarker. Hence, a combined score system was assigned to patients with no positive markers (score=0), only one positive marker (score=1) and both positive markers (score=2). As shown in Fig. 7**A**, those patients with score 0 have a significant better PFS than patients with score 1 or score 2 ($p = 0.0011$ and $p < 0.0001$, respectively). Accordingly, Fig. 7**B** shows that those patients with score 2 have a significantly poorer overall survival in comparison with those patients with score 0 and score 1 ($p = 0.0001$ and $p = 0.0131$, respectively).

4. Discussion

A wide range of studies have revealed the important role of miRNAs in the regulation of tumor angiogenesis, with clear clinical implications of these biomolecules as biomarkers for anti-angiogenic therapy response [23]. In the present study we found a differential circulating miRNAs profile in mCRC patients according to their response to treatment with chemotherapy plus the anti-angiogenic drug aflibercept. Notably, these miRNAs were not only related with CRC but most of them were also involved in angiogenesis signaling.

Our study also demonstrates the usefulness of combining clinical variables with circulating miRNAs to predict the response to therapy, the progression of disease and survival of patients treated with FOLFIRI plus aflibercept. Hence, our model of response predicts a very good chance of response to treatment in those patients with primary tumour located in rectum, along with minimum expression of hsa-miR-30a-3p and high expression of hsa-miR-33b-5p. Notably, the miR-30 family has been shown to regulate angiogenesis [24] and particularly, down-regulation of hsa-miR-30a-3p impairs endothelial angiogenic activity [25], while this miRNA has been reported to promote the angiogenic potential of melanoma cells [26]. Accordingly, we found a higher probability of response to FOLFIRI plus aflibercept treatment in those patients with lower circulating levels of this angiogenic miRNA. Regarding hsa-miR-33b-5p, this miRNA has been described as tumour suppressor in several cancers [27], including CRC, where high expression of miR-33b in tumours was related with better prognosis of patients [28]. Moreover, hsa-miR-33b-5p has been associated with the suppression of HMGA2 (High Mobility Group A 2) gene in several types of cancers leading to the inhibition of cancer cell growth [29] and, specifically in gastric cancer, this suppression also sensitized cancer cells to chemotherapy drugs [30]. Interestingly, HMGA2 promotes angiogenesis in several tumours [31,32] and chemoresistance to 5-FU therapy in mCRC [33]. Correspondingly, we found higher circulating levels of hsa-miR-33b-5p in those patients with better response to FOLFIRI plus aflibercept treatment, raising the possibility that miR-33b-HMGA2 signalling may be involved in the sensitivity of tumours to chemotherapy plus anti-angiogenic therapy.

Remarkably, hsa-miR-33b-5p was also one of the miRNAs identified in our predictive model of disease progression. Furthermore, our results support that this miRNA may constitute a valuable single biomarker to differentiate between responder and non-responder patients and also to predict progression-free survival in patients treated with FOLFIRI plus aflibercept. We have recently reported that circulating VEGF-A levels may constitute a valuable biomarker for predicting better outcomes in mCRC patients treated with FOLFIRI plus aflibercept [12]. Accordingly, we now demonstrate that combining circulating levels of miR-33b-5p and VEGF-A may greatly help in predicting the response of mCRC patients to FOLFIRI plus aflibercept.

In conclusion, our study supports circulating miRNAs as valuable biomarkers for predicting better outcomes in mCRC patients treated with FOLFIRI plus aflibercept. Further studies are warranted to validate the clinical utility of these biomarkers in the prediction of patient response to aflibercept. Also, we believe that our approach may contribute to identify miRNA-based predictive biomarkers for other approved anti-angiogenic drugs in mCRC.

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CRediT authorship contribution statement

M. T.-F.: Methodology, Investigation, Formal Analysis, Writing-Original Draft Preparation. MA. G.-E and E.E.: Data curation and resources, Writing - Review & Editing. C.G., P.G.-A., R.R., F. L., A.D, B.G., M.V.-A, MV. G.O., E.P., M.S, F.R., MJ. S., A.S, A.R-C, JM. T and M.C.-R.: Data curation and resources. A. R.-A.: Conceptualization, Supervision, Writing - Review & Editing. E. A.: Conceptualization, Supervision, Writing - Review & Editing, Funding Acquisition.

Conflict of interest statement

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Appendix A. Supporting information

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