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Associations of blood RNA biomarkers and circulating tumour cells in patients with previously untreated metastatic colorectal cancer

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Abstract

Background In patients with metastatic colorectal cancer, analysis of the number of basal circulating tumour cells (bCTCs) has been shown to be a strong prognostic indicator. In this study, we aim to explore the potential associations between whole blood mRNA and microRNA expression profiles and bCTC counts, tumour mutations and prognosis in untreated metastatic colorectal cancer patients.

Methods A total of 151 patients previously screened for inclusion in two clinical trials (VISNÚ1 and VISNÚ2) were enrolled in this study. Real-time quantitative PCR (qPCR) analyses were performed to determine the whole blood expression of selected RNAs (mRNAs and microRNAs) involved in the metastatic process. The CellSearch system was used to enumerate circulating tumour cells. The primary objective was to correlate RNA expression with the number of bCTCs, while the secondary objectives were to investigate the relationship between the levels of circulating RNA biomarkers in whole blood and the clinical, pathological, and molecular characteristics and prognosis of patients with metastatic colorectal cancer.

Results bCTC count was significantly associated with AGR2 mRNA in the entire cohort of 151 patients. AGR2, ADAR1 and LGR5 were associated with the number of bCTC, both in the subgroup with bCTC ≥ 3 and in the subgroup with native *RAS/BRAF/PIK3 CA* tumours. In patients with *RAS/BRAF/PIK3 CA* mutations no correlations with bCTC were detected, but an upregulation of miR-224-5p and the stemness marker LGR5 and a downregulation of immune regulatory CD274 were found. Lower levels of miR-106a-5p/miR-26a-5p were associated with shorter overall survival, with independent statistical significance in the multivariate analysis.

Conclusions A correlation was identified between the levels of a subset of whole blood RNAs, including AGR2, ADAR1, and LGR5, and the number of bCTC and *RAS/BRAF/PIK3 CA* mutational status. Furthermore, another set

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of whole blood RNAs, specifically miR-106a-5p and miR-26a-5p, was found to be associated with poor prognosis. This may be helpful for risk stratification.

Trial registration Clinical Trials Gov. NCT01640405 and NCT01640444. Registered on 13 June 2012. https://clinicaltrials.gov/.

Keywords Circulating tumour cells, Liquid biopsy, Metastatic colorectal cancer, RNA biomarkers, Prognosis

Background

Colorectal cancer (CRC) accounts for 9.6% of all cancers, with over 1.9 million new cases and 904,000 deaths reported in 2022 [1]. CRC is a heterogeneous disease classified based on its clinical and genetic characteristics, which inform prognosis and therapy [2].

Precision oncology aims to improve patient outcomes by utilizing molecular information specific to each patient and their tumour. One of the main challenges in implementing precision oncology is defining biomarkers that can predict prognosis, treatment response, and guide therapy selection [3].

Circulating biomarkers are particularly attractive because blood samples can be obtained in a minimally invasive manner, allowing for repeat sampling. Liquid biopsy in cancer patients has primarily focused on analyzing circulating tumour cells (CTCs) and circulating cell-free DNA (cfDNA), specifically circulating tumour DNA (ctDNA). Additionally, circulating RNAs (including mRNAs and microRNAs), extracellular vesicles, tumour-educated platelets, and circulating stromal cells from the tumour microenvironment are present in the bloodstream of cancer patients, providing valuable insights into the tumour. These components can be used for real-time tumour burden tracking, monitoring host response, and non-invasive characterization of metastatic heterogeneity.

The analysis of mRNAs and microRNAs (miRNAs) obtained from whole blood (WB) may be advantageous, as it detects not only those derived from circulating blood cells, including CTCs, but also those secreted in subcellular particles and those associated with RNA-binding proteins [4, 5]. Cancer-specific whole-blood RNA (WB-RNA) expression patterns are not defined by a single cell type. Studies indicate that other blood components, such as erythrocytes, platelets, immune cells, and exosomes, may contribute to the disease specificity of a WB-RNA signature [6].

The identification of RNA biomarkers in cancer has involved various methodologies. One approach is the study of global RNA expression (also known as"transcriptomics") to identify characteristic signatures associated with patient outcomes [7]. Another approach is hypothesis-driven, focusing on RNAs involved in specific molecular pathways and their prognostic relevance—for example, RNAs implicated in the metastatic process [8].

In 2012, the Spanish Cooperative Group for the Treatment of Digestive Tumours (TTD Group) launched the VISNÚ project, in which metastatic CRC (mCRC) patients with no prior systemic treatment were screened for eligibility in one of two randomized clinical trials (VISNÚ1 and VISNÚ2). Eligibility was determined based on their basal CTC (bCTC) counts, enumerated using the CellSearch System, and their *RAS* mutational status. The results of these clinical trials have been published [9–12].

In this ancillary study, we aimed to determine whether the expression of selected mRNAs and miRNAs in WB correlated with bCTC counts. The WB-RNAs analyzed in this study were selected using a hypothesis-driven approach, based on previous research [13–15] and a review of the literature [16–20]. These RNAs are associated with epithelial differentiation, stemness, immune response, epithelial-to-mesenchymal transition (EMT), angiogenesis, proliferation, and metastasis [21, 22]. We explored whether the expression profiles of these circulating RNAs were linked to bCTC levels, molecular tumour profiling, and prognosis in mCRC patients.

Methods

Patients

The participants included in this study were patients who consented to participate in the RNA substudy during the eligibility procedures for the VISNU clinical trials at 11 centres between June 2014 and November 2016. Eligibility criteria included age 18–70 years, Eastern Cooperative Oncology Group performance status (ECOG-PS) 0–1, histologically confirmed mCRC, availability of formalin-fixed paraffin-embedded (FFPE) tumour tissue, measurable disease according to RECIST 1.1, and no prior chemotherapy for advanced disease [9–12]. A total of 151 patients who met these criteria were included and are referred to as the screening cohort in Fig. 1. The 67 patients in this cohort who met specific criteria and were included in the VISNU1 ($\geq$ 3 bCTC regardless of *RAS* status) or VISNU2 (< 3 bCTC and *RAS wt*) studies constitute the per-protocol cohort (Fig. 1).

Mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3 CA* genes, as well as microsatellite instability testing (performed

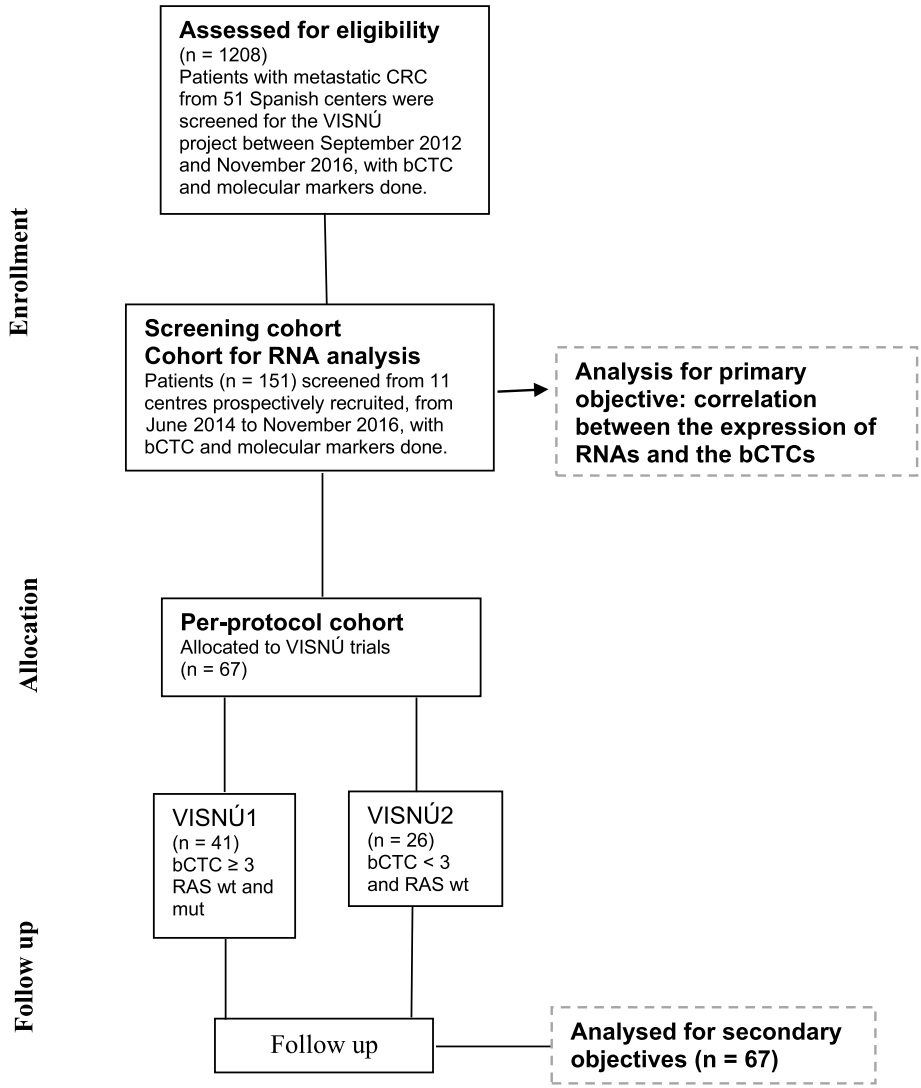

Fig. 1 Flow of participants through the study

only in the per-protocol cohort), were analyzed using tumour tissue in six reference laboratories. Detailed methods for mutational status analysis have been previously published [9–12].

Institutional review board approval was obtained prior to enrolling any patient. The study was performed in accordance with the Declaration of Helsinki and its amendments and the Good Clinical Practice Guidelines. Specific signed informed consent was obtained from all patients before study entry.

Blood samples collection and bCTCs and RNA isolation

Peripheral blood (7.5 mL) was collected in CellSave Preservative Tubes, and circulating tumour cells (CTCs) were enumerated using the CellSearch Circulating Tumour Cell Kit (Veridex LLC, Raritan, New Jersey, USA). Baseline CTCs (bCTCs) were centrally determined at a reference hospital (San Carlos Hospital).

For RNA analysis, an additional 2.5 mL of blood was collected in PAXgene Blood RNA tubes. RNA was purified using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit, following the manufacturer's protocol. During RNA extraction, 5 pM of phosphorylated *ath-miR159a* from *Arabidopsis thaliana* (Thermo) was added as an exogenous control for miRNA normalization. Total RNA was quantified using a DeNovix DS- 11 spectrophotometer.

Table 1 Classification of selected RNAs according to their association with cancer hallmarks

Hallmark	RNA	TaqMan assay ID	Accession number
Sustaining proliferative signalling, evading growth suppressors, resisting cell death	miR- 1290	hsa-miR- 1290_477895_mir	MI0006352
	miR- 224 - 5p	hsa-miR- 224_5p_483106_mir	MIMAT0000281
	miR- 133a- 3p	hsa-miR- 133a- 3p_478511_mir	MIMAT0000427
	miR- 885 - 5p	hsa-miR- 885 - 5p_478207_mir	MIMAT0004947
	miR- 15b- 5p	hsa-miR- 15b- 5p_478313_mir	MIMAT0000417
	miR- 21 - 5p	hsa-miR- 21 - 5p_477975_mir	MIMAT0000076
	miR- 193b- 3p	hsa-miR- 193b- 3p_478314_mir	MIMAT0002819
Enabling replicative immortality,	miR- 31 - 3p	hsa-miR- 31 - 3p_478012_mir	MIMAT0004504
Stemness signature	Leucine rich repeat containing G protein-coupled receptor 5 (LGR5)	Hs00969422_m1	HGNC:4504
	Doublecortin like kinase 1 (DCLK1)	Hs00178027_m1	HGNC:2700
Inducing angiogenesis	miR- 210 - 3p	hsa-miR- 210 - 3p_477970_mir	MIMAT0000267
	miR- 1229 - 5p	hsa-miR- 1229 - 5p_479548_mir	MIMAT0022942
	miR- 26a- 5p	hsa-miR- 26a- 5p_477995_mir	MIMAT0000082
Invasion and metastasis	miR- 106a- 5p	hsa-miR- 106a- 5p_478225_mir	MIMAT0000103
	miR- 200a- 3p	hsa-miR- 200a- 3p_478490_mir	MIMAT0000682
	miR- 200b- 3p	hsa-miR- 200b- 3p_477963_mir	MIMAT0000318
	miR- 200c- 3p	hsa-miR- 200c- 3p_478351_mir	MIMAT0000873
	Keratin 20 (KRT20)	Hs00300643_m1	HGNC:20,412
Inflammation and evasion of immune destruction	miR- 1248	hsa-miR- 1248_478653_mir	MIMAT0005900
	Adenosine deaminase RNA specific 1 (ADAR1)	Hs00241666_m1	HGNC:225
	Programmed cell death 1 ligand 1 (CD274)	Hs00204257_m1	HGNC:17,635
Creating the "tumour microenvironment"	miR- 20a- 5p	hsa-miR- 20a- 5p_478586_mir	MIMAT0002704
	Anterior gradient 2 (AGR2)	Hs00356521_m1	HGNC:328

Accesion number is indicated according to https://www.mirbase.org/ for microRNA or the HUGO Gene Nomenclature Committee (https://www.genenames.org/) for mRNA

Blood-derived RNA analysis

Six mRNAs and 17 miRNAs (Table 1) were selected for the analysis. Classification according to their biological and functional role in cancer and metastasis are depicted in Table 1.

For mRNA analysis, cDNA synthesis from 500 ng of total RNA was performed using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen), following the manufacturer's instructions. The levels of six mRNAs and two mRNA controls, 18S and HPRT1, used to assess RNA integrity, were then measured using a Custom TaqMan™ Gene Expression Assay (Applied Biosystems). Real-time PCR was conducted using a TaqMan™ Array Fast 96-well plate (plate 8) on the 7500 Real-Time PCR System (Applied Biosystems), with two technical replicates. The cDNA product was mixed with TaqMan Fast Advanced Master Mix (Applied Biosystems) and loaded onto the plate. mRNA levels were normalized using HPRT1.

For miRNA analysis, cDNA synthesis and pre-amplification from 10 ng of total RNA were performed using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems). The levels of 17 miRNAs (Table 1) and an exogenous control were then measured using a Custom TaqMan OpenArray Panel. qPCR was conducted using a TaqMan OpenArray Panel with TaqMan Fast Advanced Master Mix (Applied Biosystems), with three technical replicates, on the QuantStudio 12 K Flex Real-Time System (Applied Biosystems). miRNA levels were normalized using the exogenous control.

Relative expression levels were calculated using the 2^-ΔΔCt method [23] for both mRNA and miRNA analyses. The quantification cycle (Cq) threshold was set at ≤ 35. All RNA analyses were conducted in a blinded manner, without knowledge of the patients'characteristics.

Bioinformatics tools

A bioinformatic analysis was conducted to investigate the expression of the selected miRNAs in colorectal cancer, along with their functions and target genes. The CancerMIRNome database [24] was used for the interactive analysis of the selected miRNAs in colorectal cancer (CRC) datasets from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), and ArrayExpress. These analyses included differential expression (DE), Kaplan–Meier (KM) survival analysis, and functional enrichment analysis of miRNA targets.

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses were performed using the online database DIANA-miRPath v3.0 [25–27]. The"pathway intersection"option in the miRPath software was selected. Within the GO analysis, the"biological process"(BP) subcategory was chosen, and differentially expressed genes (DEGs) were identified. *p*-values were obtained using Fisher's exact test, and the false discovery rate (FDR) was estimated using the Benjamini and Hochberg (BH) method.

Statistical analysis

The study was designed as a complementary translational study, and all analyses should be considered exploratory. The primary objective was to determine the correlation between the expression of WB-RNA biomarkers and the number of bCTCs detected by the CellSearch system in the screening cohort. Secondary objectives included examining the relationship between WB-RNA biomarkers and clinical, pathological, and molecular features, as well as prognosis, in the per-protocol cohort.

bCTC counts, age, and molecular characteristics were collected at baseline for the screening cohort. The remaining clinicopathological characteristics were collected only for patients included in the per-protocol cohort.

Chi-squared or Fisher's exact tests were used to evaluate associations between bCTC or biomarker subgroups and individual clinicopathological features. Student's *t*-test, Mann–Whitney *U*, or Wilcoxon tests were used to compare the distribution of quantitative variables between groups, based on the Kolmogorov–Smirnov normality test. Box plots were generated using the DATAtab Online Statistics Calculator [28]. Statistical analyses were performed using SPSS software and the MetaboAnalyst web server [29], with a two-sided 5% significance level. Continuous variables are expressed as the mean with standard deviation (SD), while categorical data are presented as counts and percentages. Pearson correlation analyses were used to visualize the overall strength and direction of relationships between bCTC counts and WB-RNA levels.

Fold change (FC) analyses, *t*-tests, Wilcoxon tests, and volcano plots were used to assess differential RNA expression and derive the blood-based RNA profile. To correct for multiple comparisons, the FDR was estimated using the BH method in the MetaboAnalyst web server [29].

Receiver operating characteristic (ROC) curves were generated, and the area under the curve (AUC) with the respective 95% confidence intervals (95% CI) was calculated. Outcomes were evaluated on an intention-to-treat basis in the per-protocol group. Overall survival (OS), defined as the time from randomization until death from any cause, and progression-free survival (PFS), defined as the time from randomization to disease progression according to RECIST or death from any cause, were estimated using the KM method and compared with a two-sided log-rank test at a 5% significance level.

ROC curve analyses were used to identify an RNA or RNA-pair ratio capable of discriminating patients with survival times above or below the median. Survival curves were depicted using the KM method and compared using the log-rank test. Finally, a Cox proportional hazards regression model was estimated. Bootstrapping was used for internal validation. A multivariate backward stepwise method, with retention of predictors with $p < 0.05$, was performed, and the likelihood ratio test was applied.

Results

bCTC and mutational status profile

A total of 1,252 patients diagnosed with metastatic CRC from 51 Spanish centers were evaluated for eligibility in the VISNÚ clinical trials between September 2012 and November 2016. Molecular markers and bCTC counts were obtained from 1,208 of these patients. The screening cohort for this ancillary WB-RNA study consisted of 151 patients recruited prospectively from 11 centers (Fig. 1). Their clinical and molecular characteristics are summarized in Table 2.

bCTCs were evaluated in 150 patients. A total of 149 and 150 samples were suitable for mRNA and miRNA analyses, respectively. The mean bCTC count was 13.6 (range: 0–970; SD: 80.15). Ninety-nine patients (65.6%) had bCTC ≥ 1, 62 (41.1%) had bCTC ≥ 3, and 21 (14%) had bCTC ≥ 13.

Tumor mutations in the *KRAS, NRAS, BRAF,* and/or *PIK3 CA* genes were detected in 90 (63.4%) out of 142 patients evaluated. The *extended RAS* status was classified as *wild-type* (*RAS wt*) when no mutations were detected in *KRAS* exons 2, 3, and 4, as well as *NRAS* exons 2, 3, and 4 ($n = 54$ patients). However, in 13 additional cases, the *RAS wt* status was determined solely by assessing *KRAS* exons 2 and 3. *RAS* mutations *(RAS mut)*

Table 2 Clinical and molecular characteristics of patients

	n	%
Screening cohort (*n* = 151)		
Age (years) mean (StD; range)	59.7 (7.5; 38–70)	
≤ 60/> 60	72/79	47.7/52.3
Tumour molecular profle (*n* = 142)		
RAS wt[a]*/RAS mut*	67/75	47.2/52.8
BRAF wt/BRAF mut	116/10	92.1/7.9
PIK3 CA wt/PIK3 CA mut	110/16	87.3/12.7
RAS-BRAF-PIK3 CA wt/*mut*	52/90	36.6/63.4
bCTC Count		
< 3/7.5 mL/≥ 3/7.5 mL	88/62	58.3/41.1
Per-protocol cohort (*n* = 67)		
Gender		
Male	49	73.1
Female	18	26.9
ECOG		
0	39	58.2
1	28	41.8
Primary tumour site	67	
Right	12	17.9
Left	55	82.1
Microsatellite testing		
Stable (MSS)	66	98.5
Nº organs involved		
1	39	58.2
≥ 2	28	41.8
CEA (ng/ml)		
≤ 5	10	14.9
> 5	57	85.1
WBC count		
$< 10 \times 10^9$/l	54	80.6
$> 10 \times 10^9$/l	13	19.4
Alkaline phosphatase (U/l)		
< 300	56	83.6
> 300	11	16.4
Köhne's risk groups		
Low	39	58.2
Intermediate	23	34.3
High	5	7.5
Treatment		
FOLFIRI Bevacizumab	13	19.4
FOLFIRI Cetuximab	13	19.4
FOLFOX Bevacizumab	22	32.8
FOLFOXIRI Bevacizumab	19	28.4

[a] *RAS* was considered"wild type"(*RAS wt*) when no mutations on *KRAS* 2, 3 and 4 and *NRAS* 2, 3 and 4 were found (*n* = 54 patients). In other 13 cases the *RAS wt* status were based only in *KRAS* exon 2 and 3 assessment

were present in 75 (52.8%) of the 142 analyzed patients. Overall, the *KRAS, NRAS, BRAF,* and *PIK3 CA* genes were wild-type (quadruple *wt*) in 52 (36.6%) of the 142 patients. The detailed mutational status profile is provided in *Supplementary Information, Table S1*. The distribution of *RAS, BRAF,* and *PIK3 CA* mutations, along with bCTC counts and patient disposition in both the overall eligibility assessment group (*n* = 1,252) and the screening cohort (*n* = 151), is shown in *Supplementary Table 2*.

The number of bCTC was higher in the *RAS wt* group (mean 23.9; SD 118.8) comparing to *RAS mut* group (mean 4.8; SD 10.4; $p = 0.018$). The mean bCTC count was 29.5 (SD 134.6) in the quadruple *wt* and 4.7 (SD 1.04) in any *RAS*/*BRAF*/*PIK3 CA* mutated tumours ($p = 0.016$). In the group with quadruple *RAS*/*BRAF*/*PIK3 CAwt* tumours (*n* = 52), a bCTC ≥ 3 was found in 28 patients (53.8%). However, in the group with any mutation in *RAS*/*BRAF*/*PIK3 CA* genes (*n* = 90), only 31 (34.8%) patients had a bCTC ≥ 3 ($p = 0.034$).

mRNA and miRNA blood expression analyses

We evaluated the blood of 149 patients with mCRC for the presence of six mRNAs known to play a role in metastasis. Two immune-regulatory mRNAs, ADAR1 and CD274, were detected in the blood of all patients. ADAR1 and CD274 showed a significant correlation ($r = 0.314$, $p < 0.001$). Expression of stem cell mRNAs (LGR5 and/or DCLK1) was detected in 43.3% of patients (66/149), KRT- 20 mRNA in 94% of cases and AGR2 mRNA in 40.3% of patients (60/149).

The presence of stem cell markers LGR5 and/or DCLK1 in the bloodstream (referred to as the"stemness signature") was detected in 64.5% (40/74) of *RAS mut* patients and in 33.3% (22/66) of *RAS wt* patients ($p = 0.017$). This stemness signature was observed in 51.1% (45/88) of patients with any mutation in the *RAS*/*BRAF*/*PIK3 CA* genes compared to 32.7% (17/52) of quadruple *wt* patients ($p = 0.037$). However, there were no significant differences in mRNA expression levels based on the clinical characteristics studied. The significant associations between deregulated WB-miRNAs and clinical characteristics in the per-protocol cohort are shown in Table 3.

RNA biomarkers and bCTC: correlations and differential expression analysis

The bCTC count was significantly correlated with AGR2 ($r = 0.981$; $p < 0.001$). In the subgroup with bCTC ≥ 3, the bCTC were correlated with AGR2 ($r = 0.986$; $p < 0.001$), ADAR1 ($r = 0.317$; $p = 0.028$) and LGR5 ($r = 0.265$; $p = 0.039$).

Box plots of differentially expressed RNAs are shown in Fig. 2. Descriptive statistics for the box plots are presented in Table S3. The differential expression of

Table 3 Significant associations observed between the relative expression of blood microRNAs and clinical characteristics

microRNA	ECOG	N	Mean	StD	*p*
miR- 106a- 5p	0	38	0.768	0.491	
	1	28	0.559	0.484	0.027
	Primary tumour site				
miR- 200b	Left	55	0.000	0.000	
	Right	11	0.011	0.037	0.025
miR- 224	Left	55	0.002	0.011	
	Right	11	0.006	0.012	0.0006
	Nº organs involved				
miR- 15b	1	39	23.92	24.25	
	≥ 2	27	14.68	20.12	0.027
	White blood cell (WBC) count				
miR- 106a- 5p	$< 10 \times 10^9$/l	54	0.610	0.429	
	$> 10 \times 10^9$/l	12	0.992	0.659	0.036
	Alkaline phosphatase (AP) U/l				
miR- 200c	< 300	56	0.001	0.003	
	> 300	10	0.002	0.003	0.019
miR- 20a	< 300	56	0.573	0.799	
	> 300	10	1.242	1.042	0.0036
miR- 21	< 300	56	0.251	0.277	
	> 300	10	0.517	0.459	0.014
miR- 224	< 300	56	0.002	0.011	
	> 300	10	0.006	0.012	0.0003
miR- 26a	< 300	56	2.942	2.009	
	> 300	10	5.500	3.803	0.03
miR- 15b	< 300	56	17.93	20.9	
	> 300	10	32.5	30.61	0.031
	Köhne's risk groups				***p****
miR- 20a	Low	39	0.774	0.978	0.024
	Intermediate	23	0.378	0.458	
	High	4	1.402	1.036	
miR- 200c	Low	39	0.001	0.004	0.015
	Intermediate	23	0.000	0.001	
	High	4	0.004	0.005	
miR- 15b	Low	39	23.922	24.246	0.025
	Intermediate	23	12.084	17.208	
	High	4	29.579	31.469	

SD Standard Deviation, *ECOG* Eastern cooperative oncology group performance status, *WBC* White blood cells

p, Mann–Whitney *U* test

*p**, Kruskal Wallis test

WB-RNA markers was identified by performing a volcano plot and based on the mutational status profile and the number of circulating tumour cells (Table S4 and Fig. S1).

The RNA profiles associated with higher CTC burden, as defined by its mean (bCTC ≥ 13), showed higher expression of AGR2 ($p = 0.016$) and miR- 1248 ($p = 0.009$). Conversely, there was downregulation of miR-200a- 3p ($p = 0.019$) and miR- 200c- 3p ($p = 0.005$). Details are shown in Fig. 2a.

We also analysed the correlation between WB-RNA expression levels and bCTC count according to the *RAS*/*BRAF*/*PIK3 CA* mutational status. In patients with quadruple *wt* tumours, the bCTC count demonstrated a strong correlation with the levels of AGR2 ($r = 0.989$; $p < 0.0001$), ADAR1 ($r = 0.3$; $p = 0.031$) and LGR5 ($r = 0.314$; $p = 0.023$).

In the *RAS*/*BRAF*/*PIK3 CA mut* subgroup, the most significant finding (Fig. 2b) was the upregulation of miR-224 ($p = 9.46 \times 10–^5$) and, LGR5 ($p = 0.013$) and downregulation of CD274 ($p = 0.028$). See Tables S3 and S4 for details. However, no significant correlations were found between bCTC counts and the levels of any of the WB-RNAs analysed in this group.

The ROC curve and AUC analyses were used to assess the performance of WB-RNA biomarkers in classifying patients based on bCTC count (< 3 bCTCs/≥ 3 bCTCs) and according to *RAS*/*BRAF*/*PIK3 CA* mutational status (Table 5S). In the subgroup of *RAS* and *BRAF wt* patients, the best sorters were AGR2 (AUC = 0.69; 95% CI 0.537–0.809; $p = 0.02$) and miR106a- 5p/AGR2 (AUC = 0.68; 95% CI 0.547–0.807; $p = 0.02$) (Fig. 3a and b). In the *RAS*/*BRAF*/*PIK3 CA wt* subgroup the best marker for differentiating between bCTC < 3 and ≥ 3 was the ADAR1/AGR2 ratio (AUC = 0.67; 95% CI 0.517–0.803; $p = 0.03$) (Fig. 3c). There were no significant predictors in the *RAS*/*BRAF*/*PIK3 CA mut* subgroup (Table S5).

Survival analysis

Outcomes were evaluated on an intention-to-treat basis in the per-protocol group ($n = 67$). The median OS was 24.8 months (95% CI 17.4—32.2). According to the number of bCTCs, the median OS was 30.5 (95% CI, 17—43.9) and 23.8 (95% CI, 18.4—29.1) months for patients with bCTC < 3 and bCTC ≥ 3 ($p = 0.44$), respectively. The median PFS was 12.9 (95% CI 9.2—16.6) months, without any differences ($p = 0.796$) between the patients with bCTC < 3 (10.5 months; 95% CI 7.4—13.6) and bCTC ≥ 3 (13.9 months; 95% CI 11.2—16.6). The median OS was 30.87 months (95% CI 22.33—39.42) for patients with bCTC < 13 and 14.3 months (95% CI 6.22—22.39) for patients with bCTC ≥ 13 ($p = 0.049$).

The ROC curves analysis for RNA biomarkers to categorize patients according to their median OS and PFS are shown in Table S5. None of the RNAs identified in the ROC curves for PFS were found to be statistically significant in the KM analyses and log-rank tests (data not shown). The most effective RNA-based classifier of OS was the miR- 106a- 5p/miR- 26a- 5p ratio (AUC = 0.743, 95% CI, 0.617–0.859, $p < 0.01$), as demonstrated in

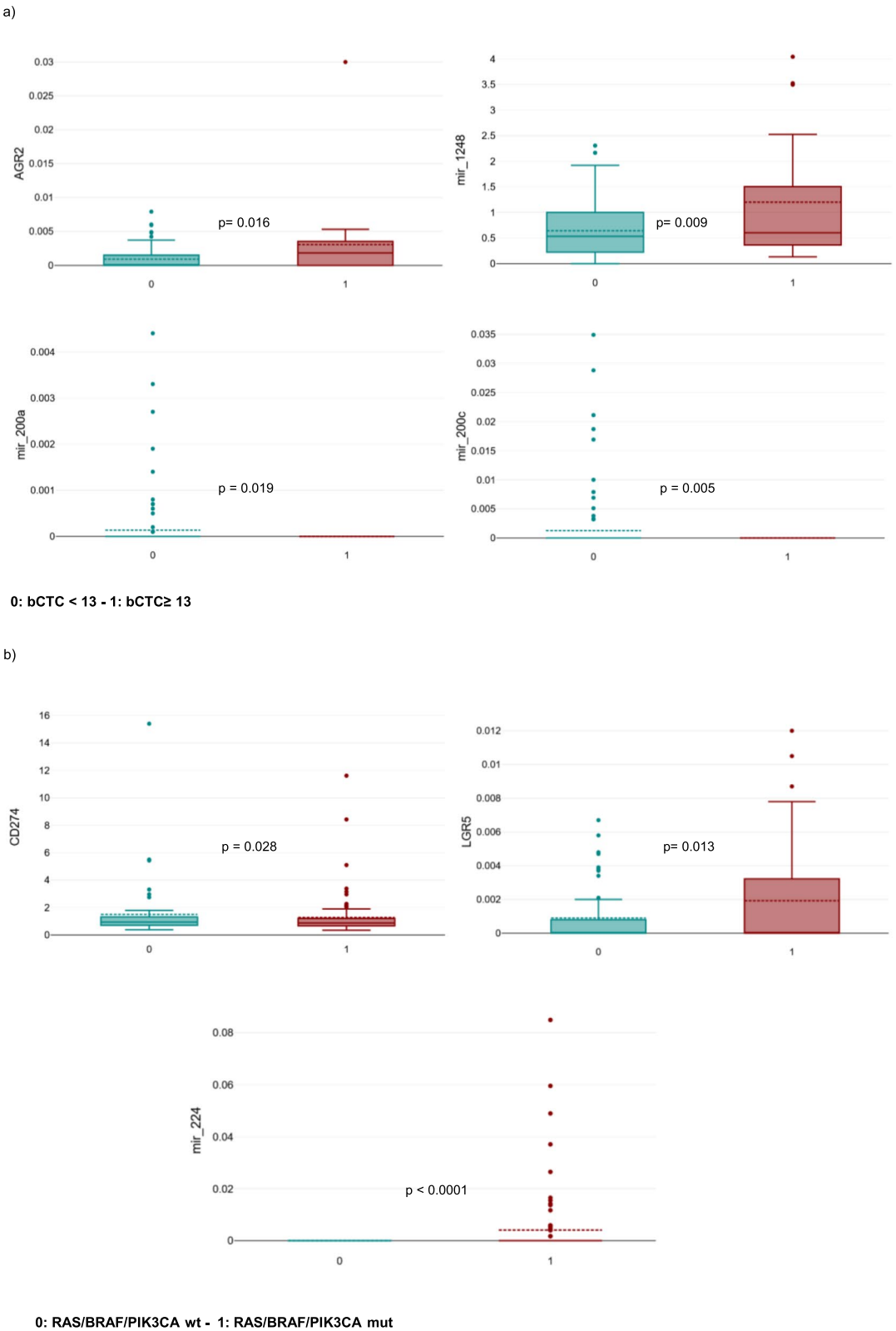

Fig. 2 The box plots illustrate the differential expression of RNA, with the expression levels presented on the y-axis in relative units. **a** The differential expression of RNA in patients who have been classified according to the bCTC mean (bCTC ≥ 13); (**b**) The differential expression of RNA in the *RAS*, *BRAF* and *PIK3 CA* sequence variant tumour. The significance of these differences was determined using the Wilcoxon test

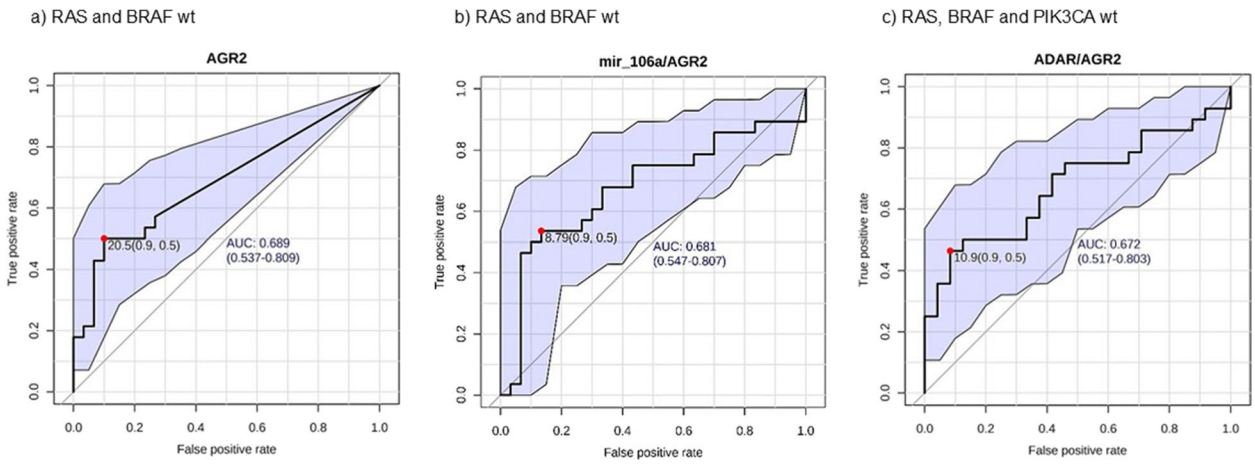

Fig. 3 ROC and AUC were used to evaluate the performance of blood RNA biomarkers in predicting basal CTC, < 3 bCTCs and ≥ 3 bCTCs according to *RAS*, *BRAF* and *PIK3 CA* sequence variant tumour subgroups: (**a**, **b**) *RAS* and *BRAF* wild-type; (**c**) *RAS/BRAF/PIK3 CA* wild-type

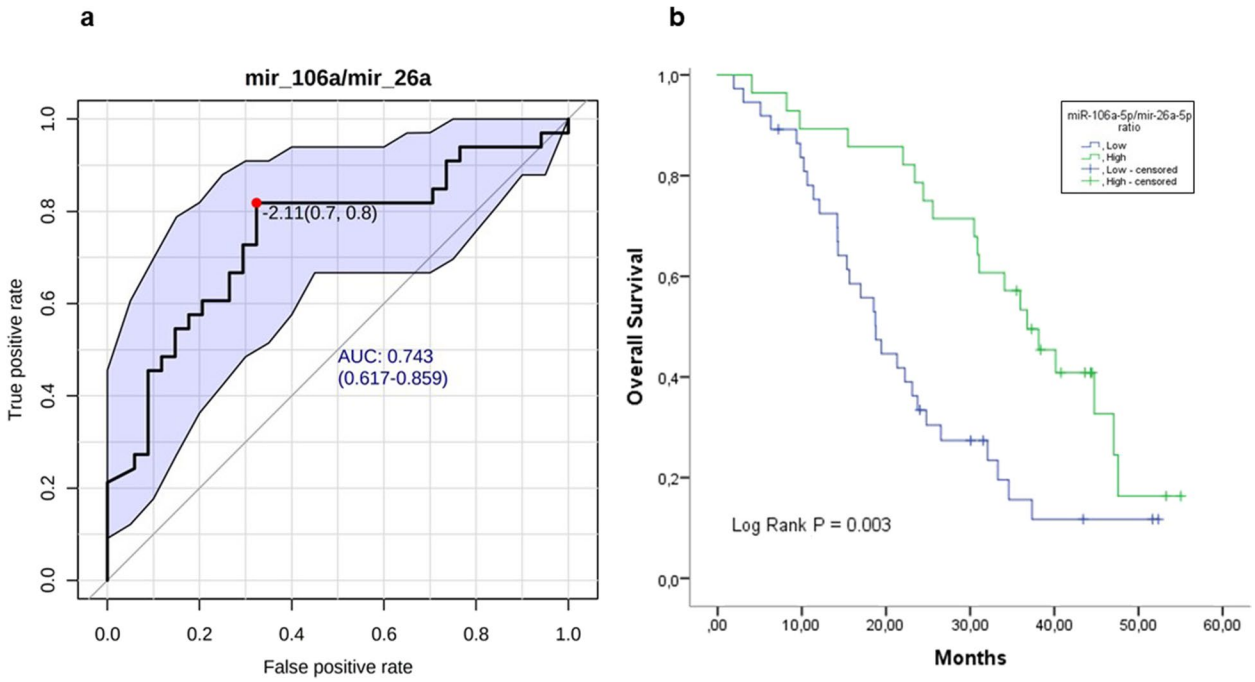

Fig. 4 Discriminative ability of RNA biomarkers to classify patients according to their overall survival: a) the best RNA-based classifier in the ROC curve analysis was the miR- 106a/miR- 26a ratio (AUC = 0.743; 95% CI, 0.617–0.859; $p < 0.01$); b) Kaplan–Meier analysis showed that a miR- 106a/miR- 26a ratio higher than the mean was associated with a longer median overall survival of 36.8 months (95% CI, 29.8–43.7) and 18.8 months (95% CI, 15.2–22.4; $p = 0.003$), respectively

Fig. 4a. Using the mean miR- 106a- 5p/miR- 26a- 5p ratio as a cut-off, KM analysis (Fig. 4b) showed that a higher miR- 106a- 5p/miR- 26a- 5p ratio associated with longer OS (36.8 months; 95% CI, 29.8–43.7 versus 18.8 months 95% CI, 15.2–22.4; $p = 0.003$). This high-risk miR- 106a-5p/miR- 26a- 5p ratio was found in 55.6% (84/151) and 55.2% (37/67) of the patients in the screening and the per-protocol cohorts, respectively. Considering miR-106a- 5p/miR- 26a- 5p ratio as a continuous variable, increasing ratios were associated with a lower risk of death (HR 0.034; 95% CI, 0.002—0.602; $p = 0.021$. Bootstrap validation, $p = 0.03$). Multivariate analysis was performed to investigate the impact of several variables on OS, including ECOG-PS, primary tumour location (left

Table 4 Predictive model of survival of patients, per-protocol group ($n = 67$): multivariate analysis

	Variables	B	SE	Wald	p	Exp(B)	95% CI Exp(B)	
							Lower	Upper
Step 1	miR- 106a/miR- 26a (lower)	0.621	0.336	3.414	0.065	1.861	0.963	3.595
	Primary tumour site (right)	1.814	0.41	19.56	0.000	6.136	2.746	13.709
	Number of metastatic sites (> 1)	- 0.51	0.318	2.558	0.11	0.601	0.322	1.122
	ECOG-PS (1)	0.572	0.329	3.022	0.082	1.772	0.93	3.377
Step 2	miR- 106a/miR- 26a ratio (lower)	0.652	0.338	3.712	0.054	1.919	0.989	3.723
	Primary tumour site (right)	1.598	0.38	17.686	0.000	4.943	2.347	10.41
	ECOG-PS (1)	0.482	0.326	2.184	0.139	1619	0.855	3.069
Step 3	miR- 106a/miR- 26a ratio (lower)	0.846	0.309	7.509	0.006	2.331	1.273	4.27
	Primary tumour site (right)	1.596	0.38	17.673	0.000	4.932	2.344	10.378

Reference category: ECOG-PS (0), Primary tumour site (left), Number of metastatic sites (1), miR- 106a- 5p/miR- 26a- 5p ratio > mean value

B Coefficient to calculate hazard ratio, *S.E.* Standard Error, *Wald* Wald statistic, Exp (B), hazard ratio, *C.I.* Confidence interval, Degrees of freedom = 1

or right), number of metastatic sites (1 *versus* more than 1) as previously reported [12] and miR- 106a- 5p/miR- 26a- 5p ratio. In the optimal model, the factors that were independently associated with a higher risk of death were primary tumour location on the right side and a low ratio of miR- 106a- 5p to miR- 26a- 5p (Table 4).

The miR- 106a- 5p/miR- 26a- 5p ratio was not associated with any of the evaluated clinical or molecular characteristic including gender/sex, age > 60 years or ≤ 60 years, primary tumour location, Köhne´s prognostic subgroup, *RAS*/*BRAF*/*PIK3 CA* mutations, white blood cells count (WBC), alkaline phosphatase (AP), Carcinoembryonic Antigen (CEA), bCTC ≥ 1 or bCTC count ≥ 3. Only a higher number of bCTC (bCTC ≥ 13 CTC) was associated with a low miR- 106a- 5p/miR- 26a- 5p ratio ($p = 0.031$).

Bioinformatics analysis

The TCGA profiles of miR- 106a- 5p and miR- 26a- 5p in the colorectal project (COAD) were obtained [24]. DE analysis was used for testing differences between miRNA expression in colorectal adenocarcinoma ($n = 444$) and normal colorectal ($n = 8$) samples. Only 63 cases (14.19%) were stage IV. In CRC samples, miR- 106a- 5p and miR- 26a- 5p were highly expressed, with median counts per million (CPM) of 4.16 and 10.61, respectively. In addition, both miR- 106a- 5p ($p = 3.26 \times 10^{-6}$) and miR- 26a- 5p ($p = 3.26 \times 10^{-6}$) were found to be significantly upregulated in tumours when compared to normal colonic samples. The survival probability was significantly higher in patients with low tumour miR- 26a- 5p expression (HR 1.57; 95% CI, 1.06–2.31; $p = 0.027$). However, the tumour expression of miR- 106a- 5p was not associated with survival (HR 1.27; 95% CI, 0.86–1.88; $p = 0.22$).

We performed a functional enrichment analysis of the miR- 106a- 5p and miR- 26a- 5p targets throughout the CancerMIRNome. KEGG pathways [26] of the miR-106a- 5p targets were enriched among others in *TP53* signalling pathway (BH. adj. $p = 1.86 \times 10^{-4}$), colorectal cancer (BH. adj. $p = 1.86 \times 10^{-4}$), *FoxO* signaling pathway (BH. adj. $p = 3.01 \times 10^{-4}$) *and transforming growth factor-β (TGFB)* signalling pathway (BH. adj. $p = 4.69 \times 10^{-3}$). For miR- 26a- 5p targets, the enriched KEGG pathways included cellular senescence (BH. adj. $p = 2.72 \times 10^{-4}$), transcriptional regulation by *TP53* (BH. adj. $p = 5.32 \times 10^{-4}$), *MAPK* family signaling cascades (BH. adj. $p = 1.82^{-2}$) and cellular responses to stress (BH. adj. $p = 1.11 \times 10^{-3}$).

KEGG and GO enrichment using DIANA-miRPath v3.0 were performed [25–27]. KEGG analysis revealed that most of the target genes of both miR- 106a- 5p and miR- 26a- 5p were involved in the *Hippo* signalling pathway. (42 genes), proteoglycans in cancer (51 genes), protein processing in endoplasmic reticulum (47 genes), and *TGFB* signalling (26 genes). The GO categories containing only enriched biological process with significant results for both miRNAs were mitotic cell cycle (GO:0000278; $p < 1 \times 10^{-325}$), cellular protein modification process (GO:0006464; $p < 1 \times 10^{-325}$) and response to stress (GO:0006950; $p < 1 \times 10^{-325}$).

Discussion

This study analyzed mRNA and miRNA expression patterns involved in metastasis in WB from mCRC patients who had not received prior systemic treatment. It assessed their correlation with bCTC and molecular characteristics. RNA alterations in mCRC patients'blood, such as stemness and immune biomarkers, indicate the presence and burden of CTCs. However, these signatures

are reliable CTC indicators only when analyzed alongside the *RAS*/*BRAF*/*PIK3 CA* mutational profile. Furthermore, our study identified a novel RNA biomarker, the miR- 106a- 5p/miR- 26a- 5p ratio, which distinguishes a subgroup of mCRC patients with poorer overall survival.

Whole blood RNA profiling, including microRNAs, has emerged as a promising strategy for detecting and predicting prognosis in digestive tumours, including pancreatic [30], liver [31, 32], and bile duct cancers [33]. WB offers higher microRNA concentrations [34] and minimizes handling issues associated with serum and plasma, making it advantageous for multicenter studies [35]. It also allows the assessment of tumour-secreted RNA and RNA generated by the host response. However, few systematic evaluations have explored WB-RNA's diagnostic and prognostic utility in CRC [36, 37]. The findings of the present study may serve as a basis for future research incorporating measurements of miR- 106a- 5p, miR-26a- 5p, and other relevant miRNAs in whole blood, combined with clinicopathological factors, to develop prognostic models in mCRC [38].

Our findings show that some RNA markers correlate with bCTC but are influenced by *RAS*/*BRAF*/*PIK3 CA* mutations. AGR2 expression was strongly associated with bCTC counts, especially in patients with RAS wild-type or quadruple wild-type tumours. Additionally, ADAR1 and LGR5 levels increased with rising CTC numbers.

We identified an RNA panel (AGR2, miR- 106a- 5p/AGR2, and ADAR1/AGR2 ratios) capable of differentiating patients with bCTC <3 and ≥ 3, but only within the *RAS*/*BRAF*/*PIK3 CA* mutation context. However, ROC analysis showed moderate predictive accuracy (AUC 0.67–0.69) for bCTC count classification.

The potential prognostic value of the described WB-RNA profiles needs to be demonstrated in external validation studies (preferably multicentre studies) including different patient populations to confirm the accuracy, reliability and generalisability of the biomarker, always taking into account the clinical characteristics and tumour mutation profile.

AGR2 has previously been recognized as a potential CTC marker in CRC [14, 39, 40]. Overexpression of AGR2 is linked to poor prognosis in gastrointestinal cancers [14, 41] and plays a key role in inflammation and tumour progression, notably through *Hippo* signaling regulation [42]. Recent research showed AGR2 upregulation in plasma from lung and breast cancer patients, but not in non-cancer controls, suggesting its classification as a tumour-specific transcript detectable in plasma and blood cell-derived RNA [43].

A blood RNA signature associated with high bCTC counts included overexpression of AGR2 and miR- 1248, and downregulation of miR- 200a- 3p and miR- 200c- 3p. There is limited data on miR- 1248 in CRC. It has been associated with cell proliferation and invasion, acting as a tumour suppressor, and its expression decreases in lymph node metastatic CRC [44]. However, bioinformatics analyses suggest miR- 1248 overexpression correlates with poor survival in stage I-IV CRC [20]. In older adults, the levels of miR- 1248 in the blood were significantly reduced. In addition, pathways associated with inflammation, such as the cytokines IL6 and IL8, were downregulated in cells that overproduced miR- 1248 [45]. Our results link high bCTC counts with elevated miR- 1248 and reduced miR- 200a- 3p/miR- 200c- 3p, which could indicate a dysregulation of EMT and the involvement of the inflammatory pathway in the circulation of tumour cells in the bloodstream.

The miR- 200 family supports epithelial phenotypes by suppressing EMT [46]. Studies have shown a modest but significant increase in circulating miR- 200 s in metastatic CRC patients compared to controls [47, 48]. Our results showed that elevated blood miR- 200c- 3p was associated with poor prognostic factors, including high alkaline phosphatase (AP >300) and a high-risk Köhne subgroup. However, at least a subset of CTCs might have a mesenchymal phenotype, as lower levels of miR- 200a-3p and miR- 200c- 3p were observed in patients with higher bCTC counts.

Stemness signatures characterised by LGR5 and/or DCLK1 expression in WB-RNA were strongly associated with *RAS*/*BRAF*/*PIK3 CA* mutations. Upregulation of these markers was detected in patients with any of these tumour mutations, suggesting a rare CTC subpopulation expressing stem cell markers. These CTCs may not be detectable by conventional EpCAM/cytokeratin-based systems like CellSearch [39, 49]. Indeed, subsets of EpCAM-negative CTCs with stem cell properties have been reported [50–52].

Patients with *RAS*/*BRAF*/*PIK3 CA* mutations showed increased miR- 224 -5p and LGR5 levels, along with reduced CD274 expression. This pattern resembles that seen in CRC tumour budding [53]. MiR- 224 overexpression has been linked to decreased CDH1 and SMAD4 levels, promoting metastasis [54]. It also enhances CRC proliferation and invasion by suppressing *Wnt/β-catenin* signaling [55]. In our study, miR- 224 -5p upregulation correlated with poor prognosis, including right-sided tumours, high AP levels, and *RAS*/*BRAF*/*PIK3 CA* mutations.

CTCs are known to be heterogeneous and to interact with immune and haematopoietic cells, influencing transcriptional profiles in both direction. Immune-related mRNAs (CD274 and ADAR1) were detected in all blood samples, with the highest relative expression levels

compared to other RNAs. Both ADAR1 and CD274 are expressed on various circulating cells, including immune, myeloid and tumour cells. A previous study identified PD-L1 (CD274) mRNA in plasma circulating RNA (ctRNA) in 45% of patients with mCRC [56], although its correlation with tumour molecular alterations was not defined. Our findings revealed lower CD274 expression in *RAS*/*BRAF*/*PIK3 CA* mutated patients. PD-L1 regulation is complex, involving multiple pathways such as *RAS*/RAF/MAPK and *PI3 K/PTEN/Akt/mTOR*. MicroRNAs like miR- 20b, miR- 21, and miR- 130b can upregulate PD-L1 by suppressing *PTEN* [57].

We observed that ADAR1 expression was associated with the number of bCTC, but only in patients with ≥3 bCTCs and wild-type *RAS*/*BRAF*/*PIK3 CA* tumours. ADAR1 has been implicated in CRC immunity via its A-to-I RNA editing activity [58]. In our study, deregulation of ADAR1 and CD274 suggests an association between CRC driver mutations, CTC burden, and immune-related factors.

Patients with ≥3 bCTCs did not have significantly different OS compared to those with <3 bCTCs. However, a high bCTC burden (≥ 13/7.5 mL) was linked to shorter OS. Several miRNAs, including the miR- 106a- 5p/miR- 26a- 5p ratio, were associated with poor prognosis. The miR- 106a- 5p/miR- 26a- 5p ratio emerged as the strongest miRNA predictor of OS (AUC =0.743, $p<0.01$) and an independent prognostic factor. Notably, a higher bCTC count correlated with a lower miR- 106a- 5p/miR- 26a- 5p ratio. Downregulation of miR- 106a in tumours has been linked to shorter survival in colon cancer [59]. miR- 106a, a member of the miR- 17–92 cluster, promotes CRC cell migration and invasion by suppressing TGF-β receptor- 2 [60]. Plasmatic miR- 106a was expressed in mCRC patients and was upregulated in patients who failed to respond to treatment with 5-FU and oxaliplatin [61]. In other studies, miR- 106a- 5p was included in a miRNA signature predictive of survival [62] and associated with liver metastasis and poor prognosis in CRC patients [63].

The role of miR- 26 in CRC remains unclear, with reports of both oncogenic and tumour-suppressive functions [64]. miR- 26a promotes oncogenesis by repressing *PTEN* [65] and is upregulated under hypoxia via *HIF- 1* [66]. In our study, high miR- 26a- 5p levels correlated with AP >300. Bioinformatic analysis showed patients with low tumour miR- 26a- 5p expression had better survival outcomes. miR- 106a- 5p and miR- 26a- 5p target genes are involved in *Hippo* [67] and *TGF-β* signalling [68]. This highlights their interaction and role in CRC metastasis..

A single microRNA can influence multiple cancer pathways, depending on the specific cellular and microenvironmental contexts [69]. Our results indicate that the integration of different miRNAs biomarkers in WB has the potential to improve prognostic stratification. In addition, miRNAs, including miR- 106a- 5p/miR- 26a- 5p ratio, may serve to select poor prognosis mCRC patients candidates for intensive first-line therapy.

Further studies investigating the potential of serial testing of blood miRNAs as a means to adjust therapy would also be of interest. Future studies could adopt a systematic approach to characterising non-coding RNA expression in WB. In a discovery cohort, high-throughput sequencing or microarray could be used to identify novel RNA expression patterns; then, in a validation cohort, the results could be confirmed using targeted qPCR. In parallel, other regulatory non-coding RNAs, such as circular RNAs, should also be analysed.

This study is exploratory, with limitations including the exclusion of patients over 70 years old and those with bCTC <3 and *RAS* mutations in the VISNÚ trials. Additionally, we analyzed a predefined RNA set without primary tumour transcriptomic data. Blood transcriptomic changes may not always reflect tumour changes [37], and our data do not clarify the cell or tissue origin of transcripts. Variability in sample types and analytical approaches (e.g., qPCR, RNA-seq, hybridization) complicates comparisons across studies.

The objective of this study was to enhance understanding of WB-RNA alterations associated with CTCs in mCRC. Our findings highlight the prognostic potential of WB-RNA analysis rather than the validation of specific biomarkers. With additional validation in larger cohorts, WB-RNA profiling has the potential to enhance prognostic evaluations and guide personalized treatment strategies for mCRC. Future research on WB-miRNAs should consider CRC subgroups and molecular differences, and comparisons with other liquid biopsy approaches such as cfDNA, exosomes, or tumour-educated platelets. In addition to this, the development of WB-RNA markers as candidate diagnostic or prognostic tools for CRC could benefit from machine learning approaches [70]. This would allow them to be integrated into algorithms alongside clinical and systemic features, as well as other immune biomarkers.

Supplementary Information

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Supplementary Material 1

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Authors' contributions
Conception/design: MV-A. Provision of study material or patients: all authors. Collection and/or assembly of data: all authors. Data analysis and interpretation: MV-A, MT-F, and AR-A. Drafting the work or revising it critically for important intellectual content: all authors. Final approval of manuscript: all authors.

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The funder of the study, Roche Farma SA had no role in study design, data collection, data analysis, or data interpretation. Data were collected and monitored by Pivotal.

Data availability
Data are available on reasonable request. The study data (ie, deidentified participant data, and datasets analysed for the present manuscript) are available from the corresponding author, MV-A (email: mvalaye@icloud.com), on reasonable request and subject to approval from the lead investigators. Additional, related documents will also be available (study protocol, statistical analysis plan, informed consent form).

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with Good Clinical Practice guidelines, and the Declaration of Helsinki and its subsequent amendments. The study was approved by the Research Ethics Committee CEIC Hospital Clínico San Carlos (Madrid), which served as the Institutional Review Board for all participating centres. The study protocol was also approved by the Spanish Agency of Medicines and Medical Devices.

Consent for publication
Not required.

Competing interests
MV-A has received grants and personal fees from Roche, personal fees from Merck, Amgen, Sanofi, Servier, Celgene, MSD and Bayer and honoraria for speaker/consulting roles from Amgen, Bayer, Merck, MSD, Roche, Sanofi and Servier. BGS has received travel grants from Amgen, Astra Zeneca, Merck, and Servier, and grants from Amgen, Sanofi, Gilead, Celgene, Bristol Myers Squibb, and Amgen. JS has done speaking honoraria for Ipsen, Lilly, Merck, MSD, Pfizer, Roche, Shire, and Servier, advisory roles for Amgen, Bayer, Bristol-Myers Squibb, Celgene, Ipsen, Merck, Roche, Sanofi, and Servier, and received travelling and accommodation support from Ipsen and Merck. FR has received payment or honoraria for lectures, presentations, speakers' bureaus, manuscript writing or educational events fromRoche, Merck-Serono, AMGEN, Sanofi-Aventis, Servier, MSD, and Support for attending meetings and/or travel from Roche, MSD, and Servier. PG-A has received payment as speaker' bureau for Amgen, Roche, Merck-Serono, MSD, Sanofi-Aventis, Pierre Fabre and Servier and received travel, accommodations and expenses from Amgen, Roche, Merck-Serono, Sanofi-Aventis, MSD, Pierre Fabre and Servier and payments from advisory activities from Amgen, Roche, Merck-Serono, MSD, Sanofi-Aventis, Pierre Fabre and Servier. RLL has stock and other ownership interests in MTrap and Nasasbiotech SL, and received consulting or advisory payments for Bayer, Bristol-Myers Squibb, Janssen, Lilly, MSD, Roche, and Novartis, received institutional research funding from Lilly, Merck, and Roche, and r travel, accommodation, and expenses from Pierre Fabre, Roche and Tesaro. CG-P has received travel, accommodation, and expenses from Sanofi/Aventis. ED-R has done consulting or advisory roles for Amgen, Bayer, Genomica, Merck Serono and Servier, speakers' bureaus for MSD and Servier, and received research funding from Amgen, AstraZeneca, Merck Serono, Roche and Sysmex. EA has received honoraria for advisory role from Amgen, Bayer, Sanofi, Incyte and Pierre Fabre. MT-F, PJ-F, GP-C, SG, AS, MS, AR-A, JMV declare no potential conflicts of interest.

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