Article

Potential utility of a panel of four-miRNAs as biomarkers in the clinical management of obese and non-obese Colorectal Cancer patients

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Simple Summary: Colorectal cancer (CRC) is one of the main tumor pathologies in our society due to its incidence and mortality. Various authors have linked the development of CRC with overweight and obesity. However, no molecular markers have been defined to connect both pathologies and that can be assessed in serum for diagnostic and/or prognostic purposes. The main objective of this work is to analyze and correlate the expression levels of a panel of four miRNAs previously associated with cancer and/or obesity in obese and non-obese patients affected by CRC, as well as in a control group without cancer. The main novelty of this study consists in the variety of samples investigated: adipose tissues, omental and subcutaneous; serum; and tumor and non-tumor tissues in the case of CRC patients. Results from this work allow us to conclude about the utility mainly of the miR-181a-5p in the clinical management of CRC.

Abstract: This work aims to investigate the expression levels of four preselected miRNAs previously linked to Cancer and/or Obesity, with the purpose of finding potential biomarkers in the clinical management of Colorectal Cancer (CRC) developed by obese and non-obese patients. We analyzed samples from a total of 65 subjects, 43 affected by CRC and 22 without cancer. Serum and both subcutaneous and omental adipose tissues (SAT and OAT) were analyzed, as well as tumor and non-tumor colorectal tissues in the case of the CRC patients. The relative expression ($2^{-\Delta\Delta Ct}$) levels of 4 miRNAs (miR-181a-5p, miR-143-3p, miR-132-3p and miR-23a-3p) were measured by RT-qPCR. Serum, SAT and OAT expression levels of these miRNAs showed significant differences between subjects with and without CRC, especially in the group of overweight/obese subjects. In CRC serum levels of miR-181a-5p, miR-143-3p and miR-23a-3p correlated with their levels in both SAT and OAT. Moreover, in the case of miR-181a-5p, these correlations were significantly influenced by the body mass index (BMI). From these data, we can conclude that both adiposity and CRC induce changes in the expression of the miRNAs investigated, demonstrating the potential utility of this panel of miRNAs in the diagnosis and prognosis of CRC patients.

Keywords: colorectal cancer; adipose tissue; miRNAs; serum biomarkers; obesity

1. Introduction

Colorectal cancer (CRC) is still nowadays the third most common malignancy and the second cause of cancer-related mortality worldwide (1). Primary tumor location conditions the molecular

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characteristics and the prognosis of CRC, being the most notable differences in right-colon cancers with respect to left-colon and rectal cancers. CRC development is believed to be a multi-step process involving both genetic and environmental factors, several related to lifestyle (2). Obesity is a known risk factor for CRC and is related to worse clinical outcomes, although the relationship between both diseases is yet not fully understood. Moreover, subcutaneous and visceral adiposity seem to affect differently to the tumor process (3).

MicroRNAs (miRNAs) are small single-stranded noncoding RNAs that are involved in gene expression regulation mechanisms, mainly at the translational level. Since their discovery, miRNAs have been related to several processes including cell proliferation or apoptosis, and their dysregulation has been linked to different types of cancer including CRC (4). Also, miRNAs are secreted to blood in exosomes or bound to lipoproteins, where they are particularly stable and resistant to ribonuclease degradation. Thus, circulating miRNAs detectable in serum or plasma are easily accessible potential biomarkers for CRC detection and the prediction of CRC prognosis (5). Furthermore, recent studies relate adipogenesis and obesity with altered miRNA levels. However, the role of the miRNAs as a possible link between obesity and cancer needs further investigation (6).

Herein we present a study in which the main objective is to evaluate a panel of four miRNAs, miR-181a-5p, miR-143-3p, miR-132-3p and miR-23a-3p, which have been previously related to obesity and/or to CRC. Particularly, miR-181a-5p was reported to target the tumor suppressor WIF-1 and has been linked to tumor growth, liver metastasis and poorer overall survival in CRC (7,8), and was proven to promote angiogenesis in both *in vitro* and *in vivo* assays by indirectly activating the SRC/VEGF pathway (9). Similarly, miR-23a-3p seems to enhance tumor and vascular growth in CRC via the STAT3/miR-23a-3p/SEMA6D axis induced by IL-17C (10). However, both miRNAs were found to be downregulated in adipose tissues from obese patients with respect to non-obese patients and reduced the TNFα-induced insulin resistance (11). On the other hand, miR-143-3p and miR-132-2p have been related to tumor suppression. As part of the miR-143/145 cluster, miR-143-3p is significantly decreased in CRC tissues and has oncostatic functions (12), as it prevents tumor growth via the inhibition of the KRAS oncogene translation (13), and it targets ITGA6 and ASAP3 inhibiting metastasis (14). The miR-132-3p inhibits the expression of ZEB2, a regulator of the epithelial–mesenchymal (EMT) transition, and its downregulation correlates with tumor growth, invasion and metastasis (15). With respect to obesity, miR-143-3p was positively correlated to insuline-mediated lipogenesis in subcutaneous adipose tissue (16) and induced insuline resistance in mouse models by inhibiting the AKT pathway (17).

Our work aims to analyze the expression levels of the four chosen miRNAs in serum and tissues from obese and non-obese CRC subjects, as well as in a control group without cancer, with the purpose of assessing their potential as biomarkers in CRC. To our knowledge, this is the first work that jointly evaluates the effect of both diseases, CRC and obesity, in the expression of selected miRNAs in human samples.

2. Materials and Methods

2.1. Patients and samples

The population for the study included 43 colorectal cancer (CRC) patients who underwent a cancer-related surgery between 2021 and 2022, as well as 22 subjects without cancer (control group) who underwent other surgeries not related to this disease. In both cases, patients were operated in the San Carlos Hospital (Madrid, Spain). Written informed consent was obtained from patients prior to investigation. In addition, written approval to develop this study was obtained from the Clinical Research Ethics Committee of the San Carlos Hospital (C.I. 19/549-E_BC, 27/12/2019), assuring the confidentiality of data to patients.

Age, gender and BMI values from the total of subjects considered, as well as CRC features (tumor location and TNM stage) are shown in Table 1.

Patients were classified according to their BMI values, following the criteria of the World Health Organization (WHO). Patients with BMI ≤ 24.9 Kg/m^2 were defined as normal weight (11 cases, 8

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CRC patients and 3 controls); patients with BMI ≥ 25 kg/m² and ≤ 29.9 kg/m², as overweight (25 cases, 23 with CRC and 2 controls); and the ones with BMI ≥ 30 kg/m² were considered as with obesity (29 cases, 12 CRC patients and 17 controls). Recruitment was done with independence from gender, age of the patient or tumor stage, in the case of CRC subjects. No CRC patient had received chemo or radiotherapy before the surgery and inclusion in the study, and exclusion criteria included a previous digestive surgery, inflammatory diseases and antibiotic treatment one month before the surgical intervention.

Table 1. Clinico-pathological features of subjects with and without colorectal cancer.

Variable	CRC* group (N = 43 patients)	Control group (N = 22 patients)
Mean age (years) ± standard error	71.65 ± 1.91	55.50 ± 2.87
Gender, N (%)		
Male	29 (67.44)	8 (36.36)
Female	14 (32.56)	14 (63.64)
BMI≠ group, N (value, mean ± standard error)		
Normal weight (BMI≠ ≤ 24,9 Kg/m²)	8 (23.02 ± 0.62)	3 (23.55 ± 0.53)
Overweight (BMI≠ ≥ 25 kg/m² and ≤ 29.9 kg/m²)	23 (27.15 ± 0.30)	2 (25.90 ± 0.90)
Obesity (BMI≠ ≥ 30 kg/m²)	12 (31.96 ± 0.46)	17 (39.30 ± 1.67)
Tumor location, N (%)		
Right colon	23 (53.49)	----------
Left colon	13 (30.23)	----------
Rectum	7 (16.28)	----------
TNM stage, N (%)		
I	6 (13.95)	----------
II	16 (37.21)	----------
III	18 (41.86)	----------
IV	3 (6.98)	----------

*Colorectal Cancer; ≠Body Mass Index.

A total number of 195 samples of serum and paired abdominal subcutaneous and omental adipose tissue (SAT and OAT, respectively) were collected prospectively from both groups of patients included in the study. Moreover, in the case of CRC subjects, also tumor and paired non-tumor colorectal tissue samples were obtained. For serum samples, blood was collected the day of the surgery after an overnight fast and centrifuged within 10 minutes to 1 hour after the extraction (10 minutes and 1300xg of speed). Serum was then separated and stored at -80ºC until analysis. Adipose tissue samples were collected during the surgery and immediately submerged in 1mL of RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany), stored 24 hours at 4ºC and subsequently frozen to -80ºC until processing. Finally, all colorectal tissues from CRC patients were provided by the San Carlos Hospital Biobank (B.0000725) via the project PT2020/00074, subsidized by the Carlos III Institute of Health (ISCIII) and co-funded by the European Union through the European Regional Development Fund (ERDF). The said Biobank belongs to the San Carlos Health Research Institute (IdISSC) and is part of the national network of Biobanks. After their extraction

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during surgery, colorectal tissue samples were instantly embedded in Tissue-Tek OCT and frozen in liquid nitrogen at -80ºC. Tumor samples were cryostat sectioned, H&E stained and examined microscopically by two independent pathologists to confirm the presence of ≥ 80% tumor cells. CRC staging was done following the NCCN guidelines (National Comprehensive Cancer Network) v 2.2022. Paired normal tissues from the same patient were also obtained and confirmed microscopically.

2.2. RNA extraction and microRNA (miRNA) expression analysis

Total RNA in serum samples was extracted using the miRNeasy Serum/ Plasma Advanced Kit (Qiagen), following the manufacturer's protocol, from a starting volume of 200µL per sample and eluting in 20µL of RNase Free Water (RFW). RNA recovery was increased by adding 1 µg of MS2 carrier RNA (Roche) to the sample before the extraction. Adipose tissues were cut and washed in 1mL PBS 1x to remove the RNA Later, and briefly homogenized in 700µL of QIAzol™ Lysis Reagent (Qiagen) using the Ultra-Turrax™ homogenizer. Next, total RNA extraction was performed using the miRNeasy Mini Kit (Qiagen), according to the manufacturer's instructions and with a final elution volume of 30µL of RFW. To optimize the extraction, 5PRIME Phase Lock Gel (PLG) Heavy tubes (VWR International Eurolab, Barcelona, Spain) were used during the phase separation step. OCT from colorectal tissue sections was also removed by washing with 1mL PBS 1x and centrifuging at 3000 rpm during 5-10 minutes at room temperature, prior to homogenization in 700µL of QIAzol™ Lysis Reagent and RNA extraction following the same protocol as for adipose tissues.

After the extraction, a reverse transcription reaction was performed in all the samples to convert total RNA into cDNA, by using the miRCURY® LNA® RT Kit (Qiagen) for a final reaction volume of 10µL per sample. Following the kit's protocol, 10ng of template RNA from each tissue sample were used for the reaction. For serum samples, 2µL of the extracted RNA were used directly. The incubation was performed in the Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Madrid, Spain). Afterwards, cDNA was stored at -20ºC until use.

miRNA expression was determined by real-time quantitative PCR (qPCR), using the miRCURY LNA SYBR® Green PCR Kit (Qiagen) as well as an individual miRCURY LNA miRNA PCR Assay (Qiagen) for each of the four miRNAs analyzed (miR-181a-5p, miR-143-3p, miR-132-3p and miR-23a-3p) and for miR-103-3p, used as an internal control. Reactions were performed in 0.1 ml MicroAmp® Fast Optical 96-Well Reaction Plates. Prior to the PCR reaction, cDNA was diluted to 1:60 (for tissue samples) or to 1:30 (for serum samples). Reaction setup was performed for a 10µL/well reaction, and ROX reference dye was added as a 20x concentrate (0.5µL per well) as manufacturer recommended in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The relative expression (RQ) calculation was done following the $2^{-\Delta\Delta Ct}$ method.

2.3. Statistical analysis

Statistical analyses were performed using the IBM® SPSS® Statistics software package version 27 (IBM Inc.). The normality of the data was assessed using the Shapiro-Wilk ($n < 50$) or the Kolmogorov–Smirnov ($n \geq 50$) tests, and the Levene's test for equality of variances was used to analyze the homoscedasticity conditions of the variables. Correlations between quantitative variables were done with Pearson (parametric variables) and Spearman (non-parametric variables) tests. To compare the means of two related variables, the Wilcoxon signed-rank test was performed. Finally, the mean values of the quantitative data between two or more study groups were compared using whether parametric tests (Student's T test for 2 categories and ANOVA for 3 or more categories) or non-parametric tests (Mann-Whitney U test for 2 categories and Kruskal-Wallis test for 3 or more categories). In any case, p values < 0.05 were considered as statistically significant.

3. Results

3.1. Differences in miRNA expression in serum and adipose tissues between subjects with and without CRC. Relationship to the BMI values.

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Table 2 shows the differences in mean serum and both SAT and OAT levels of the four miRNAs analyzed (miR-181a-5p, miR-143-3p, miR-132-3p and miR-23a-3p) between CRC patients and control subjects. As it can be observed, most of the adipose tissue expressions were higher in the control group with respect to the CRC cases. Particularly, OAT expression was significantly diminished in patients with CRC for three of the four miRNAs studied (miR-181a-5p, $P < 0.001$, miR-143-3p, $P = 0.031$, and miR-23a-3p, $P < 0.001$), whereas SAT expression was higher in controls for miR-132-3p ($P = 0.005$) and miR-23a-3p ($P = 0.044$).

Table 2. Mean relative miRNA expression in colorectal cancer and control subjects.

	Mean relative miRNA expression ($2^{-\Delta\Delta Ct}$) ± standard error		
miRNA samples	**CRC**[3]	**Controls**	**P**[4]
miR-181a-5p			
Serum	0.23 ± 0.059	0.25 ± 0.037	0.128
SAT [1]	0.66 ± 0.073	0.84 ± 0.164	0.365
OAT [2]	0.63 ± 0.067	1.25 ± 0.232	< 0.001
miR-143-3p			
Serum	0.04 ± 0.009	0.02 ± 0.003	0.380
SAT [1]	0.29 ± 0.025	0.39 ± 0.110	0.840
OAT [2]	0.24 ± 0.019	0.46 ± 0.105	0.031
miR-132-3p			
Serum	0.18 ± 0.031	0.18 ± 0.078	0.482
SAT [1]	0.18 ± 0.018	0.44 ± 0.139	0.005
OAT [2]	0.22 ± 0.023	0.49 ± 0.165	0.119
miR-23a-3p			
Serum	0.74 ± 0.126	1.21 ± 0.453	0.242
SAT [1]	0.75 ± 0.083	1.99 ± 0.824	0.044
OAT [2]	0.54 ± 0.057	1.42 ± 0.353	< 0.001

[1]Subcutaneous Adipose Tissue; [2]Omental Adipose Tissue; [3]Colorectal Cancer; [4]Mann-Whitney U test.

In Table 3, we show the mean miRNA expression levels in relation to BMI of subjects. Thus, regarding the groups of normal weight (BMI ≤ 24,9 kg/m^2) and overweight/obese (BMI ≥ 25 kg/m^2), the main differences were found between CRC and controls from the overweight/obese group. Specifically, the four miRNA analyzed in this work were significantly higher in OAT from subjects without cancer showing BMI values ≥ 25 kg/m^2. Also, miR-132-3p SAT expression was still significantly diminished in the overweight/obese CRC group ($P = 0.008$). However, in the group of normal weight, the differences in miRNA expression levels were not as evident and did not follow a specific profile. Moreover, significantly higher levels in serum were only observed for miR-181a-5p and miR-23a-3p in the normal weight group without cancer ($P = 0.008$ and $P = 0.034$, respectively).

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Table 3. Mean relative miRNA expression comparison between colorectal cancer patients and controls according to the Body Mass Index.

	Mean relative miRNA expression ($2^{-\Delta\Delta Ct}$) ± standard error					
	Normal weight		P[4,5]	Overweight/obese		P[4,5]
miRNA samples	**CRC**[3]	**Control**		**CRC**[3]	**Control**	
miR-181a-5p						
Serum	0.08 ± 0.028	0.29 ± 0.041	0.008[4]	0.26 ± 0.068	0.24 ± 0.046	0.677[5]
SAT[1]	0.63 ± 0.078	0.70 ± 0.097	0.602[4]	0.67 ± 0.087	0.87 ± 0.197	0.403[5]
OAT[2]	0.73 ± 0.161	0.86 ± 0.157	0.640[4]	0.61 ± 0.075	1.33 ± 0.272	< 0.001[5]
miR-143-3p						
Serum	0.01 ± 0.004	0.03 ± 0.010	0.117[4]	0.04 ± 0.011	0.01 ± 0.002	0.071[5]
SAT[1]	0.25 ± 0.056	0.36 ± 0.102	0.340[4]	0.29 ± 0.028	0.40 ± 0.132	0.538[5]
OAT[2]	0.23 ± 0.029	0.30 ± 0.088	0.371[4]	0.25 ± 0.022	0.49 ± 0.122	0.032[5]
miR-132-3p						
Serum	0.08 ± 0.025	0.46 ± 0.361	0.289[5]	0.20 ± 0.036	0.10 ± 0.013	0.137[5]
SAT[1]	0.15 ± 0.031	0.22 ± 0.072	0.315[4]	0.18 ± 0.021	0.48 ± 0.164	0.008[5]
OAT[2]	0.21 ± 0.013	0.15 ± 0.025	0.054[4]	0.22 ± 0.028	0.55 ± 0.190	0.043[5]
miR-23a-3p						
Serum	0.26 ± 0.111	3.10 ± 1.921	0.034[5]	0.83 ± 0.141	0.70 ± 0.119	0.921[5]
SAT[1]	0.50 ± 0.171	1.09 ± 0.024	0.086[4]	0.80 ± 0.091	2.17 ± 0.987	0.187[5]
OAT[2]	0.42 ± 0.130	0.79 ± 0.075	0.141[4]	0.57 ± 0.063	1.54 ± 0.410	0.002[5]

[1]Subcutaneous Adipose Tissue; [2]Omental Adipose Tissue; [3]Colorectal Cancer; [4]Student's T test; [5]Mann-Whitney U test.

Only considering CRC patients, our data indicated remarkable differences in miRNA serum expression levels in relation to the BMI values (Table 4). More specifically, serum levels of the miRNAs studied in this work increased in overweight/obese CRC patients with respect to the normal weight group. These differences could be noticed in the four miRNAs analyzed, although they were only significant for miR-181a-5p (P = 0.045), and were borderline-significant for miR-143-3p (P = 0.054), miR 132-3p (P = 0.088), and miR-23a-3p (P = 0.054).

Table 4. Mean miRNA levels in serum of colorectal cancer patients according to their body mass index.

	Serum mean relative miRNA expression ($2^{-\Delta\Delta Ct}$) ± standard error		
miRNA	**Normal weight CRC[1] patients**	**Overweight/obese CRC[1] patients**	**P[2]**
miR-181a-5p	0.08 ± 0.028	0.26 ± 0.068	0.045
miR-143-3p	0.01 ± 0.004	0.04 ± 0.011	0.054
miR-132-3p	0.08 ± 0.025	0.20 ± 0.036	0.088
miR-23a-3p	0.26 ± 0.111	0.83 ± 0.141	0.054

[1]Colorectal cancer; [2]Mann-Whitney U test.

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Serum miRNA expression levels in subjects without cancer (control group) with different BMI did no report significant differences (Table 5), although a downward trend was observed for most of the miRNA included in the study. Therefore, in the control group, the trend was the opposite of that detected in the cases with CRC, with a decrease in miRNAs serum levels in the group of over-weight/obese patients without cancer.

Table 5. Mean miRNA levels in serum of control subjects according to their body mass index.

	Serum mean relative miRNA expression ($2^{-\Delta\Delta Ct}$) ± standard error		
miRNA	**Normal weight controls**	**Overweight/obese controls**	**P[1]**
miR-181a-5p	0.29 ± 0.041	0.24 ± 0.046	0.073
miR-143-3p	0.03 ± 0.010	0.01 ± 0.002	0.052
miR-132-3p	0.46 ± 0.361	0.10 ± 0.013	0.243
miR-23a-3p	3.10 ± 1.921	0.70 ± 0.119	0.073

[1]Mann-Whitney U test

3.2. Correlations between serum and adipose tissue miRNA expression in subjects with and without CRC.

Comparing serum and tissue miRNA levels in patients with CRC, there were significant positive correlations between miR-181a-5p, miR-143-3p and miR-23a-3p levels in serum and their levels in both SAT ($P < 0.001$ for the three miRNAs) and OAT ($P < 0.001$ for miR-181a-5p and miR-143-3p and $P = 0.005$ for miR-23a-3p), as shown in Figure 1.

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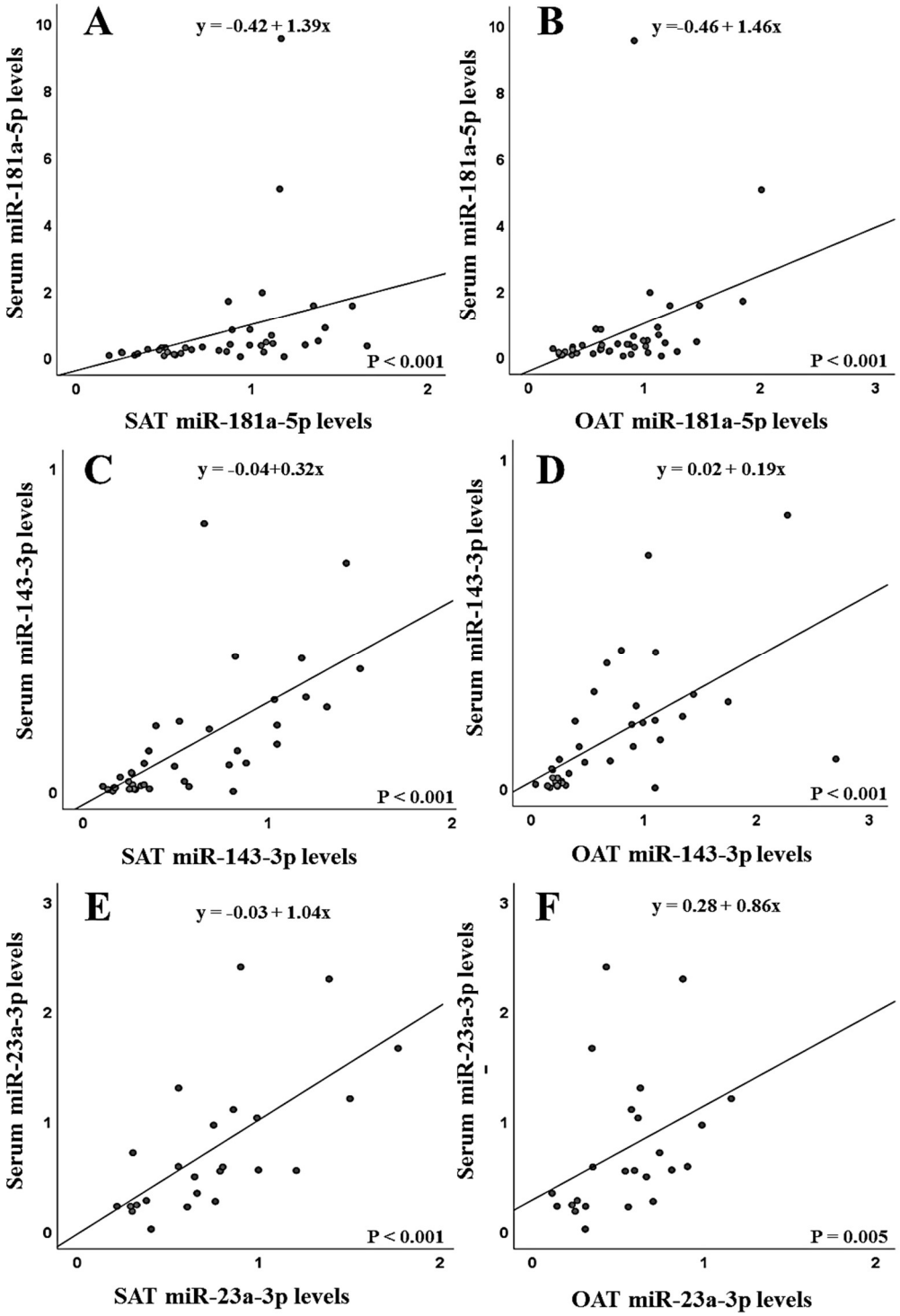

Figure 1. Correlations (Spearman test) between miRNA levels in serum and adipose tissues (Subcutaneous, SAT; and Omental, OAT) of Colorectal Cancer patients, for miR-181a-5p (**A, B**), miR-143-3p (**C, D**) and miR-23a-3p (**E, F**).

When patients were divided according to their BMI (Table 6), both serum-SAT and serum-OAT correlations kept in the three BMI groups for miR-143-3p (Serum-SAT correlation: P = 0.004 in normal weight patients, P < 0.001 in overweight patients and P = 0.023 in obese patients; Serum-OAT correlation: 0.028 in normal weight patients and P < 0.001 in overweight and obese patients), whereas for miR-181a-5p they disappeared in normal weight patients and were only significant in the overweight and obese groups (Serum-SAT correlation: P = 0.030 in overweight patients, P = 0.001 in obese

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patients; Serum-OAT correlation: P = 0.003 in overweight patients, P < 0.001 in obese patients). For miR-132-3p, serum-SAT and serum-OAT correlations were significant only in the normal weight group of patients (P = 0.047 and P = 0.045). Finally, for miR-23a-3p only a serum-SAT correlation in overweight patients was found (P = 0.008).

Table 6. Correlations between miRNA levels in serum and adipose tissues (subcutaneous and omental) of colorectal cancer patients (P values).

	Serum-adipose tissue correlations in miRNA levels							
	miR-181a-5p		**miR-143-3p**		**miR-132-3p**		**miR-23a-3p**	
BMI[1] group	**S[2]-SAT[3]**	**S[2]-OAT[4]**	**S[2]-SAT[3]**	**S[2]-OAT[4]**	**S[2]-SAT[3]**	**S[2]-OAT[4]**	**S[2]-SAT[3]**	**S[2]-OAT[4]**
Normal weight	0.102[5]	0.651[5]	0.004[5]	0.028[5]	0.047[5]	0.045[6]	0.218[6]	0.197[6]
Overweight	0.030[5]	0.003[5]	< 0.001[5]	< 0.001[5]	0.904[5]	0.671[5]	0.008[5]	0.364[5]
Obese	0.001[6]	< 0.001[6]	0.023[5]	< 0.001[5]	0.321[5]	0.262[5]	0.654[6]	0.150[6]

[1]Body Mass Index; [2]Serum; [3]Subcutaneous Adipose Tissue; [4]Omental Adipose Tissue; [5]Spearman's correlation test; [6]Pearson's correlation test.

Besides the correlation with serum, in CRC patients there was a positive correlation between the expression levels of both adipose tissues (SAT and OAT), which was significant for the 4 miRNAs analyzed (Figure 2, P < 0.001 for miR-181a-5p, miR-143-3p and miR-132-2p, P = 0.028 for miR-23a-3p). When patients were divided according to their BMI (Table 7), the SAT-OAT correlation kept in the 3 groups for miR-143-3p (P = 0.004 in normal weight, P < 0.001 in overweight and P = 0.023 in obese) and for miR-132-3p (P = 0.010 in normal weight, P < 0.001 in overweight and P = 0.007 in obese). For miR-181a-5p, SAT-OAT correlation disappeared in normal weight CRC patients and was significant only in overweight (P = 0.030) and obese (P = 0.002) patients.

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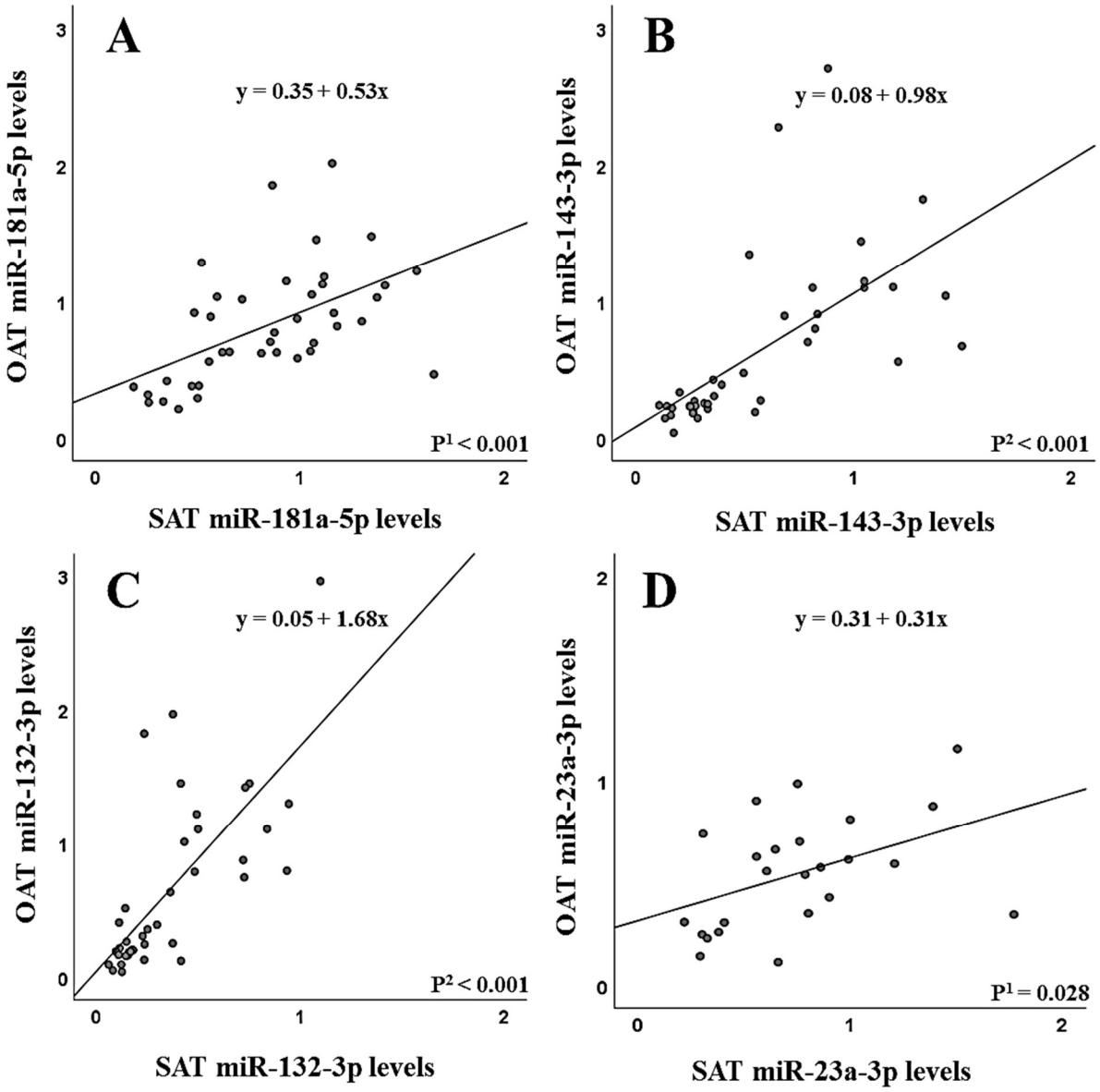

Figure 2. Correlations in miRNA levels between both adipose tissues (subcutaneous and omental) of colorectal cancer patients, for miR-181a-5p (A), miR-143-3p (B), miR-132-3p (C) and miR-23a-3p (D). [1]Pearson's correlation test; [2]Spearman's correlation test. .

Table 7. Correlations in miRNA levels between both adipose tissues (subcutaneous and omental) of colorectal cancer patients (P values).

	SAT[1]-OAT[2] correlations in miRNA expression			
BMI[3] group	**miR-181a-5p**	**miR-143-3p**	**miR-132-3p**	**miR-23a-3p**
Normal weight	0.090[4]	0.004[5]	0.010[5]	0.600[5]
Overweight	0.030[4]	< 0.001[5]	< 0.001[5]	0.298[4]
Obese	0.002[4]	0.023[5]	0.007[5]	0.344[4]

[1]Subcutaneous Adipose Tissue; [2]Omental Adipose Tissue; [3]Body Mass Index; [4]Pearson's correlation test; [5]Spearman's correlation test.

Interestingly, all the correlations indicated in this section for the group of patients affected by CRC were not observed in the control cases without cancer.

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3.3. miRNA expression levels in tumor and non-tumor tissues from CRC patients. Differences in relation to tumor location.

Relative expression levels of the studied miRNAs were compared between tumor and paired non-tumor tissues from CRC patients (Table 8). Levels of miR-143-3p, miR-132-3p and miR-23a-3p were significantly lower in colorectal tumor samples with respect to their matched non-tumor tissues ($P < 0.001$ for miR-143-3p, $P = 0.031$ for miR-132-2p and $P = 0.021$ for miR-23a-3p).

Table 8. Comparison of miRNA levels between tumor and paired non-tumor colorectal tissues of colorectal cancer patients.

	Mean relative miRNA expression ($2^{-\Delta\Delta Ct}$) ± standard error		
miRNA	**Tumor tissue**	**Non-tumor tissue**	**P[1]**
miR-181a-5p	1.02 ± 0.092	1.26 ± 0.137	0.278
miR-143-3p	0.59 ± 0.152	2.30 ± 0.456	< 0.001
miR-132-3p	0.97 ± 0.124	1.38 ± 0.164	0.031
miR-23a-3p	1.17 ± 0.221	1.46 ± 0.176	0.021

[1]Wilcoxon signed-rank test.

Comparison of the mean miRNA expression levels between samples from CRC patients with different primary tumor location revealed important differences, most of them found between right and left colon cancer patients (Table 9). More particularly, tumor/non tumor expression ratio for miR-181a-5p was significantly lower in right colon cancers compared to left colon cancers ($P = 0.002$). Interestingly, this was also found when right colon cancers were compared to rectal cancers (mean T/N ratio ± standard error: 0.58 ± 0.076 in right colon cancers and 1.02 ± 0.134 in rectal cancers, $P = 0.019$ in Students' T test). On the other hand, non-tumor tissue expression levels were higher in right colon cancers than in left colon cancers for miR-181a-5p, miR-132-3p and miR-23a-3p ($P = 0.044$, $P = 0.028$ and $P = 0.016$, respectively). There were also differences in adipose tissue levels of miR-23a-3p: both SAT and OAT expressions from right-sided CRC patients were significantly increased with respect to the ones from left-sided CRC patients ($P = 0.003$ and $P = 0.011$, respectively).

Table 9. Comparison of the mean miRNA levels between tumors from the right-colon and the left-colon.

	Mean relative miRNA expression ($2^{-\Delta\Delta Ct}$) ± standard error		
miRNA samples	**Tumors from the right colon**	**Tumors from the left colon**	**P[4,5]**
miR-181a-5p			
Tumor tissue	0.88 ± 0.136	1.30 ± 0.242	0.119[4]
Non-tumor tissue	1.61 ± 0.192	0.96 ± 0.210	0.044[4]
T/N[1]	0.58 ± 0.076	1.64 ± 0.331	0.002[5]
Serum	0.19 ± 0.034	0.16 ± 0.032	0.536[4]
SAT[2]	0.78 ± 0.124	0.53 ± 0.063	0.133[4]
OAT[3]	0.66 ± 0.097	0.58 ± 0.093	0.591[4]
miR-143-3p			
Tumor tissue	0.48 ± 0.244	0.67 ± 0.320	0.673[5]
Non-tumor tissue	2.51 ± 0.948	2.46 ± 1.118	0.800[5]

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T/N[1]	2.36 ± 2.233	2.46 ± 2.045	0.735[5]
Serum	0.05 ± 0.017	0.01 ± 0.002	0.105[5]
SAT[2]	0.33 ± 0.042	0.23 ± 0.029	0.100[4]
OAT[3]	0.27 ± 0.026	0.21 ± 0.026	0.161[4]
miR-132-3p			
Tumor tissue	0.98 ± 0.285	0.94 ± 0.277	0.800[5]
Non-tumor tissue	1.81 ± 0.350	0.79 ± 0.139	0.028[5]
T/N[1]	1.20 ± 0.749	1.57 ± 0.731	0.272[5]
Serum	0.21 ± 0.043	0.16 ± 0.071	0.247[5]
SAT[2]	0.22 ± 0.034	0.13 ± 0.008	0.083[5]
OAT[3]	0.26 ± 0.037	0.18 ± 0.038	0.133[4]
miR-23a-3p			
Tumor tissue	1.38 ± 0.387	0.83 ± 0.160	0.398[5]
Non-tumor tissue	1.85 ± 0.249	0.89 ± 0.189	0.016[4]
T/N[1]	0.87 ± 0.316	1.36 ± 0.578	0.205[5]
Serum	0.92 ± 0.175	0.47 ± 0.134	0.054[5]
SAT[2]	1.01 ± 0.127	0.44 ± 0.071	0.003[4]
OAT[3]	0.71 ± 0.079	0.38 ± 0.079	0.011[4]

[1]Tumor/Non-tumor tissue expression ratio; [2]Subcutaneous Adipose Tissue; [3]Omental Adipose Tissue; [4]Student's T test; [5]Mann-Whitney U test.

4. Discussion

In this study we analysed the expression levels of four miRNAs (miR-181a-5p, miR-143-3p, miR-132-3p and miR-23a-3p), previously related to cancer and/or obesity, in serum and tissues from obese and non-obese subjects with and without CRC. Our results show noticeable differences between subjects affected by CRC and the control group without cancer, as well as in relation to the BMI values of subjects and tumor location.

Comparing the relative miRNA expressions with relation to the cancer process, adipose tissues (SAT and OAT) from the subjects affected by CRC had diminished miRNA levels with respect to the ones from the control group, particularly in OAT. Most of the differences preserved when patients with BMI values ≥ 25 kg/m^2 (either overweight or obese) were considered. Other studies highlighted the role of different miRNAs in favouring different diseases, like cancer or metabolic processes, allowing the authors to conclude about the possible pathophysiological role of miRNAs. Thus, miR-132-3p levels were found to be decreased in serum and omental adipose tissue from obese patients with respect to non-obese individuals, and its presence in omental adipose tissue was negatively correlated to visceral fat area and macrophage infiltration (18,19). However, these authors did not showed results from CRC patients.

When the levels of the studied miRNAs were compared in CRC patients with relation to the BMI, serum levels of the four miRNAs were increased in patients with higher BMI values (overweight or obese) with respect to the group of normal weight. These differences were statistically significant in the case of miR-181a-5p, and bordered significance for the other three miRNAs studied (miR-143-3p, miR-132-2p and miR-23a-3p). As previously mentioned, miR-181a-5p has been involved in CRC mainly playing an oncogenic role (4). Its levels in the colon cancer cells have been related to a metabolic shift from oxidative to glycolytic (20), as well as to increased tumor growth and to both angiogenic and metastatic properties (7-9). Conversely, obesity has been linked to a reduction in the adipose tissue levels of this miRNA (11), so both diseases could be affecting miR-181a-5p expression in different directions. The miRNAs as an interplay between obesity and CRC are still poorly studied.

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Also, little is known about what happens to the circulating miRNA levels in the context of these two pathologies (21). Given the results shown in our study, the increment of miR-181a-5p levels in the serum of overweight and obese CRC patients probably reflects the joint deregulation that both adiposity and the cancer process cause on the expression of this molecule in the tissues, giving a clue to the molecular interactions between both conditions and their impact on cancer prognosis.

In CRC patients, circulating levels of miR-181a-5p, miR-143-3p and miR-23a-3p, detected in serum, were positively correlated to the levels detected in adipose tissues, both SAT and OAT. Thomou *et al*. found that adipose tissue disfunction resulted in a significant decrease in blood exosomal miRNAs, demonstrating a considerable contribution of this type of tissue to the pool of circulating miRNAs that can exert their effects in distant locations (22). In line with these findings, the miRNAs detected in the serum of the patients included in our study seem to be mainly secreted by the adipose tissue, and they may have an effect in the tumor microenvironment of CRC. Moreover, when our CRC patients were evaluated separately according to their BMI, some serum-adipose tissue correlations, particularly the ones for miR-181a-5p, were only present in patients with overweight and obesity, suggesting an influence of BMI on the relationship between both miRNA levels. On the other hand, the four miRNAs analyzed showed a positive correlation between their expressions in SAT and OAT from CRC patients, which were maintained in the three BMI groups for miR-143-3p and miR-132-3p but only in patients with BMI ≥ 25kg/m^2 in the case of miR-181a-5p. Although there is accumulating evidence supporting that subcutaneous and visceral adipose tissues have different metabolic and inflammatory characteristics and expression profiles (23,24), both fat depots show coordinated molecular adaptations to obesity and metabolic syndrome (25). That being said, it is possible that the expression of certain molecules such as miRNAs is connected between both adipose tissues and related to the cancer process. Also in CRC patients, tumor tissues showed significantly lower expressions of miR-143-3p, miR-132-3p and miR-23a-3p with respect to their paired non-tumor tissues. Cancer has been associated with a global defect in miRNA production, possibly due to genetic or epigenetic changes affecting the miRNA genes, or because of alterations in some components of the miRNA biogenesis pathway such as Drosha or Dicer (4,26). Moreover, matching the results shown in this study, miR-143-3p has previously been reported to be downregulated in tumor samples with respect to marginal normal mucosa of CRC patients (27), and miR-132-3p levels were found to be lower in CRC cell lines with respect to normal colonic cells, as well as in CRC tumor samples when compared to their paired normal tissue (15,28,29).

Finally, several studies have already mentioned differences in the miRNA expression profiles between CRCs with different primary tumor location (30-32), also showing a site-specific impact of these molecules on cancer survival (33). Our results indicated that right colon cancers had a lower tumor/non-tumor expression ratio for miR-181a-5p when compared to left colon and rectal cancers. Although tumor levels alone for this miRNA were not significantly different between cancer sites, non-tumor tissue levels were significantly higher in the right colon. Thus, a decreased T/N ratio reflects better the reduction of miR-181a-5p in cancer cells with respect to their original tissue, which could be linked to the distinctive molecular nature of the right-sided CRCs. Furthermore, in the case of miR-23a-3p, the levels in both SAT and OAT were increased in patients in whom the cancer was located in the right colon, with respect to the ones in individuals with left-colon cancer. Recent evidence supports a crosstalk between adipose tissue and cancer cells, through which cancer cells may induce an "activated" phenotype in the surrounding adipocytes that in turn secrete several molecules, including some miRNAs, capable of promoting tumor development and metastasis (34). Stromal cells from the visceral adipose tissue neighbouring colorectal tumors were proved to promote vasculogenesis and metastasis in obese CRC patients (35). Moreover, miR-23a/b can be transferred through exosomes from adipocytes to hepatocellular carcinoma cells, where they increase cancer growth and chemoresistance (36). In this context and given the oncogenic role of miR-23a-3p in CRC (10), increased adipose tissue levels of this miRNA in right colon cancers could be indirectly related to the cancer characteristics and even drug resistance.

5. Conclusions

Tissue expressions of the four miRNAs investigated in this work appear to be deregulated in CRC. Indeed, decreased levels were found in adipose tissues of patients affected by CRC, when compared to the control group, mainly in OAT from CRC patients showing BMI values ≥ 25 Kg/m^2. Moreover, expression levels of miR-143-3p, miR-132-3p and miR-23a-3p were significantly lower in tumor samples with respect to their matched non-tumor tissues, which corroborates the deregulation of these markers in CRC.

Of particular interest are the data obtained from miR-181a-5p analyses. In fact, this miRNA was significantly increased in serum from the overweight/obese CRC patients, showing a significant correlation among serum and OAT levels only in that group of cases. Therefore, miR-181a-5p could be considered as a biomarker in patients with BMI ≥ 25 Kg/m^2 affected by CRC. In addition, this miRNA emerges as a tumor location marker.

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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