RESEARCH ARTICLE

MicroRNAs expression in normal and malignant colon tissues as biomarkers of colorectal cancer and in response to pomegranate extracts consumption: Critical issues to discern between modulatory effects and potential artefacts

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Scope: MicroRNAs (miRs) are proposed as colorectal cancer (CRC) biomarkers. Pomegranate ellagic acid and their microbiota metabolites urolithins exert anticancer effects in preclinical CRC models, and target normal and malignant colon tissues in CRC patients. Herein, we investigated whether the intake of pomegranate extract (PE) modified miRs expression in surgical colon tissues versus biopsies from CRC patients.

Methods and results: We conducted a randomized, double-blind, controlled trial. Thirty-five CRC patients consumed 900 mg PE daily before surgery. Control CRC patients (no PE intake, $n = 10$) were included. Our results revealed: (1) significant differences for specific miRs between malignant and normal tissues modifiable by the surgical protocols; (2) opposed trends between -5p and -3p isomolecules; (3) general induction of miRs attributable to the surgery; (4) moderate modulation of various miRs following the PE intake, and (5) no association between tissue urolithins and the observed miRs changes.

Conclusion: PE consumption appears to affect specific colon tissue miRs but surgery critically alters miRs levels hindering the discrimination of significant changes caused by dietary factors and the establishment of genuine differences between malignant and normal tissues as biomarkers. The components responsible for the PE effects and the clinical relevance of these observations deserve further research.

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Abbreviations: **BRB**, black raspberry; **CRC**, colorectal cancer; **EA**, ellagic acid; **FAP**, familial adenomatous polyposis; **Mb**, malignant tissue from biopsy; **miR**, microRNA; **Ms**, malignant tissue from surgery; **Nb**, normal tissue from biopsy; **Ns**, normal tissue from surgery; **NVR**, normal values range; **PE**, pomegranate extract

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1 Introduction

Colorectal cancer (CRC) is a complex disease caused by the interaction of genetic and environmental factors. Around 85% of cases develop through the sporadic adenoma-carcinoma pathway in which the normal colonic mucosa is transformed into invasive cancer [1]. The slow progression from premalignant lesions and the implementation of mass screening programs is reducing the burden of this cancer by detecting early stages of the disease [2]. Nevertheless, both prevalence and mortality are high since this malignancy remains the third most common cancer in men (10% of the total) and the second in women (around 9% of the total) worldwide [2]. Consequently, an important current area of research focuses on the elucidation of the molecular events occurring during carcinogenesis with the aim of establishing new biomarkers that would improve tumor diagnosis, therapy, and survival [1]. A number of proteins, DNA mutations, mRNAs, and microRNAs (miRs), metabolites, volatile organic compounds, inflammatory mediators, and gut microbiota dysbiosis determined in various types of samples (blood, urine, feces, and tissues) have all been proposed as potential biomarkers for cancer and could address a wide range of clinical needs [3]. It is worth noting the increasing number of studies looking at the specific role of miRs in CRC [4]. miRs are small, evolutionary conserved, noncoding RNAs that negatively regulate the expression of protein-coding genes at the posttranscriptional level by degrading target mRNAs and/or inhibiting their translation into proteins. miRs contribute to the regulation of many cell processes including differentiation, proliferation, apoptosis, inflammation, and stress response, and have been reported to be involved in different hallmarks of cell malignancy either suppressing or promoting the expression of target genes implicated in cancer [5].

Physical inactivity, cigarette smoking, alcohol abuse, obesity, and unbalanced diets are well-known CRC risk factors. Among other recommendations, the implementation of a healthy diet is considered a key element of comprehensive primary prevention strategies [1]. Concerning this, adherence to a Mediterranean diet has been reported to be correlated with a significant reduction in the risk of CRC by 14% [6], and diets rich in fruits, vegetables, nuts, fish, fiber, and brown rice have been associated with a lower risk of colorectal polyps and CRC [7]. Nevertheless, the identification of specific dietary compounds or derived molecules as well as the mechanisms responsible for their cancer preventive effects remains elusive. Dietary polyphenols, abundant in fruits and vegetables, have been acknowledged with a wide range of colon anticancer effects both in cellular and animal studies, however, the current evidence that supports their role as CRC chemopreventive compounds in humans is still very poor [8] neither it is known the specific molecular targets affected by these compounds within the human body. Among other potential mechanisms of action, polyphenols have been suggested to target the epigenome and modulate miRs [9] although this is supported mostly by in vitro and animal studies. In humans, only one study has shown the decrease of methylated transcription start sites for some miRs in adenomas from patients with familial adenomatous polyposis (FAP) following the application of rectal suppositories containing freeze-dried black raspberry powder rich in anthocyanins and ellagic acid (EA). No further effect was observed by additional oral intake of the extract [10]. To the best of our knowledge, there are no studies looking at the effect of dietary polyphenols on miRs expression in CRC patients [8].

Pomegranate, its main polyphenolic compounds (EA and ellagitannins) as well as their derived gut microbiota metabolites (urolithins) have all been reported to exert protecting effects in CRC cell lines and in animal models of colon cancer [8] but the molecular mechanisms are not yet known. At present, only one clinical trial has been published where the metabolic profiling of pomegranate polyphenols and urolithins has been described in CRC patients evidencing that EA, various EA conjugates as well as gallic acid and up to 12 different urolithin metabolites can be found in colon normal and malignant tissues following the intake of pomegranate extracts [11]. We hypothesize that, in humans, the ellagitannin and EA derived conjugates and urolithins accumulated in the colon tissues may contribute to protect against CRC by interfering with the molecular events taking place in these tissues, more specifically, with miRs known to be critically involved in the regulation of the cancerous process. The aim of this study was thus to investigate whether the daily consumption of pomegranate extracts containing ellagitannins and EA causes distinct and significant changes in the levels of various miRs both in normal and malignant colonic tissues from CRC patients and, whether we could find an association between the observed changes and the presence of pomegranate metabolites in the colon mucosa from these patients. We further examined changes in these miRs attributable to the surgical protocol and how these changes can interfere with the modulation of miRs expression levels by dietary factors and with the use of differential expression for specific miRs as potential biomarkers for CRC.

2 Materials and methods

2.1 Pomegranate extracts

Pomegranate extracts (PE-1 and PE-2) were kindly supplied by Laboratorios Admira S.L. (Alcantarilla, Murcia, Spain). Extracts differed in their punicalagin/EA ratio and were processed and analyzed as described previously [11]. Briefly, PE-1 contained 2 mg/g punicalin, 72 mg/g punicalagin, and 294 mg/g EA derivatives, whereas PE-2 contained 5.4 mg/g punicalin, 155 mg/g punicalagin, and 28 mg/g EA derivatives.

2.2 Study design and intervention

The results presented here were obtained from the ongoing trial "*Pomegranate Extract Supplementation in Colorectal Cancer Patients (POMEcolon)*," which was registered at clinicaltrials.gov as NCT01916239. Trial protocol (reference 03/2011) was approved by the Clinical Ethics Committee at Reina Sofia University Hospital (Murcia, Spain) and by the Spanish National Research Council's Bioethics Committee (Madrid, Spain). The trial was included in the Spanish National Research Project AGL2011-22447 and conformed to ethical guidelines outlined in the Declaration of Helsinki and its amendments. The tissue samples analyzed in the present study were obtained from October 2013 to March 2015. Figure 1 shows the design of the study. Inclusion criteria were as follows: patients with age over 18 years, confirmed CRC diagnosis with resectable tumor and programmed surgery, WHO performance status between 0 and 3, hemoglobin >10 g/dL, ALT >2.5-fold above the normal values range (NVR), serum bilirubin >1.5-fold above NVR and creatinine <140 mM. Exclusion criteria were: patients who did not satisfy inclusion criteria and (or), treatment with chemotherapy or radiotherapy one month prior to recruitment, active peptic ulcer, pregnancy/breastfeeding, alcoholism, treatment with steroids or other anti-inflammatory drugs one week prior to recruitment, habitual intake of food supplements (herbal preparations, nutraceuticals, etc.).

Patients gave their written informed consent prior to participate. From 2501 eligible patients, 57 patients with diagnosed CRC and tumors programmed for surgical resection were enrolled. Two samples from normal (Nb) and four samples from malignant (Mb) endoscopic colon biopsies (Fig. 1) were rapidly taken into RNAlater stabilization reagent (Qiagen, Madrid, Spain) and stored at –80°C until use. After colonoscopy, patients were randomly allocated into three groups, i.e. control group ($n = 14$) and patients that consumed pomegranate extract (PE) (PE-1, $n = 22$ or PE-2, $n = 21$). Patients from both PE groups consumed two hard plant-based capsules containing 450 mg of either PE-1 or PE-2 daily until surgery and returned the remaining capsules the day before surgery. The last dose was consumed approximately 10–12 h before surgery. Patients were instructed to annotate possible incidences or adverse effects (digestive discomforts, constipation, diarrhea, allergic reactions, etc.). Forty-five patients completed the trial (ten from control group, 19 from PE-1, and 16 from PE-2) (Fig. 1). At surgery, malignant (Ms) and normal (Ns, ~10 cm adjacent to the tumor) tissues were resected halfway during the intervention (approximately 2 h after initiating the surgical procedure). Colon tissues were rapidly transferred to the Anatomical Pathology Service for their examination and classification. Portions from both types of tissue were cut out, immersed into RNA later stabilization reagent, and stored at –80°C until use.

2.3 RNA extraction and processing

Colon tissue samples (0.02 g obtained from a pool of different pieces of each sample) were homogenized with lysis buffer (Qiagen) using an IKA T10 Ultra-Turrax equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24 000 rpm at 4°C for 1 min. Total RNA, including miRs, was isolated using an RNeasyPlus mini kit (Qiagen) following the manufacturer's recommendations and quantified in a NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was checked using the Nanodrop and the Bioanalyzer 2100 expert (Agilent). Only samples with a ratio Abs_{260}/Abs_{280} between 1.8 and 2.1 and RNA integrity number values above 6.0 that indicate acceptable RNA integrity for RT-PCR assays [12] were used for miR expression analysis.

2.4 miRs analyses

RNA was reverse-transcribed using the universal cDNA synthesis kit II (Exiqon) and miRs were quantified by quantitative real-time PCR (qRT-PCR) using the Exilent SYBR Green master mix (Exiqon) on a 790HT fast RT-PCR system (Applied Biosystems, Madrid, Spain). For the initial screening we used the miRNome miR ready-to-use PCR panels that contain 752 mature miRs and their controls (Exiqon) following the manufacturer's instruction. Human miRNome analysis was assessed in a subgroup of five patients. SNORD38B, SNORD49, and/or U6 RNAs were used for normalization. Initial data analysis was assessed using the Exiqon GenEx qPCR analysis software (MultiD Analyses) following the manufacturer´s protocols. For individual miR quantification Specific LNA-primers for each miR were used (Exiqon). All assays for a particular miR were performed at the same time under identical conditions and in duplicate. Relative expression was calculated using the comparative C_t method and presented as relative expression ratio ($2^{-\Delta\Delta Ct}$). Fold-change values ≥1.2 and ≤1.2 were considered indicative of upregulation and downregulation, respectively.

2.5 In silico analysis of potential target genes

Validated target genes of selected miRs were obtained using miRWalk [13] and miRTarBase [14] databases. In silico

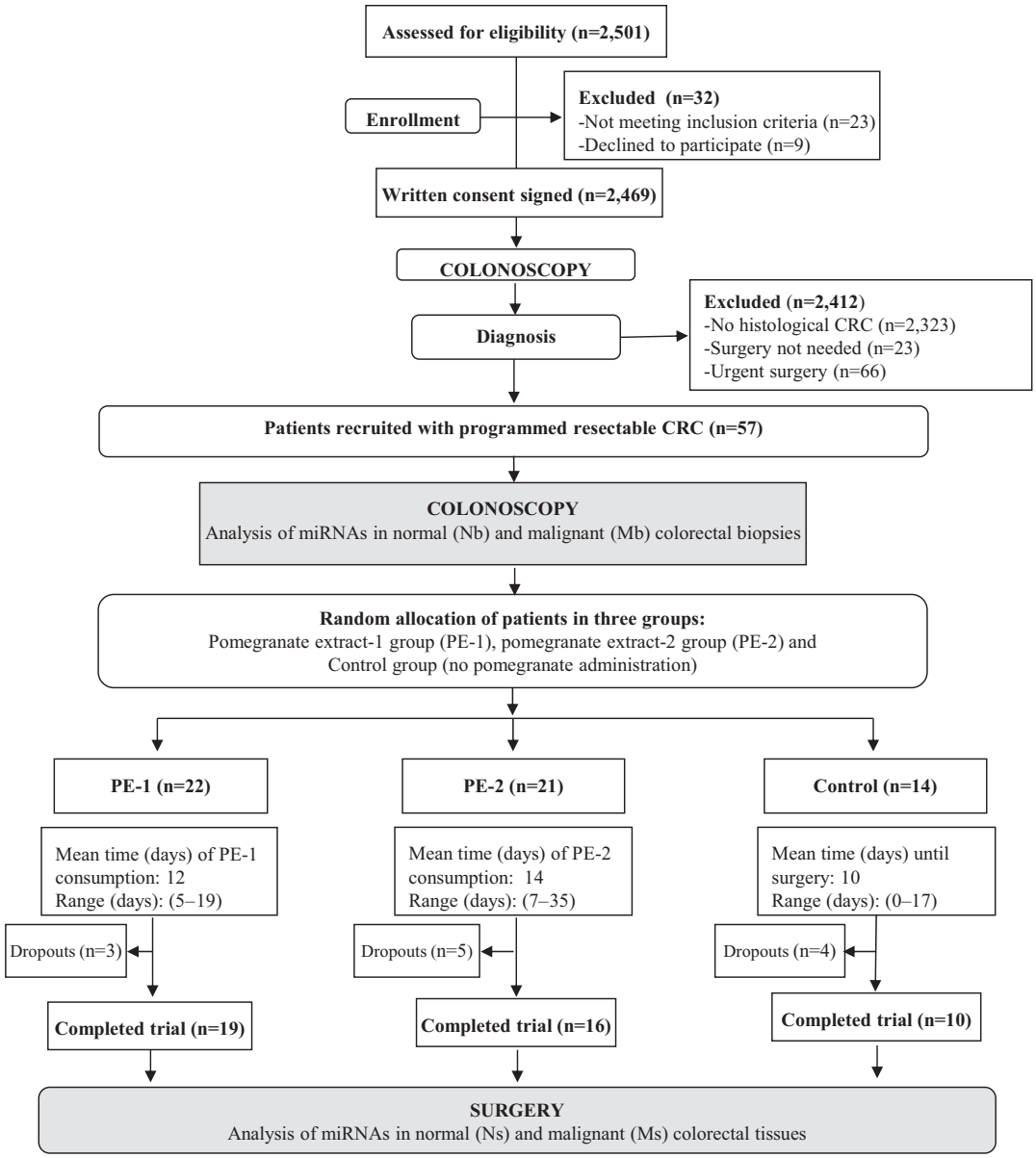

Figure 1. CONSORT flow diagram of patients through the trial.

pathway analysis of these genes was carried out using Genecodis database [15]

2.6 Statistical analysis

GenEX v.2.6.4. software (MultiD Analyses AB, Göteborg, Sweden) was used to manage the qRT-PCR data. Expression data were analyzed using the SPSS Software, version 21.0 (SPSS Inc., Chicago, IL, USA). Chi-square test was used for baseline comparison of categorical variables. The Shapiro–Wilk test indicated lack of normality for miRs expression data distribution. The nonparametric Wilcoxon signed rank and Mann–Whitney *U* tests were used to compare paired and unpaired data, respectively. The accepted level of statistical significance was 0.05 but marginal statistical differences ($0.05 < P < 0.1$) are also indicated.

3 Results

3.1 Characteristics of patients and surgical protocols

Table 1 shows the main demographic and clinical characteristics of CRC patients. Seventeen obese (BMI $\geq$ 30 kg/m^2), 13 overweight (25 kg/m^2 < BMI < 30 kg/m^2), and 15 normoweight (BMI $\leq$ 25 kg/m^2) patients participated in this trial. Patients' ages ranged from 51 to 92 years old. Most of the recruited patients were either asymptomatic ($n = 15$) or

Table 1. Clinical, demographic, and tumor features of patients that completed the trial ($n = 45$)

Patients		[a)]PE-1 group ($n = 19$)	PE-2 group ($n = 16$)	Control group ($n = 10$)
Mean age and range		75.2 ± 10.8 (51–89)	75.2 ± 9.3 (53–88)	71.3 ± 12.9 (51–92)
Mean BMI (kg/m^2) and range		28 ± 5 (21–40)	30 ± 4 (24–42)	31 ± 8 (23–45)
Males/females		10/9	9/7	3/7
[b)]WHO Status (0/1/2/3)		7/7/3/2	6/6/4/0	2/3/3/2
Hypertension (Yes/No)		10/9	8/8	7/3
Dyslipidemia (Yes/No)		7/12	8/8	6/4
Diabetes mellitus (Yes/No)		5/14	6/10	4/6
Tumor				
Location	Rectum/sigmoid	5	4	5
	Sigmoid colon	5	8	2
	Hepatic flexure/transverse colon	1	2	1
	Ascending	6	1	1
	Cecum	2	1	1
[c)]Histology	Differentiation, poor/moderate/high	2/16/1	3/12/1	0/9/1
[d)]TNM classification	T1/T2/T3/T4	0/4/10/5	3/5/7/1	0/2/8/0
	N0/N1/N2/N3	11/6/2/0	12/2/2/0	7/2/1/0
	M0/M1	19/0	15/1	10/0

a) PE-1, pomegranate extract-1 (low punicalagin/EA ratio); PE-2, pomegranate extract2 (high punicalagin/EA ratio).
b) WHO status score: 0, asymptomatic (fully active); 1, patient can do everything except heavy physical work; 2, symptomatic but less than 50% in bed during the day; 3, symptomatic and more than 50% in bed during the day; 4, bedbound (completely disabled); and 5, death.
c) TNM: T is referred to the growth of the primary tumors and whether it has grown into nearby areas; N (node) describes the extent of spread to regional lymph nodes; M (metastasis) indicates if the tumors have metastasized to distant organs such as liver or lungs.
d) Histology: highly differentiated >95% is gland forming, moderately differentiated from 50 to 95% gland formation, and poorly differentiated <50% gland formation (mostly solid).

limited for heavy physical activity ($n = 16$) according to the WHO status, i.e. 0 and 1, respectively (Table 1).

Main tumor characteristics in the three groups, after randomized allocation of patients, were moderate differentiation and TNM stage II (T3N0M0, i.e. *muscularis propria* invaded by the tumor but no regional lymph node metastasis and no distant metastasis) (Table 1).

After 12 drop-outs, 45 patients completed the trial (PE-1, $n = 19$; PE-2, $n = 16$, and Control, $n = 10$) (Fig. 1). No side effects were reported by the patients upon consumption of either PE-1 or PE-2 (dyspepsia, allergic reactions, etc.). No clinically relevant effects were detected in the serobiochemical variables determined to monitor kidney and liver functions (results not shown).

The mean time of surgery including anesthesia was 189 ± 59 min and ranged from 60 to 360 min depending on: (i) tumor localization, (ii) previous surgery in the patient, (iii) urgent or programmed surgery, and (iv) obese or normoweight patient. We did not find any apparent correlation between miR expression data and the tumor location, time of surgery, tumor differentiation, or TMN classification of our samples (results not shown).

3.2 Selection of miRs

We first carried out a screening analysis searching for miRs differentially expressed between the malignant and normal biopsies obtained from a subgroup of patients ($n = 5$) during colonoscopy (prior to PE intervention). The results of this screening are accessible from Supporting Information Material (Supporting Information Table 1). A total of 15 miRs were selected for further analysis (Table 2). We included various miRs whose expression resulted significantly different between Mb and Nb: (i) miR-18a-3p, miR-135b-3p, miR-92b-5p, miR-181c-3p, and miR-145-3p for which some association between some members of the corresponding miRs family and CRC has been reported and (ii) miR-514a-3p, miR-1248, and miR-664a-3p not yet previously associated with CRC. To further validate the preliminary screening, a number of miRs for which we did not detect significant differences were also carried forward into the study: (i) miR-646, miR-765, and miR-490-5p reported to be related to CRC and (ii) miR-1249, miR-496, and miR-621 for which specific association to CRC has not yet been described. Finally, and since miRs-specific passenger strands can have an impact on vertebrates regulatory networks and might contribute to disease state [16], we further included the mature miR 5p arm for miR-135b to allow for the comparison between miR-135b-3p and miR-135b-5p isomiRs.

3.3 miRs expression in human samples: Comparison between tumor and normal biopsies

The percentage of individuals in the sample population exhibiting upregulation and downregulation in the levels of the selected miRs between malignant and normal initial biopsies is presented in Table 3 (column initial biopsies, Mb versus Nb, $n = 45$) and Fig. 2A. Of the 15 miRs examined, miR-1249, miR-135b-5p, miR-92b-5p, and miR-18a-3p were mostly upregulated in malignant biopsies as compared to normal ones

Table 2. miRs selected and included in the study

miRs	Literature search and references
miRs that exhibited a significant difference between malignant and normal colon biopsies (Mb versus Nb) in the preliminary screening and that have some association with CRC	
miR-18a-3p	miR-18a overexpressed in CRC tissues [23]. Also, miR-18a-5p significantly reduces the hazard of dying of colon and rectal cancers regardless of tumor site [38].
miR-135b-3p	miR-135b is associated with the progression of normal epithelium to adenoma and carcinoma and with the expression of APC in colon tissue [24].
miR-92b-5p	miR-92a is significantly overexpressed in tumor samples in CRC patients [25] and a key component of the oncogenic miR-17-92 cluster in colon cancer.
miR-181c-3p	Reduced miR-181c is correlated with increased target RAS oncogenes and KRAS protein [39]. miR-181a is associated with poor clinical outcome in patients with CRC treated with EGFR inhibitor [40].
miR-145-3p	miR-145 is deregulated in CR adenomas and CRC [19]. Whereas miR-145-3p is highly associated with CRC survival and with various target pathways and genes (KRAS, EGFR, TP53, MYC, and CDKN1A) miR-145-5p is increased with more advanced CRC stage [38].
miRs not yet associated with CRC but that exhibited a significant difference between malignant and normal colonic biopsies (Mb versus Nb) in the preliminary screening	
miR-514a-3p	Downregulated in metastatic renal cell carcinoma. It may play a role in tumor recurrence [41].
miR-1248	It has an effect on the modulation of TYMS in nonsmall cell lung cancer [42].
miR-664a-3p	It promotes cell proliferation, migration, and invasion in T-cell acute lymphoblastic leukaemia cells and targets PLP2 (proteolipid protein 2 that may play a role in cell differentiation in the intestinal epithelium) [43].
miRs that have been reported to be associated with CRC but that exhibited no significant difference between malignant and normal colon biopsies (Mb versus Nb) in the preliminary screening	
miR-646	A genomic deletion has been identified in mir-646 in CRC patients [44].
miR-765	miR-765 is significantly upregulated in rectal cancer patients in response to chemo-radiotherapy [45].
miR-490-5p	miR-490-5p was selected as important miRs in CRC diagnosis [46].
miRs not yet associated with CRC and that exhibited no significant difference between malignant and normal colonic biopsies in the preliminary screening	
miR-1249	It shows significant correlation with tumor relapse in small cell carcinoma of the esophagus [47]. It was significantly modulated by a grape seed procyanidins extract in isolated rat pancreatic islets [48].
miR-496	Reported to be involved in promoter's methylation processes and subsequent gene transcription regulation in breast cancer cell lines [49].
miR-621	It might be involved in the regulation of critical cell-cycle control genes in hepatocellular carcinoma [50].

(>60% of the subjects exhibited upregulation) whereas miR-135b-3p, miR-145-3p, miR-490-5p, miR-1248, miR-664a-3p, and miR-514a-3p were predominantly downregulated (>53% of the participants exhibited downregulation) in malignant biopsies versus normal biopsies. The rest of the miRs investigated did not exhibit such a clear tendency toward induction or downregulation when comparing the two types of biopsies. Estimation of the magnitude of the changes in the levels of these miRs between malignant and normal biopsies is presented as the fold change median and *p*-values in Table 4 (column initial biopsies at colonoscopy, Mb versus Nb, $n = 45$). For miR-135b-5p, miR-92b-5p, and miR-18a-3p the fold change was significantly upregulated: (4.7-, 1.4-, and 2.1-fold change, respectively) whereas miR-145-3p, miR-1248, miR-664a-3p, and miR-514a-3p were all significantly downregulated in malignant biopsies versus normal ones (fold change values between –1.8 and –1.4). These results corroborated the trend observed in the percentage of individuals exhibiting the change (Table 3 and Fig. 2A). Also in agreement with the % results, miR-1249 was upregulated (fold change: 1.5) and miR-135b-3p downregulated (fold change: –1.5) although these values did not reach significance. Those miRs that were found to be up- or downregulated in a similar proportion of participants exhibited a nonsignificant change and (or) a fold change <1.2 or >–1.2 (indicative of no change). However, we also found that miR-490-5p that was predominantly downregulated in the sample population (53% ↓) resulted in a median fold change of 1.1 and did not reach significance. It is also interesting to note that the isomolecules miR-135b-3p and miR-135b-5p showed an opposed tendency and whereas the 5p form was mostly upregulated, the 3p form tended to be downregulated in malignant biopsies versus normal ones.

3.4 miRs variations in human colon control samples: Effects of the surgical protocol

We next investigated the changes in the levels of miRs that might be attributed to the surgical protocol. For this purpose, we compared the 15 miRs in surgical specimens against initial biopsies (both for malignant and normal tissues) obtained from control patients that did not consume the pomegranate extracts ($n = 10$). The percentage of individuals in the sample

Table 3. Comparison between samples obtained during initial colonoscopy (biopsies, b) or during surgical resection (s), both from malignant (M) and normal tissues (N)[a)]

miRs	Initial biopsies ($n = 45$)	Control individuals ($n = 10$)		Pomegranate extract consumers ($n = 35$)	
	Mb versus Nb	Ms versus Mb	Ns versus Nb	Ms versus Mb	Ns versus Nb
miR-646	40% ↑	60% ↑	70% ↑	23% ↑	37% ↑
	49% ↓	30% ↓	30% ↓	69% ↓	63% ↓
miR-1249	62% ↑	70% ↑	70% ↑	17% ↑	43%↑
	27% ↓	10% ↓	30% ↓	66% ↓	43%↓
miR-135b-5p*	87% ↑	70% ↑	70% ↑	40% ↑	40% ↑
	13% ↓	30% ↓	10% ↓	60% ↓	40% ↓
miR-135b-3p	31% ↑	60% ↑	60% ↑	34% ↑	29% ↑
	56% ↓	40% ↓	40% ↓	40% ↓	57%↓
miR-92b-5p	62% ↑	80% ↑	80% ↑	20%↑	60% ↑
	18% ↓	10% ↓	10% ↓	63% ↓	34% ↓
miR-765	40% ↑	80% ↑	90% ↑	29% ↑	54% ↑
	40% ↓	20% ↓	10% ↓	60% ↓	34% ↓
miR-496	40% ↑	60% ↑	80% ↑	31% ↑	54% ↑
	42% ↓	40% ↓	20% ↓	63% ↓	37% ↓
miR-181c-3p	40% ↑	90% ↑	80% ↑	51% ↑	37%↑
	38% ↓	10% ↓	10% ↓	31% ↓	54%↓
miR-18a-3p	78% ↑	80% ↑	90% ↑	49% ↑	57% ↑
	18% ↓	20% ↓	10% ↓	20% ↓	29% ↓
miR-145-3p	27% ↑	90% ↑	90% ↑	63% ↑	71% ↑
	60% ↓	10% ↓	10% ↓	34% ↓	20% ↓
miR-490-5p	40% ↑	90% ↑	90% ↑	71% ↑	74% ↑
	53% ↓	0% ↓	10% ↓	26% ↓	23% ↓
miR-1248	27% ↑	90% ↑	60% ↑	74% ↑	80% ↑
	62% ↓	10% ↓	20% ↓	14% ↓	9% ↓
miR-664a-3p	24% ↑	100% ↑	100% ↑	86% ↑	80% ↑
	58% ↓	0% ↓	0% ↓	11% ↓	3% ↓
miR-514a-3p	20% ↑	40% ↑	50% ↑	26% ↑	20% ↑
	64% ↓	60% ↓	50% ↓	71% ↓	74% ↓
miR-621	47% ↑	30% ↑	40% ↑	23% ↑	34% ↑
	31% ↓	70% ↓	60% ↓	63% ↓	57% ↓

a) Results are shown as percentage of patients exhibiting upregulation (↑) or downregulation (↓) for each specific miR. Values above 50% were considered as indicators of a trend and are shown in light gray color (up-) or dark gray color (down-). *mir-135b-5p ($n = 5$ in PE consumers, $n = 10$ in controls).

population exhibiting upregulation and downregulation between surgical specimen and biopsy for each miR is presented in Table 3 (column control individuals $n = 10$, Ms versus Mb, Ns versus Nb). Overall, the levels of the miRs examined were induced in the surgical samples compared to the initial biopsies as 13 of these molecules were upregulated in most of the individuals and only miR-514a-3p and miR-621 were downregulated in the postsurgical samples. These changes affected similarly to malignant and normal tissues. The corresponding fold change median and *p*-values are included in Table 4 (column control patients, $n = 10$, Ms versus Mb and Ns versus Nb) and backed up most of the results in Table 3. Importantly, when we compared the differences between malignant and normal postsurgical specimens for these miRs, we observed a general dysregulation (Fig. 2B) as compared to Mb versus Nb (Fig. 2A). As a result, the ratio malignant versus normal was critically modified. This is further illustrated in Fig. 3A for miR-1248 which was significantly downregulated in Mb versus Nb (–1.4, $p = 0.003$; Table 4) whereas in the postsurgical control samples this miR resulted upregulated in malignant versus normal tissue (2.4, $p = 0.070$; Table 4). The opposite was observed for miR-92b-5p (Fig. 3B). In addition to the general miRs upregulation, the surgical protocol also resulted in larger interindividual variability of miRs expression as illustrated in the case of miR-490-5p (Supporting Information Fig. 1), more markedly in normal tissues.

3.5 miRs changes in human colon samples attributed to the consumption of pomegranate extracts

A preliminary analysis (data not shown) revealed that there were not differences between those patients that consumed the PE-1 and those that consumed the PE-2 and thus results are presented jointly. As depicted for control patients in

Table 4. Estimation of fold change (median) and significance (p-value) for miR expression differences in colonic tissues from CRC patients[a)]

miR	Initial biopsies ($n = 45$)		Control patients ($n = 10$)						Patients that consumed PE ($n = 35$)					
	Mb versus Nb		Ms versus Mb		Ns versus Nb		Ms versus Ns		Ms versus Mb		Ns versus Nb		Ms versus Ns	
	-Fold	p	-Fold	p	-Fold	p	-Fold	p	-Fold	p	-Fold	p	-Fold	p
miR-646	−1.3	0.548	1.8	**0.007**	7.4	**0.001**	−2.5	**0.049**	−1.9	**0.012**	−2.0	0.190	−1.2	0.130
miR-1249	1.5	0.082	1.8	**0.038**	3.9	**0.005**	−1.6	0.326	−1.8	**0.002**	−1.1	0.950	−1.3	0.104
miR-135b-3p	−1.5	0.285	2.3	**0.004**	5.8	**0.028**	−1.2	0.650	1.1	0.063	−2.0	**0.033**	−1.1	0.421
miR-135b-5p*	4.7	**0.004**	12.8	**0.030**	2.0	**0.016**	6.5	**0.024**	−2.0	0.843	1.0	0.965	8.0	0.060
miR-92b-5p	1.4	**0.049**	2.0	0.176	4.9	**0.001**	−2.0	0.077	−1.7	**0.011**	1.5	0.080	−1.8	**0.013**
miR-765	1.1	0.824	3.0	**0.022**	18.4	**<0.001**	−2.5	0.171	−1.8	**0.024**	1.5	0.081	−1.9	**<0.001**
miR-496	−1.3	0.799	2.0	**0.042**	6.1	**<0.001**	−1.9	0.364	−1.5	**0.009**	1.4	0.424	−1.6	**0.001**
miR-181c-3p	−1.2	0.485	8.7	**<0.001**	18.8	**<0.001**	1.3	0.762	1.4	0.738	−1.3	1.0	1.3	0.747
miR-18a-3p	2.1	**<0.001**	2.8	0.275	5.4	**<0.001**	1.0	0.597	1.2	0.235	1.3	0.066	1.8	**0.001**
miR-145-3p	−1.4	**0.010**	4.2	**0.001**	11.5	**<0.001**	−2.8	0.151	1.8	0.089	3.0	**<0.001**	−2.8	**<0.001**
miR-490-5p	1.1	0.678	31.3	**<0.001**	14.0	**<0.001**	−1.2	0.821	2.9	**<0.001**	3.5	**<0.001**	−1.6	0.220
miR-1248	−1.4	**0.003**	4.8	**<0.001**	2.0	**0.040**	2.4	0.070	1.8	**<0.001**	3.0	**<0.001**	−2.8	0.477
miR-664a-3p	−1.5	**0.018**	21.0	**<0.001**	14.0	**<0.001**	1.2	0.940	2.7	**<0.001**	2.1	**<0.001**	−1.3	0.344
miR-514a-3p	−1.8	**0.002**	−2.2	0.206	1.0	0.956	−2.8	0.069	−2.2	**0.001**	−2.4	**<0.001**	−1.6	**0.040**
miR-621	−1.1	0.315	−8.6	**0.016**	−1.9	0.556	−1.8	0.226	−1.9	**0.006**	−1.4	0.199	1.2	0.986

a) Nb, normal biopsy; Mb, malignant biopsy; Ns, normal tissue after surgery; Ms, malignant tissue after surgery; CT, control patients; PE, patients that consumed pomegranate extracts. The statistical analysis was carried out as described in materials and methods. * miR-135b-5p ($n = 5$ in PE consumers, $n = 10$ in controls).

Fig. 2B, a general dysregulation of the studied miRs was also observed in the Ms and Ns of patients consuming the PEs (Fig. 2C) suggesting not apparent differences between control and treated patients. However, when we determined the percentage of individuals exhibiting upregulation or downregulation between surgical specimens (post-PE intake) and biopsies (previous to PE intake) for each miR in those patients that consumed a PE (Table 3), we found that the induction of miR-646, miR-1249, miR-135b-5p, miR-135b-3p, miR-92b-5p, miR-765, miR-496, miR-181c-3p, and miR-18a-3p observed in the control patients and attributed to the surgical protocol was mitigated or even upturned. The rest of the miRs did not show a substantial alteration in the proportion of individuals with up- and downregulation. These results suggested a modulatory effect of the pomegranate extracts on specific miRs. Statistical analysis of the fold changes between tissues is also included in Table 4 (column patients that consumed a PE: Ms versus Mb and Ns versus Nb) and generally support the corresponding results in Table 3. To further exemplify these results, the changes observed for miR-646 and miR-496 are depicted in Fig. 4. The induction associated with the surgical protocol was observed in Ns (more noticeably) and Ms in control patients. The PEs counteracted these effects, more significantly in the malignant tissues.

3.6 Pomegranate extract consumption may affect cancer-related miRs and their downstream effectors

To get some insight into the potential in vivo effects of those miRs significantly altered in response to the PEs consumption, we performed a preliminary in silico analysis to search for their target genes using miRs-validated target databases [13, 14]. We found that in normal tissues 134 genes (listed on Supporting Information Table 2) were direct or indirect targets of miR-181c-3p, miR-765, miR-135b-3p, and miR-646. In silico pathway analysis of these genes marked out various common pathways in cancer (Kegg: 05200) including CRC and jak-STAT signaling pathways (Kegg: 04630) (Supporting Information Fig. 2A) as potentially affected downstream pathways. We carried out a similar analysis for those miRs significantly altered in malignant tissues by the PE intake: miR-1249, miR-92b-5p, miR-765, miR-496, and miR-646. A total of 60 validated target genes were found (listed on Supporting Information Table 2). Pathway analysis (Supporting Information Fig. 3B) of these validated targets also pointed toward the potential modulation of common cancer pathways (Kegg: 05200).

4 Discussion

This study first evidences the in vivo specific but moderate modulation of various miRs in human malignant and normal colon tissues from CRC patients following the intake of PEs enriched in EA or ellagitannins (punicalagin). The

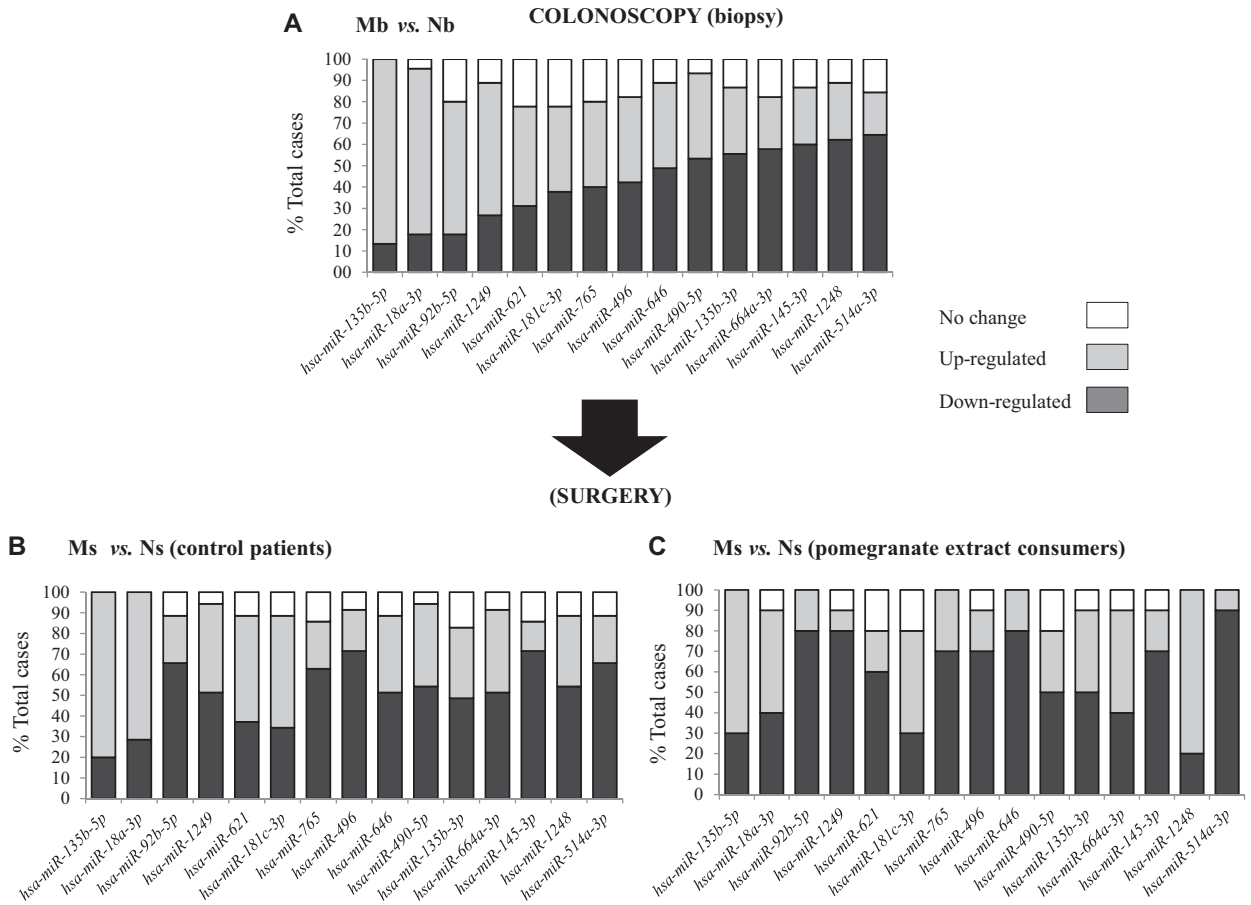

Figure 2. Distribution (% of total cases) of patients exhibiting downregulation (dark gray), upregulation (light gray), or no change (white) in colon malignant tissue (M) versus normal tissue (N) for the selection miRs. (A) Malignant biopsies (Mb) versus normal biopsies (Nb) ($n = 45$ patients). (B) Malignant surgical specimens (Ms) versus normal surgical specimens (Ns) in control patients ($n = 10$). (C) Malignant surgical specimens (Ms) versus normal surgical specimens (Ns) in patients that consumed pomegranate extracts ($n = 35$).

metabolites derived from these polyphenols and detected in those tissues do not appear to be associated with the observed effects. Importantly, we have considered and are further discussed here some critical factors that need to be taken into account in order to truly discriminate between potential artefacts and: (i) real miRs expression differences between malignant and normal tissue as potential CRC biomarkers, (ii) the modulatory effects induced on those miRs by the diet, such as PE consumption.

Literature is overwhelmed with "potential" markers that need further prospective validation before they can go into clinical practice. Despite the proliferation of publications claiming for the discovery of molecular biomarkers useful for CRC, *KRAS* mutation is the only validated marker adopted into routine clinical management of CRC patients for anti-EGFR directed therapy [17].

miRs have emerged as potential CRC biomarkers due to their participation in many pathways related to CRC as well as to their remarkable stability in biological samples [18]. miRs are well preserved in paraffin-embedded colonic tissues and this has potentiated the study of miRs expression levels in malignant and normal colonic tissues using post-surgical samples from hospital biobanks [19]. The dysregulation of various miRs between malignant and normal tissues has now been repeatedly reported and postulated as potential biomarkers for CRC [4, 18]. Nevertheless, the levels of miRs may be altered by experimental conditions such as the time to freezing following sample extraction [20]. These authors reported the induction of 56 miRs in breast cancer tissues and attributed it to the ischemia elapsing between sampling and snap-freezing. Taking this into consideration, we examined the effect of the whole surgical protocol used in our study in a group of control patients and corroborated that the levels of all the miRs examined were modified in the surgical specimens in comparison with the initial colonic biopsies. Of note, and in good agreement with the study by Borgan et al. [20], most miRs, and in particular miR-765, were upregulated by the surgical procedure. Our results also evidenced that the induction caused by the surgical protocol affected differently the levels of some miRs in malignant and normal

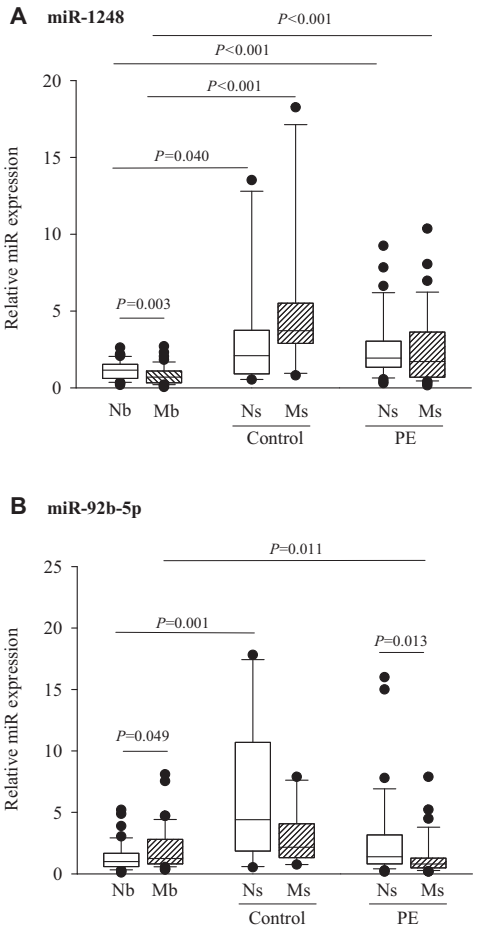

Figure 3. Effect of surgical protocols on miR expression. (A) miR-1248 and (B) miR-92b-5p are shown as representative examples. Significant differences ($P < 0.05$) are indicated. Nb, normal biopsy; Mb, malignant biopsy; Ns, normal tissue after surgery; and Ms, malignant tissue after surgery, in controls and PE consumers (patients that consumed pomegranate extracts).

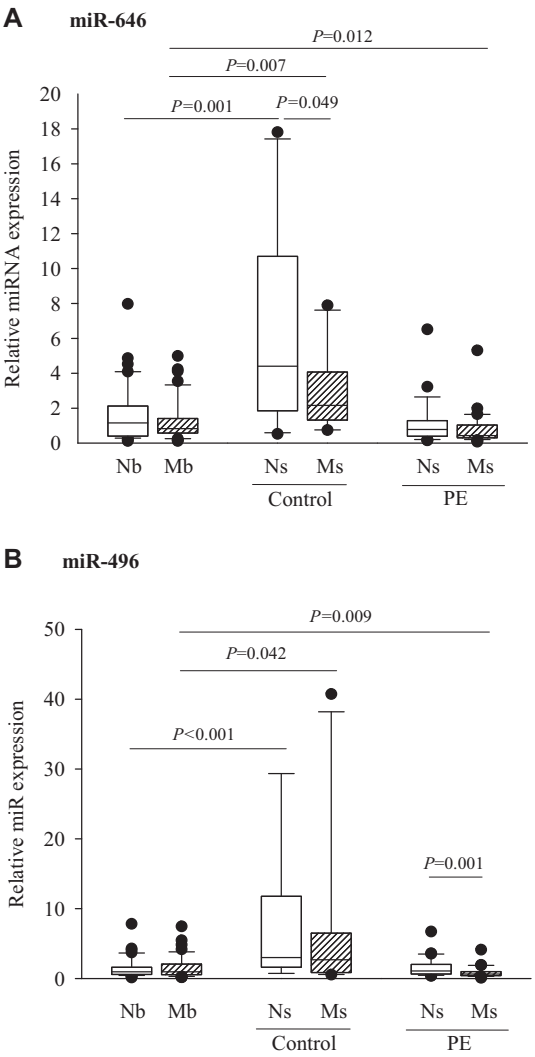

Figure 4. Effect of PE consumption on miR expression. (A) miR-646 and (B) miR-496 are shown as representative examples. Significant differences ($P < 0.05$) are indicated. Nb, normal biopsy; Mb, malignant biopsy; Ns, normal tissue after surgery; and Ms, malignant tissue after surgery, in controls and PE consumers.

tissue and thus, the differential expression between cancer and healthy samples was altered or even upturned. This was exemplified by miR-92b-5p and miR-1248 for which differential expression between malignant and normal biopsies was opposite to that determined in surgical specimens. Therefore, and in support of Borgan et al. [20] previous recommendations, caution is needed when proposing the differential expression between malignant versus normal tissue for a specific miR as a biomarker for CRC since it may depend largely on the surgical protocol, tissue removal, and storage procedure.

CRC adenocarcinomas are mainly characterized by glandular formation and tissue heterogeneous composition that can vary depending on the location and differentiation of the adenocarcinoma. Since the expression of many miRs is tissue specific [21], two additional important factors can contribute to the variability of miRs expression data: (i) histological differences between normal and malignant CRC tissues and (ii) the heterogeneity of cell types present in each biopsy or surgical sample. Further, miRs differential expression has been reported to be affected by tumor location and tumor subtype [22].

Our results corroborated that miR-18a-3p, miR-135b-5p, and miR-92b-5p were mostly upregulated and miR-145-3p downregulated in malignant versus normal adjacent biopsies in consonance with previous differential expression reported for miR-18a [23], miR-135b [24], miR-92a [25], and miR-145 [19]. We noticed that the designation of miRs can vary between reports and that often, the miR strand (-3p or -5p) or the family (i.e. a, b, c, etc.) is not indicated [26] preventing the comparison, reproducibility, and validation of studies. miRs strand selection is a highly regulated process that shows

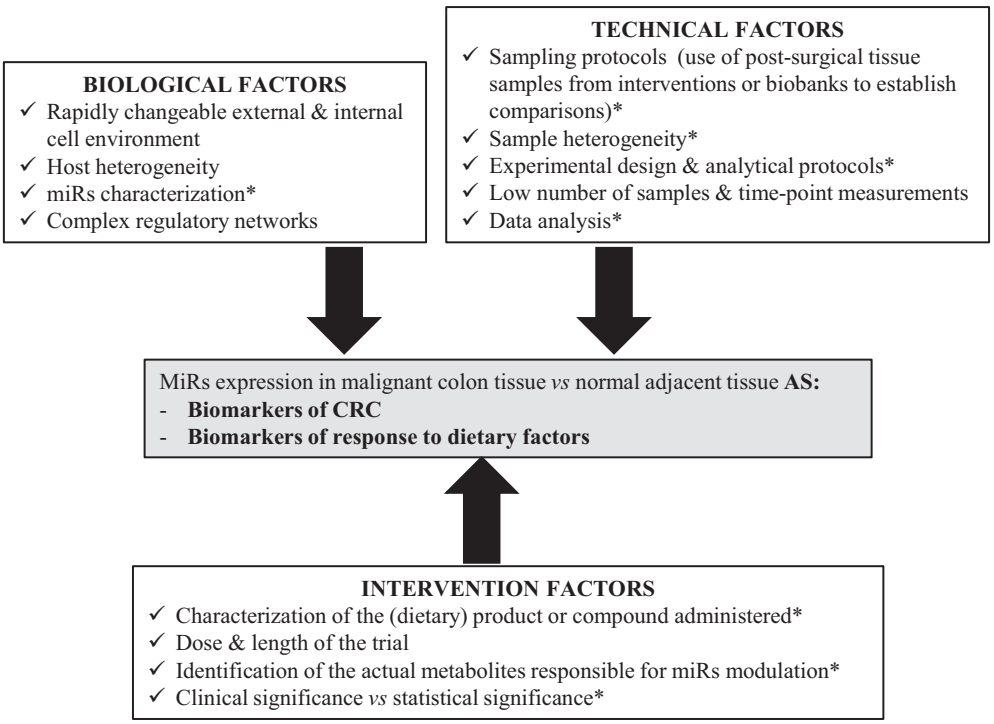

Figure 5. Factors that confer variability to colon tissue miRs expression data and that hamper the adequate estimation of expression changes as CRC biomarkers or in response to treatment. *Factors further discussed in the text.

developmental stage, tissue, cell type, and condition-specific modulation [16] and thus, it is essential to indicate the precise strand being investigated. In our study, we specifically compared the two strands for miR-135b and found that while the -5p strand was upregulated the -3p strand was downregulated in Mb versus Nb. Although the surgical procedure induced the expression of both strands, the response to the PE intake also differed between strands, i.e. the PE significantly repressed the expression of the -3p strand in normal tissues, while the -5p strand resulted unchanged. These findings support that miR-specific strand expression can be influenced by malignancy and also suggests that can be modulated by dietary interventions. Even though the targets for the -3p and -5p strands might be similar for certain miRs [27], different sequences usually gives different targets [28, 29]. Indeed, the -3p and -5p species of some miRs are frequently coexpressed in CRC, and might cross-target different genes in the same pathway [29]. There is also evidence that a different strand can have a complete different effect in CRC cells [28]. Thus, in order to allow for direct comparison studies, miR-specific strands as well as the exact family nomenclature should be carefully evaluated when searching for miR-based expression regulators.

An additional and critical issue to reflect on is the analysis and presentation of miRs expression data. Recent publications in the field show a variety of approaches: (i) parametric assumptions and bar plots indicating mean values ± SD or SEM [18], (ii) nonparametric assumptions and box-plots displaying median values and the interquartile range [30], or (iii) plots of grouped individual data and matched cases representing upregulation or downregulation for each individual case [31, 32]. In an effort to improve our analysis and data presentation we have applied robust nonparametric tests (our analysis indicated the absence of normality for the expression data) and presented the results as (i) the % of patients exhibiting up- or downregulation for each miR (Table 3) and (ii) the estimation of the magnitude and significance of the expression difference between cases (fold change and *p*-value; Table 4). In our experience this procedure reflects best the large variability in tissue miR expression and expression changes in response to experimental protocols and (or) treatment (Supporting Information Fig. 1). It also evidences that for very heterogeneous populations, as it is the case of CRC patients, the mean or the median values may not be appropriate estimators of the population average unless a high percentage of individuals (~ >55–60%) behave or respond in the same direction and the magnitudes of the changes are not very disparate.

The clinical evidence that supports the contribution of dietary polyphenols to the prevention or reduction of CRC is still very weak and the molecular mechanisms of action are not yet known [8]. Among other potential mechanisms, the modulation by polyphenols of cancer-related pathways altering the levels of transcription factors and (or) miRs is postulated [9]. Most research looking at the effects of polyphenols on miRs and CRC has been conducted in vitro

[33, 34] showing significant associations between cell responses and specific miRs expression changes. Although the translation of these results to the in vivo situation remains yet to demonstrate, a few studies using CRC animal models have now shown a link between tumor inhibition by pomegranate juice or resveratrol intake and specific intestinal miR expression changes [35,36], giving first in vivo evidence of the potential capacity of polyphenols to protect against CRC via miRs expression regulation. In patients with FAP, the daily administration of black raspberries (BRBs) powder-containing suppositories was able to reduce the burden of rectal polyps and cellular proliferation [10]. In this study, the authors reported the demethylation on the transcription start sites for miRs in BRB-treated adenomas from responders but there was no additional effect from oral supplementation of the BRB or reported changes in miRs expression levels.

Our study first evidences the specific modulation of various miRs in human colon tissue from CRC patients following the intake of polyphenol containing PEs. One critical point that needs to be elucidated is the potential in vivo biological link between the modulation of established CRC biomarkers and the actual specific polyphenols or derived metabolites responsible for such modulation. In an attempt to find an association between the changes attributed to the intake of the PEs and the ellagitannin-derived metabolites, EA, and urolithins, detected in the same colon tissues [11], we have analyzed the potential correlation between the specific miRs expression data both in malignant and normal tissues and the metabolic profile of the individuals taking part in the study. These patients were classified as producers of only urolithin A (phenotype A), urolithin A plus iso-urolithin A, and urolithin B (phenotype B) or nonproducers of urolithins (phenotype 0) [37]. We did not find any significant association between the observed changes in miRs expression data and these phenotypes or the amount of urolithins detected in the tissues (data not shown). Further, the modulatory effects attributed to the consumption of the PEs were independent of the duration of the intervention and were indistinctly observed with PE-1 (rich in EA) or PE-2 (rich in punicalagin). All these results suggest that, under our specific trial design and experimental conditions there was no direct association between the observed changes in specific miRs expression and the presence of ellagitannin metabolites in the colon tissues and thus, we were not able to establish a potential cause-effect relationship. Our results suggest that other common components of the two PE extracts may be involved in the observed changes. The identification of these other components needs to be further investigated.

Overall, our study shows the specific and moderate modulation of various miRs in human colon tissues from CRC patients following the intake of polyphenol-containing PEs and evidences the difficulty in discriminating these effects from those attributed to surgical protocols. Although our in silico analyses indicated a potential involvement of the modulated miRs with cancer-related genes and pathways, the relevance of these results in relation to cancer prevention is not yet understood. Since it appears that diet can have a significant impact on the levels of oncogenic mucosal miRs and might contribute to control the incidence of CRC, the validation of miRs as biomarkers of CRC and their regulation by dietary factors deserves extensive investigation. The final aim is that "statistically significant changes" from well-designed intervention studies can be translated into "useful clinical applications." A summary of the factors investigated and discussed here as well as other technical and biological-reported factors that confer variability to miRs expression analysis, increasing the chances of false positives and hampering their establishment as biomarkers for CRC and of response to dietary factors is depicted in Fig. 5. These issues need to be addressed and more comprehensive analyses in standardized larger studies are required before miR expression changes can be proven reliable CRC biomarkers.

J.C.E. designed the study; A.D., P.C.A., F.V., M.A.N.S., and A.G.S. performed miR expression analyses; M.A.N.S. performed the statistical analysis; T.M.S., N.V.G.T., M.B.G.S., and C.S.A. recruited and coordinated CRC patients; A.M.G.A. and F.J.R.G. collected biopsy samples; M.R.M. collected surgical samples; F.A.P.Q. and F.M.D. performed the histopathological analyses; A.D., F.A.T.B., M.T.G.C., and J.C.E. contributed to the discussion of the manuscript; A.D., A.G.S., M.T.G.C., and J.C.E. contributed to the writing. All authors have read and approved the final manuscript.

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The authors have declared no conflict of interest.

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