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Original article

Modulation of faecal miRNAs highlights the preventive effects of a Mediterranean low-inflammatory dietary intervention

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SUMMARY

Background: Dietary interventions have been proposed as therapeutic approaches for several diseases, including cancer. A low-inflammatory Mediterranean dietary intervention, conducted as a pilot study in subjects with Familial Adenomatous Polyposis (FAP), reduced markers of local and systemic inflammation. We aim to determine whether this diet may modulate faecal microRNA (miRNA) and gene expression in the gut.

Methods: Changes in the faecal miRNome were evaluated by small RNA sequencing at baseline (T0), after the three-month intervention (T1), and after an additional three months (T2). Changes in the transcriptome of healthy rectal mucosa and adenomas were evaluated by RNA sequencing at T0 and T2. The identification of validated miRNA-gene interactions and functional analysis of miRNA targets were performed using *in silico* approaches.

Results: Twenty-seven subjects were included in this study. It was observed that the diet modulated 29 faecal miRNAs ($p < 0.01$; $|\log2 \text{ Fold Change}|>1$), and this modulation persisted for three months after the intervention. Levels of miR-3612-3p and miR-941 correlated with the adherence to the diet, miR-3670 and miR-4252-5p with faecal calprotectin, and miR-3670 and miR-6867 with serum calprotectin.

Seventy genes were differentially expressed between adenoma and normal tissue, and most were different before the dietary intervention but reached similar levels after the diet. Functional enrichment analysis identified the proinflammatory ERK1/2, cell cycle regulation, and nutrient response pathways as commonly regulated by the modulated miRNAs and genes.

Conclusions: Faecal miRNAs modulated by the dietary intervention target genes that participate in inflammation. Changes in levels of miRNAs and genes with oncogenic and tumour suppressor functions further support the potential cancer-preventive effect of the low-inflammatory Mediterranean diet.

Clinical trial number registration: NCT04552405, Registered in ClinicalTrials.gov.

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1. Introduction

Familial Adenomatous Polyposis (FAP) is a rare premalignant hereditary autosomal dominant condition caused by germline mutations in the Adenomatous Polyposis Coli (*APC*) gene. FAP is characterised by the development of multiple adenomatous polyps in the colon and progression to cancer if not treated with early prophylactic colectomy with ileorectal anastomosis [1]. Lifelong endoscopic surveillance is required to reduce the risk of duodenal and rectal stump cancers [2]. There are no preventive recommendations for subjects with FAP. Nonsteroidal anti-inflammatory drugs have been associated with the regression of colorectal adenomas; however, their continuous use increases cardiovascular risk [3].

We conducted a non-randomized pilot study in subjects with FAP to assess whether a three-month low-inflammatory dietary intervention based on principles and recipes of the traditional Italian Mediterranean diet, but with lower consumption of pro-inflammatory foods and enriched with fermented foods from Japanese tradition, could reduce gastrointestinal markers of inflammation. Stool and serum levels of the inflammatory marker calprotectin decreased after the intervention [4,5], and this was accompanied by a significant decrease in the number of diarrheal discharges, increasing the subject's quality of life [6].

Dietary habits and components influences the regulation of different biological processes, including immune and metabolic pathways [7,8]. The diet also regulates gene and microRNA (miRNA) expression in the gut [9], and the faecal miRNA profile is associated with specific dietary habits and colorectal cancer (CRC) onset [10–12]. Components of the Mediterranean diet, including foods and molecules with anti-inflammatory and anti-tumoural properties, are often associated with disease prevention and can modulate miRNA expression [13,14].

The present work aimed to evaluate whether the three-month low-inflammatory Mediterranean dietary intervention in subjects with FAP induced changes in faecal miRNA levels and intestinal tissue expression and whether these changes may be associated with the observed beneficial effects.

2. Materials and methods

2.1. Study design

We performed a prospective nonrandomized pilot single-arm trial aimed at evaluating the effects of a low-inflammatory Mediterranean diet on subjects with FAP. Information on the characteristics of the diet, dietary intervention and evaluation of participant adherence has been reported previously [4] and is described in Supplementary Methods. The study included subjects diagnosed with FAP, carrying a mutation in the *APC* gene, who underwent prophylactic total colectomy/ileorectal anastomosis and are currently involved in the surveillance program at the Fondazione IRCCS Istituto Nazionale dei Tumori di Milano (INT). Participants signed an informed consent and were asked to donate blood and stool samples at baseline (T0), at the end of the three-month dietary intervention (T1), and at six months (T2). Subjects underwent endoscopy at T0 and T2, during which samples of healthy rectal mucosa and adenomas (when present) were collected as formalin-fixed and paraffin-embedded (FFPE) specimens (Supplementary Fig. 1). Finally, they were asked to complete a validated questionnaire to evaluate adherence to the Mediterranean diet (Mediterranean Diet Adherence Screener, MEDAS) [15]. Subjects treated with nonsteroidal anti-inflammatory drugs, omega-3 products, or that failed to comply with the endoscopic surveillance to which subjects with FAP are subjected every six months (in accordance with the standard procedure at INT) were excluded from the study. The study was approved by the INT Ethics Committee (INT 78/2017) and registered as interventional study #NCT04552405 (ClinicalTrials.gov). The 27 participants who provided stool samples at all three time points were considered for the present study (Supplementary Fig. 1).

2.2. Small RNA sequencing (small RNA-seq) and analysis

Total RNA was extracted with the Stool Total RNA Purification Kit (Norgen Biotek, Canada), and libraries for small RNA-seq were prepared with NEBNext Multiplex Small RNA Library Prep for Illumina (New England Biolabs, USA) as detailed in Supplementary Methods. Small RNA-seq data analysis was performed as in Tarallo et al. 2022 [11]. Names of novel mature miRNAs are reported in italics.

DESeq2 package (v.1.28.1) for the statistical software R (v.4.0.2) was used for miRNA level normalisation [16]. A miRNA was defined as differentially expressed (DE) if associated with $p < 0.01$ in a paired Wilcoxon-rank sum test, had a median>15, and a |log2 Fold Change|>1 (log2FC). Functional analysis of miRNA targets was performed using RbiomirGS v.0.2.12.

2.3. Total RNA sequencing (RNA-seq) and analysis

Total RNA was extracted from FFPE sections using Maxwell® RSC RNA FFPE Kit (Promega, USA). Normal rectal tissue was available from 8 subjects at T0 and 8 at T2, while adenomas were available from 10 subjects at T0 and 10 at T2. Samples were not all paired. RNA library preparation is reported in Supplementary Methods.

Only genes with a median Transcripts Per Million (TPM) > 1 in both tissue types at both time points were analysed. Genes with an adjusted $p < 0.05$ in a Wald test performed with the *DESeq2* package were considered DE. Genes were considered to be modulated by the dietary intervention when they were: a) DE between T2 and T0 in either tissue; b) DE between adenoma and normal tissues at T0 but not at T2; or c) DE between adenoma and normal tissues at T2 but not at T0.

3. Results

3.1. Study population

Twenty-seven individuals diagnosed with FAP participated in the dietary intervention study and provided stool samples at T0, T1, and T2. All subjects underwent prophylactic colectomy before the intervention (mean age at colonoscopy: 27 years, range: 16 to 42). The median age at the dietary intervention was 40 years (range: 18 to 77), and 48% of participants were female (Supplementary Table 1A) [4]. Twenty-two subjects had a precancerous lesion at T0 (classified as adenoma, microadenoma, hyperplastic polyp, or hyperplasia) and seventeen at T2.

3.2. Stool miRNAs modulated by the diet

On average, 12.9 million reads were obtained for each sample, of which 88.7 ± 5.0% passed the quality control and pre-processing steps (Supplementary Table 1B), yielding 646.1 ± 67.7 miRNAs per sample. Twenty-nine miRNAs were DE in at least one comparison among time points ($p < 0.01$, |log2FC|>1, and median reads>15) (Fig. 1A,B, Supplementary Fig. 2A–B, Supplementary Tables 2A–B). Among them, 14 miRNAs were DE between T1 and T0, another 14 were between T2 and T0, and five were between T2 and T1. Faecal miRNA levels were more similar between T1 and T2 (rho (ρ) = 0.73,

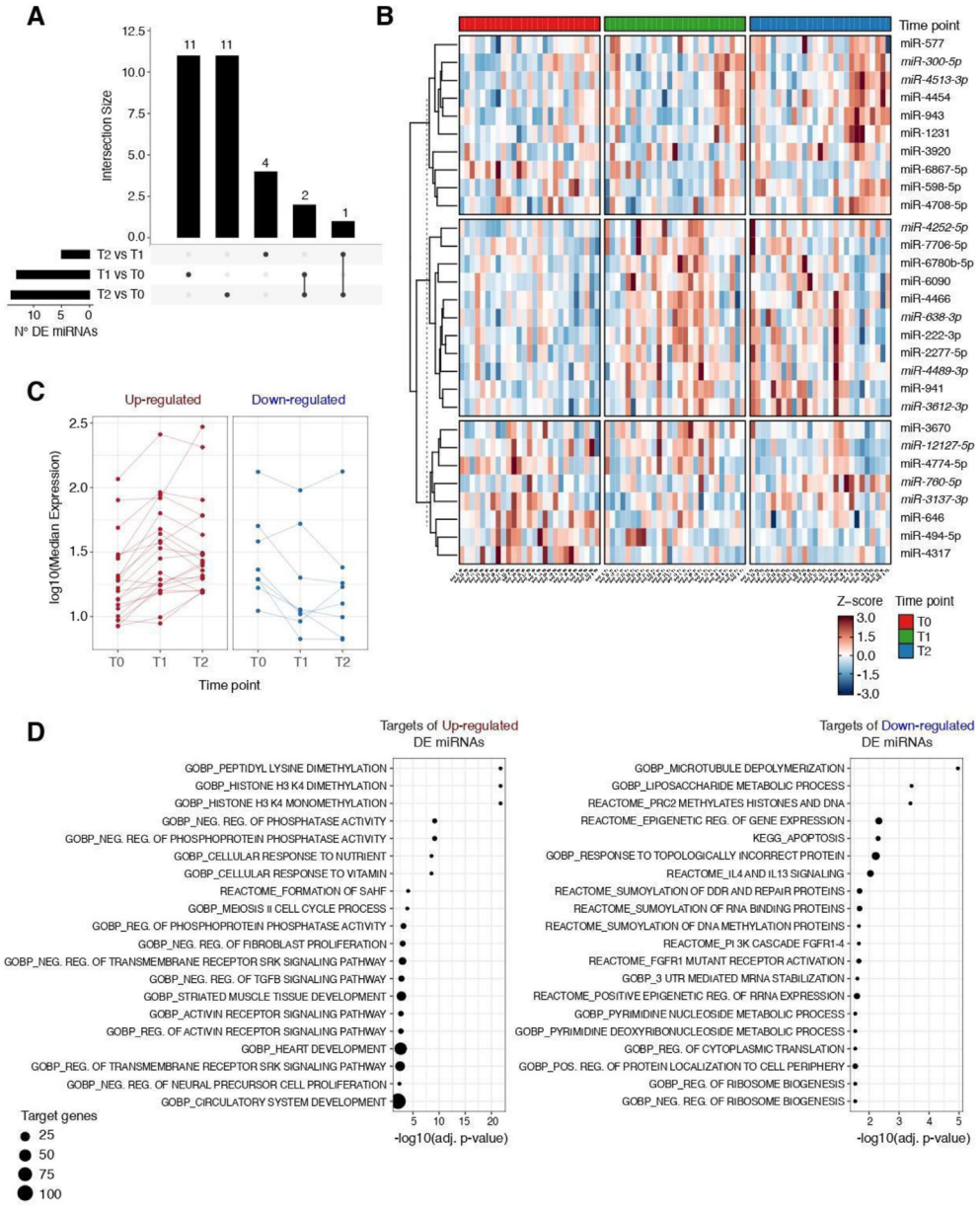

Fig. 1. Identification and characterisation of the miRNAs regulated by the diet. **A.** Upset plot reporting the number of differentially expressed (DE) faecal miRNAs detected in each time comparison (T0 = at the beginning of the intervention, T1 = 3 months after starting the intervention, T2 = 6 months after the intervention). **B.** Heat map representing the normalised DE miRNA expression levels in each sample according to time points. **C.** Line plots representing the median levels of DE miRNAs (on the left, the up-regulated, and, on the right, the down-regulated) at the three different time points. **D.** Dot plot representing the results of the functional enrichment analysis of the validated DE miRNA target genes (on the left, the up-regulated and, on the right, the down-regulated). The size of the dots represents the number of target genes included in each enriched process.

$p < 0.001$) than between T1 and T0 or T2 and T0 (Fig. 1C). After comparing DE miRNAs with public miRNA-target annotation databases, 3,557 validated miRNA-target interactions involving 1,934 genes and 19 DE miRNAs were found (Supplementary Table 3A). The miRNA-target interactions validated in the greatest number of studies were considered the most supported. Among miRNAs up-regulated after the intervention, the most supported interactions were miR-222-3p-*CDKN1B* (reported by 33 studies), miR-222-3p-*PTEN* (14 studies), miR-222-3p-*CDKN1C* (10 studies), miR-577-*ZBTB22* (10 studies) and miR-941-*HSPA6* (9 studies). Among the down-regulated miRNAs, we found miR-646-*OSCAR* (reported by 20 studies), miR-646-*PRR3* (10 studies), miR-6867-5p-*BDH1* (10 studies), miR-6867-5p-*DBT* (8 studies) and miR-646-*PPP1R11* (8 studies) (Supplementary Table 3B). Genes targeted by miRNAs whose levels increased after the diet were related to the cellular response to nutrients and vitamins, regulation of the Transforming Growth Factor β (TGFβ) family, and the SRC Proto-oncogene non-receptor tyrosine-protein kinase Src (SRC), among others. Conversely, genes targeted by miRNAs that decreased after the diet were mostly associated with apoptosis, regulation of gene expression, and inflammation (Fig. 1D, Supplementary Table 3C). A set of sncRNAs different from miRNAs was also found to be altered after the diet (Supplementary Results).

3.3. Correlation of stool miRNAs with MEDAS score and calprotectin levels

We assessed whether stool DE miRNA were correlated with previously reported changes in the MEDAS score and in calprotectin levels [4,5]. Three relevant ($\rho s \geq 0.50$) correlations were identified at

T1: miR-941 (ρs = 0.53) and miR-3612-3p (ρs = 0.53) were positively correlated with MEDAS score, while miR-3670 (ρs = −0.50) was negatively correlated with faecal calprotectin. Three other relevant correlations were observed at T2: miR-4252-5p and miR-3670 were negatively correlated respectively with faecal calprotectin (ρs = −0.57) and serum calprotectin (ρs = −0.60), while miR-6867 was positively correlated with serum calprotectin (ρs = 0.50) (Fig. 2A).

3.4. Comparison with stool miRNAs modulated by vegan and vegetarian diets

Faecal DE miRNA profiles were compared with those derived from a study we conducted on 120 healthy subjects with different dietary habits (40 omnivores, 40 vegetarians, and 40 vegans) for which detailed information on food and specific nutrient intake by validated questionnaires were also available (Supplementary Methods) [11]. Twelve miRNAs from the present study (41% of DE miRNAs) were also DE in subjects following vegetarian or vegan diets compared with omnivores. Nine of them were similarly modulated in both studies (Fig. 2B, Supplementary Table 2C).

Based on the correlations between miRNA profiles and nutrient intake retrieved from a validated food-frequency questionnaire [11], the correlation between the twelve common DE miRNAs and nutrient intake was computed, identifying 142 miRNA-nutrient associations (adj. p < 0.05, Fig. 2C, Supplementary Table 2D). The network representation of these associations showed miR-941 as the miRNA with the highest number of correlated nutrients (22), while cholesterol, total proteins, and animal lipids intake correlated with the highest number of miRNAs (10 each). The faecal miRNAs whose levels increased after the dietary intervention were

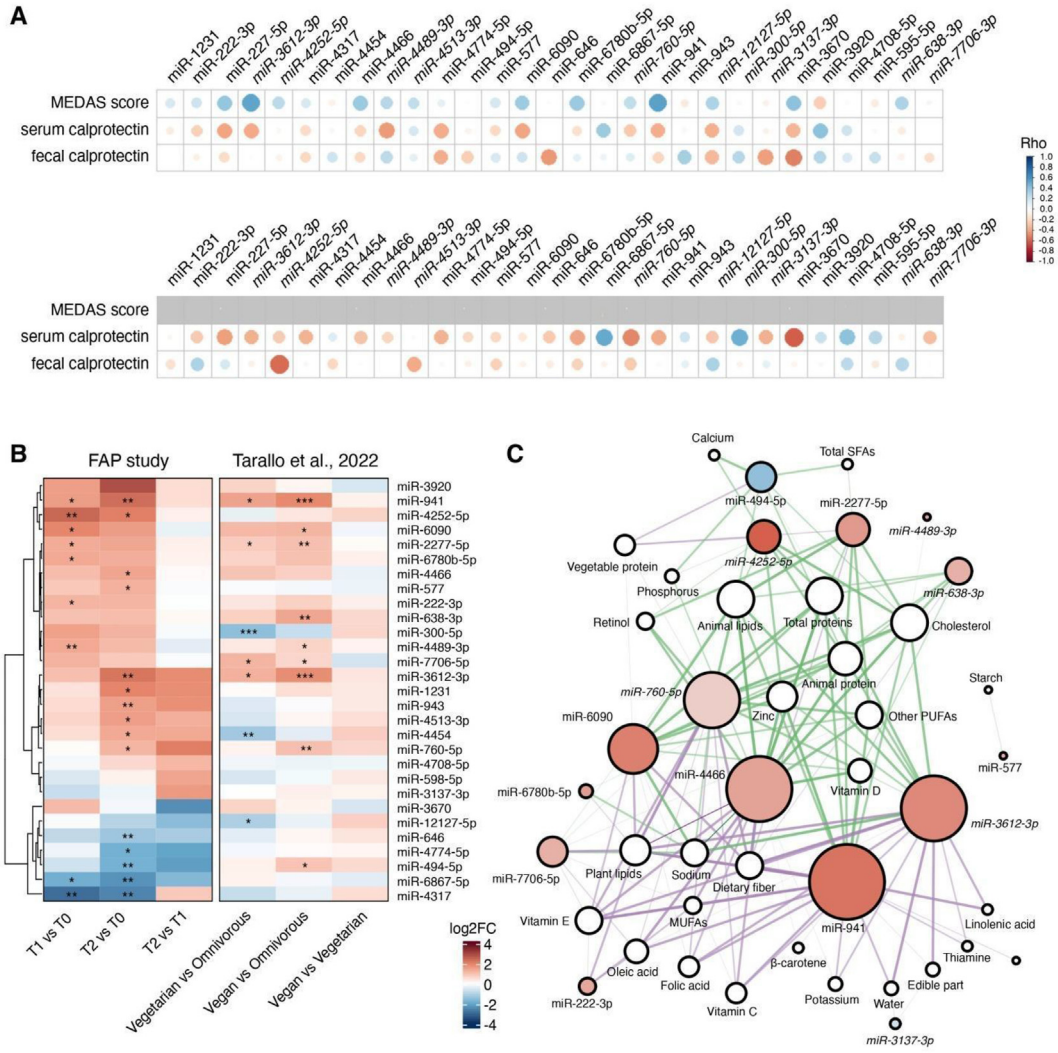

Fig. 2. Differentially expressed miRNAs and their correlation with clinical data and nutrient intake. A. Correlograms depicting the correlation strength (in terms of Spearman correlation coefficient, ρs) between each DE miRNA and the MEDAS score, faecal, and serum levels of calprotectin at T1 (up) and T2 (down). Size is determined by the ρs value, colour indicates positive (blue) and negative (red) correlations. **B.** Heat map reporting the log2 fold changes (log2FC) of stool DE miRNAs in subjects with FAP upon the dietary intervention (left panel) and in stool samples of vegan/vegetarian individuals with respect to omnivores as found in Tarallo et al., 2022. Adjusted p-value from DESeq2 analysis. ***adj. p < 0.001; **adj. p < 0.01; *adj. p < 0.05. **C.** Correlation network between DE miRNA levels and estimated nutrient intake from Tarallo et al., 2022. The node size represents the total node degree, while the edge width represents the correlation significance. miRNAs or nutrients characterised by the highest number of associations (network hubs) are represented as nodes with the largest size. Only significant correlations are reported (adj. p < 0.05). The miRNA node colour refers to the average log2FC computed between T1/T2 and T0, while the edge colour represents the correlation coefficient (green and purple for negative and positive correlations, respectively). Faecal miRNAs whose levels increased in samples collected after the dietary intervention (coloured in red) were positively associated (purple edges) with nutrients whose intake increased in a plant-based diet. Conversely, nutrients whose intake decreased in a plant-based diet were negatively correlated (green edges) with miRNA levels.

positively correlated with dietary fibres, plant lipids, and vitamins E and C, among others. Conversely, they were negatively correlated with total proteins, animal proteins, cholesterol, animal lipids, sodium, and zinc (Fig. 2C).

3.5. Genes modulated by the diet in rectal mucosa and adenoma

RNA-seq analysis evidenced seventy genes (adj. $p < 0.05$) DE between adenoma and normal tissue either before (64 genes) or after (22 genes) the intervention. Forty-eight genes were DE only at T0 and six only at T2, while 16 were similarly DE at both times (Fig. 3A,B, Supplementary Table 4A). The genes with the highest difference between T0 and T2 (|Δ log2FC T2 vs T0|>0.5) are reported in Table 1 and Fig. 3C.

The analysis also identified 10 DE genes between T0 and T2 in either adenoma or normal tissues (Supplementary Table 4A). DE genes were enriched in cell cycle regulation, ERK1/2 signalling, cellular homeostasis, and response to nutrient pathways. Comparison with cell type and disease-specific signatures showed that DE genes are related to cellular populations of the normal intestine

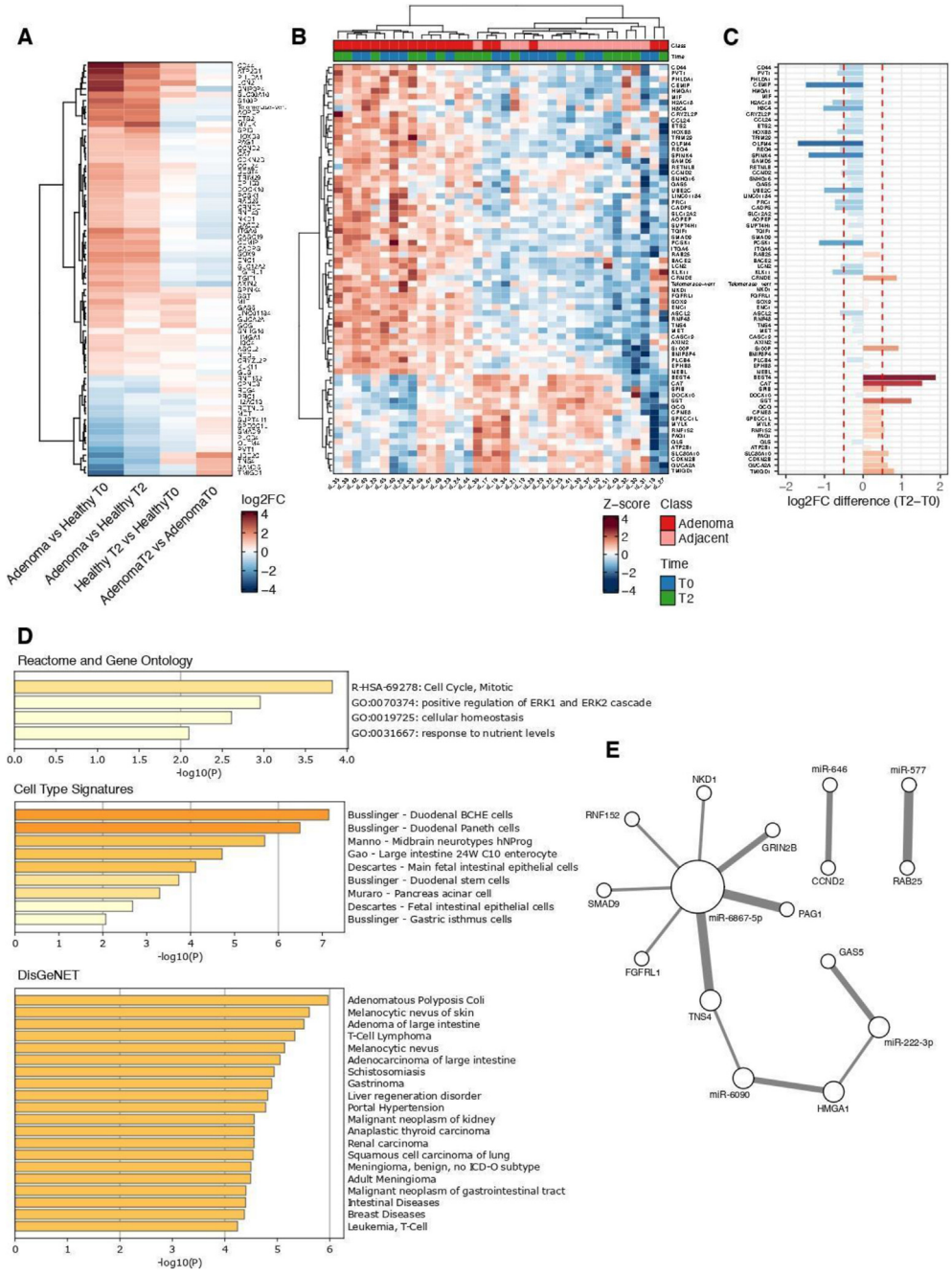

Fig. 3. Identification and characterisation of genes regulated by the diet and their association with miRNAs. A-B. Heatmaps reporting the log2FC (**A**) and the normalised levels (**B**) of DE genes identified between adenoma and normal colonic mucosa before or after the dietary intervention. **C.** Bar plot showing differences between adenoma vs adjacent mucosa. Bar height corresponds to the log2 of the T2 vs. T0 fold change (Δ log2FC T2 vs. T0), colour indicates a decrease (blue) or an increase (red) at T2 when compared to T0. The red dashed lines represent the threshold of 0.5. **D.** Bar plots showing biological processes (top), cell type-specific signatures (middle), and disease-specific gene sets (bottom) significantly enriched in the DE gene set. **E.** Network representation of the validated miRNA-target interactions involving the DE miRNAs and genes identified in the present study. The node size is proportional to the total node degree. The tightness of the edges is proportional to the number of evidence supporting the interaction.

Table 1
List of the differentially expressed genes between Adenoma and Normal tissues that lost significance after the dietary intervention (T2)[a].

Symbol	log2FC[b] at different time points			Adjusted p-value	
	T0	T2	|ΔT2-T0|	T0	T2
BEST4	−3.05	−1.17	1.88	<0.001	1
CA7	−2.64	−1.11	1.53	0.019	1
SST	−2.54	−1.28	1.25	0.001	0.921
TMIGD1	−2.07	−1.27	0.80	0.010	0.791
GUCA2A	−1.63	−0.98	0.64	0.021	1
SPIB	−1.60	−0.99	0.61	<0.001	0.200
SLC30A10	−1.48	−0.82	0.66	0.033	1
CPNE8	−1.34	−0.79	0.54	0.019	1
CDKN2B	−1.29	−0.77	0.52	0.018	0.875
RNF152	−1.05	−0.49	0.56	0.012	1
H3C4	0.94	−0.09	1.03	0.015	1
H2AC13	0.96	0.17	0.79	0.039	1
PRC1	1.04	0.30	0.73	0.023	1
CD44	1.14	0.52	0.62	0.002	1
CADPS	1.19	0.46	0.73	0.049	1
UBE2C	1.47	0.46	1.01	0.036	1
HOXB8	1.78	1.11	0.67	0.003	0.552
ASCL2	2.10	1.50	0.60	0.003	0.184
LCN2	2.15	1.57	0.58	0.014	0.360
KLK11	3.07	2.28	0.79	0.031	0.247
OLFM4	3.30	1.61	1.69	0.003	1
SPINK4	3.54	2.12	1.41	<0.001	0.279
CRNDE	1.95	2.81	0.87	0.420	0.042
S100P	0.90	1.82	0.92	1	0.011

[a] Only genes with |log2FC|>0.5 (computed considering both median and mean expression levels) are shown in this table. Full results are reported in Supplementary Table 4A.
[b] FC = fold change computed using the median expression levels.

and gastrointestinal cancers, including FAP-derived tumours (Fig. 3D).

3.6. Potential interactions of DE miRNAs and DE genes

Eleven DE genes presented at least one validated interaction with a DE miRNA (Supplementary Table 3B). A network representation of these interactions evidenced that miR-6867-5p was interacting with the highest number of DE genes (7), while *HMGA1* and *TNS4* mRNAs interacted with two miRNAs each. The most supported interactions were miR-577-*RAB25,* miR-6867-5p-*PAG1,* and miR-6867-5p-*TNS4* (three studies each) (Fig. 3E).

4. Discussion

We explored whether a dietary intervention conducted on subjects with FAP changed faecal miRNA profiles. Faecal miRNA levels indeed changed after the intervention, and changes were maintained for three months, with miR-941 and miR-3612-3p expression being correlated to the MEDAS score. Functional enrichment analysis of the validated miRNA-target interactions associated DE miRNAs with genes participating in the cellular response to vitamins and nutrients. miRNAs whose levels increased after the diet interact with genes involved in TGFβ regulation, while those decreasing after the diet interact with genes regulating DNA damage, apoptosis, and epigenetic regulation.

Some DE miRNAs have been previously reported to be regulated by dietary elements. Among these, miR-3920 and *miR-638-3p* increased after the intervention. Accordingly, miR-3920 expression was increased by the Mediterranean diet in coronary heart disease subjects [17], while *miR-638-3p* increased in celiac disease patients following a strict gluten-free diet [10]. Similarly, miR-646 decreased after the dietary intervention and was down-regulated in CRC patients consuming pomegranate juice [18].

The intervention also increased the levels of tumour suppressors miR-577, miR-4454, and miR-941 [19–21] and decreased miR-4317, which is highly expressed in CRC [22]. But, it also decreased the tumour-suppressor miR-646, and miR-1231, which is down-regulated in a CRC mouse model [23,24].

DE miRNAs up-regulated by the diet target genes associated with cell proliferation and apoptosis, while genes targeted by down-regulated miRNAs were involved in inflammatory response, ketogenesis, protein breakdown, and cell-to-cell adhesion. Altered miRNAs may also protect against other nutrition-related detrimental effects. The product of *ZBTB22*, targeted by up-regulated miRNAs, is a protein promoting insulin resistance and hyperglycemia [25], which in turn induces inflammation through Extracellular Signal-Regulated Kinases (ERK) 1/2 - Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [26]. Instead, *BDH1* mRNA, targeted by down-regulated miRNAs, codes for a protein inhibiting the overproduction of proinflammatory reactive oxidative species [27].

To analyse the relationship between faecal miRNA levels and nutrient consumption, we compared our results with those of a previous study on healthy vegan, vegetarian, or omnivorous subjects [11]. Some of the miRNAs hereby identified were also DE in vegan and vegetarian subject stool samples compared with omnivores. Most of these miRNAs were increased in both studies, including miR-941 and *miR-3612-3p*, which also correlated with the MEDAS score. Commonly regulated miRNAs were positively correlated with nutrients enriched in the diet (dietary fibre, plant lipids, and vitamins) and negatively with those reduced (animal proteins and lipids, cholesterol, and sodium) [4]. Thus, miRNA regulation by the low-inflammatory diet presented important similarities with that observed in vegans and vegetarians, possibly driven by common features.

miR-941 is particularly interesting, as it is increased by the diet and positively correlated with either MEDAS score and plant-derived nutrients. Its most supported validated target is *HSPA6*, which is correlated with advanced disease stages [28]. The observed regulation suggests that nutrients enriched in the low inflammatory diet can regulate miRNAs involved in cancer development.

Piwi-interacting RNAs (piRNAs), post-transcriptional silencers of transposable elements [29], were the most represented species among non-miRNAs sncRNAs altered after the diet. Recent evidence suggests that piRNAs may also modulate cancer development, having a role in colorectal neoplasms [30]. Given the predisposition of subjects with FAP to develop CRC, piRNAs possible role in modulating cancer development may be of interest for future research. A few years ago a study postulated the possible survival of exogenous miRNAs derived from food that can reach the intestine and be absorbed and ultimately exert a cross-kingdom gene expression regulation [31], although other researchers have subsequently challenged such findings [32]. We explored this hypothesis in a previous work, and the contribution of exogenous miRNAs to the faecal miRNome was indeed minimal [11]. Considering that extracellular miRNAs must reach a minimum concentration within a target cell to exert a biological effect [33,34], their contribution to the changes we observed was expected to be negligible.

Furthermore, the best way to protect exogenous miRNAs from degradation is to carry them via extracellular vesicles (EV), allowing them to survive the digestive tract. We did not evaluate the miRNAs contained in EVs but the freely circulating molecules primarily released in faeces by the host gut or the microbiome [35]. To further investigate whether the DE miRNAs identified in stool were expressed in intestinal tissue, we explored the chromatin states of duodenum, small intestine, sigmoid colon, colonic and rectal

mucosa, or colon smooth muscle cells as derived from Roadmap Epigenomics data [36]. The analysis confirmed that 22 out of the 33 identified DE miRNA genes were associated with an active epigenetic state (i.e., active TSS, strong/weak transcription, active enhancer) in almost all the samples analysed (Supplementary Table 5). Although further analyses are needed to evaluate such epigenetic regulation in the colonic tissue of FAP patients, this data provides evidence of the intestinal expression of the miRNAs detectable in stool as previously demonstrated in mice models [31].

Regarding the changes in mRNAs after the dietary intervention, we identified DE genes between normal and adenoma tissue and between time points in the same tissue type most of which are associated with immune response and inflammation. The DE gene profiles also coincided with those of several gastrointestinal cancers supporting the analysis results. Interestingly, most of the DE genes between adenoma and normal tissue at T0 reached similar expression levels at T2 suggesting that the diet reduces their expression differences. The tumour suppressor genes *SPIB*, *BEST4*, *CA7*, *SST,* and *GUCA2A,* coding for the precursor of guanylin, which activates the tumour suppressor *GUCY2C* [37–41], decreased in adenoma at T0 but showed similar levels in normal tissue and adenoma at T2. Conversely, the *HOXB8, ASCL2,* and *CADPS* genes, whose products are known to promote CRC proliferation and invasion, were overexpressed in adenoma at T0 but not at T2. Finally, the pro-inflammatory protein-coding genes *SLC30A10*, *CDKN2B,* and *RNF152*, associated with (Mitogen-Activated Protein Kinase) MAPK/ERK signalling, presented higher levels in normal tissue than in adenoma at T0 but decreased after the diet.

Two genes (*OLFM4* and *SPINK4*) coding for markers of normal epithelia pluripotent cells and goblet cells [42–44] had lower expression levels in normal tissue at T0 but reached similar levels as in adenomas after the intervention. Finally, the genes coding for markers of plasmacytoid dendritic cells and epithelial absorptive cells (*SPIB* and *BEST4*) [45,46] had lower expression levels in adenoma at T0 but reached similar levels as in normal tissue after the intervention. This suggests that the diet changes the gut epithelium cellular composition and increased populations depleted in inflammatory conditions and cancer. Finally, the levels of the oncogenic lncRNA *CRNDE* [47] and of the S100 Calcium Binding Protein P coding gene (*S100P*), which inhibits P53 [48], decreased in the normal tissue only at T2. Changes in DE genes suggest that the diet induces a less favourable environment for tumour development.

Our results support the hypothesis that miRNAs found in faeces may regulate gene expression in intestinal tissue since several of the DE genes are validated targets of stool DE miRNAs. In addition, functional analyses highlighted that up-regulated DE miRNAs and down-regulated DE genes are enriched in pathways associated with inflammation, cell cycle regulation, and response to nutrients. Validated interactions between DE miRNAs and DE genes further support an effect on inflammation as the genes involved are associated with Ras/ERK1/2 pathway, TGFβ, Wnt, and colon hypersensitivity.

We are aware of some limitations of the present study including the fact that tissue samples were available only at the beginning of the study (T0) and after six months (T2) as these were biopsies collected during endoscopies performed every six months following the standard INT procedure for surveillance of subjects with FAP. Moreover, the sampling of biopsies allowed us to perform only RNA-seq on a subset of subjects, and miRNAs were evaluated only in stool. Thus, we could not directly experimentally validate the interactions between DE miRNAs and DE genes in our samples. On the other hand, their possible interactions and functional analyses were assessed using bioinformatic tools based exclusively on validated miRNA-target interactions experimentally tested in other studies.

Finally, to our knowledge, this was the first dietary intervention in subjects with FAP and, accordingly, was designed as a pilot feasibility study [4]. As the study was primarily designed to determine the intervention feasibility, a one-arm pre-post design was considered suitable to measure possible modulations induced by the dietary intervention. In this design, measurements were taken in the same subject before (basal condition) and after the diet.

We are also aware that the low number of subjects included could impact the study relevance. However, the estimated sample size for the FAP-pilot trial can be considered appropriate according to both logistical and timeline issues and according to the general rule of thumb [4,49,50]. Based on the encouraging results of this pilot study in assessing the feasibility of the diet intervention, as well as the observed reduction of inflammation and the improvement of the quality of life of subjects with FAP, we have now planned a larger two-arm randomised clinical trial in FAP subjects to explore the association between diet and inflammation and to better understand the mechanisms by which diet effects may influence disease progression.

To conclude, we have shown that a low-inflammatory diet regulates miRNAs and gene expression in the gut and its effect persists even after the dietary intervention. The diet reduces inflammation and regulates miRNAs and genes with oncogenic and tumour-suppressing functions suggesting a preventive effect on developing adenomas and cancer. The present pilot study highlights the importance of specific dietary modifications as an adjunct to existing management options for subjects with FAP.

Availability of data and material

Datasets are available in a public, open-access repository. Raw small RNA-seq data were deposited on Gene Expression Omnibus (GEO identifier: GSE218823). Raw RNA-seq data were deposited on GEO (identifier: GSE222298).

Authors' contributions

OI, GF, LR, PV, PP, MV, AN and MG conceptualization; OI, GF, AB, BP, ST, SN, ED, DM investigation; OI, GF, CMC, FC, PV and MG Formal Analysis; SS, AM, MV, LC and MM Resources; OI, GF, BP, AN and MG Writing – original draft. Writing – review & editing the final manuscript: all authors. AN, MG, MV funding acquisition.

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Conflicts of interest

No relevant conflicts of interest exist for all the authors. No funding sources had a role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and the decision to submit the manuscript for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2024.02.023.

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