

Exploring the faecal miRNA profile in familial Mediterranean fever patients and the role of miR-21-5p in inflammatory pathways associated with FMF pathogenesis

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Abstract

Objective

Beyond MEFV mutations, epigenetics seems to play an important role in the manifestation of familial Mediterranean fever (FMF). This study aimed to evaluate the differential expression of selected miRNAs in stool samples from FMF patients, and to investigate the functional role of miR-21-5p in FMF-related inflammatory pathways.

Methods

Thirty-four FMF patients, 15 patients with Clostridioides difficile infection (CDI), and 25 healthy controls were enrolled in this study. The faecal levels of 27 miRNAs associated with inflammation, apoptosis, and autophagy were quantified by qRT-PCR. The functional role of miR-21-5p in regulating NF-κB and pyrin inflammasome pathways was investigated in LPS-stimulated THP-1 derived macrophages transfected with an anti-mir-21-5p inhibitor via western blot.

Results

Among the 27 analysed miRNAs, eight were significantly upregulated in FMF patients compared with controls, with miR-21-5p, miR-30e-3p, and miR-148a-3p uniquely elevated in FMF. Downregulation of the anti-inflammatory miR-186-5p and miR-374b-5p was observed particularly in M694V homozygous patients, while let-7d-5p, and miR-148b-3p were associated with higher disease severity. Functional inhibition of miR-21-5p in LPS-stimulated THP-1-derived macrophages reduced NF-κB activation and decreased the expression of inflammasome-associated proteins except ASC, consistent with ASC-independent modulation of inflammasome signalling, potentially through direct caspase-1 activation.

Conclusion

Our findings highlight the role of miRNAs in intestinal inflammation and their potential as diagnostic and prognostic biomarkers in FMF. Our functional data underscore the regulatory role of miR-21-5p in FMF-related inflammatory pathways and warrant further studies to elucidate its therapeutic potential, particularly in patients refractory to colchicine or as an adjunct to existing treatments.

Key words

familial Mediterranean fever, epigenetics, microRNA, severity, inflammasome, NF-κB

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Introduction

Familial Mediterranean fever (FMF) is the most prevalent autoinflammatory disease worldwide, affecting predominantly Eastern Mediterranean populations (1, 2). It is an autosomal recessive disease caused by mutations in the *MEFV* (Mediterranean FeVer) gene on chromosome 16 (16p13.3), which encodes the pyrin protein. Pyrin plays a central role in innate immunity, with its mutated form leading to dysregulated inflammasome activation, excessive pro-inflammatory cytokine release, mainly IL-1 β , and persistent subclinical inflammation (3-5).

Clinically, FMF is characterised by a spectrum of symptoms ranging from fever, abdominal pain, arthralgia, arthritis, and thoracic pain to the most severe FMF complication, amyloidosis (6). In this respect, monitoring and controlling inflammation is important to avoid long-term consequences (7), with colchicine being the mainstream therapeutic option for FMF patients (8). Biologics, such as IL-1 β inhibitors (anakinra and canakinumab), are alternatives to colchicine resistant/intolerant patients (9).

Clinical and treatment-response variability in FMF is largely attributed to *MEFV* allelic heterogeneity, with M694V associated with the most severe phenotype (10, 11). Environmental factors (12), genetic modifiers (13), and epigenetic mechanisms may further contribute to disease heterogeneity (14). Epigenetic regulation modulates gene expression without altering the DNA sequence (15). Among its key mechanisms, microRNAs (miRNAs) are small, conserved, non-coding RNAs that regulate gene expression through RNA interference (16). They also act as signalling molecules that mediate intercellular communication by being secreted and transferred between cells (17).

In recent years, several studies have investigated the possible roles of miRNAs in FMF. miRNAs have been proposed as potential biomarkers for FMF and were associated with key pathological processes, including inflammation, apoptosis, and autophagy (14). Nevertheless, these studies have been conducted predominately using

blood, serum, or plasma samples and have yielded inconsistent results. To date, the faecal miRNA profile remains unexplored, despite the frequent gastrointestinal manifestations of FMF, making stool a relevant sample for investigating disease-related molecular changes. Therefore, the objectives of this research are to: (1) evaluate the differential expression of 27 selected miRNAs, implicated in inflammation, apoptosis, and autophagy, in stool samples of FMF patients and controls, and analyse their association with patients' genotypes and disease severity; (2) assess the levels of pro-inflammatory cytokines IL-1 β , IL-8, IL-6, and TNF- α in faecal samples of FMF patients; and (3) investigate the functional role of miR-21-5p, a candidate miRNA, in FMF-related inflammatory pathways.

Materials and methods

Study design and participants

Thirty-four clinically confirmed Lebanese FMF patients, aged 18-65 years, along with 25 age- and gender-matched healthy controls were included in this study. Healthy individuals were free of chronic diseases or inflammation and were not taking any medication or antibiotics. Additionally, 15 patients with *Clostridioides difficile* infection (CDI) were included as a comparative group to establish miRNA specific profile to FMF patients.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Lebanese American University (LAU) (IRB #: LAU.SAS.JI2.6/Apr/2023). Written informed consent was obtained from all participants prior to enrolment. All collected data were de-identified and maintained anonymously to ensure confidentiality.

Data collection

FMF patients completed a structured questionnaire designed to collect information on sociodemographic characteristics, genetic and family background, age of disease onset, clinical manifestations, comorbidities and treatment modalities.

MEFV genotyping was performed using Sanger sequencing, and disease

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severity was assessed using the International Severity Scoring System for FMF (ISSF) (18). The ISSF is a 10-item scale with a maximum score of 10, categorising patients into mild (≤ 2), intermediate (3–5), and severe disease (≥ 6).

Stool samples collection and faecal miRNA extraction

Fresh stool samples (20–30 g) were collected at LAU Medical Center-Rizk Hospital, stored at 4°C, and transferred to -80°C within 24 h. Approximately 200–300 mg of stool was homogenised in 1 mL TRIzol LS reagent using an RNase-free pestle and vortexing, followed by the addition of 300 μ L chloroform. Total RNA enriched in miRNAs was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and eluted in 40 μ L nuclease-free water. RNA integrity was assessed by gel electrophoresis, while concentration and purity were measured using NanoDrop 2000 and Qubit (260/280 and 260/230 ratios).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

miRNA expression in stool was measured by qRT-PCR. cDNA was synthesised using the miRCURY LNA RT kit (Qiagen) and amplified with the miRCURY LNA SYBR Green PCR Kit using specific primers (Supplementary Table S1). Cycling conditions were 2 minutes at 95°C (initial activation), followed by 40 cycles at 95°C for 10 seconds and 56°C for 1 minute. At the end, a melting curve was performed to verify amplification specificity. Expression levels were normalised to the most stable miRNA, showing the least variability among samples, and reported as fold change relative to healthy individuals or other FMF subgroups, using the $2^{-\Delta\Delta CT}$ method.

Supernatants collection and cytokine measurement

Cytokines levels in faecal samples were assessed by diluting 2g of stool in 1:2 PBS containing PMSF (1 mg/mL) and soy trypsin inhibitor (1 mg/mL). Following centrifugation at 10,000g for 15 minutes at 4°C, the supernatants

Table I. Differentially expressed miRNAs in FMF patients compared with healthy controls and *Clostridioides difficile* infection (CDI) patients.

miRNAs	Fold change relative to controls	p-value	Fold change relative to CDI patients	p-value
miR-30e-3p	5.62 $\pm$ 39.78	0.0379*	8.37 $\pm$ 48.10	0.0233*
miR-144-3p	4.41 $\pm$ 13.33	0.0489*	0.20 $\pm$ 3.65	0.065
miR-21-5p	2.68 $\pm$ 3.12	0.0001***	3.34 $\pm$ 6.87	0.0032**
miR-320d	2.50 $\pm$ 4.22	0.0025**	1.27 $\pm$ 2.41	0.633
miR-148a-3p	2.28 $\pm$ 2.75	0.0378*	1.99 $\pm$ 4.03	0.0109*
miR-29b-3p	1.78 $\pm$ 2.04	0.0479*	0.43 $\pm$ 1.31	0.570
miR-29c-3p	1.75 $\pm$ 1.35	0.0266*	1.28 $\pm$ 0.92	0.590
miR-148b-3p	1.52 $\pm$ 1.36	0.0246*	0.40 $\pm$ 0.78	0.731

Expression levels were normalised using miR-574-3p as a reference.

Values are presented as mean $\pm$ SD. Comparisons between groups were performed using the non-parametric Mann-Whitney test, with significance indicated as follows: * $p < 0.05$; ** $p < 0.01$; or *** $p < 0.001$. miRNAs highlighted in bold are uniquely regulated in FMF patients.

were filtered and stored at -80°C. IL-1 β , IL-8, IL-6, and TNF- α levels were measured in duplicate using Enzyme-Linked Immunosorbent Assay (ELISA) kits according to the manufacturer's instructions (PeproTech).

Cell culture and transfection

THP-1 cells, a human monocytic cell line, were used as an *in vitro* model of monocyte/macrophage-mediated inflammatory responses relevant to FMF (19). Cells were cultured in RPMI with 10% FBS and 1% MEM non-essential amino acids. THP-1 cells were seeded at 3×10^5 cells/well in 24-well plates and differentiated into macrophage-like cells with 50 ng/mL PMA overnight. Cells were then transfected with 50 nM anti-hsa-miR-21-5p or miRNA inhibitor control using Lipofectamine 2000 in Opti-MEM. Six hours post-transfection, cells were stimulated with 1 μ g/mL LPS and incubated for 2, 6, and 24h at 37 °C with 5% CO₂.

Western blot analysis

Western blots were performed to assess NF- κ B and pyrin inflammasome protein after miR-21-5p inhibition. At 2, 6, and 24h post-LPS stimulation, cells were lysed in RIPA buffer with protease and phosphatase inhibitors. Proteins were quantified using the DC protein quantification kit (Bio-Rad), separated by SDS-PAGE, and then transferred to PVDF membranes. Membranes were blocked with 5% fat free milk or 5% BSA in 1X TBST-0.1% Tween 20 for 1h and then incubated overnight at 4°C with primary

Table II. Differential expression of miRNAs in M694V homozygous FMF patients with severe disease relative to intermediate severity.

miRNAs	Fold change (severe vs. intermediate)
miR-130a-3p	4.53 $\pm$ 18.02
miR-4281	2.91 $\pm$ 4.93
miR-4285	2.30 $\pm$ 0.08
miR-320e	1.97 $\pm$ 0.27
miR-222-5p	1.96 $\pm$ 2.75
let-7d-5p	1.93 $\pm$ 0.02
miR-320d	1.82 $\pm$ 0.66
miR-144-3p	0.14 $\pm$ 0.31
miR-146a-5p	0.46 $\pm$ 0.38

Values are represented as mean $\pm$ SD.

antibodies against I κ B α (1:1000, Cell Signalling Technology (CST)), p-NF- κ B p65 (1:200, Santa Cruz Biotechnology), pyrin (1:1000, Proteintech), ASC (1:1000, CST), IL-1 β (1:1000, CST), cleaved IL-1 β (1:1000, CST), caspase-1 (1:1000, CST), cleaved caspase-1 (1:1000, CST), and β -actin (1:1000, CST). Membranes were then incubated with HRP-conjugated anti-rabbit (CST) or anti-mouse (Santa Cruz Biotechnology) secondary antibodies. Protein bands were visualised by enhanced chemiluminescence using the Clarity™ Western ECL Blotting Substrate and ChemiDoc XRS+ system. Bands were quantified using the Image Lab Software, and expression ratios were calculated relative to the loading control (β -actin).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 10. Data for ex-

periments performed at least twice is presented as mean $\pm$ standard deviation (SD), and a p -value of <0.05 was considered significant. The statistical tests used were the non-parametric Mann Whitney and Kruskal-Wallis one-way ANOVA.

Results

Demographic and clinical features

No significant differences in age or gender were observed between FMF patients and controls. The mean age at diagnosis among FMF patients was 12.77 ± 9.97 years. Abdominal pain was the most common clinical manifestation (94.1%). Other gastrointestinal symptoms included constipation (44.1%), diarrhoea (37.3%), and vomiting (29.4%). Notably, three patients were diagnosed with amyloidosis, and most patients (88.2%) were receiving colchicine treatment, with daily doses ranging from 0.5 to 2 mg. Regarding disease severity, 14.7% of patients had mild disease, whereas 70.6% and 14.7% exhibited intermediate and severe phenotypes, respectively (Suppl. Table S2A).

Genetic analysis revealed homozygous *MEFV* mutations in 17 patients, including nine homozygous for the M694V variant. Compound heterozygosity was identified in 10 patients, while the remaining seven carried either a single pathogenic variation ($n=5$) or a variant of uncertain significance (VUS) ($n=2$) (Suppl. Table S2B).

miR-574-3p as a reference gene for faecal miRNA quantification

No universally accepted reference miRNA exists for normalisation in human faecal samples, likely due to stool's heterogeneous composition, which comprises the gut microbiome and shed colonocytes (20). To identify the most appropriate reference miRNA for normalisation, commonly used endogenous controls, including *U6*, *RNU5G*, *SNORA66*, and *GAPDH*, were initially evaluated but were not detected in our samples. The expression stability of the 27 selected miRNAs was then assessed to identify the most stable candidate, ensuring more accurate normalisation than exogenous or poorly expressed

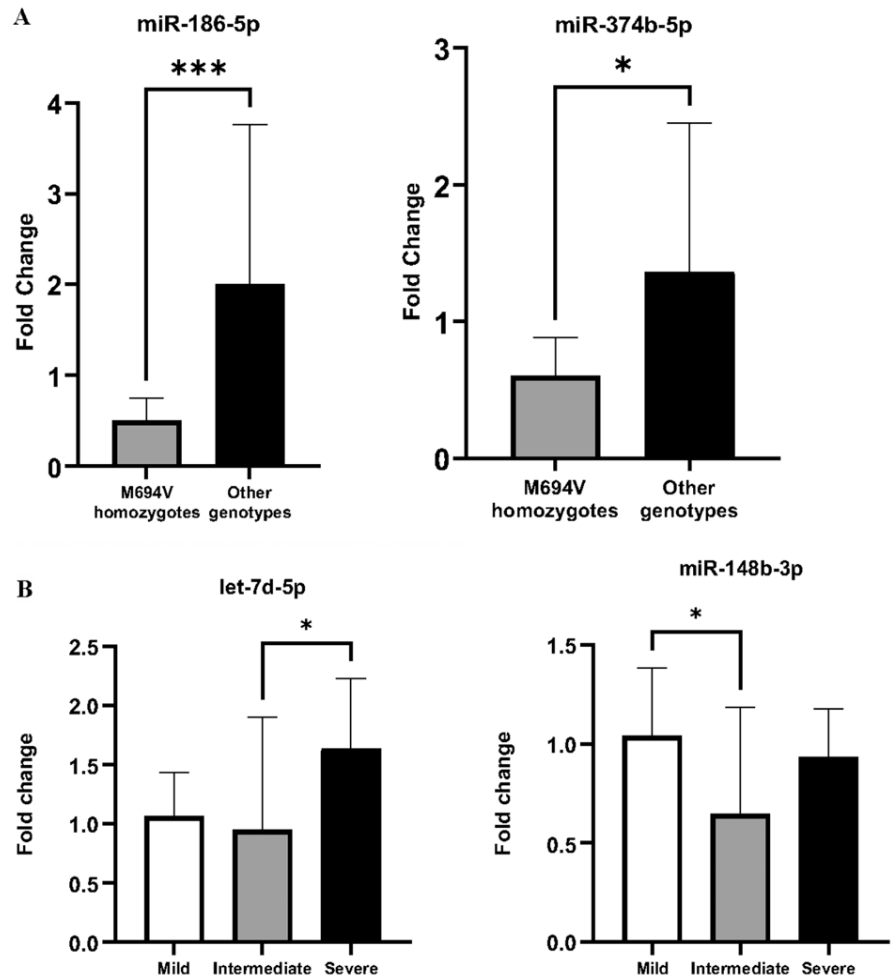


Fig. 1. Differentially expressed miRNAs in FMF patients according to (A) *MEFV* genotype and (B) disease Severity. **A.** miR-186-5p and miR-374b-5p were downregulated by 4.1- and 2.3-fold, respectively, in M694V homozygotes ($n=9$) compared with other *MEFV* genotypes ($n=25$). **B.** Compared to the intermediate phenotype group ($n=24$), patients with severe disease ($n=5$) showed a 1.7-fold upregulation of let-7d-5p, whereas those with mild disease ($n=5$) exhibited a 1.6-fold upregulation of miR-148b-3p. Data are presented as mean $\pm$ SD. Statistical comparisons among groups were performed using the non-parametric Mann-Whitney test or Kruskal-Wallis test followed by Dunn's multiple comparison test. Significance is indicated as $*p<0.05$ and $***p<0.0001$.

small RNAs (21). The analysis revealed that miR-574-3p exhibited the least variability across FMF patients, CDI patients, and healthy subjects, with a coefficient of variation (CV) of 5.37%, indicating high expression stability, and was therefore chosen as the reference gene in our cohort.

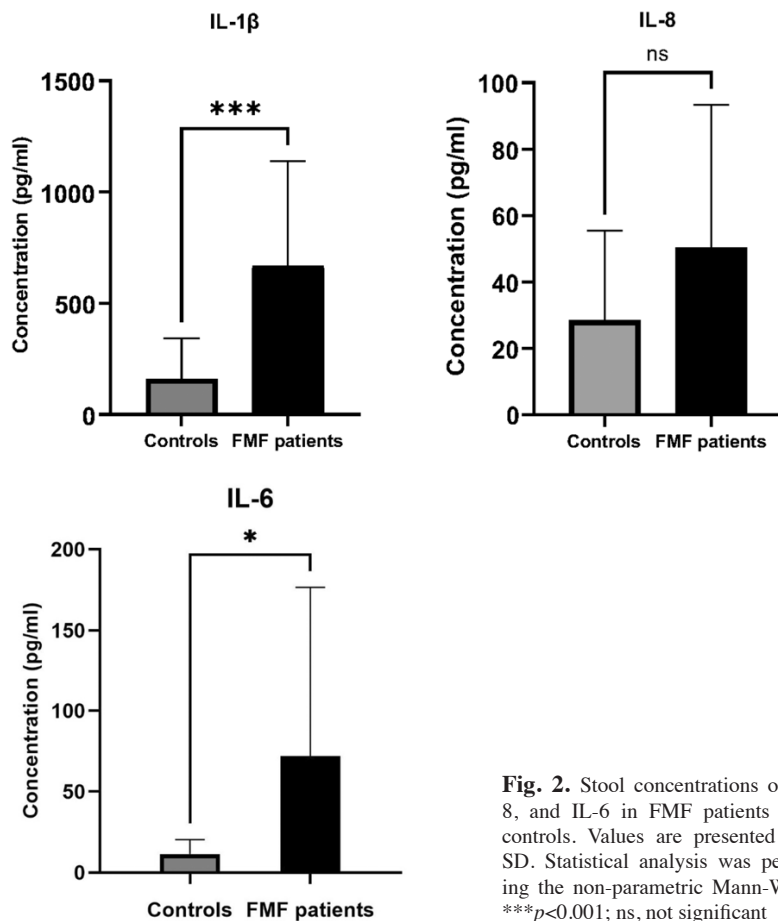
Distinct miRNA expression profile in FMF patients compared to healthy subjects and CDI patients

Among the 27 analysed miRNAs, miR-181-3p and miR-130b-5p had Cq values >36 and were excluded from further analysis, as they were not detected across all tested groups. Of the remaining 25 miRNAs, miR-30e-3p, miR-

144-3p, miR-21-5p, miR-320d, miR-148a-3p, miR-29c-3p, miR-29b-3p, and miR-148b-3p were significantly upregulated in FMF patients compared to controls. Among these, only miR-30e-3p, miR-148a-3p, and miR-21-5p, remained significantly upregulated when FMF patients were compared with CDI patients. Notably, miR-30e-3p showed the highest increase in FMF patients (8.4-fold), followed by miR-21-5p (3.3-fold) and miR-148a-3p (2-fold) (Table I).

Distinct miRNA expression patterns across MEFV genotypes and FMF severity

To assess associations with *MEFV*



genotype and disease severity, patients were stratified into subgroups. Given its association with severe FMF, M694V homozygotes ($n=9$) were compared with patients with other genotypes ($n=25$). Of the 25 analysed miRNAs, only miR-186-5p and miR-374b-5p were significantly downregulated in M694V homozygotes (Fig. 1A).

Patients were also categorised by disease severity into mild ($n=5$), intermediate ($n=24$), and severe ($n=5$) using the ISSF. Two miRNAs, let-7d-5p and miR-148b-3p, showed significant variation across disease severity groups. Specifically, let-7d-5p was 1.6-fold upregulated in severe compared with intermediate patients, whereas miR-148b-3p showed a 1.7-fold increase in mild compared with intermediate patients (Fig. 1B).

Notably, among M694V homozygotes, seven patients had intermediate and two had severe disease. Their miRNA profiles were compared, revealing seven upregulated miRNAs, miR-130a-3p, miR-4281, miR-4285, miR-320e, miR-222-5p, let-7d-5p and miR-320d, and two downregulated miRNAs, miR-144-

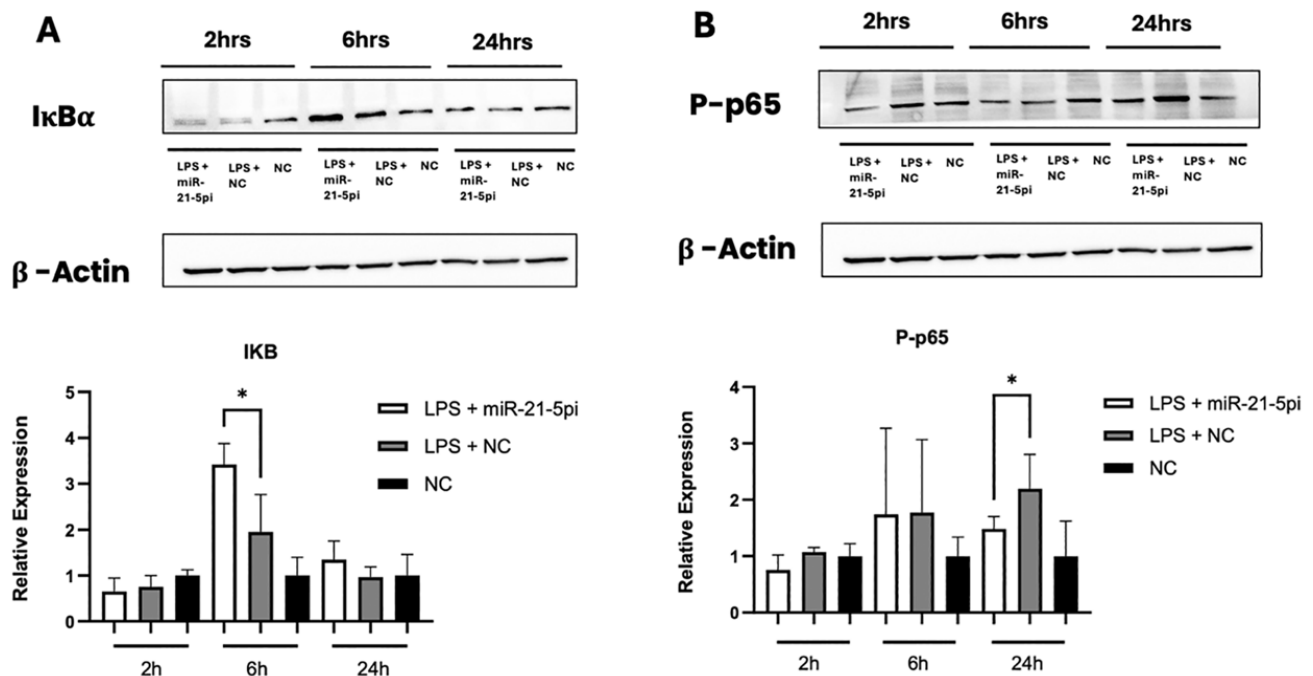
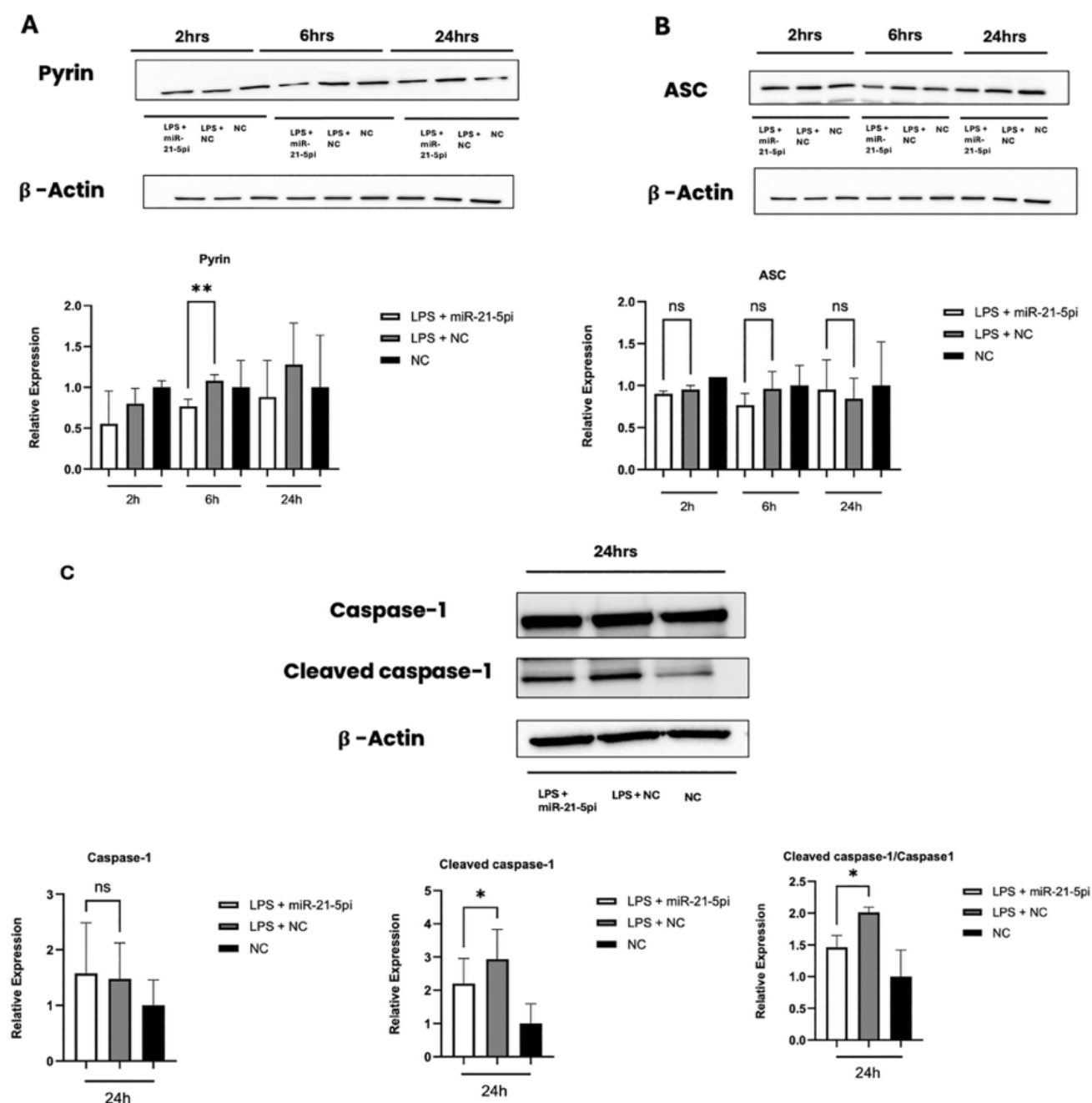


Fig. 3. Effect of miR-21-5p knockdown on IkB α and p-p65 protein expression in THP-1-derived macrophages. Inhibition of miR-21-5p resulted in a 1.8-fold increase in IkB α levels at 6h (A) and a 1.5-fold decrease in p-p65 levels at 24h (B) after LPS stimulation compared with LPS-stimulated cells transfected with control (anti-NC). β -actin was used as a loading control. NC (non-stimulated cells transfected with anti-NC) provides a baseline reference to account for effects of transfection and LPS stimulation. Western Blot experiments were performed independently three times ($n=3$). Statistical analysis was conducted using the non-parametric Mann-Whitney test to compare untreated and treated conditions. Significant differences are indicated as * p <0.05



3p and miR-146a-5p in patients with severe disease compared to those with intermediate severity (Table II).

Increased IL-1 β and IL-6 concentrations in stool samples of FMF patients

IL-1 β , IL-8, IL-6, and TNF- α levels were measured in the supernatants of stool samples from FMF patients and healthy controls. IL-1 β and IL-6 levels were significantly higher in FMF patients (668.3 $\pm$ 470 pg/ml and 72 $\pm$ 104 pg/mL, respectively) compared with con-

trols (160.2 $\pm$ 182 pg/ml and 11.41 $\pm$ 8.95 pg/mL, respectively). In contrast, IL-8 levels were relatively low and did not differ significantly between patients and controls, with mean concentrations of 50.4 $\pm$ 38.8 pg/mL and 28.5 $\pm$ 25.4 pg/mL, respectively. TNF- α levels were below the detection limit and were therefore undetectable in both groups (Fig. 2).

miR-21-5p modulates NF- κ B and pyrin inflammasome activity in FMF pathogenesis

The increased expression of miR-21-

5p in FMF patients, relative to both healthy controls and CDI patients, suggests a potential role for this miRNA in modulating inflammation in FMF pathogenesis. While other miRNAs exhibited variability mainly within patient subgroups, miR-21-5p was consistently and significantly elevated across all FMF patients and was therefore selected for further investigation. To examine its inflammatory role, THP-1-derived macrophages were transfected with anti-miR-21-5p and subsequently stimulated with LPS at 2, 6, and 24h to cap-

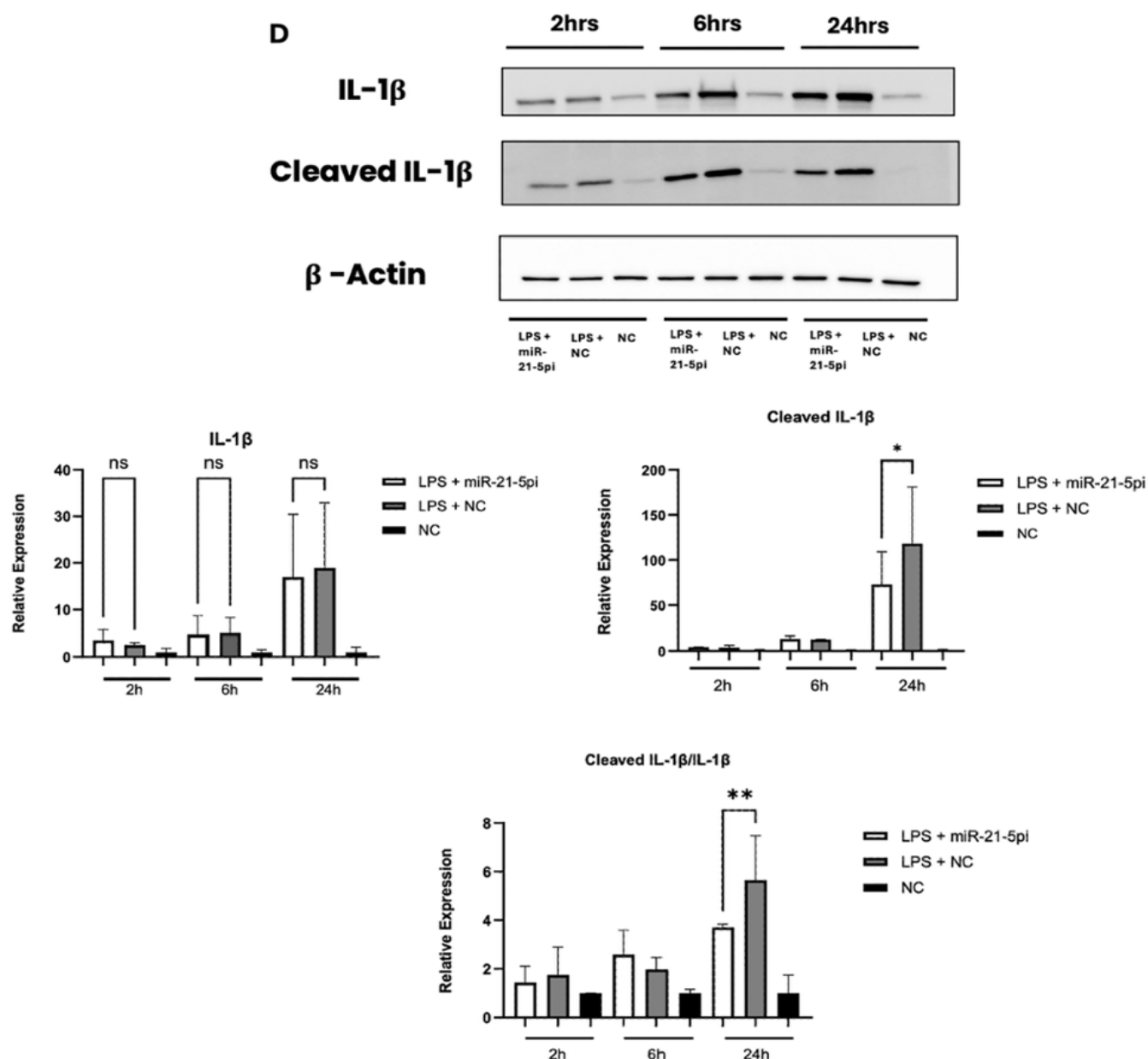


Fig. 4. Effect of miR-21-5p knockdown on inflammasome-related protein expression in THP-1 derived macrophages assessed. miR-21-5p knockdown resulted in a 1.4-fold decrease in pyrin expression at 6h post-LPS stimulation (A) with no significant change in ASC (B); a 1.4- and 1.5-fold decrease in the cleaved/total caspase-1 (C) and cleaved/total IL-1 β (D) ratios at 24h post-LPS stimulation. β -actin was used as a loading control. NC (non-stimulated cells transfected with anti-NC) served as a baseline reference. Western Blot experiments were performed independently three times. Statistical analysis was conducted using the non-parametric Mann-Whitney test to compare untreated and treated conditions. Significant differences are indicated as * $p < 0.05$ and ** $p < 0.01$; ns, not significant.

ture the kinetics of protein activation and processing. Post-stimulation, the levels of key proteins involved in NF- κ B and pyrin inflammasome pathways were assessed.

Western blot analysis revealed a significant 1.8-fold increase in I κ B α expression at 6h and a 1.5-fold reduction in p-p65 levels at 24h post-LPS stimulation in cells transfected with anti-miR-21-5p compared to those transfected with anti-NC (Fig. 3).

Regarding inflammasome activity, miR-21-5p inhibition significantly decreased pyrin protein levels by 1.4-fold at 6h post-LPS stimulation. At 24h, the cleaved caspase-1/total caspase-1 ratio was reduced by 1.4-fold, indicating decreased caspase-1 activation. Consistently, the cleaved IL-1 β /total IL-1 β ratio showed a 1.5-fold decrease at the same time point. In contrast, ASC expression remained unchanged following miR-21-5p inhibition (Fig. 4).

Discussion

In recent years, miRNAs have emerged as key modulators in FMF, with studies reporting distinct or inconsistent expression profiles in blood, plasma, or serum. However, their expression in alternative biological samples, such as faeces, remains unexplored, and their relationship with *MEFV* mutations, clinical variability, and underlying inflammatory pathways is poorly understood. Given the predominance of gastrointestinal symp-

toms in FMF, faecal miRNA profiling offers a novel approach to investigate intestinal inflammation and its molecular underpinnings. Faecal miRNAs are stable biomarkers in gastrointestinal disorders, including inflammatory bowel disease and colorectal cancer (22, 23), owing to their protection within extracellular vesicles or RNA-binding proteins (24), supporting their investigation in FMF.

Out of the 27 selected miRNAs, eight (miR-30e-3p, miR-144-3p, miR-21-5p, miR-320d, miR-148a-3p, miR-29c-3p, miR-29b-3p and miR-148b-3p) were significantly upregulated in FMF patients compared to controls. Several findings are consistent with previous reports in other biological matrices, as miR-144-3p, miR-320d, and miR-29b-3p were shown to be elevated in PB-MCs, serum, and whole blood, respectively (25-27). In contrast, miR-29c-3p and miR-148b-3p, upregulated in our faecal samples, were previously reported as downregulated in blood and PB-MCs (25, 27), while miR-21-5p showed inconsistent patterns across studies (27, 28). These discrepancies likely reflect differences in sample type and study design, but may also highlight the contribution of the gut microenvironment, where faecal miRNAs can capture localised host-microbiota interactions (29).

To further assess the FMF-associated miRNA profile, we compared its expression with CDI patients, a relevant inflammatory control characterised by toxin-mediated activation of the pyrin inflammasome (30). Among the eight miRNAs elevated in FMF *versus* healthy controls, only three, miR-21-5p, miR-30e-3p, and miR-148a-3p, remained significantly upregulated when compared to CDI patients, suggesting a more disease-specific signature. IBD represents another relevant comparison given its chronic intestinal inflammation. Although IBD patients were not included in our cohort, studies have reported dysregulation of faecal miRNAs, including miR-16, miR-21, miR-155, miR-223, and miR-1246 (22, 31, 32). Notably, faecal miR-21-5p was increased in ulcerative colitis (31), consistent with our FMF findings. However, the other significantly upregulated

miRNAs in FMF, miR-30e-3p, miR-144-3p, miR-320d, miR-148a-3p, miR-29c-3p, miR-29b-3p and miR-148b-3p, have not been consistently reported in major IBD faecal miRNA studies. Together, the CDI and IBD comparisons suggest that the FMF-associated profile extends beyond that observed in intestinal inflammatory conditions. The potential contribution of these three FMF-associated miRNAs to disease pathogenesis is further supported by their reported roles in inflammatory and immune regulatory pathways. miR-30e-3p directly targets IL-1 β and reduces its expression in systemic autoimmune-inflammatory diseases, including FMF, CAPS, TRAPS, and MKD (33), while miR-148a suppresses inflammation via inhibition of the TLR4/NF- κ B pathway in LPS-induced endometritis (34). Their upregulation may reflect a compensatory response to limit excessive inflammation, potentially contributing, together with colchicine treatment, to resolution of inflammatory flares and maintenance of asymptomatic periods. Conversely, colchicine may also influence the expression of these miRNAs in our cohort, suggesting a bidirectional interplay that warrants further investigation. In contrast, miR-21-5p has been widely associated with pro-inflammatory effects, including activation of the IL-6/STAT3 pathway in ulcerative colitis (35) and promotion of NLRP3 inflammasome activity in both mice and human myeloid cells (36). The concurrent upregulation of miRNAs with opposing regulatory roles may reflect the complex interplay between inflammatory activation and counter-regulatory mechanisms in FMF.

Interestingly, stratified analyses revealed distinct miRNA expression patterns across genetic and clinical subgroups of FMF. When grouped according to *MEFV* genotype, miR-186-5p and miR-374b-5p were significantly downregulated in patients homozygous for the M694V mutation compared with those harbouring other genotypes. Classification based on clinical severity showed upregulation of let-7d-5p and downregulation of miR-148b-3p in patients with more severe disease. miR-186-5p and miR-374b-5p have previ-

ously been reported to be more highly expressed in patients with milder FMF compared with those presenting other more severe autoinflammatory diseases associated with high IL-1 β production. Gene ontology enrichment analyses further implicate these miRNAs in cytokine-mediated signalling pathways and the regulation of apoptotic processes (37). Functionally, miR-148b-3p exerts anti-inflammatory effects through broad immunomodulatory functions, including the regulation of immune cell proliferation, differentiation, polarisation, migration, and apoptosis, as well as the modulation of multiple signalling pathways across different disease contexts (38-40). Moreover, let-7d-5p, which is reported to be upregulated in Crohn's disease and ulcerative colitis, has been associated with resistance to apoptosis and the persistence of chronic inflammation (41). Collectively, these findings support a shift toward a pro-inflammatory miRNA profile that parallels the heightened inflammatory state and increased clinical severity observed in M694V homozygous patients. Notably, the increased expression of let-7d-5p in M694V homozygous patients with severe disease compared with those with an intermediate phenotype suggests that, while *MEFV* mutations play a major role in disease severity, they likely act in concert with miRNA-mediated regulatory mechanisms and other epigenetic factors to shape the overall inflammatory response and contribute to clinical heterogeneity. These findings underscore the potential utility of miRNA signatures for patient stratification, prognostic assessment, and disease management.

The marked increase in faecal IL-1 β and IL-6 suggest an altered intestinal inflammatory profile in FMF patients and a potential contribution of inflammasome-mediated signalling. Given the role of the pyrin inflammasome in caspase-1-mediated IL-1 β maturation, the pronounced IL-1 β elevation may be consistent with activation of this pathway, while the increase in IL-6 may reflect the broader downstream inflammatory response. In contrast, the relatively low IL-8 and undetectable TNF- α levels may reflect more transient expression

and variable stability and detectability in stool, as reported for these cytokines across different conditions (42, 43). Moreover, the consistently elevated expression of miR-21-5p across all FMF patients, together with its documented role in the activation of NF- κ B and inflammasome pathways and in promoting IL-1 β production, prompted further mechanistic investigation of miR-21-5p as a potential FMF-related epigenetic biomarker and therapeutic target. Notably, our findings revealed that miR-21-5p contributes to NF- κ B pathway activation, as evidenced by the significant increase in I κ B expression and the decrease in phosphorylated p65 following miR-21-5p inhibition. Additionally, inhibition of miR-21-5p resulted in reduced expression of pyrin, cleaved caspase-1, and cleaved IL-1 β , without a significant change in ASC expression, suggesting that miR-21-5p regulates pyrin inflammasome activation through an ASC-independent mechanism, consistent with the reported capacity of the pyrin B30.2 domain to interact directly with caspase-1 (44).

Despite the significance of our findings, several limitations should be acknowledged. First, although the prevalence of FMF is increasing in Lebanon, patient recruitment and faecal sample collection remained challenging, which limited our sample size and, consequently, the generalisability of our results. Second, most patients were receiving colchicine therapy, the effect of which on miRNA expression remains incompletely understood. Third, the majority of patients included in this study were in remission at the time of sampling; therefore, a different miRNA profile may be observed during acute FMF attacks. Furthermore, although the diagnostic and therapeutic potential of miRNAs is increasingly recognised, practical challenges, including ensuring the stability of faecal miRNAs and achieving efficient delivery, warrant further investigation for clinical application.

Conclusion

Our study provides new insights into the epigenetic landscape of FMF by identifying, for the first time, a distinct faecal miRNA signature in FMF patients,

characterised by the specific upregulation of miR-30e-3p, miR-148a-3p, and miR-21-5p. Importantly, these findings establish a potential molecular link between FMF and intestinal involvement, particularly abdominal pain. Distinct miRNA patterns associated with genotype and disease severity further highlight the clinical heterogeneity of FMF and support the existence of additional layers of disease regulation beyond genetic mutations alone.

miR-21-5p was identified as a key regulator of FMF-related NF- κ B and pyrin inflammasome signalling. Given its consistent upregulation in FMF patients, miR-21-5p represents a potential diagnostic biomarker and therapeutic target. To further elucidate its role in FMF pathogenesis, future studies should identify its molecular targets using *in silico* analyses followed by experimental validation. Its role in FMF inflammation should also be investigated *ex vivo* using PBMCs from FMF patients to further confirm its association with disease pathogenesis and inflammatory responses.

Beyond inflammatory pathways, additional investigations are warranted to explore the involvement of miR-21-5p in other cellular processes relevant to FMF, including apoptosis, autophagy, and cell migration. Similarly, functional characterisation of other dysregulated miRNAs may enable the identification of subgroup-specific biomarkers and contribute to a more refined understanding of FMF heterogeneity. Ultimately, such efforts may support the development of personalised therapeutic strategies and encourage translational studies aimed at evaluating the feasibility of miRNA-based interventions, including optimised delivery systems.

Collectively, these findings support the concept of FMF as a multifactorial autoinflammatory disease in which epigenetic regulation, alongside genetic background, contributes to shaping disease expression.

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