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Expression of miR-155 in monocytes of people with migraine: association with phenotype, disease severity and inflammatory profile

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Abstract

Background miR-155 is involved in the generation and maintenance of inflammation and pain, endothelial function and immune system homeostasis, all functions that are relevant for migraine. The present study aims to assess the levels of miR-155 in migraine subtypes (episodic and chronic) in comparison to age- and sex-matched healthy controls.

Methods This is a cross-sectional, controlled, study involving three study groups: I) episodic migraine ($n=52$, EM), II) chronic migraine with medication overuse ($n=44$, CM-MO), and III) healthy controls ($n=32$, HCs). We assessed the interictal gene expression levels of miR-155, IL-1β, TNF-α, and IL-10 in peripheral blood monocytes using rtPCR. The monocytic differentiation toward the M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes was assessed in circulating monocytes with flow cytometry analysis and cell sorting.

Results miR-155 gene expression was higher in CM-MO group (2.68 ± 2.47 Relative Quantification - RQ) when compared to EM group (1.46 ± 0.85 RQ, $p=0.006$) and HCs (0.44 ± 0.18 RQ, $p=0.001$). In addition, miR-155 gene expression was higher in EM group when compared to HCs ($p=0.001$). A multivariate analysis confirmed the difference between EM and CM-MO groups after correction for age, sex, smoking habit, preventive treatment, aura, presence of psychiatric or other pain conditions. We found higher gene expression of IL-1β, TNF-α, and lower gene expression of IL-10 in migraine participants when compared to HCs ($p=0.001$ for all comparisons). TNF-α and IL-10 genes alterations were more prominent in CM-MO when compared to EM participants ($p=0.001$). miR-155 positively correlated with IL-1β ($p=0.001$) and TNF-α ($p=0.001$) expression levels. Finally, in people with CM-MO, we described an up-regulated percentage of events in both M1 and M2 monocytic profiles.

Conclusions Our study shows for the first time a specific profile of activation of miR-155 gene expression levels in monocytes of selected migraine subpopulations, more pronounced in subjects with CM-MO. Interestingly, mir-155 expression correlated with markers of activation of the inflammatory and immune systems. The CM-MO

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subpopulation showed a peculiar increase of both pro-inflammatory and anti-inflammatory monocytes which worths further investigation.

Trial registration www.clinicaltrials.gov. (NCT05891808).

Keywords MiR-155, Epigenetics, microRNAs, Neuroinflammation, Migraine pathophysiology, Monocytes differentiation, Migraine spectrum

Background

Increasing scientific evidence supports a role of epigenetic mechanisms in migraine pathophysiology [1]. Among different epigenetic mediators, microRNAs (miRNAs) have gained attention as they may be involved in migraine pain generation and chronification [2]. miRNAs are small endogenous noncoding RNAs that are ~22 nucleotides in length that operate as post-transcriptional gene expression regulators by promoting messenger RNA (mRNA) degradation or repressing mRNA translation, and may reflect the environmental influence on gene expression regulation [3–5]. The regulation process performed by miRNAs is complex and articulated since an individual miRNA might target hundreds of different mRNAs, and conversely, each mRNA may be regulated by multiple miRNAs [6]. Furthermore, miRNAs can influence microglia and astrocytes activation, as well as peripheral immune cells activity during the neuroinflammatory process [7–9].

miR-155 is considered a key regulator of inflammation due to its ability to modulate many inflammatory mediators, including interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), alarmins, nuclear factor erythroid 2-related factor 2 (NRF2), and toll-like receptors (TLR) in various cell types [9–11]. miR-155 is involved in endothelium-dependent vasodilation through the regulation of the nitric oxide synthase pathway [12]. In addition, an overexpression of miR-155 in classic monocytes has been shown to result in the production of pro-inflammatory cytokines (TNFα, IL-6, IL-8 and IL-1β) and chemokines in autoimmune diseases [13]. In the case of migraine, it is worth noting that Cheng et al. described increased miR-155 levels in the serum of people with episodic migraine (EM), suggesting a role of this specific miRNA in migraine biology [14].

Several microRNAs, including miR-155, are potent modulators of monocytes differentiation and of the immune system [15–17]. Alterations in the immune system, and specifically in monocytes, were described in the inter-ictal and ictal migraine phases [18]. The inter-ictal migraine phase seems characterized by increased nitric oxide production and prostaglandin E2 release from peripheral monocytes [19]. Furthermore, acute and preventive migraine medications may influence monocyte function, since propranolol and acetylsalicylic acid showed to inhibit monocyte chemotaxis [20].

Two hours from a migraine attack onset, nuclear factor-kappa B activity increased in monocytes in internal jugular blood with an upregulation of nitric oxide synthase that persisted up to 6 hours [21].

It is known that miR-155 interferes with the immune system by modulating several transcription factors and molecular pathways, ultimately resulting in the promotion of pro-inflammatory monocytes (M1) and the inhibition of the anti-inflammatory monocytes (M2) [22–25].

In the present study, we assessed the miR-155 gene expression in peripheral blood monocytes of participants with EM or chronic migraine (CM), and in age- and sex-matched healthy controls (HCs).

As exploratory outcomes we evaluated: I) mRNA levels of pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory (IL-10) cytokines in circulating monocytes; and II) the differentiation of monocytes into M1/ M2 phenotypes.

Methods

Study design

This is a cross-sectional controlled study with three different groups: EM, CM with medication overuse (CM-MO) and HCs. Blood samples were collected in the inter-ictal phase for migraine participants. The study protocol was pre-registered at www.clinicaltrials.gov (NCT05891808).

Study population

Participants with migraine between 18 and 65 years of age were enrolled among consecutive subjects attending the Headache Science & Neurorehabilitation Unit of the IRCCS Mondino Foundation of Pavia (Italy).

Inclusion criteria for EM were: diagnosis of "1.1 migraine without aura" according to ICHD-III [26]; monthly migraine days between 2 and 14; episodic pattern stable for at least 10 years at screening (to reduce the likelihood to enroll subjects transitioning to CM in the future), and negative lifetime history of CM.

Inclusion criteria for CM-MO were: diagnosis of "1.3 chronic migraine" and "8.2 medication overuse headache" according to ICHD-III [26]; documented history of CM for at least 1 year prior to enrollment.

Inclusion criteria for HCs were: absence of diagnosis of primary and/or secondary headache according to ICHD-III (with the only exception of sporadic tension-type headache); absence of chronic pain conditions.

General exclusion criteria were: concomitant diagnosis of neurological, psychiatric, or other pathologies deemed clinically relevant by the researcher, such as systemic autoimmune diseases; pregnant and lactating women; intake of non-steroidal anti-inflammatory drugs, triptans, gepants or opiates in the 24 hours preceding blood sampling. For EM and CM-MO groups, a concomitant diagnosis of "1.2 migraine with aura" did not represent an exclusion criterion.

All patients underwent a screening visit with a neurologist of the Headache Science & Neurorehabilitation Unit during which clinical/demographic data were collected, paper headache diary was revised, and inclusion/exclusion criteria were verified. If the criteria were met, subjects were scheduled for a second appointment to complete the study procedures during which we performed: an evaluation with a psychologist of the Headache Science & Neurorehabilitation Unit of the IRCCS Mondino Foundation of Pavia to assess presence of anxious and/or depressive symptoms, and a peripheral venipuncture for the measurement of miRNAS and cytokines gene expression in monocytes and the phenotypic characterization of the latter.

All participants with migraine were tested in an interictal phase defined as follow:

- for the EM group: no ongoing headache and no headache or acute medication intake in the previous 24 h;
- for the CM-MO group: no headache or a mild headache (namely less than 3 on 0 to 10 visual analogue scale) that did not qualify for a "migraine day" and did not require the intake of acute antimigraine drugs in the previous 24 h [27].

All participants had been overnight fasting before collection of the blood samples. The blood samples vials from migraine participants and HCs were labelled with codes before being sent to the laboratory, so that the biologists who performed all the biochemical determinations were blinded to subject's diagnosis and clinical data.

Peripheral blood mononuclear cells (PBMCs) isolation

Blood samples (18 mL) from the cubital vein were collected in sterile tubes, between 8:00 and 12:00 am.

PBMCs were first isolated immediately after blood collection as described in Greco et al. 2022 [28]. Briefly, blood samples were collected within ethylenediamine tetra-acetic acid (EDTA) containing tube and diluted in 1:1 ratio with phosphate buffer saline (PBS) (Sigma Aldrich, Milan, Italy). Then, diluted blood samples were slowly loaded into Ficoll separating solution (15 ml) (Sigma Aldrich, Milan, Italy) and centrifuged at 800 g for 30 min at room temperature. PBMCs, accumulated as the middle white monolayer, were washed twice in sterile PBS at 300 g for 15 min.

Monocyte isolation

Untouched monocytes were isolated from PBMCs, collected as described above, using the Pan Monocyte Isolation Kit and a MidiMACS™ Separator (MiltenyiBiotec). To this end, PBMCs were resuspended in PBS containing 0.5% bovine serum albumin and 2 mM EDTA. Non-monocytes, such as T cells, NK cells, B cells, dendritic cells, and basophils, were indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies along with a Fc receptor blocking reagent and Anti-Biotin MicroBeads. Depletion of the magnetically labeled cells allowed the isolation of high-purity unlabeled monocytes. Subsequently, monocytes were washed with buffer, pelleted by centrifugation and resuspended in PBS for analyses by flow cytometry (for characterization of surface markers) and by rtPCR for cytokine gene expression.

Gene expression

To investigate the cytokine gene expression, total RNA (including all small non-coding RNAs) was extracted from monocytes within 2 weeks using the Direct-zol RNA Mini prep plus (Zymo Research from Aurogene, Rome, Italy. RNA quality was determined by an optical density (OD) 260/280 ratio $\geq$ 1.9 and OD 260/230 ratio $\geq$ 1.5 by using a NanoDrop Spectrophotometer (Nanodrop™ Thermo Fisher Scientific, Euroclone Milano, Italy). The synthesis of cDNA was performed by using MirXMirna First strand Synthesis (Takara-Diatech Labline, Jesi-Ancona, Italy) and TB Green q-Rt PCR was used (Takara-Diatech, Labline Jesi-Ancona, Italy) to determine expression levels of miRNA-155. miRNA expression was normalized with U6 (a type of small nuclear RNA), used as housekeeping gene. The primer for this miRNA was selected from the Prime 3 software and synthesized by Sigma Aldrich (Milan, Italy).

The gene expression of inflammatory cytokines was analyzed using the Fast Eva Green Supermix (Bio-Rad). The primer sequences are reported in Supplementary materials [29]. Ubiquitin C, whose expression remained constant in all experimental groups, was used as housekeeping gene. The amplification was performed with a light Cycler 480 Instrument rtPCR Detection System (Roche) following the supplier's instructions. All samples were assayed in triplicate and gene expression levels were calculated according to the $2^{-\Delta\Delta Ct} = 2^{-(Ct}$

gene−Ct housekeeping gene) formula by using Ct values (Relative Quantification - RQ).

Flow Cytometry Analysis and Cell Sorting (FACS)

The monocytes were centrifuged at 300×g for 15 min and resuspended in FACS buffer. Live cells were counted using an automated cell counter with trypan blue staining. A total of 1×10^6 monocytes were aliquoted into each tube and preincubated with a human FcR blocking reagent (MiltenyiBiotec) for 10 min to block non-specific binding to Fc receptors. Subsequently, they were incubated for 30 min at 4 °C in the dark with all of the following monoclonal antibodies: Peridinin Chlorophyll Protein Complex (PerCP)-conjugated anti-human CD14 (BD Biosciences, 20µL per 1×10^6 cells), R-phyco-erythrin-cyanine7 (PE-Cy7)-conjugated anti-human CD16 (BD Biosciences, 5µL per 1×106 cells), and R-phycoerythrin (PE)-conjugated CD163 (BD Biosciences, 5µL per 1×106 cells) or R-phycoerythrin (PE)-conjugated CD80 (BD Biosciences, 5µL per 1×106 cells). After incubation, the samples were washed in FACS buffer and filtered using a 70 µm sterile cell strainer from BD Biosciences.

Sample analysis was performed using the BD FACS Melody Cell Sorter. To optimize the performance of the sorter, the Cytometer Setup and Tracking bead calibration was performed. First of all, monocytes were recognized as CD14+(expressing on their cell surface the lipopolysaccharide co-receptor) and CD16+(expressing on their surface the activatory Fc gamma receptor III). After that, the monocytic phenotypes were identified as follows: CD14+/CD16− expression, "classical" monocytes, and CD14+/CD16+expression, "non classical-intermediate" monocytes. As M1 monocytes are characterized by the expression of CD80 and M2 monocytes by the expression of CD163, CD80+and CD163+expressions were used to identify pro-inflammatory "M1" monocytes and anti-inflammatory "M2" monocytes, respectively.

By means of FACS, we calculated 10,000 consecutive events for each subject. Each monocytic subpopulation, as previously described, was expressed as the percentage of events out of the 10,000 consecutive events recorded at FACS.

Statistical analysis

The minimum sample size was calculated from available data suggesting a difference in miR-155 levels of 1.69 RQ, with standard deviations of 1.15, and 2.69 RQ among HCs and EM subjects respectively [14].

Therefore, assuming a statistical power of 80 percent, a significance level of 95 percent and taking in account a Bonferroni correction, we needed a sample size of 24 subjects for each of the three experimental groups (HCs, CM-MO and EM). Considering possible variability in dosing and other methodological biases compared to previous results, we aimed to enroll a population of at least 30 subjects per group.

For the exploratory outcome, which involves a flow cytometric evaluation of monocytes, we planned to analyze a sub-population composed of at least 15 subjects for each group (CM-MO, EM and HCs).

Clinical and demographic features were compared among the three study groups with Fisher exact test or Chi-square test depending on the distribution of categorical variables.

The difference in miRNAs and cytokine expression among EM, CM-MO and HCs was assessed using non parametric tests, the Mann–Whitney U test or the Kruskal–Wallis test, according to the distribution of data. Statistical significance levels were further corrected using the Bonferroni method to account for multiple comparisons. Non-parametric correlations were used to measure the relationship between biochemical variables and clinical variables in CM-MO and EM patients. Post-hoc multivariate analyses were performed according to the results of the univariate analysis using a logistic regression. Statistical significance was set at the 5% level ($p<0.05$).

Results

Clinical and demographic characteristics

A total of 126 participants were consecutively enrolled and divided among the three study groups.

EM participants ($n=52$; 75% females; 41.0 ± 10.5 years) reported 6.0 ± 3.7 monthly migraine days (MMDs) and 5.9 ± 3.8 monthly days of acute drugs intake (6.9 ± 5.2 doses of acute drugs/month). CM-MO participants ($n=44$; 84.1% females; 45.8 ± 10.7 years) reported 22.5 ± 6.3 MMDs and 22.4 ± 6.5 monthly days of acute drugs intake (36.0 ± 23.1 doses of acute drugs/month). Thirty HCs (66.7% female; 42.9 ± 14.8 years) were enrolled as control group.

The three study groups did not differ in terms of age and sex ($p=0.140$ and $p=0.217$, respectively). As expected in a migraine population managed at a third level headache center, ongoing pharmacological prevention rate was similar between EM (32%) and CM-MO (34.9%) patients ($p=0.827$). CM-MO participants had higher rates of other co-existing diseases (61.4%) when compared to either the EM group (34.6%) or the HCs (6.7%) ($p=0.001$). The most frequent co-existing diseases were depressive and anxious symptoms, both of which were more frequent in the CM-MO group (52.3% and 70.4%, respectively) when compares to EM patients (19% and 27%, respectively; $p=0.001$ for both comparisons). As regards non-psychological co-existing diseases, the most frequent was asthma followed by hypertension,

hypothyroidism, dyslipidemia and polycystic ovary syndrome. Clinical and demographic features are reported in Table 1.

miR-155 levels

miRNA-155 gene expression was higher in CM-MO participants (2.68 ± 2.47 RQ) when compared to either the EM group (1.46 ± 0.85 RQ, $p = 0.006$) and the HCs (0.44 ± 0.18 RQ, $p = 0.001$). The gene expression of miR-155 was also higher in monocytes of EM participants when compared to HCs ($p = 0.001$) (Fig. 1).

miR-155 gene expression in monocytes was not associated with use of prevention therapy ($p = 0.717$), other pain conditions ($p = 0.878$), anxious symptoms ($p = 0.309$), depressive symptoms ($p = 0.331$), co-existing diseases ($p = 0.069$), insomnia ($p = 0.565$) or cigarette smoking ($p = 0.698$). miR-155 gene expression in monocytes positively correlated with monthly headache days and MMDs (for both: Spearman's rho = 0.36, $p = 0.001$), with monthly days of acute drugs intake (Spearman's rho = 0.36, $p = 0.001$) and monthly doses of acute drugs (Spearman's rho = 0.34, $p = 0.001$) (Fig. 2). In a logistic regression model, after correcting for age, sex, ongoing prevention, presence of aura, presence of anxious or depressive symptoms, presence of other pain conditions, and smoking habit, miR-155

Table 1 Clinical and demographic characteristics of study population

	CM-MO	EM	HCs	*p*-value
n	44	52	30	
Age (years)	45.8 ± 10.7	41 ± 10.5	42.9 ± 14.82	0.140
BMI (kg/m^2)	24.6 ± 5.3	22.3 ± 4.1	23.5 ± 2.9	0.389
Female	37 (84.1%)	39 (75%)	20 (66.7%)	0.217
Aura	13 (29.5%)	6 (11.5%)	-	0.042
Disease duration (years)	29.6 ± 12.0	26.0 ± 11.0	-	0.137
Ongoing prevention	15 (34.1%)	16 (30.7%)	-	0.827
Previously failed preventions	2.9 ± 2.5	1.0 ± 1.6	-	0.001
MHDs	25.2 ± 6.1	6.4 ± 3.8	-	0.001
MMDs	22.5 ± 6.3	6.0 ± 3.7	-	0.001
Days of acute drugs intake	22.4 ± 6.5	5.9 ± 3.8	-	0.001
Doses of acute drugs (monthly)	35.9 ± 23.1	6.9 ± 5.2	-	0.001
Acute treatments				
NSAIDs	14 (31.8%)	23 (44.2%)	-	0.280
Triptans	5 (11.4%)	7 (13.5%)	-	
Combination	3 (6.8%)	6 (11.5%)	-	
Multiple drug classes	22 (50.0%)	16 (30.8%)	-	
NRS	7.3 ± 0.9	7.4 ± 0.7	-	0.388
MIDAS	79.0 ± 50.1	33.6 ± 18.2	-	0.008
HIT-6	64.6 ± 10.9	58.4 ± 10.5	-	0.132
ASC-12	5.5 ± 3.7	2.1 ± 2.2	-	0.013
Anxious symptoms	31 (70.4%)	14 (27%)	-	0.001
Depressive symptoms	23 (52.3%)	10 (19%)	-	0.001
Other co-existing diseases	27 (61.4%)	18 (34.6%)	2 (6.7%)	0.001
Asthma	4 (9%)	4 (7.7%)	1 (3.3%)	
Hypertension	4 (9%)	3 (5.8%)	1 (3.3%)	
Hypothyroidism	4 (9%)	3 (5.8%)	-	
Dyslipidemia	1 (2.3%)	3 (5.8%)	-	
Polycystic ovary syndrome	-	3 (5.8%)	-	
Insomnia	18 (40.9%)	13 (25%)	5 (16.7%)	0.096
Smoking habit	9 (20.4%)	7 (13.5%)	4 (13.3%)	0.620
Other pain conditions	12 (27.3%)	8 (15.4%)	-	0.007

Legend: *CM-MO* Chronic migraine with medication overuse, *EM* Episodic migraine, *HCs* Healthy controls, *BMI* Body mass index, *MHDs* Monthly Headache Days, *MMDs* Monthly Migraine Days, *NSAIDs* Nonsteroidal anti-inflammatory drugs, *NRS* Numeric rating scale, *MIDAS* Migraine disability assessment score questionnaire, *HIT-6* Headache impact test, *ASC-12* Allodynia symptom checklist. Continuous variables are presented as mean ± standard deviation

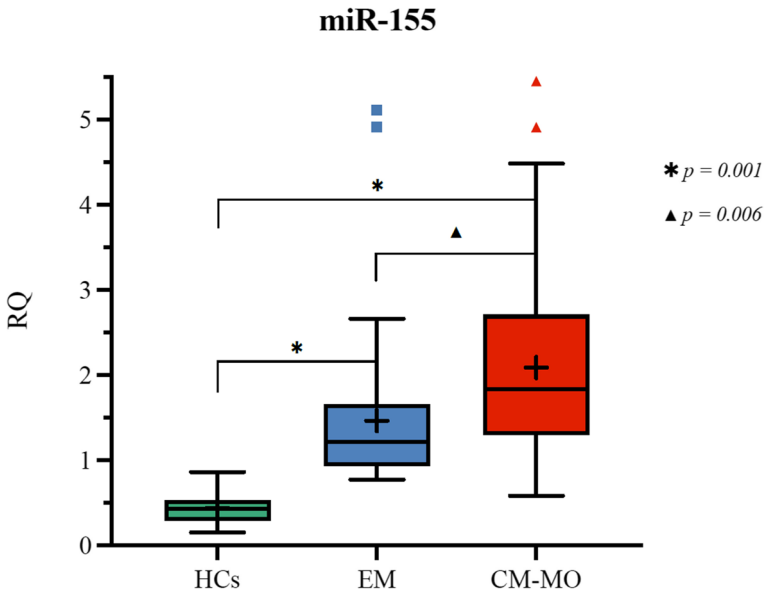

Fig. 1 miR-155 expression levels in peripheral blood monocytes among the three study groups. Legend: *CM-MO:* chronic migraine with medication overuse, *EM:* episodic migraine, *HCs:* healthy controls. *RQ* Relative Quantification: $2-\Delta\Delta Ct = 2-(\Delta Ct \text{ gene} - \Delta Ct \text{ housekeeping gene})$; *Ct* cycle threshold. Box-plot: the range between the upper and lower border of the box indicates the interquartile range (IQR), spanning from the 25th to the 75th percentile. Within the box, the line indicates the median, and the cross denotes the mean. The upper and lower whiskers extend to the maximum and minimum values, excluding outliers. Symbols positioned above the upper whisker represent outliers, defined statistically as values beyond the 75th percentile plus 1.5 times the IQR. Kruskal–Wallis Test was used for intergroup comparisons

gene expression was still significantly associated with migraine phenotype (adjusted R-square: Exp(B) 2.32 [1.13 – 4.78], $p=0.022$) (Table 2).

Cytokines genes expression

Due to technical issues with the kit, cytokines gene expression in monocytes was not assessed in 11 participants (9 from the CM-MO group and 2 from the EM group).

IL-1β gene expression was higher in both migraine groups (CM-MO 1.74 ± 0.89 RQ; EM 1.18 ± 0.30 RQ) when compared to HCs (0.38 ± 0.12 RQ, $p=0.001$ for all comparisons). IL-1β gene expression did not differ between EM and CM-MO groups ($p=0.567$) (Fig. 3 – Panel A). TNF-α gene expression was higher in both migraine groups (CM-MO 1.82 ± 0.63 RQ; EM 1.09 ± 0.26 RQ, $p=0.001$) when compared to HCs (0.39 ± 0.15 RQ, $p=0.001$ for all comparisons). It was also higher in CM-MO compared to EM ($p=0.001$) (Fig. 3 – Panel B).

IL-10 gene expression was lower in both migraine groups (CM-MO 0.64 ± 0.22 RQ; EM 0.82 ± 0.25 RQ) when compared to the HCs (1.54 ± 0.59 RQ, $p=0.001$ for all comparisons). The gene expression of IL-10 was also lower in the CM-MO group compared to the EM group ($p=0.037$ (Fig. 3 – Panel C).

Correlations of miR-155 levels with cytokines expression

miR-155 levels positively correlated with IL-1β (Spearman's rho = 0.68, $p=0.001$) and TNF-α (Spearman's rho = 0.67, $p=0.001$) relative expression levels (Fig. 2). Contrariwise, miR-155 levels in monocytes negatively correlated with IL-10 gene expression (Spearman's rho = -0.45, $p=0.001$) (Fig. 2).

Monocytes differentiation

In a subset of the overall study population composed of 59 participants (18 EM, 24 CM-MO and 17 HCs), we assessed monocytes differentiation by means of FACS. Clinical and demographic features of this subpopulation are summarized in Supplementary materials.

The percentage of M1/classical (CD80 + /CD14 +) monocytes FACS events was higher in the CM-MO group (40.8 ± 4.0 RQ) when compared to either the EM group (32.7 ± 3.3 RQ, $p=0.001$) or HCs (27.5 ± 5.8 RQ, $p=0.001$). The percentage of M1/classical monocytes FACS events did not differ between EM participants and HCs ($p=0.153$) (Fig. 4—Panel A).

The percentage of M1/non classical-intermediate (CD80 + /CD16 +) monocytes FACS events was higher in the CM-MO group (38.5 ± 3.8 RQ) when compared to either the EM group (31.8 ± 4.9 RQ, $p=0.005$) or HCs (29.0 ± 4.5 RQ, $p=0.001$). The percentage of M1/

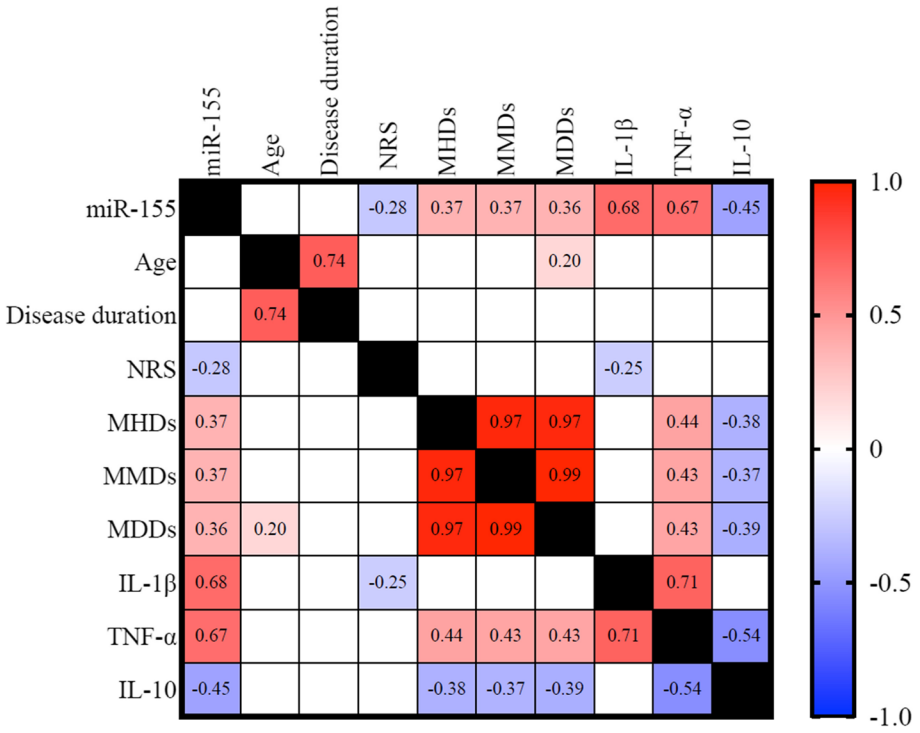

Fig. 2 Square matrix showing the correlation analysis among miR-155 and the other study variables. Legend: miR-155 is expressed as "Relative Quantification (RQ)", Age and Disease duration are expressed in "Years", *MHDs*: Monthly Headache Days, *MMDs:* Monthly Migraine Days, *MDDs:* Days of acute drugs intake per month, *NRS:* 0 to 10 numeric rating scale. Correlation matrix: Spearman's rank correlation coefficients are plot in an heatmap where the direction of the relationship between two variables is displayed by color (red for positive correlations, and blue for negative correlations, with intensity of color representing the strength of correlation from 1 to -1). Blank cells represent correlations that did not reach statistical significance

Table 2 Results of logistic regression model for dependent variables: EM vs CM-MO

	B	S.E	Wald	df	Sig	Exp(B)	95% CI for Exp(B)	
							Inferior	Superior
miR-155 (RQ)	0.84	0.368	5.238	1	0.022	2.32	1.13	4.78
Sex (Male)	-0.17	0.744	0.055	1	0.815	0.84	0.19	3.61
Age (years)	0.05	0.032	2.347	1	0.126	1.05	0.99	1.12
Presence of aura	2.00	0.789	6.451	1	0.011	7.43	1.58	34.88
Ongoing prevention	-0.20	0.643	0.099	1	0.753	0.82	0.23	2.88
Presence of other pain conditions	1.00	0.741	1.835	1	0.176	2.73	0.64	11.65
Presence of anxious symptoms	2.43	0.722	11.369	1	0.001	11.42	2.77	47.03
Presence of depressive symptoms	0.52	0.676	0.601	1	0.438	1.69	0.45	6.36
Presence of at least one co-existing disease	0.81	0.614	1.745	1	0.187	2.25	0.68	7.49
Smoking habit	1.14	0.794	2.071	1	0.150	3.13	0.66	14.84
Constant	-6.14	2.020	9.238	1	0.002	0.002	-	

Legend: *CM-MO* Chronic migraine with medication overuse, *EM* Episodic migraine. *B* is the coefficient in log-odds units used in predicting the dependent variable (*EM* versus *CM-MO*) from the independent variable. *S.E.* represents standard errors associated with *B* coefficients, while *Wald* signifies the Wald chi-square value, with *df* representing degrees of freedom. *Exp(B)* indicates odds ratios for the predictors, obtained through exponentiation of the coefficients. *95% CI* or *95% confidence intervals* denotes the confidence intervals for the odds ratios *Exp(B)*

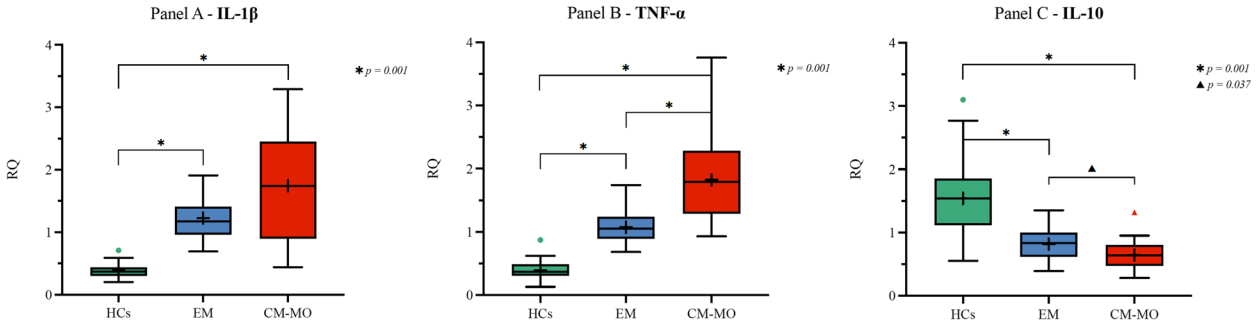

Fig. 3 mRNA expression levels of pro-inflammatory (IL-1 β, TNF-α) and anti-inflammatory (IL-10) in peripheral blood monocytes among the three study groups. Legend: *CM-MO:* chronic migraine with medication overuse, *EM:* episodic migraine, *HCs:* healthy controls. *RQ*: Relative Quantification: $2-\Delta\Delta Ct = 2-(\Delta Ct\ gene - \Delta Ct\ housekeeping\ gene)$; Ct: cycle threshold. Box-plot: the range between the upper and lower border of the box indicates the interquartile range (IQR), spanning from the 25th to the 75th percentile. Within the box, the line indicates the median, and the cross denotes the mean. The upper and lower whiskers extend to the maximum and minimum values, excluding outliers. Symbols positioned above the upper whisker represent outliers, defined statistically as values beyond the 75th percentile plus 1.5 times the IQR. Kruskal–Wallis Test was used for intergroup comparisons

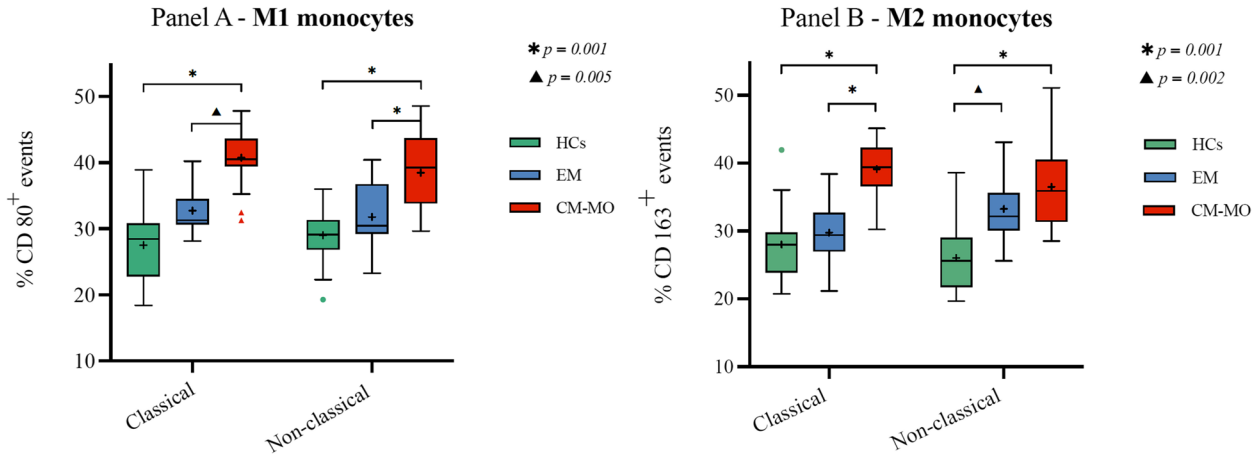

Fig. 4 Percentage of events recorded for the different monocytic sub-populations among the three study groups. Legend: Panel **A**: percentage of M1 monocytes (CD80 +) sub-populations. Panel **B**: percentage of M2 monocytes (CD163 +) sub-populations. *CM-MO:* chronic migraine with medication overuse, *EM:* episodic migraine, *HCs:* healthy controls, *classical:* "classical" monocytes (CD14 + /CD16– expression), *non-classical:* "non classical-intermediate" monocytes (CD14 + /CD16 + expression), *M1:* pro-inflammatory "M1" monocytes (CD80 + expression), *M2*: anti-inflammatory "M2" monocytes (CD163 + expression). Box-plot: the range between the upper and lower border of the box indicates the interquartile range (IQR), spanning from the 25th to the 75th percentile. Within the box, the line indicates the median, and the cross denotes the mean. The upper and lower whiskers extend to the maximum and minimum values, excluding outliers. Symbols positioned above the upper whisker represent outliers, defined statistically as values beyond the 75th percentile plus 1.5 times the IQR. Kruskal–Wallis Test was used for intergroup comparisons

non classical-intermediate monocytes FACS events did not differ between EM participants and HCs ($p = 0.369$) (Fig. 4—Panel A).

The percentage of M2/classical (CD163 + /CD14 +) monocytes FACS events was higher in the CM-MO group (39.1 ± 3.8 RQ) when compared to either the EM group (29.7 ± 4.5 RQ, $p = 0.001$) or HCs subjects (28.0 ± 5.2 RQ, $p = 0.001$). The percentage of M2/classical monocytes FACS events did not differ between EM participants and HCs ($p = 1.000$) (Fig. 4—Panel B).

The percentage of M2/non classical-intermediate (CD163 + /CD16 +) monocytes FACS events was higher in the CM-MO group (36.5 ± 5.8 RQ) and in the EM group (33.2 ± 4.9 RQ) when compared to HCs (26.0 ± 5.1 RQ; $p = 0.001$ vs. CM-MO and $p = 0.002$ vs. EM). The percentage of M2/non classical-intermediate monocytes events did not differ between EM and CM-MO groups ($p = 0.443$) (Fig. 4—Panel B).

Discussion

The present study detailed the miR-155 gene expression profile in peripheral monocytes of people with EM or CM-MO, and HCs. Our main results may be summarized as follows: I) miR-155 expression is higher in people with migraine when compared to HCs, II) miR-155 expression is higher in people with CM-MO than in people with EM, and III) the difference in miR-155 monocytic expression between the EM and the CM-MO group is confirmed after correction for several clinical and demographic variables known to affect mR-155 expression, including ongoing prevention, concomitant psychological pain conditions and smoking habit [30–34].

We also described an alteration of cytokines gene expression in people with migraine, which was more pronounced in people with CM-MO, consistent with an ongoing pro-inflammatory state. Finally, in a subset of people with migraine, we described a disruption of the physiological M1 and M2 monocytes differentiation, leading to an increase in both pro-inflammatory and anti-inflammatory profiles in people with CM-MO.

At state of art, data about the role of miR-155 in migraine is limited. Pre-clinical data demonstrated increased miR-155 levels in the nitroglycerin (NTG) migraine model in rats and mice [35, 36]. It is worth noting that administration of a miR-155 antagonist (antagomir) reduced the microglial activation and the inflammatory response, while a miR-155 agonist (agomir) enhanced central sensitization [36]. In addition, olcegepant, an anti-CGRP receptor antagonist, reduced NTG-induced trigeminal hyperalgesia in parallel with miR-155 expression in rat trigeminal ganglion, brainstem, and cervical spinal cord [35].

Cheng et al. suggested an endothelial dysfunction in migraine as demonstrated by increased levels of miR-155, miR-126, and let-7g in subjects with episodic migraine without cardiovascular risk [14].

The pro-inflammatory actions of myeloid cells appear to be regulated by several miRNAs [37]. miR-155 is elevated in myeloid cells of patients with inflammatory diseases and can enhance monocytes and macrophages differentiation toward a pro-inflammatory profile [38]. miR-155 plays a pro-inflammatory function during microglia activation by blocking anti-inflammatory proteins such as SIRT1, SOCS1, SHIP1, and others [39]. In addition, miR-155 may modulate the adaptive immune response. Overexpression of miR-155 in monocytes induced an immunoregulatory response characterized by co-expression of stimulatory and inhibitory molecules [40].

The role of inflammation in migraine pathophysiology is undisputed. Antidromic release of CGRP from trigemino-vascular fibers in the meninges is believed to induce plasma protein extravasation and mast cell degranulation with the development of a sterile inflammation state, leading to activation and sensitization of the peripheral and central components of the trigeminovascular system [8, 41, 42]. Cytokines are important regulators of inflammatory and immunological responses, and several of them have been directly linked to pain sensitization by acting on both peripheral nociceptive nerve terminals and sensory ganglia, as well as central sensitization. IL-1β and TNF-α have a pro-nociceptive function in peripheral and central pain pathways [43]. The sensitization phenomenon represents a typical migraine feature, becoming more pronounced as migraine frequency increases [44, 45].

Our results align well with the role of inflammation in migraine pathogenesis. miR-155 gene expression in monocytes was associated with the migraine condition and paralleled the severity of disease, being higher in CM-MO than EM, and with a correlation with MMDs and other indicators of severity. The increased levels of miR-155 are not an isolated finding, indeed it seems associated to the monocytic function and polarization. Indeed, miR-155 levels positively correlated with IL-1β and TNF-α, pro-inflammatory cytokines, while they negatively correlated with IL-10, an anti-inflammatory cytokine. Monocytes of migraine participants overexpressed pro-inflammatory cytokines (IL-1β and TNF-α genes) and under-expressed IL-10. At least for TNF-α and IL-10, this alteration showed a continuum across the migraine spectrum being more severe in CM-MO.

A dysregulation of interleukins in migraine patients has been widely described, although few studies reported on the differences between people with EM and CM [46]. Our findings are consistent with the study of Gerring et al., confirming immune abnormalities by means of whole blood next-generation RNA sequencing in migraine subjects [47]. Togha et al. described increased serum levels of TNF- α in CM when compared to EM, but conflicting data are present in literature [48–50]. Cowen et al. did not find differences in cerebrospinal fluid and serum levels of IL-10 between EM and CM [50]. It should be noted that in the present study we specifically focused on the expression of interleukins in monocytes, thus the different specimen and methods largely account for the observed contradictions.

As an exploratory assessment, in a subgroup of patients, we characterized the profiles of circulating monocytes by examining CD80 + (M1 – pro-inflammatory) and CD163 + (M2 – anti-inflammatory) expression in relationship to disease severity. The surface molecules CD14 and CD16 were also used to classify monocytes. Classical monocytes, which make up 80–90% of circulating monocytes, express high levels of CD14, but not CD16.

By contrast, the population expressing CD16 includes the so-called intermediate and non classical monocytes [51]. A novel finding in our study is the overall and simultaneous increase of M1 and M2 events recorded by means of FACS in patients with CM-MO. This was confirmed in both classical and non classical monocytes populations. Recently, it was shown that non classical peripheral blood monocytes were lower in people with migraine than in controls. The Authors suggested that this could be explained by a possible migration of this subpopulation of monocytes into the endothelium of cranial vessels [18]. Indeed, CD16 + monocytes may patrol the endothelium of vascular tissues over long distances [52, 53].

In our cohort of patients, we could speculate that in the most severe migraine phenotype the dysregulated levels of miR-155 and pro-inflammatory mediator first activate the monocytes toward a M1 phenotype, with a secondary and compensatory activation of the M2 anti-inflammatory response. This exploratory analysis provides important, although preliminary, insights and deserves attention and consideration in the future. A larger sample of migraine individuals is needed to disentangle the role of monocyte subpopulations.

How this complex interaction between the immune response and epigenetic factors takes place is unclear, but it is likely to involve CGRP-initiated inflammatory mechanisms that activate miR-155 expression and other pro-inflammatory genes, creating a cycle that amplifies neuroinflammation, causing chronic pain via microglial activation [54]. In an animal model of migraine, we found that mRNA levels of miR-155-5p increased in specific regions of the central nervous system of rats, supporting this hypothesis, while the administration of olcegepant, a CGRP antagonist, considerably prevented miR-155 rise [55].

The present findings contribute to the identification of validated biomarkers of migraine, a crucial step for advancing the management of migraine in several ways: I) refining the diagnosis based on biological features; II) monitoring drug response; III) delineating distinct phases within the migraine cycle; and IV) facilitating the discovery of novel molecular targets for drug development. microRNAs inherently lack disease specificity and therefore miR-155 is probably not specific enough to qualify as a migraine biomarker, but our study may pave the way toward the identification of a migraine-specific panel including miR-155 [56].

Another potential impact of our findings regards the possibility of targeting microRNAs for therapeutic purposes. The inhibition of miRNA-155 alleviated hyperalgesia in bone cancer pain [57] and in neuropathic pain [58] in in-vivo rodent models. Nonetheless, safety concerns about miR-155 antagomir use in clinical remain to be elucidated by future studies, given the many crucial cellular processes regulated by this microRNA.

Limitations of the study

The present study has several limitations. It focused on the monocytic function and detailed the role of miR-155 and interleukins in this cell population. Thus, our results cannot be generalized to other specimens, namely plasma, serum, or CSF. Another limitation is the enrollment of people with CM with MO, as the frequent intake of analgesics may indeed have influenced the data. We already evaluated other specific microRNAs in the CM-MO group, thus we decided to collect data on a similar and comparable population for consistency. Another limitation of the study is the possible presence of confounding factors, such as absence of precise data to assess body composition, or the presence of migraine with aura. While we excluded participants with BMI > 30, we cannot rule out with certainly the possibility that a low-grade inflammation associated with an excessive fat diet or dysfunctional adipocytes activity may have influenced the results. As regards migraine with aura, we did not exclude patients bearing the condition and, although we performed a statistical correction, we cannot completely rule out a biological role of aura on the observed results. Furthermore, we did not put in place a one-to-one matching procedure during recruitment, which would have possibly reduced the risk of bias, although it might introduce a selection bias as well. We felt that an acceptable trade off was to include far more migraine subjects than the minimum required by the sample size calculation. Finally, though our study was adequately powered to test the primary outcome, confirmatory studies with larger sample size are necessary to strengthen our findings. This is particularly important for the exploratory outcomes, which must be considered purely hypothesis generating and will require further specifically focused studies.

Conclusion

Our study shows for the first time a specific profile of activation of miR-155 gene expression levels in selected migraine subpopulations, more pronounced in subjects with CM-MO. Interestingly, mir-155 expression correlates with markers of activation of the inflammatory and immune systems. The CM-MO subpopulation showed a peculiar increase of both pro-inflammatory and anti-inflammatory monocytes worth further investigation.

Abbreviations

miRNAs	microRNAs
mRNA	Messenger RNA
IL-1β	Interleukin 1β
TNF-α	Tumor necrosis factor alpha
NRF2	Nuclear factor erythroid 2-related factor 2

TLR	Toll-like receptors
miR-155	microRNA-155
EM	Episodic migraine
CM-MO	Chronic migraine with medication overuse
HCs	Healthy controls
PBMCs	Peripheral blood mononuclear cells
EDTA	Ethylenediamine tetra-acetic acid
PBS	Phosphate buffer saline
RQ	Relative quantification
CD14	Cluster of differentiation 14
CD16	Cluster of differentiation 16
CD163	Cluster of differentiation 163
CD80	Cluster of differentiation 80
FACS	Flow cytometry analysis and cell sorting
MHDs	Monthly headache days
MMDs	Monthly migraine days
NSAIDs	Nonsteroidal anti-inflammatory drugs
CGRP	Calcitonin gene-related peptide
CSF	Cerebrospinal fluid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10194-024-01842-y.

Supplementary Material 1.

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Authors' contributions
This study was designed by RG an RD. AMZ, SF, CD, and MF performed monocyte extraction and assessments. RD performed statistical analysis. RD and FB wrote the original draft of the manuscript. FB, MC, FC, AA, EM, MP, and VG performed clinical evaluations, and provided critical revision of results and manuscript. MA, DM, EG, NG, and GS enrolled patients and provided critical revision of results and manuscript. SB performed the psychological evaluations and provided critical revision of the manuscript. RG critically revised the draft. CT provided supervision and critical revision of the manuscript. All the authors read the final version of this paper and approved it.

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Availability of data and materials
The dataset generated and/or analysed during the current study is available in the Zenodo repository, at https://doi.org/https://doi.org/10.5281/zenodo.10617867. The dataset is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The research received approval from the local Ethics Committee (p-20200048922), and all participants provided written informed consent upon enrollment. The present study adhered to the principles outlined in the Declaration of Helsinki as well as prevailing national ethics regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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