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### **Altered miR-193a-5p expression in children with cow's milk allergy**

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**Short Title:** miRNoma features in cow's milk allergy

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## Abstract

**Background:** Cow's milk allergy (CMA) is one of the most common food allergies in children. Epigenetic mechanisms have been suggested to play a role in CMA pathogenesis. We shown that DNA methylation of Th1/Th2 cytokine genes and FoxP3 affects CMA disease course. Preliminary evidence suggest that also the miRNome could be implicated in the pathogenesis of allergy. Main study outcome was to comparatively evaluate miRNome in children with CMA and in healthy controls. **Methods:** Peripheral blood mononuclear cells were obtained from children aged 4-18 months: 10 CMA patients, 9 CMA patients who outgrew CMA, and 11 healthy controls. Small RNA libraries were sequenced using a next-generation sequencing-based approach. Functional assessment of IL-4 expression was also performed. **Results:** Among the miRNAs differently expressed, 2 were up-regulated and 14 were down-regulated in children with active CMA compared to healthy controls. miR-193a-5p resulted the most down-regulated miRNA in children with active CMA compared to healthy controls. The predicted targets of miR-193a-5p resulted up-regulated in CMA patients compared to healthy controls. Peripheral blood CD4<sup>+</sup> T cells transfected with a miR193a-5 inhibitor showed a significant up-regulation of IL-4 mRNA and its protein expression. Children who outgrew CMA showed miRNA-193a-5p level, and its related targets expression, similar to that observed in healthy controls. **Conclusions:** Our results suggest that miR-193a-5p is a post-transcriptional regulator of IL-4 expression and could have a role in IgE-mediated CMA. This miRNA could be a novel diagnostic and therapeutic target for this common form of food allergy in childhood.

**Keywords:** epigenetics, food allergy, IL-4, miRNome

## Background

Cow's milk allergy (CMA) is one of the most common food allergy (FA) in early childhood, with a significant increase in prevalence, persistence, severity of clinical manifestations and a consequent negative impact on quality of life and medical care costs (1). The evidence that FA could derive from a complex gene-environment interaction that induces epigenetic changes at immune system level (2,3) suggests that epigenetic mechanisms could represent a potential target of intervention. This leads to the importance of a better definition of the epigenetic mechanisms involved in CMA (4). Several allergy-related genes, including those related to T-effector pathways, namely IFN- $\gamma$  and IL-4, have been found to be susceptible to epigenetic regulation (4,5). An epigenome-wide association study in children with CMA revealed alterations of methylation status in Th1-Th2 response mediator genes (i.e., IL1RL1, IL5RA, IL4, CCL18 and STAT4) in whole blood DNA, and their association with the disease (6). We demonstrated that epigenetic mechanisms, involving DNA methylation of the promoter region of the Type 1 helper (Th1) and Th2 cytokine genes and Treg-specific demethylated region of FoxP3 play a role in tuning the CMA disease course, and that dietary strategies could influence these mechanisms differentially (2,3). In addition to DNA methylation, there are also other epigenetic networks including micro RNAs (miRNAs). They are small endogenous RNAs that shape gene expression and are transcribed from intergenic or intronic genomic loci which inhibit target gene expression by mRNA degradation or translational repression (7). Preliminary results suggest that also the miRNAome could be involved in the pathogenesis of allergy. Profiling studies of miRNAs in human biopsy specimens and in mouse models of allergy including asthma, eosinophilic esophagitis and contact dermatitis show differential expression in ~10–20% of miRNAs (8–12). Selected miRNAs have been implicated in several regulatory mechanisms of Th2 response, including sphingosine-1-phosphate receptor gene (S1pr1) expression and

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suppression of Th1 differentiation (13-15). Other miRNAs have been implicated in allergy prevention through the induction of FoxP3<sup>+</sup> Treg differentiation (16). miRNome in CMA is still largely unexplored. Identification of specific miRNAs and their targets will both facilitate our understanding of the regulation of key CMA determinants as well as offer the opportunity to identify novel targets for innovative diagnostic and therapeutic strategies. To address this issue, we comparatively investigated miRNA profile in CMA children and healthy controls using a next-generation sequencing (NGS)-based approach.

## Methods

### Study subjects

IgE-mediated CMA children (aged 4-18 months), consecutively referred to our tertiary Pediatric Allergy Center from December 2012 to April 2014, for a diagnostic oral food challenge because recent evidence of suspected CMA signs and symptoms, were invited to participate into the study.

All patients underwent a double-blind placebo-controlled oral food challenge (DBPCFC), as described previously (17). All oral food challenges took place at the Center on two separate days with a 1-week interval. Parents of children taking antihistamine were advised to withhold these medications for at least 72 h before and during the challenge. Randomization and preparation of the challenges were performed by experienced FA dieticians not directly involved in the procedures. Full emergency equipment and medications (epinephrine, antihistamines, and steroids) were available. The results were assessed simultaneously by three experienced pediatric allergists. Clinical symptoms occurring within 2 h of administering the highest dose were defined as “IgE-mediated reactions.” Only subjects with positive oral challenge were enrolled in the study.

During the same study period, subjects with a sure diagnosis of IgE-mediated CMA (aged 4-18 months), visiting the Center to assess the possible occurrence of immune tolerance by the result of DBPCFC were also evaluated. Only subjects with negative oral food challenge were enrolled in the study. During the challenge procedure, a venous blood sample (4 ml) was obtained from all these patients.

We excluded patients with concomitant suspected or confirmed diagnosis, according to validated criteria, of the following conditions: allergic disorders or food allergies other than CMA, eosinophilic disorders of the gastrointestinal tract, chronic systemic diseases, congenital cardiac defects, active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, celiac disease, cystic fibrosis, metabolic diseases, food intolerance, malignancy, chronic pulmonary diseases, and malformations of the gastrointestinal tract. During the same study period, consecutive healthy children (aged 4-18 months), not at risk for atopic disorders (namely, without any first degree family member affected by allergy), attending our Department for minimal surgical procedures (phimosis, inguinal hernia repair, or cyst excisions), were enrolled as healthy controls. A venous blood sample (4 ml) was collected during pre-surgery evaluation procedures also from these subjects. They were assessed for the presence of FA and other allergic diseases at enrollment and 6 months after blood sampling by pediatric allergists at our Center. The same exclusion criteria were applied to healthy controls. In addition, all study subjects were also followed at our center for at least 12 months after blood sampling with the aim to confirm the absence of all exclusion criteria.

The parents or guardians of the children enrolled gave their written informed consent to the study. The study was approved by the Ethics Committee of the University of Naples Federico II (Protocol number: NCT02062476).

### **Total serum IgE and specific IgE against cow's milk proteins**

Serum was obtained by centrifugation for 15 min. Serum was flash frozen and stored at  $-80^{\circ}\text{C}$  until analysis. Serum total IgE and specific IgE against cow's milk proteins (alpha-lactalbumin, beta-lactoglobulin, bovine serum albumin, casein, lactoferrin) were analyzed by enzymatic immunoassay (Phadia 100 ThermoFisher Scientific CAP system, Rodano Milano, Italy). Results were expressed as kilounits per liter (kU/l).

### **RNA isolation and small RNA sequencing**

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral whole blood samples using the Ficoll-Paque (GE Healthcare, Uppsala, Sweden) method. Total RNA was isolated from PBMC samples using the Trizol Reagent kit (Invitrogen, Carlsbad, CA, USA) and quantified with the Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA quality and integrity were assessed with the Experion RNA Standard Sense kit (Biorad, Hercules, CA, USA). All RNA samples had an RNA Quality Index  $\geq 8$ . Small RNA library preparation was performed using the TruSeq Small RNA Sample Prep kit, according to the manufacturer's indications (Illumina, San Diego, CA, USA), as previously described (18,19). Briefly, 1  $\mu\text{g}$  of total RNA from each sample was ligated to specific adapters, reverse transcribed and amplified to obtain a population of double-stranded cDNAs. During PCR amplification, barcode-tagged primer pairs were used to univocally tag each sample. The resulting cDNAs were size-selected on a 6% PAGE gel. In detail, the gel band corresponding to fragments ranging from 140 to 160 bp was excised, ethanol-precipitated and eluted in ultra-pure water. The libraries were then evaluated for quality on the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Eleven libraries were pooled in equimolar amounts. Sequencing reactions were carried out using the MiSeq instrument (Illumina). The study population was analyzed in two separate sequencing experiments.

## **Bio-informatics analysis**

Each deep sequencing library was processed independently. The first step in the analysis workflow, after adapter trimming (Cutadapt tool v1.7.1) and sequence quality assessment (FastQC tool v0.11.2), was the alignment of the obtained reads to multiple human sequencing databases with the STAR tool v 2.3.1.2 (20) to the reference ncRNAs (fRNAdb, <http://www.ncrna.org/frnadb/download>) and the hg19 build of the human genome (UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/>). After trimming the adaptor, the reads shorter than 15 and greater than 32 nucleotides were discarded. The reads were aligned to the reference miRNA precursors (miRBase v.21, <http://www.mirbase.org/>) with the SHiRMP tool (21). To identify and count mature miRNAs, the reads that mapped to miRNA precursors were aligned to the reference mature miRNA (miRBase v.21, <http://www.mirbase.org/>).

Differential miRNA expression was evaluated with the DESeq2 tool (adjusted  $P < 0.05$ ); assuming a typical DESeq2 data frame, the miRNA read counts were normalized using the DESeq2 normalization method 'sizeFactors' (22). The putative miR-193a-5p target genes were predicted by DIANA-MICROT software ([http://diana.imis.athena-innovation.gr/DianaTools/index.php?r= microT\\_CDS/index](http://diana.imis.athena-innovation.gr/DianaTools/index.php?r= microT_CDS/index)), and the experimentally validated miR-193a-5p target genes and the miRNA-Target interaction network were obtained from miRTarBase database (<http://mirtarbase.mbc.nctu.edu.tw/>) (23).

## **Quantitative real time PCR analysis**

Quantitative Real Time PCR (qRT-PCR) analysis of the miR-193a-5p, and of the RPL35A, TP73, ZC3H7B and C/EBP $\alpha$  genes was performed with the TaqMan miRNA assay kit and the TaqMan gene expression assay kit, respectively (both from Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. Samples were run in duplicate at 95°C for 15 seconds and 60°C for 1 minute using an ABI Prism® 7900 Sequence

Detection System (Applied Biosystems). Data analysis was performed with the comparative threshold cycle (CT) method. In order to identify the most appropriate reference miRNA to use for data normalization, we evaluated the RNU6, miR-130 and miR-301b miRNAs, which were the most stable miRNAs also in sequencing raw data. The relative quantity of each miRNA in samples was calculated using the equation  $\Delta CT = [CT \text{ miRNA (target)} - CT \text{ miR-301 (endogenous control)}]$ . We used the GAPDH gene to normalize the level of mRNA expression. Student's and ANOVA t-tests were used to determine differences among means. The StatView 5 software was used to generate the bar plot.

### **Functional assessment of IL-4 expression depending on miR-193a-5p inhibition**

Ten ml of fresh heparinized peripheral blood were collected from a healthy human donor to evaluate IL-4 expression in CD4<sup>+</sup> T-cells (mRNA) and supernatant (protein). Donor signed an agreement acknowledging that their blood may be used for research. After, red blood cells were lysed with ammonium chloride solution, peripheral blood cells were washed, immediately stained using anti-CD4 antibody (BD Biosciences, San Jose, California, USA) and sorted for CD4<sup>+</sup> T-cell population selection. The isolated CD4<sup>+</sup> T-cells were resuspended in Opti-MEM™ medium (Invitrogen – Life Technologies, Carlsbad, California, USA) for transfection experiment. In particular, 300 x 10<sup>3</sup> cells were cultured in one well of a 24-well plate and transfected with 10 μM of miR193a-5 inhibitor (mirVana™ miRNA Inhibitors, Ambion, Austin, TX, USA) combined with Lipofectamine RNAiMax (Invitrogen - Life Technologies, Carlsbad, California, USA), following the manufacture's instructions. As negative control, we used 300 x 10<sup>3</sup> not transfected cells. After 16 hours of incubation at 37°C in a humidified atmosphere of air containing 5% CO<sub>2</sub>, T cell activation experiments were performed. In particular, transfection medium was replaced with Iscove's Modified Dulbecco's culture Medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 20%

heat inactivated fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 4mM GlutaMAX™ (Invitrogen - Life Technologies, Carlsbad, California, USA), 100 U/ml penicillin and 100 µg/ml streptomycin and only miR193a-5 inhibitor-transfected cells were stimulated with Dynabeads® Human T-Activator CD3/CD28 (Invitrogen - Life Technologies, Carlsbad, California, USA) for 3 days. After the 3 days of incubation, cells and supernatants derived from both transfected/stimulated state and untransfected/unstimulated condition (negative control) were collected to measure IL-4 at mRNA and protein level using respectively qPCR (TaqMan gene expression assay kit, Applied Biosystems, Grand Island, NY, USA) and ELISA (IL-4 ELISA Kit, human, Life Technologies, Carlsbad, California, USA), according to the manufactures' instructions. The assay was repeated twice.

## Results

The main demographic and clinical features of children enrolled in the study are reported in Table 1. Small RNA deep sequencing generated a total of 27,155,643 high-quality filtered reads, of which 19,957,521 were successfully mapped, and 4,672,481 of these were uniquely mapped reads. Almost 85% of all uniquely mapped reads fall within the miRNA category.

The size distribution pattern of these mapped reads was similar in all samples and the average read length was 22.6 nt (Table 2). We performed a differential expression analysis to assess differences between active CMA subjects and healthy controls. The heatmap shows the expression of miRNAs in all samples after unsupervised hierarchical clustering analysis of normalized data. Samples from the same study group were largely clustered together (Fig. 1A). Accordingly, the principal component analysis revealed a separation between the two groups (Fig. 1B). Then, we used the DESeq2 tool ([www.bioconductor.org](http://www.bioconductor.org)) to estimate the differential expression of miRNAs between groups. Among the miRNAs most differentially expressed between active CMA children and healthy controls, 2 were up-regulated and 14

were down-regulated (Table 3), and miR-193a-5p resulted the most down-regulated in active CMA children compared to healthy controls. Quantitative PCR confirmed that miR-193a-5p expression was significantly lower ( $P < 0.05$ ) in active CMA patients than in healthy controls (Fig. 1C).

With the aim to investigate the functional consequences of miRNA-193a-5p down-regulation in active CMA, we identify the following four targets predicted by DIANA-MICROT software: RPL35A, TP73, ZC3H7B and C/EBP $\alpha$  (Fig. 2A). These genes encode, respectively: a component of the 60S ribosomal subunit; a member of the p53 family of transcription factors; a protein that contains a RNA-binding domain; and a transcription factor with a basic leucine zipper (bZIP) domain that recognizes the CCAAT motif in the promoters of target genes. ([www.genecards.org](http://www.genecards.org)).

Quantitative PCR showed that the expression of three out of these 4 targets (RPL35A, ZC3H7B, TP73) was significantly up-regulated in active CMA patients if compared to healthy controls (see Fig. 2C-E). The C/EBP $\alpha$  transcript showed only a trend towards up-regulation in active CMA patients versus healthy controls (see Fig. 2B).

Finally, to investigate whether miR-193a-5p could be involved in the regulation of Th2 response in CMA, we assessed the effects of the inhibition of this particular miRNA on IL-4 production by CD4<sup>+</sup> T cells from a healthy donor. Cells were transfected with 10  $\mu$ M of miR193a-5p inhibitor, and a significant up-regulation of IL-4 mRNA expression and protein production were observed (Fig. 3).

Children who outgrew CMA showed miRNA-193a-5p expression profile similar to that observed in healthy controls. Quantitative PCR showed that the expression of the 4 targets (RPL35A,ZC3H7B,TP73) of this miRNA resulted also similar (data not shown).

## Discussion

Using a NGS-based approach, we investigated the “miRNA signature” associated with IgE-mediated CMA. This signature was characterized by a miR-193a-5p four-fold decrease in children with active disease. Our results are in line with previous findings in allergic dermatitis (24), and with evidence on the role of miRNA-193a-3p in regulating the expression of pro-inflammatory cytokines in human gut and PBMCs (25,26). The pivotal role of miR-193a-5p in IgE-mediated allergic response is also supported by functional data on IL-4 RNA expression and protein synthesis. These data open the road to new therapeutic opportunities targeting the miR-193a-5p in the context of CMA. Interestingly, children who acquired immune tolerance showed miR-193a-5p level similar to that observed in healthy controls, suggesting that the expression of this miRNA could be influenced by the disease state. If this result will be confirmed in future studies, miR-193a-5p could become an easily accessible biomarker for monitoring the CMA disease course.

The strengths of our study are related to a well characterized study population and to the adoption of high quality and accurate testing. The main limitations are related to the relatively small number of observations, the cross-sectional design, and the PBMC-based evaluation. Longitudinal studies in larger cohorts of CMA children, from symptom onset to immune tolerance acquisition, including simultaneous investigations on different tissue and plasma samples could be useful to further confirm our results. Finally, the miRNome network involves a large number of regulatory pathways potentially involved in CMA, and further studies are advocated to assess the effect of miRNAs interaction on CMA disease course.

In conclusion, our results suggest that miR-193a-5p is a post-transcriptional regulator of IL-4 gene expression and could have a role in restraining IgE-mediated CMA. This miRNA could be a novel, possibly therapeutic, target for this very common form of FA in childhood.

## Abbreviations

CMA: cow's milk allergy;

DBPCFC: double blind placebo-controlled food challenge;

EHCF + LGG: extensively hydrolyzed casein formula containing the probiotic *Lactobacillus rhamnosus* GG;

IgE: immunoglobulin E;

Th: T helper;

Th1: Type 1 helper;

Th2: Type 2 helper;

IL-4: interleukin-4;

IL-5:interleukin-5;

IL-10: interleukin-10;

INF- $\gamma$ : interferon- $\gamma$ ;

PBMCs: peripheral blood mononuclear cells

miRNome: full spectrum of miRNAs expressed in a specific genome;

NGS: next generation sequencing;

miRNA: micro RNA;

EoE: eosinophilic esophagitis.

### **Authors' contribution**

V.Da, V.De, L.P, FS and R.B.C. designed the study, coordinated the research team and wrote the first draft of the report. R.N. and R.B.C. cared for the patients and evaluation of their health status. V.Da, V.De, L.P, F.D.E.D, F.D, F.V, V.Di and L.D performed laboratory activities. V.Da, V.De and F.S. performed bioinformatics and statistical analysis and interpretation of analytical data. All authors revised and approved the final version of the article.

### **Availability of data**

The data set supporting the results of this article are under submission to the NCBI SRA database.

### **Conflicts of interests**

The authors have no other conflict of interests that are directly relevant to the content of this paper, which remains their sole responsibility.

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**Table 1.** Main demographic and clinical features of the study population

	<b>Active CMA patients</b>	<b>Healthy controls</b>	<b>Subjects who outgrew CMA*</b>
N.	10	11	9
Male, N. (%)	7 (70)	5 (45.4)	6 (66.7)
Age, months (SD)	5.5 (0.7)	8.2 (4.3)	17 (0.9)
Body weight, kg (SD)	7.385 (964.7)	8.644 (3.570)	12.185 (554.9)
Spontaneous delivery, N (%)	5 (50)	5 (45.5)	2 (22.2)
Breast fed, $\leq$ 8 weeks, N (%)	10 (100)	11 (100)	9 (100)
Symptoms at CMA onset:			
<i>Gastrointestinal, N (%)</i>	4 (40)	-	4 (44.4)
<i>Cutaneous, N (%)</i>	8 (80)	-	8 (88.9)
<i>Respiratory, N (%)</i>	1 (10)	-	3 (33.3)
Total serum IgE, kU/l (SD)	260.6 (230.9)	0.2 (0.1)	272.3 (244.3)
alpha-lactalbumin, kUA/l (SD)	6 (11.2)	-	0.7 (0.7)
beta-lactoglobulin, kUA/l (SD)	4.5 (7.1)	-	2.5 (3.6)
bovine serum albumin, kUA/l (SD)	5.4 (8.7)	-	1 (1.9)
casein, kUA/l (SD)	22.7 (39.2)	-	0.5 (0.4)
Lactoferrin, kUA/l (SD)	2 (6.1)	-	0

\*In all these patients acquisition of immune tolerance, after treatment with extensively hydrolyzed casein formula containing the probiotic *L. rhamnosus* GG (Nutramigen LGG, Mead Johnson Nutrition, Evansville, IN, USA), was demonstrated by the results of oral food challenge.

**Table 2.** Small RNAs deep sequencing results. The table summarizes the sequencing raw data obtained for each analyzed sample, including the total number of reads (and their average length) and the number of mapped and uniquely mapped reads. The low-quality discarded reads are reported for each sample.

Sample ID	Study group	Reads length (average)	Total reads (N)	Mapped reads (N)	Uniquely mapped reads (N, %)	Trimmed reads (%)	Reads too short/long (N)
1	Control	22.7	809,299	754,324	200,221 (24.74)	1.90%	2,314/52,661
2	Control	22.64	977,404	943,305	30,386 (3.10)	1.20%	8,561/25,538
3	Control	22.41	913,509	869,306	161,905 (17.72)	1.40%	4,716/39,487
4	Control	22.36	1,010,877	956,963	117,079 (11.58)	1.40%	5,850/48,064
5	Control	22.17	723,265	629,458	63,901 (8.83)	1.00%	2,769/91,038
6	Control	22.8	615,729	553,280	37,997 (6.17)	1.20%	4,810/57,639
7	Control	22.85	591,532	471,934	75,287 (12.72)	1.60%	3,409/116,189
8	Control	22.48	937,594	782,855	191,169 (20.38)	1.70%	826/153,913
9	Control	22.84	2,094,121	1,498,354	450,670 (21.52)	1.50%	2,120/593,647
10	Control	22.6	1,471,312	1,194,340	263,734 (17.92)	1.70%	768/276,204
11	Control	22.94	3,694,589	3,049,716	956,642 (25.89)	1.80%	1,987/642,886
12	CMA	22.39	1,163,198	1,046,515	329,420 (28.32)	1.60%	1,630/115,053
13	CMA	22.75	1,335,833	1,049,824	205,514 (15.38)	1.50%	6,320/279,689
14	CMA	22.35	694,018	656,621	44,682 (6.43)	2.20%	1,1298/26,099
15	CMA	23.55	3,004,754	295,705	55,113 (1.83)	0.40%	197/2,708,852
16	CMA	22.41	509,618	493,502	81,052 (15.90)	1.40%	2,902/13,214
17	CMA	22.55	844,123	756,700	86,758 (10.27)	1.70%	8,472/78,951
18	CMA	22.59	1,309,81	1,167,87	286,678	2.00%	3,013/138,92

			4	2	(21.88)		9
<b>19</b>	CMA	22.72	1,701,66	1,568,06	477,284	2.00%	2,979/130,62
			7	7	(28.04)		1
<b>20</b>	CMA	22.87	1,305,61	1,192,97	193,008	1.70%	11,528/101,1
			4	8	(14.78)		08
<b>21</b>	CMA	22.51	1,447,77	1,272,98	363,981	1.70%	982/173,804
			3	7	(25.14)		

ID, identifiers; N, number.

**Table 3.** Most significant down-regulated and up-regulated miRNAs in children with active cow's milk allergy versus healthy controls.

Mature*	Chr	Strand	Log2(FC)	pvalue (adj)
miR-193a-5p	chr17	1	-1.84	0.000276
miR-197-3p	chr1	1	-1.49	0.000062
miR-423-5p	chr17	1	-1.26	0.000276
let-7b-5p	chr22	1	-1.14	0.004
miR-486-5p	chr8	1	-1.08	0.0425
miR-92b-3p	chr1	1	-1.07	0.022
let-7b-3p	chr22	1	-1.05	0.0425
miR-574-3p	chr4	1	-1.04	0.0163
miR-125a-5p	chr19	1	-0.99	0.00381
miR-93-3p	chr7	-1	-0.94	0.0385
miR-3615	chr17	1	-0.84	0.0385
miR-423-3p	chr17	1	-0.73	0.00172
miR-320a	chr8	-1	-0.70	0.0289
miR-191-5p	chr3	-1	-0.64	0.00625
miR-30a-5p	chr6	-1	1.08	0.00625
miR-224-5p	chrX	-1	1.13	0.0144

\*Mature: '5p' and '3p' indicate the DNA arm of the miRNA precursor (from the 5' or from the 3', respectively) according to miRNA nomenclature (<http://www.mirbase.org/help/nomenclature.shtml>); Chr: chromosome; Log2 (FC): Log2 (FoldChange); p value (adj): p value adjusted by Benjamini, Hochberg method. The DESeq2 tool was used to estimate the differential expression of miRNAs between the two groups based on normalized read counts (p value adjusted $\leq$ 0.1).

## FIGURE LEGENDS

**Figure 1. miRNome differential analysis between active CMA patients and healthy controls.**

(A) Heat map representation showing hierarchical clustering of active CMA patients and controls on the basis of the high differentially expressed miRNAs. On the heat map vertical axis are reported the names of the most significant miRNAs differentially expressed between the two groups (adjusted  $p$  value  $<0.1$ ) on the heat map horizontal axis, are the sample identification numbers. (B) Principal component analysis plot, based on miRNA expression of active CMA patients and healthy children, showing that almost all samples belong to the same condition are clustered together. (C) The relative expression of miR-193a-5p was confirmed by qPCR showing significant down-regulation in active CMA patients versus healthy controls ( $P<0.05$ ). The horizontal bars represent the mean values and the range.

**Figure 2. Evaluation of miR-193a-5p targets.**

(A) Interaction network of miR-193a-5p-targets obtained from miRTarBase database showing that it has four targets: RPL35A, TP73, ZC3H7B and C/EBP $\alpha$ . Junction:11; Edge:11. The blue junctions represent an interaction between the edges, for which there is strong evidence (reporter assay, western blot, qRT-PCR or qPCR); the light blue junctions represent an interaction between the edges with evidence of cross linking, ligation, and sequencing of hybrids (CLASH). The CLASH method is based on high-throughput mapping of RNA-RNA interactions. miR-193a-5p predicted targets were evaluated by qPCR in active CMA patients versus healthy controls (B) C/EBP $\alpha$  transcript shows a trend in up-regulation in active CMA patients versus controls. (C) RPL35A transcript was significantly up-regulated in active CMA patients versus controls ( $***=P<0.001$ ) (D) The relative expression of TP73 transcript shows significant up-regulation in active CMA subjects versus healthy controls ( $*=P<0.05$ ) (E) ZC3H7B transcript was significantly up-regulated in active CMA subjects versus healthy controls ( $***=P<0.001$ ).

**Figure 3. Functional assessment of IL-4 expression depending on miR-193a-5p inhibition.**

(A) IL-4 mRNA and protein levels were assessed by qPCR and ELISA, respectively, in CD4 $^{+}$  T cells from a healthy human donor. CD4 $^{+}$  T cells, transfected with miR193a-5

inhibitor, showed a higher IL-4 expression compared to unstimulated CD4+T cells (negative control) (B) Accordingly IL-4 protein, as assessed by ELISA, showed a corresponding increase in protein level in CD4+ T cells transfected with miR193a-5 inhibitor.

**Figure 1**

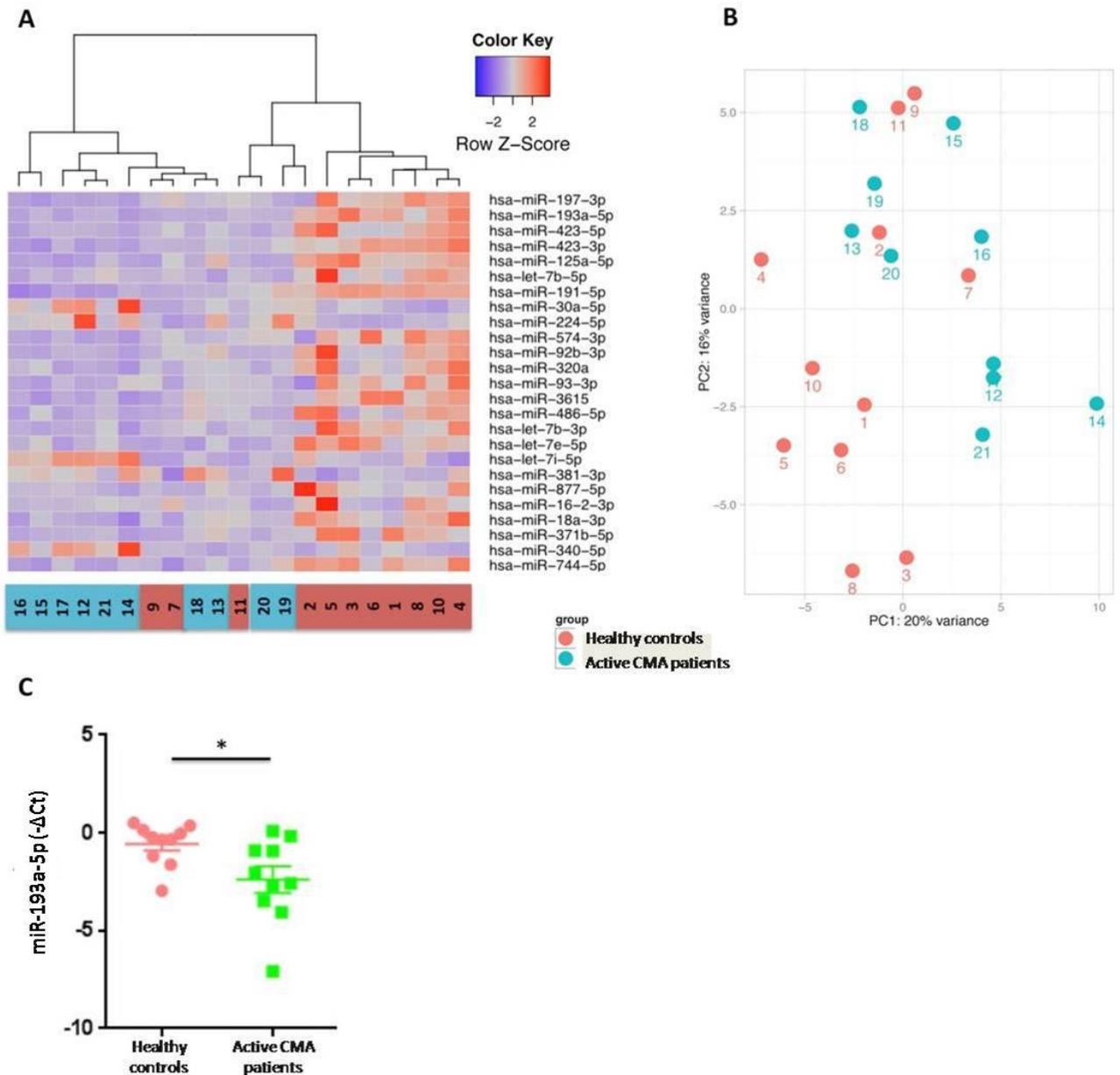


Figure 2

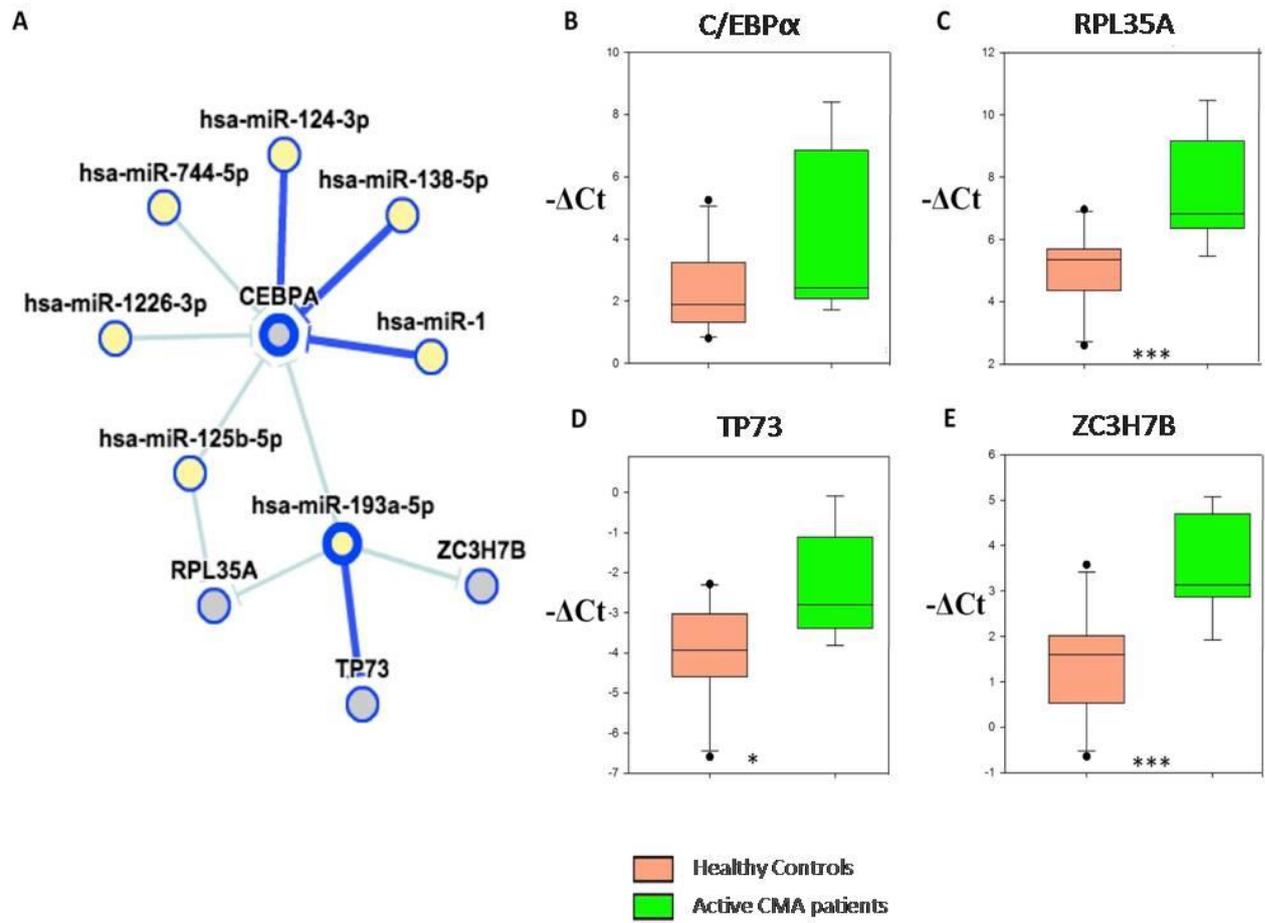


Figure 3

