

Neutrophil MiRNA-128-3p is Decreased During Active Phase of Granulomatosis with Polyangiitis

Marcin Surmiak[#], Magdalena Hubalewska-Mazgaj[#], Katarzyna Wawrzycka-Adamczyk, Jacek Musiał and Marek Sanak*

Department of Internal Medicine Jagiellonian University Medical College Krakow, Poland

Abstract: Granulomatosis with polyangiitis is a rare chronic inflammatory disease. In this multisystem autoimmune disorder neutrophils cause small vessels necrosis and infiltrate perivascular tissue to form granulomas. Progression of the disease is evaluated by the symptoms score and by a titer of anti-neutrophil cytoplasm antibodies. Despite glucocorticoid and immunosuppressive therapy, prognosis is complicated by chronic renal insufficiency, hearing loss and skin ulceration. In this preliminary study we tested the hypothesis that altered neutrophil expression of miRNAs can contribute to the cell activation, extracellular traps formation and decreased apoptosis. First we compared a profile of 728 miRNAs expressed in circulating neutrophils of patients with active disease and matched healthy donors. Subsequently, candidate miRNAs were quantified in neutrophils from 16 subjects with active disease, 16 asymptomatic patients at the remission and in 16 healthy controls. Out of 11 candidate miRNAs, only miR-128-3p was both biologically (relative quantity < 30% control or remission patients) and statistically ($p < 0.01$) decreased in the cells during active stage of the disease. This miRNA correlated with a clinical score of the disease well. A set of 10 transcripts involved in the mechanism of the disease was quantified from the same neutrophils RNA. Relative expression of *MMP9* was higher in neutrophils from the patients with active disease and correlated negatively with miR-128-3p. The opposite finding was present for *MTA1* transcripts. Despite surprisingly scarce changes in the expression of neutrophil miRNAs, miR-128-3p is the best candidate for deciphering etiology of granulomatosis with polyangiitis.



M. Sanak

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1. INTRODUCTION

Granulomatosis with polyangiitis (GPA) is a rare (1:50 000-1:100 000) systemic autoimmune disease of unknown etiology. In this form of necrotising vasculitis small and medium vessels of many tissues are affected by neutrophilic inflammation and formation of polymorphonuclear cells granulomas. The disease expands from unspecific nasal symptoms of congestion or bleeding to chronic inflammation of paranasal sinuses and the lung. Other common manifestations of GPA are eye involvement, renal insufficiency, peripheral polyneuropathy, arthralgia and skin rashes [1]. Many subjects with GPA have circulating autoantibodies against neutrophil cytoplasm, named ANCA [2]. Neutrophils are one of the key cells in pathophysiology of GPA but still little is known about molecular mechanisms of their activation. Proteinase-3 (PR3), a neutrophil granule protein, is a common antigen recognized by ANCA [3].

Expression of the surface PR3 increases upon activation of neutrophils, whereas binding of anti-PR3 antibodies perpetuates activation of the cell [4, 5]. Recently, we demonstrated that a release of circulating cell free nuclear and

mitochondrial DNA, plausibly in effect of neutrophil extracellular trap formation, is another hallmark of GPA [6]. The disease follows a protracted course of flares and remissions. The treatment requires induction of remission by a high systemic dose of glucocorticoids and additional immunosuppressive drugs, usually continued in lower doses to prevent relapses of the disease. Altered phenotype of neutrophil in GPA patients suggests a profound reprogramming of the genes expression pattern, although no genomic alterations were described except some strong genetic associations, e.g. with certain variants of *HLA-DP* locus [7, 8].

The aim of this preliminary study was a planned comparison of the peripheral blood neutrophil's miRNAs expression profile between the patients with exacerbated and inactive GPA in order to establish the role of miRNAs in neutrophil activation observed in this disease.

2. MATERIALS AND METHODS

2.1. Study Participants

We enrolled 32 patients with granulomatosis with polyangiitis to the study. Disease activity was measured with the use of Birmingham Vasculitis Activity Score (BVAS) [9, 10]. During the remission of the disease the score is 0 indicating no symptoms, whereas its maximal theoretical value is 68. Sixteen patients were in active stage of the disease (BVAS>1) and 16 patients had remission (BVAS=0). We also recruited 16 age and sex matched healthy volunteers

*Address correspondence to this author at the Department of Medicine, Jagiellonian University Medical College, Skawińska Str. 8, 31-066 Kraków, Poland; Tel/Fax: ++48-12-430-5372, +48-12-430-5203; E-mail: nfsanak@cyf-kr.edu.pl

[#] These two authors contributed equally to this work.

who served as a control group. Basic laboratory tests (CBC, CRP level, anti-PR3 IgG level, creatinine and LDH level) were ascertained in all participants at the same time the peripheral blood was collected for neutrophils isolation. Informed, written consent was obtained from all subjects in the study. This study was approved by the Jagiellonian University Ethical Committee.

2.2. Peripheral Blood Neutrophils Isolation

Neutrophils were isolated from heparinized blood using commercially available kit (EasySep Human Neutrophil Enrichment Kit, STEMCELL Technologies Inc, Vancouver, Canada). Briefly, to separate polymorphonuclear leukocytes (PMN) from mononuclear cells (PBMC), a standard procedure of Histopaque-1077 (Sigma-Aldrich Chemical Co, St Louis, USA) gradient density centrifugation was used. After centrifugation the layer of plasma and mononuclear cells was discarded and the remaining erythrocytes/granulocytes were transferred into a fresh tube for erythrocytes lysis with a hypotonic ammonium chloride solution. Obtained PMNs were used for the isolation of neutrophils according to neutrophil enrichment kit protocol provided by the manufacturer. Purity of the neutrophil fraction (>98%) was determined by a flow cytometry and the cells viability (>95%) was verified by trypan blue exclusion staining. Immediately after isolation of neutrophils, their total cellular RNA was isolated.

2.3. Neutrophils's Total RNA Isolation and Reverse Transcription

Total cellular RNA was isolated using RNAzol reagent (Sigma-Aldrich Chemical Co, St Louis, USA) as recommended by the manufacturer. Concentration and purity of isolated RNA were ascertained by spectrophotometry using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was done using TaqMan MicroRNA Reverse Transcription Kit for miRNA analysis. High Capacity cDNA Reverse Transcription Kit (both from Life Technologies, Carlsbad, CA, USA) was used for a gene expression measurements.

2.4. Measurements of miRNAs and Genes Expression

Screening for differentially expressed miRNAs in neutrophils of patients with GPA was performed using TaqMan OpenArray Human MicroRNA Panel (Life Technologies, Carlsbad, CA, USA). Expression of 728 miRNAs was analysed in a randomly selected sub-sample consisting of 3 patients with active GPA and 3 healthy controls. MiRNA species differing significantly between patients and controls were next analysed in all study participants. Selection of candidate miRNA target genes was assisted with the use of bioinformatics tools. Relative expression of miRNA and their candidate mRNA targets were measured using quantitative real-time PCR based on TaqMan probes chemistry (7900HT Fast Real Time PCR System; Life Technologies, Carlsbad, CA, USA). Quantification cycle data were normalized to RNU44 and miR-26b for miRNA analysis or to ribosomal 18S rRNA and GAPDH for gene expression analysis. Relative quantities (RQ) were calculated using $2^{-\Delta Ct}$ formula from the appropriate endogenous controls [11].

2.5. Measurements of TNF- α in Serum

Venous blood samples were collected without anticoagulant using Sarstedt S-Monovette tubes from all patients and controls. Serum was separated using a standard laboratory method, aliquoted and frozen in -80°C for further analysis. Serum level of TNF- α was measured by Luminex assay (Bio-Plex Pro™ Human Chemokine Panel, Bio-Rad, Hercules, CA, USA) using MAGPIX fluorescent-based detection system (Luminex Corp., Austin, TX, USA).

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). All comparisons were done using one-way analysis of variance (ANOVA) with Tukey's post hoc test or with Kruskal-Wallis non-parametric test with Dunn's post hoc test. Descriptive statistics was presented as a mean \pm standard deviation (SD) or median with 25th-75th percentile range, depending on the distribution. Type I statistical error $P < 0.05$ was considered significant.

3. RESULTS

3.1. Subjects of the Study

Patients, stratified according to the presence of symptoms of the exacerbated disease (BVAS>0) or remission (BVAS=0), did not differ by their age or gender distribution. In patients with active disease, higher doses of oral glucocorticoids were administered, however this difference was not significant and none received immunosuppressive drugs. Active disease was also characterized by elevated white blood cell and polymorphonuclear leukocyte count and higher levels of C-reactive protein. Serum creatinine was also higher reflecting renal involvement. Serum lactic dehydrogenase was elevated both in active and inactive GPA indicating an increased tissue damage in the disease. Details on the study groups are summarized in (Table 1).

3.2. Measurements of miRNAs

MiRNA screening was done using Megaplex (Applied Biosystems) chemistry on a sub-group of 6 study subjects and detected 224 miRNAs, the expression levels of which was measurable in at least one subject, out of 728 human miRNAs quantified. Of these detectable miRNAs, 33 were up regulated and 8 miRNAs were down regulated (fold change >2) in GPA samples. Interestingly, expression of 2 miRNAs: miR-148-5p, miR-362-5p was detectable only in GPA samples while three others: miR-128-3p, miR-659 and miR-661 were present only in control samples.

3.3. Quantification of Candidate miRNA in the Study Subjects

Eleven candidate miRNAs were selected for the measurements in all study subjects. These were 5 of 6 differentially expressed miRNAs in screening samples, and other 6 miRNAs, the expression of which was detectable in screening samples and mRNA targets were possibly linked to the leukocyte biology. The list of the candidate miRNAs with their assays ID is provided in (Table 2). Real-time quantification of miR-148-5p gave inconsistent results since quanti-

fication cycles varied in technical triplicates by more than 1 cycle. No difference in expression across the study groups was found for miR-661, miR-659-3p, miR-362-5p, miR-535-5p, miR-25 and miR-19b. There were some differences in the abundance of miR-223, miR-16 and miR-106a in neutrophils between the study groups, however they did not reach the biological significance of twofold expression change (Fig. 1). Thus, only the one miR-128-3p expression out of 11 quantified in all study subjects was different when contrasted between patients with GPA and healthy controls (Fig. 2a). MiR-128-3p expression was the lowest in patients with active GPA (3.39 times lower in comparison with controls; $p=0.005$) and 4.04 times lower than in GPA patients with remission ($p=0.002$).

Table 1. Selected characteristics of the study participants.

	Active GPA	Remission	Control
n	16	16	16
Age (mean±SD)	57.2±13.9	54.2±12.3	55.9±10.6
Gender (F/M)	9/7	7/9	9/7
BVAS (range)	6-28	0	0
GC treatment	6/10	11/5	-
GC dose(mg)	3-20	2-8	-
cANCA (range)	<20-200	<20-140	<20
CRP [mg/mL]	<5.0-140	<5.0-6.3	<5.0
PBMC [$10^3/\mu\text{L}$]	10.6±3.9*	7.6±2.49	5.7±2.5
PMN [$10^3/\mu\text{L}$]	7.6±3.9*	5.4±2.4	3.1±1.1
PLT [$10^3/\mu\text{L}$]	227.5±125.4	225±66.6	202.5±36.5
Creatinine [mg/L]	179±302*	119.2±80.5	78±15
LDH [U/L]	508±128*	510.2±101.7*	394±76

BVAS- Birmingham Vasculitis Activity Score; * $p<0.05$, in comparison with controls; GC – glucocorticosteroids.

Relative expression of miR-223 was elevated in neutrophils from patients with active or inactive granulomatosis with polyangiitis by comparison with healthy controls; miR-16 and miR-106a relative expression was decreased in patient at remission of granulomatosis with polyangiitis by comparison with active disease or with controls. Neither difference was biologically significant ($0.5 < \text{relative quantity} < 2$).

3.4. Serum TNF- α Level and the Symptoms Score in Patients with GPA

As expected, serum TNF- α level was elevated in all GPA patients (active GPA - 17.1±5 pg/mL; inactive GPA – 17.9±4 pg/mL) by comparison with controls (11.56±2.8 pg/mL; $p<0.05$), regardless of the disease activity (Fig. 2b). However, no correlation was present between this inflammatory cytokine concentration and relative expression of miR-

128-3p in all subjects studied or in any GPA subgroup. Instead, miR-128-3p relative expression correlated robustly and positively with the clinical BVAS score in the subgroup of GPA patients with active disease (Spearman's $\rho=0.71$; $p<0.05$; Fig. 2c).

Table 2. Candidate miRNAs and target mRNAs quantified in the study.

miRNA/gene	miRNA/gene TaqMan assay ID
miR-661	001606_hsa-miR-66
miR-659-3p	001514_hsa-miR-659
miR-128a	002216_hsa-miR-128a
miR-362-5p	001273_hsa-miR-362
miR-535-5p	001518_hsa-miR-532
miR-16	000391_hsa-miR-16
miR-223	002295_hsa-miR-223
miR-25	000403_hsa-miR-25
miR-106a	002169_hsa-miR-106a
miR-19b	000396_hsa-miR-19b
miR-26b	000407_hsa-miR-26b
RNU44	001094_RNU44
18s	Hs99999901_s1
GAPDH	Hs99999905_m1
MMP9	Hs00234579_m1
MTA1	Hs00950776_m1

3.5. Selected Target Transcripts Analysis

Since human miR-128-3p has at least one complementary sequence of minimal 12 base pairs length within 19294 transcripts, as predicted by RNA22v2.0 (available at <https://cm.jefferson.edu/rna22/Precomputed/>) and the miR relative expression was decreased in active GPA patients as the only one reaching biological significance, a systems biology approach to identify consequences of its downregulation was impractical. We quantified instead expression of 10 neutrophil mRNA identified previously by us to associate with GPA. These were transcripts of: *DIABLO*, *MDM2*, *MMP9*, *MPO*, *MTA1*, *NFKBI*, *PMPAIP1*, *PR3*, *PTEN* and *RUNX3*. Correlation of miR-128-3p abundance was present only for *MMP9* and *MTA1* mRNA, out of 10 quantified neutrophil transcripts. *MMP9* encodes matrix metalloproteinase 9, named also gelatinase B. *MMP9* mRNA relative expression was significantly higher in patients with active stage of GPA (3.27 fold in comparison to controls; $p<0.05$ and 2.26 fold in comparison to patients in GPA remission; $p>0.05$, Fig. 3a).

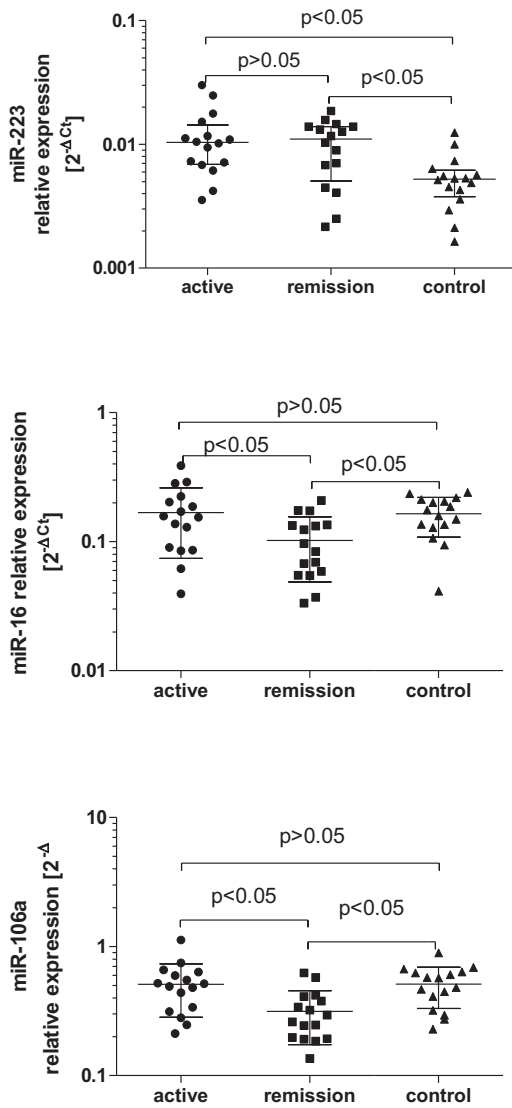


Fig. (1). Relative expression of miR-223 was elevated in neutrophils from patients with active or inactive granulomatosis with polyangiitis by comparison with healthy controls; miR-16 and miR-106a relative expression was decreased in patient at remission of granulomatosis with polyangiitis by comparison with active disease or with controls. Neither difference was biologically significant ($0.5 < \text{relative quantity} < 2$).

MTA1 gene encodes for metastasis-tumour antigen-1. Relative expression of *MTA1* gene was lower in active stage of GPA (0.39 fold lower in comparison with controls; $p < 0.05$ and 0.5 fold lower in comparison with patients in GPA remission; $p > 0.05$ Fig. 4a). There was a significant correlation between relative expression of *MMP9* mRNA and miR-128-3p ($\rho = -0.6$ $p < 0.05$, Fig. 3b) in patients with active GPA, whereas *MMP9* and *MTA1* mRNA cross-correlated negatively in the same study subgroup ($\rho = -0.51$ $p < 0.05$, Fig. 4b). No other mRNA quantified showed any relation with miR-128-3p abundance, although *PR3*, *MPO* and *PTEN* transcripts were increased in active GPA, whereas *RUNX3*, *DIABLO* and *PMPAIP1* transcripts were decreased both in active and inactive GPA (data not shown).

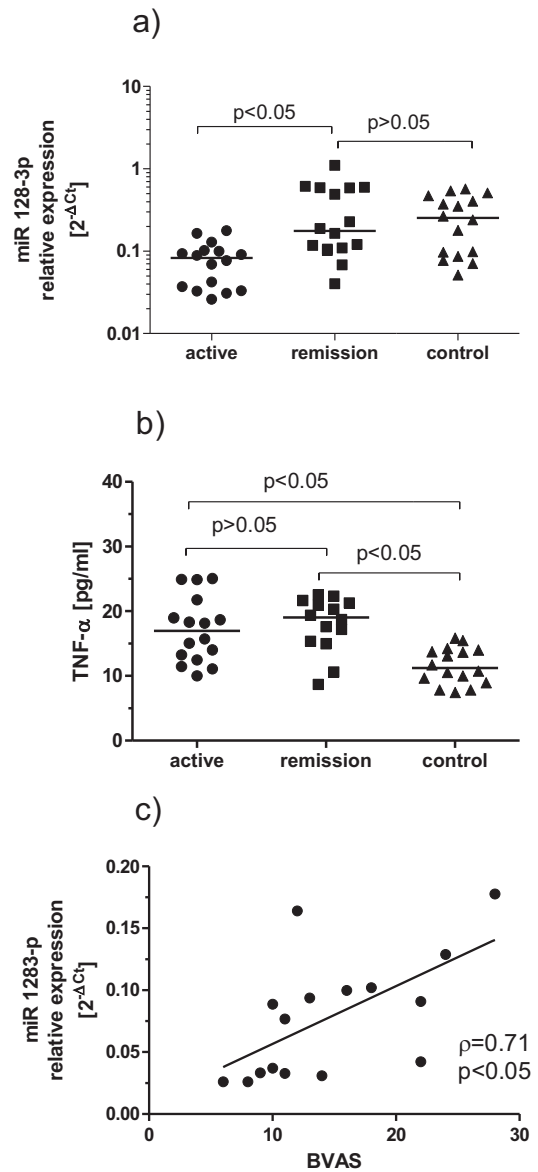


Fig. (2). Relative (to combined RNU44 and miR-26b) expression of neutrophil miR-128-3p in the study groups of: active granulomatosis with polyangiitis, patients at the remission of the disease and healthy controls ($n=16$ each group, (a)); serum level of tumor necrosis factor- α was elevated in all patients with granulomatosis with polyangiitis (b); there was a correlation between Birmingham Vasculitis Symptoms Score and relative expression of miR-128-3p (c).

4. DISCUSSION

GPA is a relatively rare but clinically important systemic inflammatory disorder of an autoimmune background. No aetiological factors were established for this disease, however, circulating anti-PR3 and less frequently anti-myeloperoxidase ones are both diagnostic and contribute to the mechanism of the disease by activation of neutrophils [2]. As to the best of our knowledge no studies on miRNAs expression in GPA have been published yet. In the current study we report on a relatively unaltered expression of neutrophil's miRNAs in GPA subjects, despite profound changes in several cellular pathways found by studies on neutrophils exposed to ANCA auto-antibodies [5]. An unbi-

ased screening for miRNA expression profile suggested initially that some species could be significantly associated with the disease. However, a scrupulous planned comparison of the expression level of the candidate miRNAs, mandatory to attain a statistical power of the study, did not confirm the initial findings except decreased expression of miR-128-3p. This miRNA was described to downregulate expression of adenosine receptor 2B (A2BAR or ADORA2B) in epithelial tissue. One of the cellular responses in colitis is a potent overexpression of this receptor in response to TNF- α . Interestingly, Kolachala *et al.* [12] using a cell culture model found that both miR-27b and miR-128-3p decreased in TNF- α pretreated T84 cells, and that overexpression of these miRNAs reduced A2BAR levels. Adenosine receptors are involved in inflammatory responses on multiple levels of the organism. In epithelia, they facilitate inflammatory cells influx, in myeloid cells affects degranulation. In a mice model of renal ischemia/reperfusion injury, adenosine protected against the tissue damage due to inhibition of TNF- α release from neutrophils, a mechanism mediated through activation of A2BAR [13]. However, these findings do not agree with the results of our current study. Deficiency of miR-128-3p in neutrophils from active GPA patients should enhance signaling by A2BAR, but similar TNF- α levels in active and inactive GPA subjects suggest that elevated TNF- α level in both subgroups is refractory to a putative inhibition by adenosine, if this mediator could modulate neutrophil degranulation and release of the cytokine. Seeking for biologically meaningful targets of miR-128-3p, we encounter two major problems. This microRNA was the only one significantly and clearly altered in active GPA patients. Its abundance was decreased, which precluded filtering out possible targets using system biology bioinformatics tools. Moreover, predicted targets list for miR-128-3p is particularly lengthy one. Thus, we relied on our previous findings of the gene expression profile of neutrophils from healthy subjects stimulated with native cANCA autoantibodies [5] and a mechanism of extracellular DNA trap formation and delayed apoptosis of neutrophils in patients with GPA [6].

Circulating neutrophils are the major source of matrix metalloproteases (MMPs), however, *MMP9* is expressed during rather early stages of neutrophil maturation, and preformed MMP9 protein is stored in cytoplasmic granules [14]. In our previous study, we showed that stimulation of neutrophils with cANCA antibodies caused up-regulation of *MMP9* expression [5]. *MMP9* expression can be regulated by several other genes including *MTA1*. Yan *et al.* [15] reported that overexpression of *MTA1* led to down-regulation of MMP9. In this study we observed similar pattern in patients with active GPA, whose expression of neutrophil MMP9 was upregulated while *MTA1* was down-regulated. *MTA1* is commonly upregulated in several types of malignancies like breast or ovarian cancer. Studies of Reddy *et al.* showed that expression of *MTA1* can be negatively regulated by miR-661 and c/EBP α [17]. Abgawad *et al.* reported in patients with ANCA-associated vasculitis increased neutrophil level of c/EBP α [18]. However in the current study we did not quantify expression of c/EBP α . Moreover, we failed to find any differences in the expression of miR-661, which could validate such a hypothesis on a suppression of *MTA1* transcripts by miR-661 and sequential induction of *MMP9*.

Another plausible indirect mechanism regulating expression of *MMP9* can link expression of miR-128-3p with polycomb complex protein (BMI-1). Venkataraman *et al.* [19] observed the presence of a negative correlation between expression of miR-128-3p and BMI-1 in medulloblastoma cells of the central nervous system tumour. Also Li *et al.* showed in hepatocellular carcinoma cells an upregulation of BMI-1 concurrently with upregulation of *MMP9* expression [20]. This may suggest that MMP9 level can be regulated indirectly by miR-128-3p, exemplary by positive influence of BMI-1. Lack of data on the expression of *BMI1* oncogene expression is a limitation of the current study, which will require investigations.

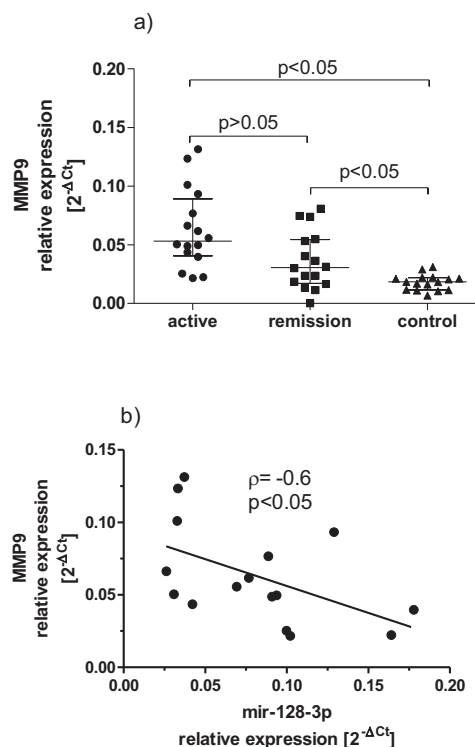


Fig. (3). Matrix metalloproteinase-9 (MMP9) mRNA was increased in neutrophils from patients with granulomatosis with polyangiitis during the active disease (a). Overexpression of *MMP9* correlated negatively with relative expression of miR-128-3p (b).

Interestingly, we found a good correlation between clinical score of GPA activity and relative expression of miR-128-3p. The score is based on the presence of general (myalgia, arthralgia, fever, weight loss) and organ limited disease symptoms. Since this correlation was positive, it precluded a simple mechanistic association between decreased neutrophil's miR-128-3p and increased activity of the disease. Moreover, during clinical remissions expression levels of miR-128-3p normalized, however, during active phase of GPA patients with more symptoms of the disease also had higher relative expression of miR-128-3p. This microRNA molecule was well studied in human malignancies and was found to be oncogenic. Among other mechanisms of action, overexpression of miR-128-3p confers resistance to Fas-mediated apoptosis in myeloid leukemia [21], a mechanism which complies with decreased apoptosis of neutrophils we observed in GPA [6]. Similarly, serum levels of miR-128a were also described elevated in patients with psoriasis, a skin disease with neutrophils' infiltrate

and arthropathy [22]. Any clear explanation about deficiency of neutrophil's miR-128-3p with activity of GPA process is another limitation of this study. We may only speculate that intensity of clinical symptoms reflects rather a secondary tissue damage than an alteration within circulating neutrophils, and these two processes are not simply parallel. These speculations might be elucidated by functional studies on neutrophil activation, recruitment and transmigration in response to altered expression of miR-128-3p.

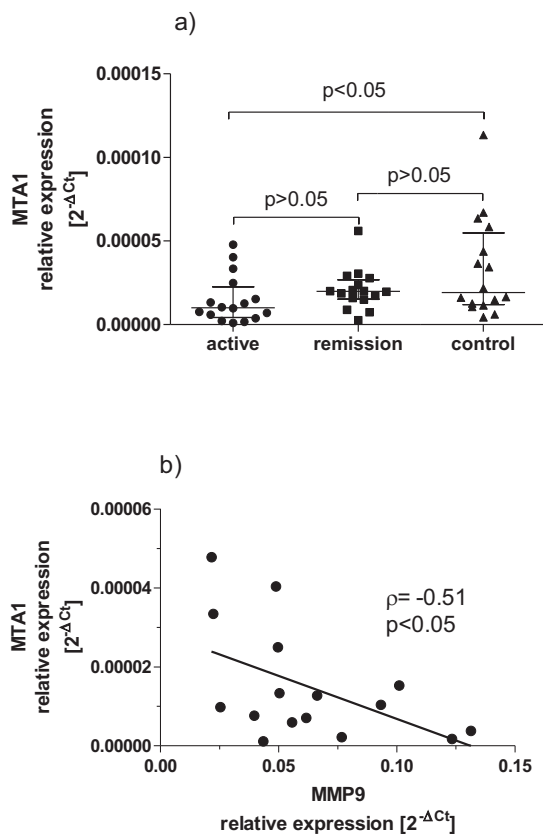


Fig. (4). Metastasis-tumour antigen-1 (MTA1) mRNA relative expression was decreased in neutrophils from the patients with active granulomatosis with polyangiitis (a). There was a negative correlation between abundance of *MTA1* and *MMP9* transcripts (b).

5. CONCLUSION

In conclusion, we describe a surprisingly scarce changes in miRNA expression profile of peripheral blood neutrophils in granulomatosis with polyangiitis patients. These key cells involved in tissue damage and granulomas formation had only one miR-128-3p decreased significantly, despite a profound alterations of inflammatory pathways induced by the circulating anti-neutrophil cytoplasm antibodies.

LIST OF ABBREVIATIONS

A2BAR	=	Adenosine receptor 2B
ANCA	=	Anti-neutrophil cytoplasmic antibodies
CBC	=	Complete blood count
c/EBP α	=	CCAAT-enhancer-binding proteins
BMI-1	=	Polycomb complex protein
BVAS	=	Birmingham Vasculitis Activity Score

cANCA	=	Cytoplasmic anti-neutrophil cytoplasmic antibodies
GC	=	Glucocorticosteroids
GPA	=	Granulomatosis with polyangiitis
miRNA	=	microRNA
MMP-9	=	Matrix metalloproteinase 9
MTA1	=	Metastasis-tumor antigen-1
PMN	=	Polymorphonuclear leukocytes
PR3	=	Proteinase 3
TNF- α	=	Tumor necrosis factor alpha

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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