The Journal of Teachers Association
ISSN 1019-8555 (Print) & ISSN 2408-8854 (Online)
Frequency: Bi-Annual
DOI: https://doi.org/10.62469/taj.v037i01.0376

OPEN ACCESS
Website: www.tajrmc.com
Email: editor@tajrmc.com
Volume-37 | Issue-1 | Jan-Jun 2024

Myeloperoxidase mRNA Expression in Neutrophils of Polyarticular and Enthesitis-related Juvenile Idiopathic Arthritis

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ABSTRACT: ***Background:*** Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic condition in childhood. ***Methods:*** This comparative cross-sectional study was conducted at the Genomic Research Laboratory, Department of Anatomy, Bangabandhu Sheikh Mujib Medical University (BSMMU) among polyarticular, enthesitis-related arthritis (ERA), and healthy children. Diagnosed patients of polyarticular JIA (15), enthesitis-related JIA children (15) and healthy children as control (15) who came for screening of arthritis directly or referred by physicians at Department of Pediatric Rheumatology and General Pediatrics Outpatient Departments aged less than 16 years were included by conveniently by purposive sampling technique. RNA was isolated from the peripheral blood before synthesizing cDNA. The MPO mRNA expression was observed with a gene specific primer in real-time quantitative PCR. The gene expression was leveled as $2^{-\Delta ct}$ relative as compared to reference gene. Chi-square test and Fisher's exact test was used for the level of significance. Clinical characteristics were described using SPSS version 24. ***Results:*** The mean age of the 45 participants with SD was 10.02±2.84 years. Then average age of onset of poly articular JIA in children was 10.5±3.1 years with male-to-female ratio was 2.78:1 in both diseased groups. In ERA group, the mean age at onset was 11.13 years and the lowest age was 8.5 years and the highest age was 14 years. A total of 13.3% of ERA children had a positive family history and only one child (6.7%) in the poly JIA group. The MPO mRNA expression was found significantly elevated (p <0.001). ***Conclusion:*** The gene expression status of MPO may be used in future to identify early changes in neutrophil activation.

Keywords: Juvenile Idiopathic Arthritis, Rheumatic Disease, mRNA Expression, Bangladeshi Children.

Original Researcher Article

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How to cite this article:
Ananya R, Nahar S, Dhiraj B, Islam MM, Banu LA; Myeloperoxidase mRNA Expression in Neutrophils of Polyarticular and Enthesitis-related Juvenile Idiopathic Arthritis. Taj 2024;37 (1): 292-299.

Article history:
Received: December 28, 2023
Accepted: February 22, 2024

Article at a glance:

Study Purpose: *The purpose of this study was to investigate myeloperoxidase mRNA expression profile in neutrophils among children with polyarticular and enthesitis-related juvenile idiopathic arthritis and healthy children.*

Key findings: *Changes in the mRNA expression profile in neutrophils of Bengali Bangladeshi children may help in producing new knowledge for development of biomarkers.*

Newer findings: *This is the first ever study conducted in Bangladesh, taking into account the race and ethnicity (Bengali Bangladeshi) in JIA children. the MPO mRNA expression was 1.6 folds in the ERA group and 1.2-fold in the poly JIA group in comparison to control group 0.001.*

Abbreviations: *ERA: enthesitis-related arthritis, JIA: juvenile idiopathic arthritis, CRP: C-reactive protein.*

INTRODUCTION

There are multiple types of juvenile idiopathic arthritis (JIA), each with distinct features. They are Oligoarticular juvenile idiopathic arthritis, Polyarticular juvenile idiopathic arthritis–rheumatoid factor negative, Polyarticular juvenile idiopathic arthritis–rheumatoid factor positive,

Enthesitis-related juvenile idiopathic arthritis, Psoriatic juvenile idiopathic arthritis, Systemic juvenile idiopathic arthritis and Undifferentiated arthritis.[1,2] Generally, they all exhibit arthritic manifestations of joint pain, inflammation, warmth, and rigidity that persist for a minimum of 6 weeks. In asian countries, Juvenile idiopathic arthritis (JIA) and enthesitis-related arthritis (ERA) types are more common. [3-5] Juvenile idiopathic arthritis (JIA) is the most prevalent chronic rheumatic condition in children. The illness can lead to prolonged inflammation, restricted movement of joints, and even loss of movements.[6, 7] Approximately 10% to 15% of children diagnosed with JIA enthesitis-related arthritis (ERA). Enthesitis refers to inflammation at tendon or ligament attachment points on bones, affecting boys more than girls. It mainly impacts larger leg joints, causing foot pain during activities, with the knees and ankles being the most commonly affected areas.[2, 7] Research conducted in a semi-urban region of Bangladesh indicates that 60.5 out of every 100,000 children are affected by JIA.[8] An imbalance of neutrophils is often found in synovial fluid during arthritis.[9] JIA may not display heightened levels of standard markers such as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR). Variations in the distribution of JIA subtypes among different ethnic groups suggest potential genetic factors may exist. Low density neutrophils (LDNs) have been recognized in autoimmune diseases, infections, and inflammation, where they are thought to play a role in the disease process. LDNs show different functions in various inflammatory disorders, highlighting the specificity of LDNs to each condition.[10,11] Identifying these biomarkers is crucial for accurate diagnosis and targeted treatment. Investigations into markers like MPO mRNA could improve the understanding of JIA-related inflammation among different populations.[12] Myeloperoxidase (MPO) mRNA expression in neutrophils of children with polyarticular and enthesitis-related JIA have significant alterations linked to disease activity and distinct transcriptional profiles, with elevated MPO mRNA levels correlating with disease severity and inflammatory markers.[13] Currently, mRNAs is identified as novel markers of the therapeutic efficacy of anti-inflammatory drugs specially in JIA.[14] Identifying changes in the expression profile of myeloperoxidase mRNA in neutrophils of Bengali Bangladeshi children with polyarticular and enthesitis-related juvenile idiopathic arthritis is essential for early intervention. Real-time PCR is a highly effective technique for detecting and measuring gene expression, even at minimal levels.[15] It offers greater sensitivity than protein analysis, enabling the identification of gene expression changes prior to alterations in protein levels. This methodology is vital for cancer diagnostics and therapeutic monitoring.[16] Exact data regarding the prevalence of juvenile idiopathic arthritis (JIA) has been scarce in Bangladesh over the last two decades, although our understanding of the genetic factors linked to JIA has significantly enhanced.

Objectives

This study aimed to investigate myeloperoxidase mRNA expression profile in neutrophils among children with polyarticular and enthesitis-related juvenile idiopathic arthritis and healthy children.

METHODS

It was a comparative cross-sectional study at Genomic Research Laboratory, Department of Anatomy, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka from September 2019 to March 2020. Diagnosed patients of polyarticular JIA (15), enthesitis-related JIA children (15) and healthy children as control (15)) who came for screening of arthritis directly or reffered by physicians at Department of Pediatric Rheumatology and General Pediatrics Outpatient Departments aged less than 16 years were included by purposive sampling technique. Patients were Bengali by ethnicity and Bangladeshi by nationality and residence. Children with comorbidities like diabetes, hepatic and renal disease, malignancy and relapse and remission cases of JIA were excluded. Parents or caregiver was given an informed consent form and an assent form for their children (when they can give at least verbal consent). Peripheral venous blood (3 ml) was collected into K3-EDTA vials from all participants using heparinized syringes with minimum possible trauma, proper aseptic measures and with psychological support. Genomic total RNA was isolated from neutrophils by using RNA extraction kit PureLink® RNA Mini Kit as per a standard operating procedure (SOP) of the genomic research laboratory. Then extraction of

total RNA from neutrophils, qualifying & quantifying mRNA was done using spectrophotometer (NanoDropTM 2000/2000c spectrophotometer; Thermo Fisher Scientific, USA). The primer was designed using Primer 3 plus software and was validated by IDT Oligo Analyzer.

The sequence of primers was- For MPO gene (target gene)
Forward primer-5/-
GGTGGGGCTGAGGTACAAAG -3/
Reverse primer-5/-
CAGCCCAGCAAGGTCCTAAG -3/

For β-actin gene (reference gene)
Forward primer-5/-
TCACCATGGATGATGATATCGC-3/
Reverse primer-5/-
ATAGGAATCCTTCTGACCCATGC-3/

Total mRNA was converted to cDNA using reverse transcription system (Thermo Scientific Verso cDNA Synthesis Kit). Validation of the amplicons using gel electrophoresis was performed and stored at -20^{0}C until used for PCR. After that, quantification of the transcripts of cDNA samples was done with qRT-PCR with qPCR master mix (PowerUp™ SYBR™ Green Master Mix). After obtaining the C_T value from the qRT-PCR, calculation of ΔC_T of the target gene and reference gene (β-actin) was done. Gene expression level was represented as $2^{-\Delta ct}$ and expressed in percentage relative to reference gene. Group wise comparison of gene expression was performed by relative expression software tool (REST) between polyarticular JIA, enthesitis-related JIA and healthy children. Gene expression of the target gene was expressed as fold changes over the control group. Data of pattern of disease and clinical history were analyzed using the SPSS software version 24. Descriptive statistics was presented by mean, median, standard deviation for numerical data and by frequencies & percentages for categorical data. Inferential statistics were performed by chi-square test and Fisher's exact test. Statistical significance was set as 95% confidence level. Ethical clearance was obtained from Institutional Review Board of BSMMU, Dhaka. (Memo-BSMMU/2020/1399, Dated-03-02-2020)

RESULTS

Table 1: Demographic and Clinical characteristics of participants between three groups (n=45)

Characteristics	Poly articular JIA n=15 f (%)	ERA n=15 f (%)	Control Group n=15 f (%)	p-value
Age (onset of disease)				
3-4 years	1 (6.7)	0 (0.0)	2 (13.3)	0.343
5-15 years	14 (93.3)	15 (100.0)	13 (86.7)	
Mean ± SD	10.5±3.1	11.1±1.6	10.0±2.8	
Gender				
Male	11 (73.3)	11 (73.3)	8 (53.3)	0.407
Female	4 (26.7)	4 (26.7)	7 (46.7)	
Male: Female	2.78:1	2.78:1	1.1:1	
Pattern of Joint involvement				
Only large joint	6 (40)	10 (66.7)	0 (0.0)	0.143
Both small and large joint	9 (60)	5 (33.3)	0 (0.0)	
Family History				
Yes	1 (6.7)	2 (13.3)	0 (0.0)	1.000
No	14 (93.3)	13 (86.7)	0 (0.0)	

Pearson's chi-square test was done to test the level of significance
*p value <0.05 was considered as statistically significant.

The mean age at onset of disease was 10.5±3.1 years in poly articular JIA and 11.1±1.6 years in ERA. The mean age of control group was 10.0±2.8 years. The age at disease onset was more than 5 years in majority of poly articular JIA children and ERA group. The age and gender

differences between groups were not statistically significant explained in Table-1. In poly articular JIA group, 6 (40%) children had only large joint involvement and 9 (60%) children had both large and small joint involvement. On the other hand, in the ERA group 10 (66.7%) children had only large joint involvement and 5 (33%) children had both large and small joint involvement. Out of 15 only 2 (13.3%) of ERA children had a positive family history of joint pain, swelling and morning stiffness among their relatives. While in poly articular JIA group only 1 (6.7%) child had a positive family history for such kind of illness described in Table 1.

Table 2: Comparison of MPO gene expression among three groups (n=45)

mRNA expression	Poly articular JIA (n=15)	ERA (n=15)	Control (n=15)	p-value
Median	139.5	152.6	0.034	<0.001*
Range	(8.304 -339.874)	(11.744-377.114)	(0.011-0.622)	
Mean rank	19.13	31.87	8.0	

Kruskal Wallis test was done to test the level of significance
*p value <0.01 is highly significant.

Mean rank of MPO mRNA expression was 19.13, 31.87 and 8 in poly articular JIA, ERA and control group respectively. Mean rank of MPO mRNA expression between groups were statistically significant (p<0.01) stated in Table 2.

Table 3: Association of MPO m RNA expression with the pattern of joint involvement in two groups (n=30)

Groups	Statistics	Large only	Small and Large	p-value
	Median	19.3	152.6	
Poly JIA (n=15)	Range	8.3-172.9	35.23-99.9	0.099
	Mean rank	5.67	9.56	
	Median	181.6	70.5	
ERA (n=15)	Range	13.8-377.1	11.7-152.6	0.057
	Mean rank	9.55	4.9	

Mann-Whitney U test was performed to test the level of significance

In poly articular JIA group, mean rank of MPO mRNA expression was 9.56 who had both small and large joint involvement and 5.67 who had only large joint involvement. In case of ERA group, mean rank of MPO mRNA expression was 9.55 who had only large joint involvement and 4.9 who had both small and large joint involvement stated in Table 3.

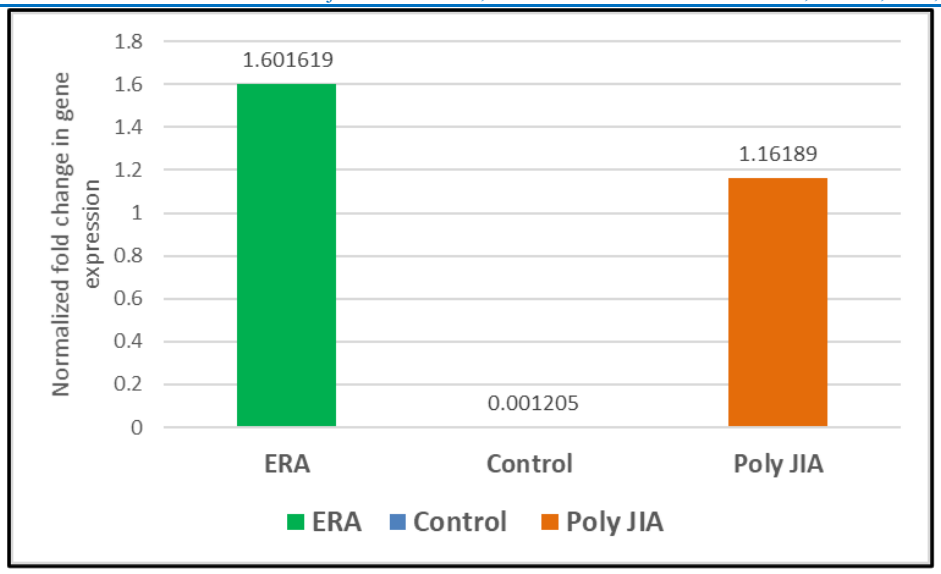

Figure 1: Fold change MPO mRNA expression in ERA and poly articular JIA group versus control group

Gene expression was recorded by mRNA expression relative to β-actin and reported as "fold change". The MPO mRNA expression was 1.6 folds in ERA group and 1.2 folds in poly articular JIA group in comparison to control group which had fold 0.001 showed in bar diagram in Figure 1.

DISCUSSION

The mean age of onset for polyarticular JIA at 10.5 ± 3.1 years, while for ERA, the mean age was 11.1 ± 1.6 years. Comparative data from a retrospective study at BSMMU reported the mean onset ages for polyarticular JIA and ERA as 12.2 years and 14 years, respectively.[17] Additionally, a study from Singapore identified median onset ages of 9.4 years for polyarticular JIA and 12.1 years for ERA, aligning closely with the results of the current study.[1] A multicenter study from Oman revealed similar onset ages, recording 8.8 ± 3.1 years for polyarticular JIA and 12.0 ± 2.6 years for ERA.[18] However, a study from Sweden reported a later onset for polyarticular JIA at 12.7 years and an earlier onset for ERA at 10.7 years, highlighting some discrepancies.[19] The earliest onset for polyarticular JIA recorded was 8.8 ± 3.1 years in Oman, whereas the latest was noted at 12.2 years in Bangladesh. Despite the prevalence of JIA, delayed diagnoses and limited awareness may contribute to the later onset age observed in Bangladesh. Most European studies tend to emphasize the incidence and prevalence of JIA, with fewer focusing on age of onset. Variations in findings across studies may result from differences in case selection and how ethnicity affects disease frequency.[20] In the current study, the male-to-female ratio was found to be 2.78:1 in both JIA groups, contrasting sharply with a 1.1:1 ratio in the control group. Research into gender differences across JIA subtypes remains limited and differs in different populations.[21] Polyarticular JIA is common in male in Asian populations revealed by several studies.[1, 22, 23] Similarly, a Bangladeshi study conducted by Rahman *et al*. explored the M:F ratio was approximately 2:1 which was very similar to the current findings.[22] Another study also found that, in asian population, among two hundred eighty-seven JIA patients, 60.6% participants were males.[1]

Understanding joint involvement aids in better patient classification and prognosis. For instance, in polyarticular juvenile idiopathic arthritis (JIA), 40% show only large joint involvement, while 60% are affected in both large and small joints.[24] In enthesitis-related arthritis (ERA), 66.7% involve large joints, with lower limb joints primarily affected, and no sacroiliac joint involvement reported.[7] Common joints affected in polyarticular JIA are the knee, ankle, and wrist, mirroring findings from over 16,000 children in Bangladesh.[25] ERA affects joints like the knee and ankle, similar to observations in China, Taiwan, and Canada. Real-time PCR has become the preferred method for gene quantification due to its sensitivity and precision.[4, 5] In this study, The

expression of MPO in neutrophils in poly articular JIA, ERA and control group were quantified using real-time PCR. This study measured myeloperoxidase (MPO) mRNA using SYBR Green, a DNA-binding dye. While effective, it poses a risk of false positives; thus, we validated our protocol with a thermal cycler and gel electrophoresis. Although less specific than probes, dyes like SYBR Green are popular for their cost-effectiveness.[26] In the present study, the results are not only confined to seeing only MPO mRNA expression in neutrophils, but it also highlights the picture of socio-demographic and disease condition related characteristics of the poly articular JIA and ERA children. Though a clear cutoff value of the MPO mRNA expression is not possible to establish from the present study, the significant rise in expression than the control group may provide a new insight to develop a gene-expression based biomarker. The MPO mRNA expression level has not shown as a whole in JIA as the study is subgroup-specific (poly articular JIA and ERA).

Limitations

As it is comparative cross-sectional study, the RF positivity or negativity was not possible to take into account and correlation of the expression with therapeutic response was not possible. Another limitation of this study is any pedigree chart was not drawn while taking history. The patient were also chosen using a convenient sampling technique which might cause the availability of a small number of patient with a positive family history. Moreover, the present study was conducted among two subtypes of JIA due to easy availability of the participants.

CONCLUSION

In the present study, the results are not only confined to see only MPO mRNA expression in neutrophils but it also highlights the picture of socio-demographic and disease condition related characteristics of the poly articular JIA and ERA children. The MPO mRNA expression level has not shown as a whole in JIA as the study is subgroup-specific (poly articular JIA and ERA). This is the first ever study conducted in Bangladesh, taking into account the race and ethnicity (Bengali Bangladeshi) in JIA children.

Ethics approval and consent to participate: Before data collection, both verbal and written informed consent was taken from patients.

Consent for publication: All authors have approved this manuscript for publication.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: This research received funding from Bangabandhu Sheikh Mujib Medical University for conduction.

Authors' contributions

RA, LAB participated in the design of the study, data interpretation and drafted the manuscript. RA, LAB contributed to the data design, data interpretation and data analysis. BD, MMI, JA did critical review of the manuscript.

Acknowledgements

We express our appreciation to the participants for their enthusiastic cooperation with this study and also to the Department of Anatomy, Genetic Research Laboratory, and the Department of Paediatrics Rheumatology of Bangabandhu Sheikh Mujib Medical University for their valuable support.

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The Journal of Teachers Association
Abbreviated Key Title: TAJ
Official Journal of Teachers Association
Rajshahi Medical College