A$_{2A}$ adenosine receptor agonist reduced MMP8 expression in healthy M2-like macrophages but not in macrophages from ankylosing spondylitis patients

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Abstract

Background. Ankylosing spondylitis (AS) is an inflammatory autoimmune disease that mostly affects different joints of the body. Macrophages are the predominant cells that mediate disease progression by secreting several pro-inflammatory mediators. Different receptors are involved in macrophages’ function including the adenosine receptors (AR). Our main objective in this study was to assess the effect of applying A$_{2A}$ adenosine receptor agonist (CGS-21,680) on the gene expression of inflammatory mediators including bone morphogenetic proteins (BMP)-2, 4 and matrix metalloproteinases (MMP)-3, 8, 9, and 13 on the macrophages from AS patients compared to healthy macrophages.

Methods. Monocytes were isolated from the whole blood of 28 individuals (AS patients and healthy controls in a 1:1 ratio). Macrophages were differentiated using macrophage colony-stimulating factor (M-CSF), and flow cytometry was performed to confirm surface markers. CGS-21,680 was used to treat cells that had been differentiated. Using SYBR green real-time PCR, relative gene expression was determined.

Results. Activating A$_{2A}$AR diminished MMP8 expression in healthy macrophages while it cannot reduce MMP8 expression in patients’ macrophages. The effect of A$_{2A}$AR activation on the expression of BMP2 and MMP9 reached statistical significance neither in healthy macrophages nor in the patients’ group. We also discovered a significant positive connection between MMP8 expression and patient scores on the Bath ankylosing spondylitis functional index (BASFI).

Conclusion. Due to the disability of A$_{2A}$AR activation in the reduction of MMP8 expression in patients’ macrophages and the correlation of MMP8 expression with BASFI index in patients, these results represent defects and dysregulations in the related signaling pathway in patients’ macrophages.

Keywords. Ankylosing spondylitis, Adenosine A$_{2A}$ receptor, Macrophages, Bone morphogenetic protein, Matrix metalloproteinase.
Introduction

Ankylosing spondylitis (AS) is an autoimmune disorder that mostly affects the sacroiliac joints and the spine. People suffering from this disease typically complain about spinal stiffness and back pain as a result of enthesitis and vertebrae fusion. AS usually starts at an early age and it is believed that one in every two hundred people is influenced by this disease, making it an important health issue [1, 2]. The exact etiology by which the disease is started is still unknown; however, The most important contributors to disease creation are supposed to be hereditary and environmental causes [3]. Diverse environmental factors, infectious diseases, and gut dysbiosis in genetically predisposed people, which is defined by possessing certain human leukocyte antigen (HLA) and non-HLA genes including HLAB27, IL23R, ERAP1, and certain TLRs alleles; can cause disease occurrence [1, 4, 5].

Different signaling pathways are involved in autoimmune diseases. One of which is the adenosinergic pathway [6]. To date, available data show that the adenosinergic pathway has a tremendous impact on immunosuppression and aids the body to recover from excessive inflammatory responses. Adenosine is a byproduct of the breakdown of an enzyme cascade, consisting of CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1, E-NTPDase1) breaks down adenosine triphosphate/diphosphate (ATP/ADP) to adenosine monophosphate (AMP) and CD73 (ecto-50-nucleotidase, NT5E) produces adenosine from AMP. Adenosine acts through G-protein-coupled cell-surface receptors which are expressed on a variety of cells, and to date four of them, A1R, A2AR, A2BR, and A3R, are recognized. These receptors are known as type 1 purinergic (P1) receptors [6–8]. There is not much information regarding the role of adenosine receptors in AS pathogenesis. However, we have previously reported that macrophages from AS patients expressed elevated levels of A2AR and diminished levels of A1R and A2BR compared to healthy macrophages [9]. Besides, our results demonstrated that A2AR activation results in a reduction in TNF-α production and an increase in IL23A expression in AS macrophages [4].

Bone morphogenetic proteins (BMPs) are important members of the transforming growth factor superfamily with multiple vital roles such as embryonic development and osteoblastic differentiation [10]. Of these, BMP-2 is an active and important member which can independently or synergistically with other signaling pathways like the Wnt/β-catenin pathway, enhance osteoblast differentiation and bone formation [10, 11]. Data regarding the level of these proteins in AS patients are incompatible; however, most of them show higher levels of these proteins in patients compared to healthy controls [12, 13].

Matrix metalloproteinases (MMPs) are a family of 23 zinc-dependent enzymes produced by a variety of cells especially immune cells in the event of an inflammatory situation. Inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and IL-6 are produced in this environment [14, 15]. These proteins are mostly involved in the degradation and remodeling of extracellular matrices in humans [16]. High levels of MMPs have been seen in inflammatory autoimmune diseases like rheumatoid arthritis (RA). Different studies showed the relation of high levels of MMP-3 and its certain single nucleotide polymorphisms (SNPs) with AS occurrence [16, 17]. Along with the studies showing the relation of MMP-3 and AS, the correlation of bath AS disease activity index (BASDAI) with a group of clustered biomarkers comprising MMP-8, MMP-9, chemokine (C-X-C motif) ligand (CXCL)-8, and hepatocyte growth factor was found [18]. MMP-8 expression is related to inflammatory cytokines and it can have cartilage destructive activities during spondyloarthopathies. One of the SNPs of this enzyme has also been found to be associated with the risk of AS. MMP-9, also called gelatinase B, along with MMP-2 (gelatinase A), are mediators of joint destruction [16, 19].

It is known from previous studies that Inflammatory lesions and the overlaying synovium of spondyloarthritides (SpA) are dominated by macrophages, which are mostly responsible for the breakdown of fibrocartilage [4, 20, 21]. The inflammatory activities of macrophages can be regulated by P1 receptors through binding to adenosine [4]. The expression of proteins responsible for the pathogenesis of AS in macrophages is an important issue to consider for the treatment and control of the disease progression, so we aimed to evaluate the expression level of MMP3, 8, 9, 13, and BMP2, and 4 in macrophages of AS patients in an untreated situation and after treatment with A2AR agonist (CGS-21,680) as no study has done this up until now.

Materials and methods

Study population

With a male/female ratio of 3.6/1 and an average age of 32±10 years, 14 AS patients were chosen. Patients were recruited from the Rheumatology Research Center’s out-patient AS clinic at Shariati Hospital at Tehran University of Medical Sciences, and they all met the modified New York categorization criteria [22]. Patients who had not undergone any disease-modifying drugs or methotrexate were identified for this investigation because certain therapies, such as methotrexate, have a considerable impact on adenosine receptor expression [23]. Simultaneously, the study recruited 14 age and sex-matched healthy persons with a gender and sex distribution similar to AS patients with an average age of 32±96 years.
There was no personal or familial history of rheumatic disorders, inflammatory diseases, or psoriasis in the control group. All participants signed a written informed consent form. The Tehran University of Medical Sciences ethical committee accepted this work. (IR.TUMS.DDRI.REC.1399.047).

**Monocyte isolation and macrophage generation**

Twenty milliliters of participants’ peripheral blood samples were collected and inserted into tubes containing ethylenediaminetetraacetic acid (EDTA). Within five hours of the time of collection, samples were diluted at 1:2 in phosphate-buffered saline (GIBCO Invitrogen) at a pH of 7.2. Ficoll (Lymphodex, Inno-Train) density gradient centrifugation was used to obtain peripheral blood mononuclear cells (PBMCs), which were then washed in PBS. Cell sorter columns were used to separate monocytes after they were treated with MACS CD14 microbeads to undergo positive CD14 selection (all from Miltenyi Biotec). Immunofluorescence labeling of the separated monocytes was done with a phycoerythrin (PE)-conjugated anti-CD14 antibody (BD bioscience) and flow cytometry results revealed a purity of 92–95% [20]. Following that, the isolated CD14 positive monocytes were cultured in the Roswell Park Memorial Institute (RPMI) at a concentration of 500,000 cells per well in 24-well plates. The media contained 2 mM L-glutamine (Biosera), 10% fetal bovine serum (FBS; Gibco BRL), 0.1 mg/ml streptomycin, and 100 U/ml penicillin (Sigma). For seven days, 0.05 µg/ml recombinant macrophage-colony stimulating factor (M-CSF; eBioscience) was added to culture media to convert monocytes into macrophages [24].

**Flow cytometry analysis of macrophage surface markers**

The macrophage-specific markers of generated macrophages were examined by flow cytometry using a CyFlow ML flow cytometer (Partec, GMBH, Munster, Germany) and FlowJo software after one week of monocytes treatment with M-CSF (Tree Star, Ashland, OR, USA) [25]. Cells incubation with fluorescein isothiocyanate (FITC)-conjugated anti-human CD163 and PE-conjugated anti-human CD206, (BD bioscience) or their relative isotype conjugated anti-CD14 antibody (BD bioscience) and PE-conjugated anti-CD163 and fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody (BD bioscience) and PE-conjugated anti-CD163 (BD bioscience) were examined by flow cytometry using a CyFlow ML flow cytometer (Partec, GMBH, Munster, Germany) and FlowJo software after one week of monocytes treatment with M-CSF (Tree Star, Ashland, OR, USA) [25].

**Table 1**  Primer sequences and product size of the studied genes

| Gene Name | Primer Sequence | Size (bp) | Reference |
|-----------|----------------|----------|-----------|
| GAPDH | F: 5’ GAGTCAACGGAATTGGTGCTG 3’<br>R: 5’ GCATCTGTCAGGTGCTGAG 3’ | 185 | [9] |
| BMP2 | F: 5’ ACTTCCAAAAGCAATGGAGA 3’<br>R: 5’ CATGCTTCTCGAAGAAAACCT 3’ | 113 | [45] |
| BMP4 | F: 5’ ATGATTCCTGTGAACCAAGA 3’<br>R: 5’ CCCGCTTCAGGTACATCAACT 3’ | 165 | [45] |
| MMP3 | F: 5’ AGCAAGGACCTCGTTTTCATT 3’<br>R: 5’ GCCATCGTCAGGTGCTGAG 3’ | 261 | [46] |
| MMP8 | F: 5’ TCTGCAAGGTTATCCCAAGG 3’<br>R: 5’ GTCAATCCCTGGAAAGTCTTCA 3’ | 154 | [47] |
| MMP9 | F: 5’ ACCTCGAATTTGCAAGGAGA 3’<br>R: 5’ GTCAGGGCGAGGACCATAG 3’ | 220 | [48] |
| MMP13 | F: 5’ GCAGTCTTTTCTCAGGTCAGG 3’<br>R: 5’ TGATATCCACCATACAGAAGC 3’ | 101 | - |

**GAPDH**: Glyceraldehyde-3-phosphate dehydrogenase, **BMP2**: Bone morphogenetic protein 2; **MMP**: Matrix metalloproteinase

 utilized to extract total RNA after 24 h (Roche). The complementary DNA (cDNA) was made from the same amount of total RNA using the CellAmp direct RNA prep kit for real-time PCR (Takara bio). StepOnePlus Real-Time PCR equipment (Applied Biosystems) and SYBR green master mix were employed to evaluate the relative expression levels of MMP3, 8, 9, 13, BMP2, and 4 genes (Ampliqon). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. The primer sequences used for BMP2 and 4 were from the Harvard Primer Bank. The site https://primer3.ut.ee/ and https://genome.ucsc.edu/ were used to design the MMP13 primers. The others were selected from previous research. The specific primer sequences and the references were shown in Table 1. For accuracy and specificity, primers were checked using the Basic Local Alignment Search Tool on the US National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and https://genome.ucsc.edu/.

The mRNA expression level was compared between CGS-21,680 treated cells and the untreated group using the comparative CT method (2^-ΔΔCT). It’s worth noting that the cytotoxic potential of CGS-21,680 was tested before the results were interpreted. In this study, the MTT test was utilized to determine the cytotoxicity of CGS-21,680. CGS-21,680 had no harmful effects in this assay, and cell viability was identical to that of untreated cells.

**Statistical analysis**

The Shapiro-Wilk test was used to ensure that all of the variables were normal. To compare non-normally distributed variables, non-parametric methods such as the Mann-Whitney U test were utilized. To compare the levels of mRNA expression in CGS-21,680 treated and untreated cells, the paired sample t-test and Wilcoxon test.
Table 2: Clinical and demographical features of healthy individuals and AS patients

| Group                      | CO individuals (n = 14) | AS patients (n = 14) |
|----------------------------|-------------------------|----------------------|
| Female/Male (%)            | 3/11 (21/79%)            | 3/11 (21/79%)        |
| Age, years                 | 32 ± 9.6                | 32 ± 10              |
| Smoking, %                 | 35                      | 35                   |
| ESR, mm/h (SD)             | 5 (3)                   | 37 (19)              |
| Disease duration, years    | -                       | 7.5 ± 6              |
| HLA-B27 positivity, %      | -                       | 71                   |
| BASMI score (SD)           | -                       | 3.7 (2.5)            |
| BASDAI score (SD)          | -                       | 6.1 (1.9)            |
| BASFI score (SD)           | -                       | 4.6 (2.7)            |
| PPGA score (SD)            | -                       | 7 (2.7)              |
| BASG score (SD)            | -                       | 7.2 (1.8)            |
| ASQoL score (SD)           | -                       | 9.5 (5.6)            |
| Biological agents, %       | 0                       | 0                    |

AS: Ankylosing spondylitis; CO: Control; ESR: Erythrocyte sedimentation rate; HLA-B27: Human leukocyte antigen (subtypes B*2701–2759); BASMI: Bath Ankylosing Spondylitis Metrology Index; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BAS-G: Bath Ankylosing Spondylitis Global Score; PGDA: Patient global disease activity; ASQoL: Ankylosing Spondylitis Quality of Life; SD: Standard deviation

Results

Demographic and clinical characteristics

Table 2 represents the demographic characteristics of enrolled patients and the healthy ones. Clinical scores showing disease severity and activity were also displayed for AS group. According to the report, none of the included patients were on biological or methotrexate therapy.

Selected genes mRNA expression

Among selected genes, MMP3, MMP13, and BMP4 were not expressed in macrophages from patients and healthy individuals and were excluded from the analysis. M2-like macrophages only expressed BMP2, MMP8, and MMP9.

BMP2 mRNA expression in M2-like macrophages of AS patients following the A2AR activation

The effect of the A2AR agonist (CGS-21,680) on BMP2 mRNA expression was determined before and after treating M2-like macrophages of AS patients and healthy controls. Untreated healthy macrophages expressed more BMP2 than AS macrophages (0.47-fold; P = 0.001, Table 3). CGS-21,680 raised BMP2 mRNA expression in AS patients’ macrophages by 1.62-fold, which was not statistically significant (P = 0.167), and it had no effect on BMP2 mRNA expression in healthy people’s macrophages (Fig. 1).

MMP8 mRNA expression in AS patients’ M2-like macrophages after A2AR activation

The expression of MMP8 did not differ between untreated healthy macrophages and AS ones (Table 3). The CGS-21,680 decreased the mRNA expression level of MMP8 in monocyte-derived macrophages of healthy individuals by 0.26-fold (P = 0.002). On the other hand, the decrease noticed in AS patients was not statistically significant (0.78-fold; P = 0.122) (Fig. 1; Table 4).

MMP9 mRNA expression in AS patients’ M2-like macrophages after A2AR activation

We did not find any significant differences in the expression of MMP9 between AS and healthy macrophages (Table 3). Besides, the A2AR agonist couldn’t significantly change MMP9 mRNA expression level in macrophages from AS patients and healthy persons. (Fig. 1; Table 4).

Correlation between the relative mRNA expression with clinical manifestations of AS patients

The link between the expression level of studied genes with clinical manifestations of AS patients was also evaluated. A significant positive correlation between MMP8 and BASFI level (Bath Ankylosing Spondylitis Functional Index) was also seen in AS patients (P = 0.034) (Table 5).

Discussion

Regulating inflammation in autoimmune diseases like SpA is very important in ameliorating disease signs. Different inflammatory molecules take part in disease progression like matrix metalloproteinases which contribute to bone and joint degeneration to a great extent [15]. Matrix metalloproteinases are one of many inflammatory mediators in AS patients. They are mostly involved...
in bone and cartilage degeneration; and are considered a good therapeutic target, especially in a group of patients that don’t respond to TNF therapies who are called non-responders to TNF inhibitor therapies [15, 27]. We investigated the gene expression levels of four matrix metalloproteinases: MMP-3, MMP-8, MMP-9, and MMP-13, which have been linked to pro-inflammatory roles in disease pathogenesis in earlier investigations. We did not detect \textit{MMP3} and \textit{MMP13} expression in isolated monocyte-derived macrophages. Same as our results, Huang et al. did not detect \textit{MMP13} expression in unstimulated differentiated macrophages and \textit{MMP3} was expressed at a low level in them [28]. It is also demonstrated that \textit{MMP3} expression is induced in primary macrophages only after LPS stimulation [29].

MMP-8 which is predominantly produced by activated neutrophils and therefore called neutrophil collagenase, besides AS plays important pathogenic roles in other inflammatory autoimmune diseases like multiple sclerosis (MS) and RA [30, 31]. The relation between certain SNPs of \textit{MMP8} and AS occurrence has also been shown in previous research [19]. In addition, the serum level

\begin{table}
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\begin{tabular}{lccc}
\hline
 & \textbf{Healthy} & \textbf{Treated healthy} & \textbf{Mean fold changes} & \textbf{P-value} \\
 & macrophages & macrophages & (95% CI) & \\
\hline
\textit{BMP2} & 0.19±0.07 & 0.19±0.10 & 0.99 (0.77, 1.26) & 0.974 \\
\textit{MMP8} & 0.11±0.09 & 0.03±0.01 & 0.26 (0.19, 0.36) & 0.002 \\
\textit{MMP9} & 4333.5±2247.9 & 5556.2±3580.8 & 1.28 (0.98, 1.68) & 0.101 \\
\hline
 & \textbf{AS} & \textbf{Treated AS} & \textbf{Mean fold changes} & \textbf{P-value} \\
 & macrophages & macrophages & (95% CI) & \\
\hline
\textit{BMP2} & 0.09±0.05 & 0.15±0.11 & 1.62 (0.96, 2.72) & 0.167 \\
\textit{MMP8} & 0.07±0.09 & 0.05±0.08 & 0.78 (0.45, 1.34) & 0.122 \\
\textit{MMP9} & 4860.4±1943.2 & 4696.05±1372.1 & 0.97 (0.84, 1.11) & 0.729 \\
\hline
\end{tabular}
\caption{The mRNA expression of \textit{BMP2}, \textit{MMP8}, and \textit{MMP9} in monocyte-derived (M2-like) macrophages from AS patients and healthy controls before and after CGS-21,680 treatment.}
\end{table}

\textit{BMP}: Bone morphogenetic protein; \textit{MMP}: Matrix metalloproteinase

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig1}
\caption{The effect of A$_2A$ adenosine receptor agonist (CGS-21,680) on the expression of \textit{BMP2}, \textit{MMP8}, and \textit{MMP9} in monocyte-generated macrophages from AS patients and controls. The data are presented as the mean±SD}
\end{figure}
of MMP-8 has been shown to be higher in AS patients and associated with disease activity [18, 32]. Here, in this study, although we didn’t see a significant difference in MMP8 gene expression between macrophages from AS patients and healthy controls, we saw a positive correlation between MMP8 expression level and BASFI index in patients. This finding is showing that higher levels of MMP8 expression in macrophages may contribute to the inflammatory process in patients and could influence patients’ ability to cope with everyday life [18]. Applying A2AR agonist, as an anti-inflammatory agent, on macrophages significantly reduced MMP8 expression in healthy controls but didn’t have this significant reduction in AS patients. Previously we have reported that AS patients’ macrophages express more A2AR than healthy people’s macrophages [19]. The fact that activation of A2AR cannot diminish the MMP8 expression in patients’ macrophages may be due to defects or dysregulations in the related signaling pathway in AS patients.

Just like MMP-8, MMP-9 is another important enzyme that plays a part in multiple autoimmune diseases [33, 34]. In AS specifically, MMP-9 has cartilage destructive activities through degrading type IV collagen in extracellular matrices and facilitating lymphocytes’ entrance into sites of inflammation [34]. Higher amounts of this enzyme have been detected in the serum of AS patients along with MMP-8 and CXCL-8 and their relevance to cellular matrices and facilitating lymphocytes’ entrance in extra.

In conclusion, the present study shows that activating A2AR on macrophages cannot reduce MMP8 expression in patients’ macrophages while it diminished MMP8 expression in healthy macrophages. These results represent a dysregulation in the related signaling pathway in AS patients. Moreover, we found a significant positive

### Table 5
Matrix of Spearman’s correlation coefficient between the mRNA expressions of BMP2, MMP8, and MMP9 genes and clinical manifestations of patients

|       | ESR    | BASMI | BASFI | BASDAI | BASG  | PGDA  | ASQoL |
|-------|--------|-------|-------|--------|-------|-------|-------|
| BMP2  | -0.133 | 0.049 | 0.132 | -0.066 | -0.025 | -0.051 | 0.037 |
| MMP8  | 0.220  | 0.148 | 0.639 | 0.498  | -0.042 | 0.395 | 0.458 |
| MMP9  | 0.082  | -0.114 | -0.025 | -0.006 | 0.014  | 0.183 | -0.150 |

**BMP**: Bone morphogenetic protein, **MMP**: Matrix metalloproteinase, **ESR**: Erythrocyte sedimentation rate, **BASMI**: Bath Ankylosing Spondylitis Metrology Index, **BASFI**: Bath Ankylosing Spondylitis Functional Index, **BASDAI**: Bath Ankylosing Spondylitis Disease Activity Index, **BASG**: Bath Ankylosing Spondylitis Global Score, **PGDA**: Patient global disease activity, **ASQoL**: Ankylosing Spondylitis Quality of Life. **SD**: Standard deviation. * p < 0.05; ** p < 0.01; *** p < 0.001
correlation between MMP8 gene expression level and BASFI score in patients, telling us that higher MMP8 expression can be associated with higher BASFI and patients' incapacitation. The effect of A2A AR activation on the expression of BMP2 and MMP9 did not reach statistical significance.

However, the small sample size and healthy controls as comparators are the limitations of the current study. Besides, other relevant gene expressions, protein levels in the supernatant, and the entire downstream pathway were not assessed in our study. Therefore, further studies with bigger sample sizes on different genes and signaling pathways in macrophages of AS patients can be useful to investigate and determine the exact effect of A2A AR on the pathogenesis of the disease.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ADP          | Adenosine diphosphate |
| AMP          | Adenosine monophosphate |
| AR           | Adenosine receptor |
| AS           | Ankylosing spondylitis |
| ATP          | Adenosine triphosphate |
| BASDAI       | Bath ankylosing spondylitis activity index |
| BASFI        | Bath Ankylosing Spondylitis Functional Index |
| BMP          | Bone morphogenetic protein |
| CD39         | Ecto-nucleoside triphosphate diphosphohydrolase 1, E-NTPDase1 |
| CD73         | Ecto-50-nucleotidase, NTSE |
| CRP          | C-reactive protein |
| CXCL         | Chemokine (C-X-C motif) ligand |
| EDTA         | Ethylenediaminetetraacetic acid |
| GAPDH        | Glyceraldehyde-3-phosphate dehydrogenase |
| IFN-γ        | Interferon-gamma |
| M-CSF        | Macrophage colony-stimulating factor |
| MMP          | Matrix metalloproteinases |
| MS           | Multiple sclerosis |
| PBMC         | Peripheral blood mononuclear cell |
| RA           | Rheumatoid arthritis |
| RPMI         | Roswell Park Memorial Institute |
| SNP          | Single nucleotide polymorphism |
| SPA          | Spondyloarthritis |
| TNF-α        | Tumor necrosis factor-alpha |

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Authors' contributions

OS, MTE, NA, and MV: Acquisition of data, interpretation of data, drafting the article, and final approval of the article. MA, AJ, EF, and MM: The conception and design of the study, analysis and interpretation of data, revising the article critically for important intellectual content, and final approval of the article.

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Data availability

Data are available on request from the corresponding authors.

Declarations

Ethics approval and consent to participate

This study was performed based on the Declaration of Helsinki guidelines and was approved by the ethics committee of the Tehran University of Medical Sciences (Approval ID: (IR.TUMS.DDRI.REC.1399.047). The written informed consent was signed by all patients before enrolling in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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