Activity of JAK/STAT and NF-κB in patients with axial spondyloarthritis*

Aktywność układu JAK/STAT oraz NF-κB u chorych na spondyloartropatie osiowe

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Summary

The etiology of axial spondyloarthritis (axSpA) is not fully elucidated. Research continues in determining the mechanisms responsible for initiation of the disease process, its maintenance and development.

The aim of this study was to evaluate the expression of transcription factors STAT (signal transducer and activator of transcription) and NF-κB (nuclear factor kappa B) as well as Janus kinase3 (JAK3) in the peripheral blood leukocytes. We also analyzed the connection between the degree of activation of transcription factors and the disease activity.

The study involved 46 patients with axSpA and 19 healthy individuals who comprised the control group. The expression of NF-κB, STAT1, STAT3, STAT4, STAT5, STAT6, and JAK3 was assessed. To determine the degree of activation of transcription factors STAT-s and NF-κB and JAK3 kinase, the immunocytochemistry method was used. For location of the factors, the primary monoclonal anti-NF-κB, anti-JAK3 and polyclonal anti-STAT-s antibodies were used (Chemicon International, USA, Abcam, Cambridge, UK), and the set of antibodies Novocastain Super ABC Kit (Novocastra, UK).

Expression of STAT1, STAT3, STAT4, STAT5, STAT6, NF-κB and JAK3 was statistically higher in the group of patients with axSpA than in the control group. There was a positive correlation with ESR values and expression of STAT4. There was no correlation between STAT, NF-κB, and JAK3 expression and ASDAS, BASDAI, and BASFI. Nine patients were treated with TNF-α inhibitors. The expression of NF-κB and STAT6 was higher in the group treated with TNF-α inhibitors, even though disease activity in these patients was shown to be lower than in those not receiving such treatment (ASDAS = 1.34±0.51 vs. 3.52±0.90, BASDAI = 2.34±1.92 vs. 5.51±2.41).

In the group of patients with axSpA compared with the control group, higher expression of the transcription factors STAT and NF-κB as well as JAK3 was observed. Due to its crucial roles in inflammation and autoimmunity, STAT4 may have promise as an effective therapeutic target for axSpA.

Keywords: axial spondyloarthritis • signal transducer and activator of transcription • nuclear factor kappa B • Janus kinase 3

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**Introduction**

Spondyloarthritis (SpA) is the name of a group of related rheumatic diseases, characterized by shared clinical symptoms and a similar genetic background. In recent years, significant developments have been made in understanding the etiopathogenesis of spondyloarthritis, as well as in its treatment. Experts from EULAR and ASAS (Assessment of SpondyloArthritis International Society) have developed new classification criteria and recommendations for diagnosis of patients with suspicion of spondyloarthritis [14,15]. Based on the dominant clinical symptoms, two diagnostic possibilities are axial and peripheral spondyloarthritis. The category of axial spondyloarthritis (axSpA) comprises non-radiographic axial spondyloarthritis (nr-axSpA) and ankylosing spondylitis (AS). Among patients with axial spondyloarthritis, the percentage of AS increases with time from onset of symptoms. Initially, when symptoms have been present for less than one year, about 2/3 of patients are diagnosed with nr-axSpA and 1/3 with AS. With time from onset between 3 and 6 years, the two diagnoses have similar frequencies, and with time from onset exceeding 10 years, 2/3 of diagnoses are AS and 1/3 are nr-axSpA [12]. A characteristic symptom of spondyloarthritis (especially axial) is chronic back pain, persisting for more than 3 months. Axial SpA manifestations include sacroiliitis, spondylitis, and inflammation of paravertebral tissues and ligaments. Peripheral joints and tendon enthese can be affected as well. Extra-articular manifestations include uveitis, aortic valve inflammation, as well as intestinal, cutaneous, and mucosal lesions. In 90% of patients, the first symptoms appear between 15 and 40 years of age. In approximately 18-30% of patients the course of the disease is severe, with significant functional disorders; in 20-30% the progression is moderate, and in 50% it is mild [1].

In axial spondyloarthritis pathogenesis, genetic, environmental and immunological factors play significant roles. The chronic inflammatory process is initiated and maintained by cytokines produced by peripheral blood cells and synovial cells. Increased production of interleukin (IL) 23, and subsequently of IL-22, IL-17 and tumor necrosis factor (TNF) is observed. The mechanisms whereby inflammation mediators, and especially cytokines, influence effector cells are not yet fully understood. One transmission pathway, considered by researchers, that would allow such influence is the Janus tyrosine kinase/Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway. So far, seven homologous members of the STAT protein family have been identified in mammalian cells (STAT1, -2, -3, -4, -5A, -5B, -6) [6]. When a cytokine molecule binds to a membrane receptor, the subunits of the latter dimerize, and the JAK tyrosine kinase is activated. Then, tyrosine sites in the cytoplasmic domain of the receptor are phosphorylated. Single subunits of STAT proteins, present in the cytoplasm, bind to these phosphorylated tyrosine sites by their SH2-pY domains and are phosphorylated by the JAK tyrosine kinase. Thus activated, STATs dimerize, and the conformational change triggers a nuclear localization signal (NLS), recognized by the specific importer protein. The STAT is transported to the nucleus, where it dissociates from the importer protein and binds to the DNA fragment that promotes the genes controlled by a given JAK/STAT system. The bound STAT is then dephosphorylated and consequently detaches from the DNA. The promoter activation results in expression of the target gene sequence [13,18]. The described mechanism, typical of STAT1, also applies, with minor modifications, to other STAT family proteins.

Most STAT proteins are commonly present in various types of cells. Each type of STAT can be activated by several ligands, but cytokines usually preferentially...
use a specific signaling system, e.g. STAT1 in the case of interferon (INF-γ) and STAT6 in the case of IL-4. Interferon receptors are known to induce JAK tyrosine kinases that activate the target site in the nucleus via STAT, which results in the transcription of between 50 and 100 genes [6]. The role of STATs in the course of synovitis is not fully understood. It is known that the proteins are pleiotropic, able to act in various ways (some even contradictory), depending on the external conditions and the target cell type. Successful attempts at synthesizing an inhibitor for one of the STAT system proteins have been made, which allows for further research on applying the understanding of this system in therapy.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) proteins are inactive in the cytoplasm, sequestered by their inhibitor, IκB. In an activated cell, IκB molecules are degraded by a specific kinase, which allows the NF-κB factor to translocate into the nucleus, bind to the appropriate genetic sequence, activate its transcription and trigger the production of proinflammatory cytokines [19].

The purpose of this study was to determine the expression of the selected transcription factors STAT (STAT1, STAT3, STAT4, STAT5, and STAT6), NF-κB as well as JAK3 in peripheral blood leukocytes of patients with axial spondyloarthritis. Furthermore, the relationship between transcription factor activation and disease activity and treatment was analyzed.

**Materials and Methods**

In total, the study included 46 Caucasian patients with axSpA, fulfilling the ASAS classification criteria [14,15], and 19 healthy controls. There were no significant age or sex differences between the two populations. Mean time from onset was 59±7.1 months, mean age of patients with axSpA was 40.6±13 years (range 24-75 years), and mean age of controls was 40.4±10.4 years (range 28-62 years). The patients’ characteristics are shown in Table 1.

Twenty percent of patients only fulfilled the criteria for nr-axSpA; in the remaining patients, AS was diagnosed using the modified New York criteria. Based on the patient’s medical history or examination, 30% of patients had tendon enthesitis, 17% had iritis, and 45% had peripheral arthropathies. Most of the axSpA patients received non-steroidal anti-inflammatory drugs (NSAIDs, 84%), while 26 (56.5%) were treated with disease-modifying anti-rheumatic drugs (DMARDs), including sulfasalazine (n=21), methotrexate (n=5) and tumor necrosis factor inhibitors (n=9). Twenty-eight percent of patients were treated with a stable dose of glucocorticosteroids (GCS). The following exclusion criteria were adopted: pregnancy or breastfeeding; clinically significant impairment of hepatic and renal function; alcohol abuse; hepatotropic viral infection; treatment-resistant infection; ongoing history of cancer if no cure was achieved; uncontrolled diabetes; patient unwilling or unable to cooperate. Clinical evaluation was based on the patient’s medical history; the number of painful and swollen joints (44-joint count); back pain intensity and global disease activity in the preceding week, as assessed by the patient on a 100-mm visual analogue scale (VAS); the duration of morning stiffness; blood tests, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), blood cell count, creatinine levels and urinalysis. Disease activity was assessed using BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) and ASDAS-CRP (Ankylosing Spondylitis Disease Activity Score). BASDAI consists of 6 questions on 5 major symptoms: spinal pain, joint pain, enthesal pain, fatigue, and morning stiffness [5]. ASDAS is a new composite index to assess disease activity in AS. It combines five disease activity variables with only partial overlap, resulting in one single score with better truth (validity), enhanced discriminative capacity and improved sensitivity to change as compared to single-item variables. Apart from the value of CRP or ESR, the four additional self-reported items included in this index are back pain (0-10 cm VAS, or 0-10 numerical rating scale [NRS]), duration of morning stiffness, peripheral pain/swelling, and patient global assessment of disease activity [9,20]. Function was assessed using the Bath Ankylosing Spondylitis Functional Index (BASFI), which comprises 10 questions on function and the patient’s ability to cope with everyday life [2]. Answers to both BASDAI and BASFI were given on a 10 cm VAS. The patients were divided into two

<table>
<thead>
<tr>
<th>Table 1. Demographic and clinical characteristics of patients with axSpA</th>
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<tbody>
<tr>
<td><strong>n = 46</strong></td>
</tr>
<tr>
<td><strong>Age [years]</strong> mean± SD (range)</td>
</tr>
<tr>
<td><strong>Male/Female</strong></td>
</tr>
<tr>
<td><strong>Time from axSpA onset [months]</strong> mean± SD (range)</td>
</tr>
<tr>
<td><strong>Age at axSpA diagnosis</strong></td>
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<tr>
<td><strong>Age at onset of first symptoms</strong></td>
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<tr>
<td><strong>Delay of diagnosis in years</strong></td>
</tr>
<tr>
<td><strong>ESR [mm/h]</strong> mean± SD (range)</td>
</tr>
<tr>
<td><strong>CRP [mg/dl]</strong> mean± SD (range)</td>
</tr>
<tr>
<td><strong>BASDAI mean± SD (range)</strong></td>
</tr>
<tr>
<td><strong>BASFI mean± SD (range)</strong></td>
</tr>
<tr>
<td><strong>ASDAS mean± SD (range)</strong></td>
</tr>
<tr>
<td><strong>Disease activity</strong></td>
</tr>
<tr>
<td>- low (ASDAS &lt; 1.3)</td>
</tr>
<tr>
<td>- moderate (1.3 &lt; ASDAS &lt; 2.1)</td>
</tr>
<tr>
<td>- high (2.1 ≤ ASDAS ≤ 3.5)</td>
</tr>
<tr>
<td>- very high (ASDAS &gt; 3.5)</td>
</tr>
<tr>
<td><strong>HLA B27 positive (%)</strong></td>
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</tbody>
</table>

ESR – erythrocyte sedimentation rate, CRP – C-reactive protein, BASDAI – Bath Ankylosing Spondylitis Disease Activity Index, ASDAS – Ankylosing Spondylitis Disease Activity Score, BASFI – Bath Ankylosing Spondylitis Functional Index, VAS – visual analogue scale, HLA – human leukocyte antigen; n – number of patients.
groups based on disease activity. One group comprised patients with low or moderate disease activity (ASDAS < 2.1), while the other comprised patients with high or very high disease activity (ASDAS ≥ 2.1). Moreover, patients with BASDAI < 4 were compared with those with BASDAI ≥ 4.

The expression of NF-κB, STAT3, STAT4, STAT5, STAT6, and JAK3 in peripheral blood leukocytes was determined. The degree of STAT and NF-κB transcription factor activation as well as JAK3 was determined using immunocytochemistry. The patients’ peripheral blood samples were collected into sodium heparin coagulant, and then centrifuged on a density gradient with the lymphocyte LSM 1077 separation medium (Cytogen, Germany) to isolate leukocytes. The obtained cells were placed on 1 mm ground-edge frosted Superfrost Plus slides from Menzel (Gerhard Menzel GmbH, Germany), using a cytocentrifuge (Cytospin Thermo Shandon, USA). The cells were fixed at room temperature using 4% paraformaldehyde solution (POCH, Gliwice, Poland). Incubating slides in 3% hydrogen peroxide solution in methanol (POCH, Gliwice, Poland) blocked the endogenous peroxidase activity. The preparations were treated with universal blocking serum (Novostain Super ABC Kit – universal, Novocastra Laboratories Ltd., Newcastle, UK). The peripheral blood cells were initially incubated with primary antibodies specific to the above-mentioned transcription factors, polyclonal rabbit anti-STATs IgG antibody, monoclonal mouse anti-JAK3 IgG antibodies (Abcam, Cambridge, UK) and monoclonal mouse anti-NF-κB p65 subunit antibodies (Chemicon International, Inc., Temecula, CA, USA) at a dilution of 1:100 of a stock solution. A negative control was applied during staining, by using PBS instead of the primary antibody.

After washing, the cells were treated with a biotinylated secondary antibody which recognized rabbit or mouse IgG (Novostain Super ABC Kit – universal). Subsequently preparations were treated with peroxidase-conjugated avidin (Novostain Super ABC Kit – universal, Novocastra). Chromogen fast diaminobenzidine (Liquid DAB Substrate Kit for peroxidase, Novocastra) was used for JAK, STATs and NF-κB staining. To improve contrast and staining of cell structures, the samples were additionally stained with hematoxylin from Vector Laboratories Inc. (USA). STATs, NF-κB and JAK3 kinase activation was evaluated using a Nikon type 120 microscope (Japan) with a video channel and appropriate computer software. The percentage of cells with stained nuclei reflected the degree of activation of the studied factors (100 cells were counted on each slide, in 3 independent observations). All patients provided written informed consent. The study was approved by the Wrocław Medical University Ethics Committee.

**Statistical analysis**

Statistical methods: normality of distribution was tested using the Kolmogorov–Smirnov test. Independent quantitative variables consistent with a normal distribution were compared using Student’s t-test. To assess the correlation between analyzed parameters, Spearman’s correlation coefficient (r) was calculated. Results at p<0.05 were considered statistically significant. All tests were performed using the STATISTICA version 10 software.

**Results**

The expression of transcription factors STATs and NF-κB as well as JAK3 in peripheral blood leukocytes was analyzed in 46 patients with axial SpA. A comparison of results between the axial SpA group and the control group showed statistically significant differences in the expression of STAT1, STAT3, STAT4, STAT5, STAT6, JAK3 and NF-κB (Table 2).

Correlations between individual transcription factors STATs and NF-κB as well as JAK3 in patients with axial SpA were analyzed. Statistically significant positive correlations were found between all the assessed parameters. Examples of such correlations are shown in figure 1a and 1b.

In the control group, the only positive correlations found were: NF-κB with STAT5 (r = 0.053, p = 0.0200); STAT1 with STAT3 (r = 0.46, p = 0.045); and JAK3 with STAT1 and STAT3 (r = 0.74, p = 0.040 and r = 0.47, p = 0.042, respectively). No significant correlations were found between the expression of STATs, JAK3, and NF-κB proteins and patients’ age, duration of SpA, age at first symptom onset or delay of SpA diagnosis.

Results were also compared between the subset of patients with nr-axSpA and the subset fulfilling the modified New York diagnostic criteria for AS. No statistically significant differences were found between the two subsets in terms of the studied parameter expression.

The expression of STATs, JAK3, and NF-κB proteins was analyzed against inflammatory parameters. No correlations between STATs and disease activity were found. A positive correlation was observed between JAK3 and CRP (r = 0.37, p = 0.032) and ESR (r = 0.43, p = 0.009).

**Table 2. Expression of STATs, JAK3, and NF-κB in patients with axial SpA and healthy volunteers**

<table>
<thead>
<tr>
<th>Parameter studied</th>
<th>Axial SpA (n=46)</th>
<th>Controls (n=19)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>NF-κB</td>
<td>9.07±7.33</td>
<td>5.00±4.77</td>
<td>0.0241</td>
</tr>
<tr>
<td>STAT1</td>
<td>8.65±7.46</td>
<td>3.89±3.25</td>
<td>0.0096</td>
</tr>
<tr>
<td>STAT3</td>
<td>8.70±6.15</td>
<td>4.48±4.71</td>
<td>0.0057</td>
</tr>
<tr>
<td>STAT4</td>
<td>7.83±6.74</td>
<td>3.33±2.03</td>
<td>0.0060</td>
</tr>
<tr>
<td>STAT5</td>
<td>8.31±7.61</td>
<td>4.56±4.68</td>
<td>0.0442</td>
</tr>
<tr>
<td>STAT6</td>
<td>8.72±6.87</td>
<td>4.37±3.08</td>
<td>0.0103</td>
</tr>
<tr>
<td>JAK3</td>
<td>8.54±7.14</td>
<td>3.89±1.97</td>
<td>0.0072</td>
</tr>
</tbody>
</table>

The results are presented as: mean ± SD.
lar results were obtained when comparing patients with BASDAI > 4 to those with BASDAI < 4. Likewise, no statistical differences in the expression of factors STATs, NF-κB as well as JAK3 were found between subsets of patients when the group was divided based on the presence or absence of concurrent peripheral arthritis or extra-articular manifestations (iritis, enthesitis). Moreover, the expression of STATs, JAK3, and NF-κB proteins with CRP or ESR were found, except for a positive correlation between ESR and STAT4 (Figure 2).

The expression of STATs, JAK3 and NF-κB proteins was also analyzed against disease activity. No statistically significant differences were found between patients with low or moderate disease activity (ASDAS < 2.1) and those with high or very high scores (ASDAS ≥ 2.1). Simi-

Fig. 1a. Correlation between the expression of NF-κB and STAT6 (r – Spearman correlation coefficient)

Fig. 1b. Correlation between the expression of STAT3 and JAK3 (r – Spearman correlation coefficient)
domains, which in turn are involved in the activation of latent cytoplasmic STAT proteins. These proteins bind to the DNA fragment containing the promoter of the genes controlled by the particular JAK/STAT system, and activate transcription [6].

No study has yet provided a definitive explanation of the JAK/STAT pathway’s role in axSpA pathogenesis. A few studies indicate increased expression of STAT1, STAT4 and JAK3 proteins in synovial cells of patients with spondyloarthritis, compared to healthy controls [21].

Increased levels of JAK1, STAT3, and STAT1 were found in synovial T cells of psoriatic arthritis patients, compared with peripheral blood of healthy controls. T lymphocyte signal transduction pathway mapping revealed enhanced activation of JAK1, STAT3, and STAT1 proteins that may drive the local inflammatory process, characterized by the in vivo expansion of T CD4(+)IL-17A-F(+) and T CD4(+)IL-23R(+) Th17 T effector cells in synovial fluid of clinically active joints of psoriatic arthritis patients [4].

Up to 75% of AS patients will have elevated ESR or CRP, which is a poor prognostic factor. We have found a positive correlation between ESR and STAT4 expression (r – Spearman correlation coefficient).

The expression of factors STATs, NF-κB as well as JAK3 was compared between patients treated and not treated with glucocorticosteroids (GCS). The expression of STAT4 was significantly higher in GCS-treated patients (11.3±10.3/6.6±4.5; p=0.03). In terms of ASDAS, all patients treated with GCS had high or very high disease activity. In terms of BASDAI, 10 out of 13 patients treated with GCS had a score higher than 4.

Nine patients were treated with TNF-α inhibitors. The expression of NF-κB and STAT6 was higher in the group treated with TNF-α inhibitors, even though disease activity in these patients was shown to be lower than in those not receiving such treatment (ASDAS = 1.34±0.51 vs. 3.52±0.90, BASDAI = 2.34±1.92 vs. 5.51±2.41) (Figure 3).

**DISCUSSION**

One of the signaling systems used at the cellular level by cytokines and growth factors that regulate a number of intracellular processes, including gene expression and cell activation, proliferation, and differentiation, is the JAK/STAT system. Cytokines bind to type I/II membrane receptors and activate JAK tyrosine kinases, involved in the phosphorylation of the receptor’s cytoplasmic proteins, which in turn are involved in the activation of latent cytoplasmic STAT proteins. These proteins bind to the DNA fragment containing the promoter of the genes controlled by the particular JAK/STAT system, and activate transcription [6].
and plays a role in the maintenance of Th17 cells, inhibits Treg cell polarization, suppresses Th2 cell differentiation and reduces Th2 cytokine levels, promotes Th1 cell differentiation, and induces production of IFN-γ in macrophages, NK cells and mast cells. In addition, STAT4-mediated signaling promoted the production of autoimmune-associated components, which are implicated in the pathogenesis of autoimmune diseases. STAT4 is the major signaling transducing STATs in response to IL-12. IL-12R is composed of two subunits termed IL-12Rβ1 and IL-12Rβ2, which are associated with tyrosine kinase 2 (TYK2) and JAK2, respectively. Also IL-23, via IL-23R dependent STAT4 phosphorylation, is able to exert biological potential similar to IL-12. IL-23 is a heterodimeric cytokine composed of IL-12/23p40 and IL-23p19 subunits [8]. Increased production of IL-17 and IL-23 has been detected in the peripheral blood, the gut, and skeletal tissue biopsies from AS patients. Greater knowledge of AS pathogenesis will help inform the future development of therapeutics and optimize the application of current therapeutics. Ustekinumab is a monoclonal antibody specific for the common IL-12/IL-23 subunit p40 that has proven effective in psoriasis. In the TOPAS study (Ustekinumab for the Treatment Of Patients with active Ankylosing Spondylitis), ASAS20 was achieved by 75% of 20 study subjects. Long-term effects of IL-12/IL-23 and IL-17 blockade in AS specifically are unknown. Due to its crucial roles in inflammation and autoimmunity, STAT4 may have promise as an effective therapeutic target for axSpA [17].

The effectiveness of disease-modifying treatment, evidenced by decreased disease activity, may be related to a significant decrease of STAT1, -4, and -6 protein expression in synovial cells. Such results were reported by Australian researchers studying patients with rheumatoid arthritis [22]. The present study evaluated the expression of STATs, JAK3, and NF-κB in peripheral blood leukocytes. Interesting results could be obtained by comparing the expression of the studied parameters before and after treatment, e.g. with TNF-α inhibitors, not only in peripheral blood, but also in synovial fluid or synovial cells. In the present study, only 9 patients were treated with TNF-α inhibitors, but despite the low disease activity achieved, the expression of STAT6 and NF-κB was higher than in patients not treated with biologic agents (Figure 3). This may be due to the small number of patients observed, and may indicate that the actual decrease in expression of these proteins upon treatment is more relevant.

Glucocorticosteroids may be involved in decreasing the activity of transcription factors. However, they are not a recommended long-term treatment for axSpA patients. We have found higher expression of STAT4 in GCS-treated than GCS-untreated patients, which may be related to high disease activity in those patients, despite treatment with GCS. Similarly, the higher STAT3 expression observed in patients treated with sulphasalazine or methotrexate may be due to the fact that these drugs are mainly used in patients with concurrent peripheral arthritis or tendon enthesitis. The proinflammatory nature of STAT3 has been described in terms of rheumatoid arthritis pathogenesis, while its role in SpA pathogenesis is not yet fully understood [3,7].
Influencing the JAK/STAT pathways is considered a promising therapeutic target, especially in the case of multidirectional action on various elements of the signaling cascade [11]. The currently available JAK kinase inhibitor tofacitinib has been approved by the FDA (Food and Drug Administration, USA) for rheumatoid arthritis treatment in cases where classical DMARDs or biological agents have proven ineffective [16]. Efficacy in psoriasis has also been demonstrated in a phase 2 trial. Such agents potentially have notable safety issues, including risks of infection, malignancy, hyperlipidemia, neutropenia, and anemia; therefore, appropriate monitoring will be required [10].

**References**


The authors have no potential conflicts of interest to declare.