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Cytokine Profile in the Synovial Fluid of Children with Legg-Calvé -Perthes Disease and Transient Synovitis of the Hip

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Abstract

Background. The nature of synovitis development in the early stages of Legg-Calvé-Perthes disease (LCPD), as well as certain aspects of the pathogenesis of subsequent osteodestructive processes, remains incompletely understood. A targeted approach to the treatment of the hip osteochondropathy should be based on an understanding of the dysregulation of osteogenesis at the molecular and cellular levels.

The aim of the study — to perform a comparative analysis of the concentrations of immunoregulatory molecules in the synovial fluid of patients with manifested Legg-Calvé-Perthes disease and those with transient synovitis of the hip.

Methods. This prospective case-control pilot study included two groups of children. We analyzed the concentrations of five mediators/chemokines/cytokines (CD40, MDC/CCL22, Fractalkine (CX3CL1), IP10/CXCL10, VEGF) in the synovial fluid of 42 children with transient synovitis of the hip (TSH), as well as in 26 children with stage II LCPD according to the Waldenström classification.

Results. The conducted study demonstrates differences in the nature of synovial inflammation with favorably occurring TSH and LCPD. The concentrations of regulatory molecules in synovial fluid depends on the predominant etiological factor and may influence the processes of osteoresorption and osteogenesis. Thus, changes in cytokine activity in patients with LCPD indicate the significance of disturbances in the coupling of angiogenesis and osteogenesis at the molecular and cellular levels. An increase in the concentration of phosphoprotein CD40, along with VEGF-induced glycoprotein proliferation, is associated with the activation of inflammation in vascular disorders. In the development of TSH, an increase in the level of cytokine IP10, which regulates the Th1 immune response, was observed.

Conclusions. In transient synovitis of the hip, the predominant factor is the immune-inflammatory response accompanied by activation of the chemokine system. The manifestation of Legg-Calvé-Perthes disease is associated with disturbances in the coupling of angiogenesis and osteogenesis at the molecular and cellular levels, as well as with increased expression of inflammatory mediators.

Keywords: Legg-Calvé-Perthes disease; osteogenesis; osteodestruction, inflammatory mediators.

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Цитокиновый профиль синовиальной жидкости детей с болезнью Легга – Кальве – Пертеса и транзиторным синовитом тазобедренного сустава

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Реферат

Актуальность. Природа развития синовита на ранних стадиях болезни Легга – Кальве – Пертеса (БЛКП), а также некоторые вопросы патогенеза дальнейших остеодеструктивных процессов остаются до конца не известными. Таргетный вариант лечения остеохондропатии тазобедренного сустава должен базироваться на понимании нарушений регуляции остеогенеза на молекулярно-клеточном уровне.

Цель исследования — выполнить сравнительный анализ концентрации иммунорегуляторных молекул в синовиальной жидкости у пациентов с манифестацией болезни Легга – Кальве – Пертеса и у пациентов с транзиторным синовитом тазобедренного сустава.

Материал и методы. В пилотном проспективном исследовании по принципу «случай–контроль» участвовало две группы детей. Выполнен анализ концентрации пяти медиаторов/хемокинов/цитокинов (CD40, MDC/CCL22, Fractalkine (CX3CL1), IP10/CXCL10, VEGF) в синовиальной жидкости у 42 детей с диагнозом «транзиторный синовит тазобедренного сустава» (ТСТС), а также у 26 пациентов с диагнозом «болезнь Легга – Кальве – Пертеса» 2-й стадии по классификации Waldenström.

Результаты. Выполненное исследование свидетельствует о различных природе и характере воспаления синовии при благоприятно протекающих ТСТС и БЛКП. Концентрация регуляторных молекул в синовиальной жидкости зависит от ведущего этиологического фактора и может влиять на процессы остеорезорбции и остеогенеза. Так, изменения активности цитокинов у пациентов с БЛКП свидетельствуют о значимости нарушений в системе сопряжения ангиогенеза и остеогенеза на молекулярно-клеточном уровне. Рост концентрации такого фосфопротеина, как CD40, на фоне индукции пролиферации гликопротеина VEGF связан с активацией воспаления при нарушениях в сосудистом русле. При развитии ТСТС отмечался рост уровня цитокина IP10, регулирующего Th1 иммунный ответ.

Заключение. При транзиторном синовите тазобедренного сустава ведущим фактором является иммуновоспалительный ответ с активацией системы хемокинов. Манифестация воспаления при болезни Легга – Кальве – Пертеса связана с нарушениями в системе сопряжения ангиогенеза и остеогенеза на молекулярно-клеточном уровне, а также с усилением экспрессии медиаторов воспаления.

Ключевые слова: болезнь Легга – Кальве – Пертеса; остеогенез; остеодеструкция; медиаторы воспаления.

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INTRODUCTION

Legg-Calvé-Perthes disease (LCPD) is a type of osteochondropathy characterized by the development of idiopathic avascular necrosis of the femoral head. Despite long-standing research, the nature of hip joint synovitis in the early stages of the disease, as well as the pathogenesis of osteodestructive processes, remains incompletely understood. An important research objective is to elucidate the patterns of molecular and cellular cascade alterations depending on the stage of LCPD [1, 2].

The onset of avascular necrosis is considered to be triggered by the formation of a hypoperfusion focus in the proximal femoral epiphysis against the background of vascular collapse. Acute tissue hypoxia is accompanied by the overexpression of hypoxia-inducible factor 1α (HIF-1α). The increased transcription of this oxygen-dependent protein is aimed at regulating processes such as angiogenesis, erythropoiesis, and energy and glucose metabolism. It also participates in numerous intercellular signaling pathways. The primary biological function of HIF-1α is cellular adaptation to hypoxic conditions. The protective role of HIF-1α is associated with its ability to activate the transcription of genes encoding erythropoietin (EPO) and vascular endothelial growth factor (VEGF) [3, 4]. Increased VEGF activity promotes angiogenesis and revascularization of the hypoperfused area.

At the same time, proteins of the HIF-1 family may act as both anti-apoptotic and pro-apoptotic factors. One of the possible mechanisms of apoptosis induction is associated with prolonged acute hypoxia and the concomitant enhancement of HIF-1α and p53 protein expression [5]. The p53 protein is a potent tumor suppressor and transcription factor that promotes the activation of genes initiating programmed cell death. The relationship between the increased expression of HIF-1α and p53 was demonstrated by co-immunoprecipitation in cells exposed to hypoxia [6].

Moreover, it has been shown that HIF-1α overexpression can activate the production of inflammatory mediators, including the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 [7, 8]. In experimental animal models of surgically induced avascular necrosis of the femoral head, HIF-1α overexpression was accompanied by the subsequent activation of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 [2, 8]. The proliferation and migration of these cytokines are associated not only with increased HIF-1α expression but also with damage-associated molecular patterns (DAMPs), whose elevated extracellular concentration results from osteocyte apoptosis under hypoxic conditions [9, 10, 11]. Alterations in the cytokine profile may play a significant role in the disruption of bone homeostasis regulation.

From this perspective, it is quite possible that the pathogenesis of LCPD manifestation may be associated with a local phenomenon of an exaggerated inflammatory response and dysregulation of immunoregulatory molecules-cytokines, chemokines, and soluble forms of transmembrane receptors-under hypoxic conditions. At first glance, similar mechanisms may underlie transient synovitis of the hip (TSH) in children. Moreover, this form of inflammatory arthropathy is highly prevalent among preschool and early school-aged children [12]. At the early stages, both diseases share similar clinical and instrumental features, characterized by the acute onset of pain and synovitis of the hip joint. However, in TSH, synovitis resolves completely, whereas in LCPD it may progress into a chronic condition. Several experimental studies have demonstrated high concentrations of IL-6 and TNF-α in synovial fluid in LCPD, confirming the phenomenon of an uncontrolled inflammatory response [8, 9].

Currently, active research continues on several regulatory molecules involved in chronic inflammation, including the soluble form of the transmembrane glycoprotein CD40, which belongs to the tumor necrosis factor receptor (TNFR) superfamily [13]; the macrophage-derived chemokine MDC/CCL22 [14, 15]; the chemokine Fractalkine (CX3CL1), one of the key molecules in the pathogenesis of systemic lupus erythematosus and juvenile rheumatoid arthritis [16, 17]; the interferon gamma-induced chemokine IP10/CXCL10, which plays an important role in the activation and regulation of inflammatory and immune responses and contributes to the development of autoinflammatory diseases [18, 19]; as well as VEGF, extensively studied in autoimmune and autoinflammatory disorders [20, 21]. All of these molecules are considered potential targets for

the development of new therapeutic approaches for autoimmune and autoinflammatory diseases. It is therefore likely that dysregulated regulatory mechanisms in the hip joint in LCPD are also linked to these mediators. Thus, understanding the patterns of molecular composition changes in synovial fluid during osteodestruction in LCPD may provide a foundation for developing new diagnostic criteria and treatment strategies for this pathology, including the use of genetically engineered biologic agents.

The aim of the study – to perform a comparative analysis of the concentrations of immunoregulatory molecules in the synovial fluid of patients with manifested Legg-Calvé-Perthes disease and those with transient synovitis of the hip.

METHODS

Study design

The study was designed as a pilot prospective case-control study.

Two groups of children participated. The first group included 42 children diagnosed with TSH, comprising 32 boys and 10 girls, with a mean age of 6.7±0.6 years. The second group included 26 children with stage II LCPD according to the Waldenström classification, comprising 21 boys and 5 girls, with a mean age of 6.1±0.8 years. All children with TSH and LCPD were treated in the Department of Traumatology and Orthopedics of Kuzbass Regional Children's Clinical Hospital named after Y.A. Atamanov (Kemerovo, Russia) between 2017 and 2022. All examined children were of Caucasian ethnicity and residents of the Kemerovo Region.

The diagnosis of LCPD was established based on X-rays (AP and Lauenstein views) and MRI of the hip joint. All LCPD cases were accompanied by pronounced synovitis. The inclusion criterion was a diagnosis of stage II LCPD according to the Waldenström classification, confirmed by instrumental studies. The exclusion criterion was the presence of other nosological entities associated with bone destruction.

The diagnosis of TSH was made after excluding surgical and orthopedic pathology and confirmed during follow-up observation. All children were examined by a rheumatologist, and no cases of juvenile idiopathic arthritis were identified. The inclusion criteria were coxalgia and limited range of motion in the hip joint developing in previously healthy children. The exclusion crite-ria were bone destruction according to instrumental studies and infectious or auto-immune inflammation of the hip synovium.

All children included in the study underwent synovial fluid aspiration during hip joint punc-ture, performed as part of femoral head decom-pression in patients with LCPD and during differential diagnosis in patients with TSH. The needle was inserted from lateral to medial, targeting the projection of the femoral head beneath the inguinal ligament, with the puncture site located away from the femoral artery pulse. The presence of synovial fluid in the syringe confirmed correct entry into the hip joint cavity. No complications occurred during the procedu-res. The preparation of synovial fluid samples involved treatment with hyaluronidase (4 mg/ml; Sigma, USA) at 37°C for 1 hour in a shaker, with the addition of 0.5% bovine serum albumin (BSA) as the final concentration. After incubation, the samples were centrifuged at 1000 g for 5 minutes at room temperature. The resulting supernatants were stored at -20°C until analysis.

In all samples from 68 patients, the concentrations of five mediators/chemokines/cytokines – CD40, MDC/CCL22, Fractalkine (CX3CL1), IP10/CXCL10, and VEGF – were measured using ELISA kits (Invitrogen, USA). The analysis of synovial fluid was performed at the City Hospital No. 40, Kurortny District (St. Petersburg, Russia). All samples were analy-zed in duplicate according to the manufacturer's instructions. The concentrations of the studied mediators were expressed in pg/ml.

Statistical analysis

To identify significant differences between groups, statistical processing was performed using Statistica 10.0 and MedCalc 17.5.2 software, applying the principles of variational statistics. Quantitative data were presented as median (Me) and 25th and 75th percentiles (P25 and P75). The Mann-Whitney U test was used to assess the significance of intergroup differences. To evaluate the role of the studied mediators in the development of LCPD, multiple linear regression with a logit transformation was applied. The dependent variable was the presence

of LCPD (score = 1) or TSH (score = 0), while the independent variables were the concentrations of all analyzed mediators. The type I error (α) was set at 5%, and the type II error (β) at 20%. Therefore, statistical significance was established at $p < 0.05$, in accordance with standard requirements. The performance of the resulting logistic model was evaluated using the area under the curve (AUC) derived from ROC analysis, which is the standard criterion for assessing the quality of binary classification. During logistic regression, potential confounding factors such as age, sex, and body weight of the patients were taken into account.

RESULTS

Hip joint aspiration was performed in all patients under local anesthesia using an anterior approach. In several cases, ultrasound guidance was utilized to ensure accurate needle placement into the joint cavity. The volume of aspirated synovial fluid ranged from 2 to 6 ml. There were no significant differences in the amount of synovial fluid obtained between the study groups.

The study revealed distinct characteristics of synovial inflammation in patients with TSH and LCPD. The predominant molecular patterns varied depending on the underlying pathology triggering the inflammatory process. The analysis of chemokine and mediator profiles in synovial fluid demonstrated significant differences for most of the studied molecules (Table 1).

As shown in Table 1, the concentrations of several molecules in the synovial fluid of patients with LCPD significantly differed from those observed in patients with TSH. In particular, CD40 and VEGF levels were elevated in LCPD, whereas children with TSH had significantly higher concentrations of IP10. No differences between groups were found for macrophage-derived chemokine (MDC) and Fractalkine. These findings suggest an important role of the soluble form of the CD40 receptor and VEGF in the development of LCPD.

To assess the interactions among the studied mediators involved in LCPD pathogenesis and to derive an equation for estimating the risk of LCPD, multiple linear regression followed by logit transformation was performed. The results are presented in Table 2. It should be noted that β-coefficients of the classifier reflect the relative influence of each predictor on the dependent variable, whereas B-coefficients represent the predictive value of the corresponding factor and can be used in the logistic function equation.

As shown in Table 2, the predictors of LCPD included elevated concentrations of soluble CD40 receptor and VEGF in the synovial fluid, while IP10 (CXCL10) and Fractalkine (CX3CL1) acted as protective factors. These results are consistent with the data in Table 1, where soluble CD40 receptor and VEGF were also identified as key molecules in the manifestation of synovial inflammation in LCPD.

Based on the B-coefficients and the logit-transformed regression results, an equation was developed to estimate the risk of LCPD formation according to deviations in mediator and chemokine levels in hip synovial fluid.

Table 1

Comparison of chemokine/mediator concentrations in synovial fluid in LCPD and TSH

Cytokines	LCPD			TSH			p
	Me	P25	P75	Me	P25	P75	
CD40, pg/ml	239.854	137.949	341.758	73.387	25.183	121.591	0.012
Fractalkine (CX3CL1), pg/ml	24.121	2.479	45.764	31.764	0.832	62.696	0.067
IP10 (CXCL10), pg/ml	10.928	3.974	17.882	64.468	25.034	103.902	0.024
MDC (CCL22), pg/ml	343.317	198.988	487.646	163.796	117.140	210.451	0.079
VEGF, pg/ml	209.804	103.329	316.279	64.336	23.973	104.699	0.043

Table 2

Immunoregulatory predictors and protectors of LCPD

Parameter	β	SE of β	B	SE of B	p
Regression intercept	–	–	0.3900	0.1223	0.0041
CD40, pg/ml — *X1*	0.4029	0.1918	0.0009	0.0004	0.0468
IP10 (CXCL10), pg/ml — *X2*	-0.2749	0.1720	-0.0018	0.0011	0.1236
VEGF, pg/ml — *X3*	0.2983	0.1692	0.0006	0.0004	0.0911
Fractalkine (CX3CL1), pg/ml — *X4*	-0.2329	0.1898	-0.0017	0.0013	0.2322

SE — standard error.

The logistic regression equation was as follows:

$$p = \frac{\exp(z)}{1 + \exp(z)} \times 100\%,$$

where z = 0.39 + 0.0009*X1 - 0.0018*X2 + 0.0006*X3 - 0.0017*X4, where X1, …, X4 are the factors listed in Table 2; 0.39 is the intercept (constant) of the logistic model; and p represents the probability of LCPD development based on inflammatory mediator levels in hip synovial fluid. The overall model was statistically significant (p = 0.0001).

To evaluate the quality of the equation, ROC analysis was performed (Figure 1). The following parameters were analyzed: the area under the curve (AUC), indicating diagnostic accuracy (0.9-1.0 = excellent; 0.8-0.9 = very good; 0.7-0.8 = good; 0.6-0.7 = moderate; < 0.6 = poor), as well as specificity and sensitivity.

The sensitivity and specificity of the developed equation for predicting the risk of LCPD based on inflammatory changes in synovial fluid exceeded 78%, indicating high diagnostic performance.The discrimination threshold was 60.47%. The AUC was significantly higher than that of a random classifier.

Thus, the proposed equation can be used as a predictive tool for assessing the risk of LCPD development based on inflammatory mediator profiles in hip joint synovial fluid.

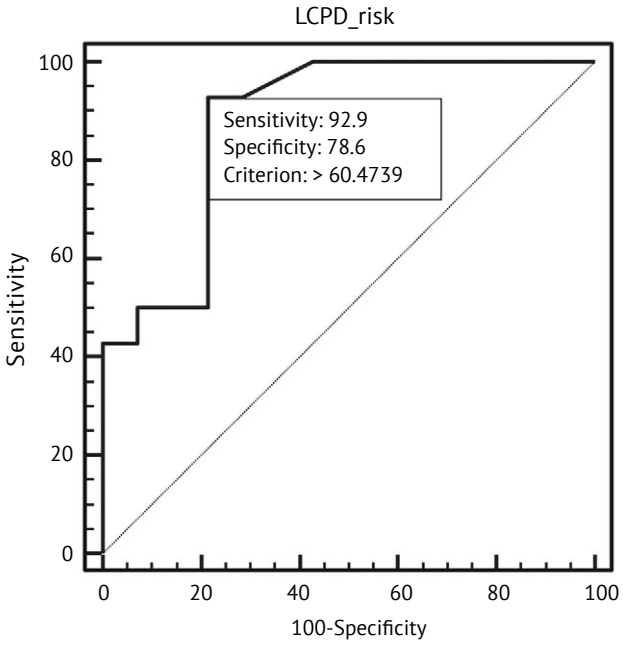

Figure 1. ROC analysis of the equation for calculating the risk of developing Legg-Calvé-Perthes disease (AUC = 0.878; p = 0.0001)

DISCUSSION

The formation of a hypoperfusion focus in the femoral head during the development of LCPD has been confirmed by numerous studies, including histological investigations, hip MRI with perfusion imaging, and Doppler ultrasonography [22, 23].

The crucial role of the vascular component in the pathogenesis of osteochondropathy at the molecular and cellular levels is evidenced by a significant increase in the concentration of VEGF, which plays a key role in stimulating angiogenesis through the activation of endothelial cell proliferation. The increase in VEGF activity may be associated with the rise of HIF-1α level in response to acute tissue hypoxia [24]. Enhanced VEGF activity can therefore be interpreted as a compensatory mechanism aimed at restoring perfusion and promoting angiogenesis under acute ischemic conditions.

Alongside VEGF proliferation, elevated levels of the phosphoprotein CD40 were observed in the synovial fluid of patients with LCPD, which may indicate the activation of inflammatory processes associated with vascular dysfunction.

Several studies have highlighted the pivotal role of the soluble form of the transmembrane receptor CD40 in the pathogenesis of chronic inflammation [25, 26]. Increased expression of CD40, including its alternatively spliced transmembrane isoform, promotes the synthesis of pro-inflammatory cytokines (IL-6, TNF-α) and regulates transcription factors such as nuclear factor kappa β (NF-κβ) [27]. Interaction between CD40 and its ligand (CD40L) induces phosphorylation of protein kinase B (Akt) and nuclear translocation of NF-κβ. Experimental studies have demonstrated that fibroblasts, when stimulated with CD40L, express CD40 and secrete pro-inflammatory cytokines such as IL-6 and TNF-α. In the present study, patients with LCPD exhibited a marked increase in the concentration of transmembrane phosphoprotein CD40 in the synovial fluid.

In addition, several studies have linked the CD40/CD40L system with enhanced thrombogenesis [13, 14]. The transmembrane CD40 receptor is expressed on the surface of platelets, endothelial cells, vascular smooth muscle cells, and B-lymphocytes. Although CD40 does not directly influence platelet aggregation, it facilitates platelet adhesion to the vascular wall [28]. Experimental studies in CD40-deficient mice demonstrated a twofold reduction in the risk of atherosclerosis compared with the mice with normal level of CD40 thrombocytes.

The etiology of impaired blood supply to the proximal femur in LCPD remains unclear. In some children, the disease follows a more aggressive course characterized by osteoarthritic changes that lead to early hip osteoarthritis [29]. Meanwhile, the increased concentration of CD40 in synovial fluid observed in patients with LCPD may indicate a higher risk of atherosclerotic plaque formation followed by the development of a hypoperfusion focus in the femoral head.

In contrast, the synovial fluid of patients with TSH showed slightly elevated levels of interferon-gamma-inducible cytokine IP10 (CXCL10) compared with LCPD. This is an important laboratory finding for understanding the mechanisms of TSH in children. IP10 (CXCL10) is a chemokine that promotes migration and adhesion of activated T-cells through binding to the CXCR3 receptor. IP10 is secreted by macrophages infected with viruses or bacteria, as well as during antigen presentation. Its secretion is upregulated by interferon-gamma stimulation of T-cells and other pro-inflammatory cytokines (IL-17, IL-23, IL-6, TNF-α, IL-1β). Moreover, IP10 regulates the Th1-type immune response and recruits leukocytes, including T-lymphocytes and natural killer (NK) cells. A positive feedback loop is thought to exist between Th1 lymphocytes producing interferon-gamma and resident cells secreting CXCL10 [30]. Elevated IP10 levels in the synovial fluid of patients with juvenile idiopathic arthritis (JIA) confirm the cytokine-mediated pathogenesis of the disease. CXCL10 is expressed by various cell types within the synovial environment in JIA, including macrophages, epithelial, and endothelial cells. Several studies have demonstrated that targeting CXCL10 or its receptor CXCR3 may represent a potential therapeutic strategy for JIA [31].

Thus, the present study demonstrates distinct nature and mechanisms of inflammation in TSH and LCPD. The concentration of regulatory molecules in synovial fluid depends on the predominant etiological factor and may influence processes of bone resorption and osteogenesis.

Specifically, cytokine activity patterns in LCPD indicate a significant disturbance in the coupling between angiogenesis and osteogenesis at the molecular and cellular levels. The increased concentration of the phosphoprotein CD40, along with VEGF-induced glycoprotein proliferation, reflects activation of inflammation associated with vascular dysfunction, whereas in TSH, an elevated level of IP10 indicates stimulation of the Th1-mediated immune response.

Study limitations

According to the presented study design, there were limitations regarding age and population. The direct interpretation of the results does not apply to children younger than 5 or older than 8 years, nor to other populations. The study was limited to children aged 5 to 8 years living in the Kemerovo Region, without severe comorbidities or obesity.

CONCLUSIONS

Changes in the concentration of immunoregulatory molecules in synovial fluid may determine the course of various diseases. The present study demonstrated a different nature of inflammation in Legg-Calvé-Perthes disease and transient synovitis of the hip. In case of transient synovitis, the leading factor is an immune-inflammatory response with the activation of the chemokine system. In contrast, the manifestation of Legg-Calvé-Perthes disease is associated with disruptions in the coupling of angiogenesis and osteogenesis at the molecular and cellular level, formation of a hypoperfusion focus in the femoral head, and subsequent induction of inflammatory mediator expression.

DISCLAIMERS

Author contribution

Shabaldin N.A. — study concept and design, data acquisition, analysis and interpretation, drafting the manuscript.
Kenis V.M. — data acquisition, analysis and interpretation, editing the manuscript.
Kozhevnikov A.N. — data acquisition, analysis and interpretation, editing the manuscript.
Kutikhin A.G. — data acquisition, analysis and interpretation, editing the manuscript.
Shabaldin A.V. — statistical data processing, editing the manuscript.
All authors have read and approved the final version of the manuscript of the article. All authors agree to bear responsibility for all aspects of the study to ensure proper consideration and resolution of all possible issues related to the correctness and reliability of any part of the work.
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Use of artificial intelligence. No generative artificial intelligence technologies were used in the preparation of this manuscript.

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