

## Innate/inflammatory bioregulation and clinical effectiveness of whole-body hyperthermia (balneotherapy) in elderly patients with osteoarthritis

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### ABSTRACT

**Objectives:** Balneotherapy with mud application (mud therapy) is a common hydrothermal intervention for the treatment and rehabilitation of elderly patients with osteoarthritis, leading to anti-inflammatory effects. The main purpose of this investigation was to study a role for regulatory T cells in these anti-inflammatory effects. The second objective was to assess whether the neutrophil-mediated innate response is affected by these anti-inflammatory effects.

**Methods:** Thirty-six elderly patients with knee osteoarthritis underwent a 10-day cycle of balneotherapy at a spa center. They received daily sessions of whole-body mud therapy at 40–42 °C, using mineral-medical water and mud. IL-8 and TGF-β serum concentrations, percentage of circulating CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> and CD8<sup>+</sup> CD28<sup>-</sup> regulatory T cells, and neutrophil phagocytic capacity were evaluated at baseline and at the end of the intervention. Clinical assessments included knee flexion and extension angle, pain, stiffness, physical function and health-related quality of life.

**Results:** All clinical outcomes significantly improved. Circulating concentrations of IL-8 and TGF-β decreased, which correlated with decreased pain and improved knee flexion, respectively. Percentage of CD4<sup>+</sup> regulatory T cells decreased, whereas CD8<sup>+</sup> regulatory T cells increased. Neutrophil functional capacity increased.

**Conclusions:** Balneotherapy with mud application was effective in the management of osteoarthritis symptoms. The anti-inflammatory effect mediated by cytokines contributed to the improvement in pain and joint function; and changes in the circulating percentage of regulatory T cells seem to be involved in the anti-inflammatory effects. Improvement in neutrophil function after mud therapy reflects an optimal bioregulatory effect on the inflammatory and innate responses.

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### KEYWORDS

Knee osteoarthritis; mud therapy; granulocytes; cytokines; regulatory T cells; inflammation

## Introduction

Osteoarthritis (OA) is the most common arthritic disease and a leading cause of pain, disability and impaired quality of life worldwide, especially among the elderly. It affects synovial joints (mainly knees and hips), in which cartilage destruction, subchondral bone remodeling, osteophyte formation and synovial inflammation is found, leading to joint stiffness, swelling, pain and loss of mobility [1,2]. Although the multifactorial pathophysiological mechanisms of OA still remain largely unknown, it is known that various inflammatory and immune processes are strongly implicated in the pathogenesis, progression and burden of OA [3]. In addition to local inflammatory events occurring within joint tissues such as the release of inflammatory mediators by cartilage, bone and synovium [4,5], low-grade systemic inflammation also plays a pivotal role in this condition. The presence of low-grade systemic inflammation could lead to the initiation and aggravation of OA, whereas locally produced inflammatory mediators, such as cytokines, might be reflected in peripheral blood and contribute to perpetuate this systemic

inflammatory status [6–8]. Patients with OA also present changes in peripheral blood T cell composition [9]. Furthermore, they have an immune-neuroendocrine dysregulation affecting the negative feedback between the inflammatory and stress responses along with the decreased functional capacity of phagocytes, thus reflecting an altered bioregulation of the innate/inflammatory responses and suppression of the immune system's defenses against pathogens [8].

Traditional pharmacologic therapies for OA focus on treating inflammation (e.g., NSAIDs, corticosteroids) but they merely palliate the symptoms rather than modifying the course of the disease. Besides, pharmacologic and surgical treatments are associated with problems including invasiveness, the high cost of the procedures and, frequently, adverse events [10]. Therefore, there is a need for developing conservative therapies for OA focusing on evidence-based mechanisms of effectiveness. Balneotherapy is the set of methods and practices which, based on scientific evidence, use medically and legally recognized mineral-medical

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waters, muds and natural gases from natural springs for therapeutic purposes inside the facilities of thermal spa centers. Particularly, muds are matured muddy suspensions composed of a complex mixture of fine-grained materials of geologic origin, mineral water and common organic compounds from the biological metabolic activity. Thus, mud therapy is a balneological intervention that consists of the external application of mud for therapeutic purposes [11]. Temperature has a central role in the effects of these therapies. Mineral-medicinal water and mud are generally applied hot as they are excellent vehicles for the transference of heat (being able to hold heat and release it slowly) so these treatments can be considered thermotherapeutic interventions [12]. The peculiarity of balneotherapy is that its beneficial effects on the organism are brought about not only by the physical properties of mineral-medicinal water and mud but also by their chemical and biological composition [11,13]. Balneotherapy, without or with mud application, is a common non-pharmacological approach for OA carried out by physiotherapists and physicians (specialists in Physical Medicine and Rehabilitation, and in Medical Hydrology). In this context, balneotherapy could not only reduce pain and stiffness, and improve joint mobility and quality of life, but also effectively prevent or delay the progression of OA [14]. Several studies have reported the beneficial effects of balneotherapy [15–18], and particularly mud therapy, on function, perceived pain, analgesic drug consumption, and quality of life in patients with OA, suggesting that they are effective and safe alternatives in the clinical management of this pathology [14,19,20]. Moreover, this strategy is also cost-effective, making it a relevant part of the public health systems of many countries within and outside Europe [21,22]. However, despite the evidence of clinical and symptomatic benefits of these therapies, their role in modern medicine is still under discussion [16], mainly because the biological and physiological mechanisms underlying these benefits have not yet been completely elucidated. It is then necessary to achieve a deeper understanding of these biological mechanisms of effectiveness, so balneotherapy can be practiced by health professionals based on scientific evidence that supports its use in patients with OA. In this context, our group has recently found systemic cytokine-mediated anti-inflammatory effects together with a better immune-neuroendocrine regulation after mud therapy in patients with OA, which constitutes a mechanism of effectiveness responsible for the amelioration of the symptoms in this pathology [14]. However, to the best of our knowledge, nothing is known about the effects of mud therapy on the cellular immune response; particularly those involved in the innate/inflammatory response.

Thus, our main objective was to evaluate the participation of regulatory T (Treg) cells in the anti-inflammatory effects of mud therapy in elderly OA patients. In contrast, and taking into account our previous observations of a reduced neutrophil-mediated innate immune response in patients with OA [8], the second objective was to assess whether the innate immune cell function is affected by the anti-inflammatory effects of this intervention. In addition, we examined the

correlation between concentrations of systemic inflammatory cytokines and clinical outcomes of the OA patients after the intervention.

## Methods

This was a prospective, controlled study conducted at the healthcare and spa center 'El Raposo' (Puebla de Sancho Pérez, Badajoz, Spain), declared a Public Utility in 1926. We evaluated the effects of a cycle of mud therapy, using mud naturally found at the mineral-medicinal spring, in a group of patients with OA participating in the Social Thermalism Programme organized by the Elderly and Social Services Institute of Spain's Ministry of Health, Social Policy, and Equality.

The volunteers assessed for eligibility in the study were 68 patients with primary knee OA. After being informed of the investigation, all volunteers from this homogenous group that complied with the inclusion and exclusion criteria were accepted. The inclusion criteria were to be  $\geq 60$  years old and to be diagnosed with primary knee OA by a rheumatologist following American College of Rheumatology (ACR) criteria [23]. The exclusion criteria were having any infection, neoplastic illness, or cardiopulmonary, vascular, inflammatory, immune or other musculoskeletal condition, having had a total or partial knee replacement, having received any physical therapy in the previous six months, having consumed NSAIDs in the previous three days, having received intra-articular injections of corticosteroids or hyaluronic acid in the previous six months, or having received oral or local corticosteroid or anticytokine therapy that could influence inflammatory and immune parameters. Forty-two patients satisfied the eligibility criteria and were included in the study. Six patients were lost to follow-up, leaving 36 patients from 62 to 80 years in age (mean age  $\pm$  SEM  $70.71 \pm 1.05$  years) for evaluation (Figure 1). All patients signed written informed consent prior to inclusion in the study. Each participant was

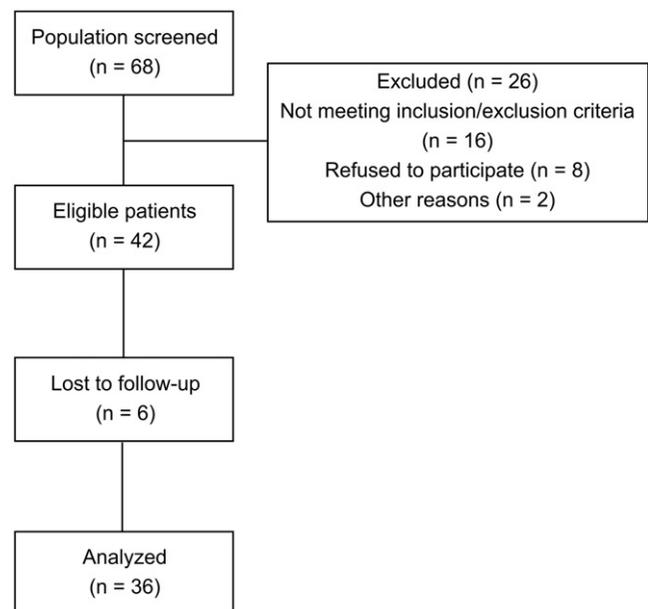


Figure 1. Flowchart of patients through the study.

**Table 1.** Anthropometric and demographic data.

	Pre-treatment (n = 36)	Post-treatment (n = 36)
Sex (women/men)	23/13	–
Age (years)	70.71 (±1.05)	–
Weight (kg)	75.22 (±2.25)	75.75 (±2.23)
BMI (kg/m <sup>2</sup> )	28.15 (±0.89)	28.35 (±0.88)
WHR	0.90 (±0.01)	0.90 (±0.01)
<b>Employment status</b>		
Blue collar	n = 12 (33.33%)	–
White collar	n = 5 (13.88%)	–
Unemployed	n = 19 (52.77%)	–

Data are expressed as mean (± SEM)

BMI: body mass index; WHR: waist-hip ratio.

identified with an alphanumeric code in order to preserve their anonymity.

Table 1 shows baseline and posttreatment anthropometric measurements along with demographic data. These parameters and other clinical variables were evaluated along with blood sampling between 8 am and 9 am. Baseline evaluation and sampling were performed one day after arrival at the spa center, before initiating the first session of mud therapy. Posttreatment sampling was carried out a day after the last session of mud therapy, thus avoiding the evaluation of the effect of an acute intervention.

The study was approved by the Ethical Committee of the University of Extremadura, Spain, in accordance with the guidelines of the European Community Council Directives and the Helsinki Declaration.

### Intervention

The spring water of the spa center ‘El Raposo’ emerges at 16.5 °C, forming a natural stream where the mud is obtained. Then, the mud is placed in a maturation tank along with the mineral-medicinal water and left to mature until it reaches optimal biological, chemical and thermophysical properties for the application.

The water of ‘El Raposo’ spa contains bicarbonate (396.5 mg/L) and calcium (130.2 mg/L) as predominant ions. The mineral-medicinal water fraction represents ~40% of the mud. Mud’s solid content (~60%) consists of a mixture of silt, clay and sand. It is basically composed of phyllosilicates (smectite and illite), quartz and calcite. The major chemical elements of the mud are SiO<sub>2</sub>, CaO, Al<sub>2</sub>O<sub>3</sub> and Fe<sub>2</sub>O<sub>3</sub> [24,25]. The predominant microalgae species present in the mud was identified morphologically as *Monoraphidium pusillum* (Spanish Bank of Algae).

Patients received a daily session of mud therapy for 10 consecutive days, according to the therapeutic procedure implemented by their healthcare professionals. The intervention was always conducted in the morning by the same physiotherapists. The sessions consisted of the whole-body application of mud at 40–42 °C by brush, followed by a drying period of 45–60 minutes in a solarium. Subsequently, patients received a mud bath (consisting of mineral-medicinal water and mud) at 38–40 °C for 15 minutes. Finally, mud remnants were removed with a thermal water jet (38–40 °C) for 2 minutes. No adverse events were reported.

### Anthropometric and clinical data

Body weight, body mass index (BMI), waist-hip ratio (WHR) and knee flexion and extension angle were measured using standardized methods.

Perceived pain intensity was assessed using a visual analogue scale (VAS) [26]. Besides, Western Ontario and McMaster Universities Arthritis Index (WOMAC) (5-point Likert format) were used to evaluate pain, stiffness, and physical function associated with OA [27]. The EuroQoL-5D (EQ-5D) questionnaire was used to measure the generic health-related quality of life [28].

### Whole blood and serum isolation

In a fasted state, peripheral blood samples were drawn by an antecubital fossa vein sterile puncture and collected in serum-separating and heparinized tubes. Blood samples for serum isolation were maintained for 15–20 minutes at room temperature after extraction to allow clotting. Then, they were centrifuged at 700 g for 10 min, and the serum samples were aliquoted and stored at –80 °C until assay.

### Determination of systemic concentration of IL-8 and TGF-β

Serum inflammatory cytokine concentrations (IL-8 and TGF-β) were measured with the Bio-Plex<sup>®</sup> 200 system and Luminex<sup>®</sup> xMAP technology (Bio-Rad Laboratories, Inc., Hercules, CA) using a high sensitivity kit (Bio-Techne, R&D Systems, Inc., Minneapolis, MN). To avoid inter-assay variations, all samples were analyzed with the same kit on the same day.

### Regulatory T cell phenotyping

Blood samples were fixed and permeabilized using the Inside Stain Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), following manufacturer instructions. Then, CD3-FITC, CD4-APC Vio770, CD25-APC, CD28-PE Vio770, CD8-PerCP and FOXP3-PE antibodies (Miltenyi Biotec GmbH) were added to 100 µL of permeabilized samples in the corresponding fluorescence order in several tubes. After 1 hour of incubation at room temperature, 1 mL of diluting buffer from intra staining kit was added and samples were spun down. Pellet was resuspended in 500 µL of PBS and samples were analyzed using a MACSQuant<sup>®</sup> Analyzer 10 (Miltenyi Biotec GmbH) flow cytometer. Twenty thousand events were acquired per sample.

### Study of the neutrophils’ phagocytic capacity

The neutrophils’ phagocytic capacity against opsonized bacteria (*Staphylococcus epidermidis*) was evaluated by flow cytometry of heparinized whole blood. This quantitative technique allows the evaluation of the percentage of active ‘phagocytic neutrophils’ and the number of bacteria ingested per cell by measuring the mean fluorescence intensity (MFI) of active phagocytic cells, which reflects the phagocytic activity of neutrophils [8].

**Table 2.** Clinical measurements and serum concentration of inflammatory cytokines IL-8 and TGF- $\beta$  in patients with OA before (pretreatment) and after (posttreatment) mud therapy intervention.

	Pretreatment	Posttreatment
Knee flexion angle ( $^{\circ}$ )	104.22 ( $\pm$ 2.40)	115.38 ( $\pm$ 2.71)***
Knee extension angle ( $^{\circ}$ )	171.72 ( $\pm$ 1.74)	176.11 ( $\pm$ 1.27)**
VAS	5.93 ( $\pm$ 0.39)	3.48 ( $\pm$ 0.58)***
WOMAC	39.08 ( $\pm$ 2.63)	25.51 ( $\pm$ 2.81)***
EQ-5D	7.64 ( $\pm$ 0.26)	6.58 ( $\pm$ 0.29)***
IL-8 (pg/mL)	409.37 ( $\pm$ 81.44)	120.66 ( $\pm$ 18.05)***
TGF- $\beta$ (pg/mL)	93 746.9 ( $\pm$ 10 835.9)	78 288.9 ( $\pm$ 8036.67)**
Inflammatory index (IL-8/TGF- $\beta$ ) ( $\times 10^{-3}$ )	4.3 ( $\pm$ 0.6)	2.1 ( $\pm$ 0.3)***

Data are expressed as mean ( $\pm$ SEM)

VAS: visual analog scale; WOMAC: Western Ontario and McMaster Universities Arthritis Index; EQ-5D: EuroQol-5D.

\*\* $p < .01$

\*\*\* $p < .001$  with respect to basal values.

In brief, bacteria were stained with fluorescein isothiocyanate (FITC) (1  $\mu$ g/ml) and opsonized with human serum. Aliquots of 200  $\mu$ l of blood from each donor were incubated (for 1 h at 37  $^{\circ}$ C in the dark with shaking) with 50  $\mu$ l of the opsonized bacteria, 10  $\mu$ g/ml of Hoechst 33342, 1  $\mu$ g/ml of 7-actinomycin-D (7AAD), 250  $\mu$ l of PBS and 2% of fetal bovine serum (FBS). The controls consisted of 100  $\mu$ l of blood combined with 10  $\mu$ g/ml of Hoechst 33342, 1  $\mu$ g/ml of 7AAD, 400  $\mu$ l of PBS and 2% FBS. Blood samples were then analyzed with a flow cytometer (MACSQuant<sup>®</sup> VYB; Miltenyi Biotec GmbH).

### Statistical analysis

The values are expressed as mean  $\pm$  standard error of the mean (SEM). The normal distribution of the variables was checked using the Kolmogorov–Smirnov normality test, followed by Student's *t*-test for paired samples and Pearson correlation coefficient for correlation analysis. The significance level was set at  $p < .05$ . Calculations were performed using the IBM<sup>®</sup> SPSS<sup>®</sup> Statistics version 22 software package (IBM Corp., Armonk, NY).

## Results

### Clinical outcomes: function, pain, stiffness and quality of life

After the cycle of mud therapy, knee flexion and extension angle significantly increased; perceived pain (VAS) and OA-related pain, stiffness and physical function (WOMAC index) significantly improved; and health-related quality of life (EQ-5D) significantly improved in the OA patients (questionnaire response rate 93%). The detailed results and statistical significance are presented in Table 2.

### Systemic inflammatory cytokine concentrations: correlation with clinical outcomes

Circulating concentrations of IL-8 and TGF- $\beta$  were markedly decreased after the intervention (IL-8,  $p < .001$ ; TGF- $\beta$ ,  $p < .01$ ) (Table 2). Using these values, an inflammatory index was calculated, indicating the pro-inflammatory cytokine IL-8 to the anti-inflammatory cytokine TGF- $\beta$  ratio. This inflammatory index decreased after the intervention in all groups,

thus showing a greater decrease in IL-8 levels than in those of TGF- $\beta$  (Table 2).

In posttreatment state, a significant positive correlation ( $p < .01$ ) between IL-8 serum concentration and perceived pain, as well as a significant negative correlation ( $p < .01$ ) between TGF- $\beta$  and knee flexion angle were found (Figure 2).

### CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> and CD8<sup>+</sup> CD28<sup>-</sup> regulatory T cells phenotypic analysis

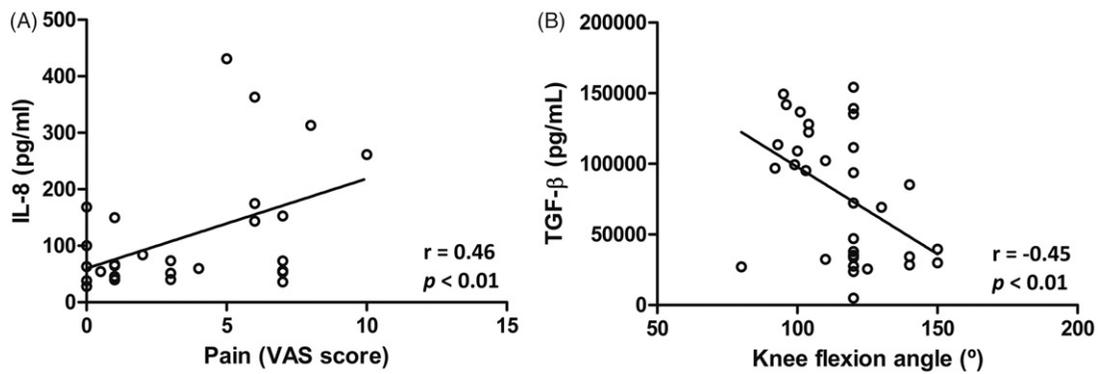
Flow cytometer phenotypic analysis revealed that the percentage of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg cells was significantly reduced after mud therapy ( $p < .01$ ) (Figure 3(A)), whereas CD8<sup>+</sup> CD28<sup>-</sup> Treg cell post-intervention percentage notably increased ( $p < .001$ ) (Figure 3(B)).

### Neutrophil phagocytic capacity

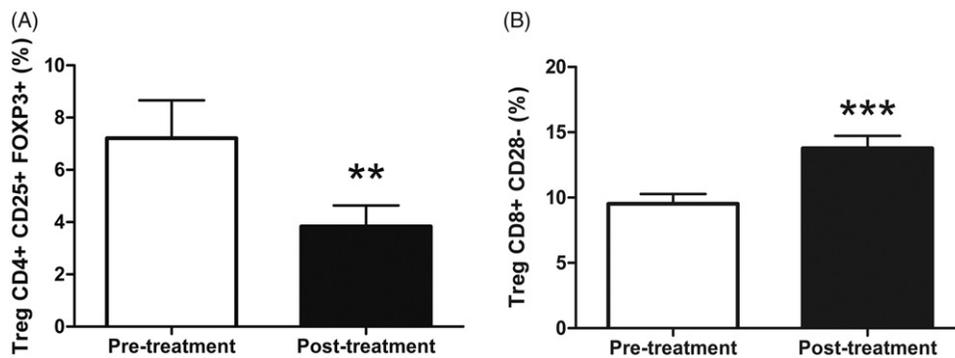
Figure 4 shows the results relating to the neutrophil functional activity. Both the percentage of 'phagocytic neutrophils' (%) (Figure 4(A),  $p < .01$ ) and the phagocytic activity (MFI) (Figure 4(B),  $p < .001$ ) of circulating neutrophils against opsonized bacteria significantly increased after mud therapy in the patients with OA.

## Discussion

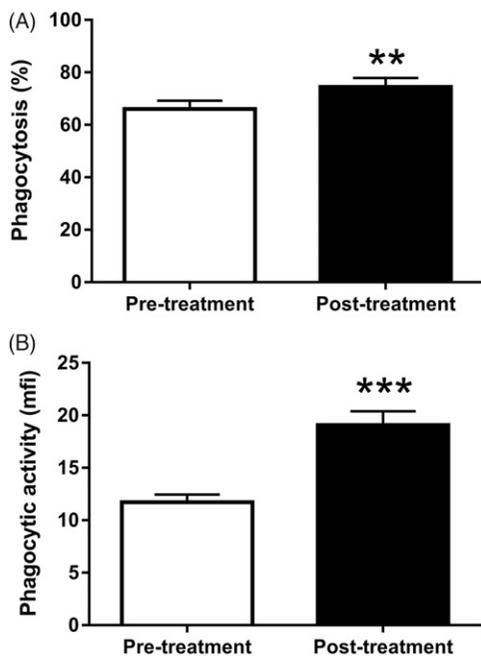
Balneotherapy with mud application is a well-tolerated, cost-effective, thermal treatment frequently used for rheumatic diseases, especially knee OA, reinforcing the suitability of this therapy in health systems [21]. The clinical effectiveness of this therapy in OA has been largely demonstrated by numerous investigations [29,30], including studies from our group in the same spa center as the present investigation [14,19]. Results presented here corroborated the clinical benefits of this therapy in OA patients, as manifested in the improvement in knee flexion and extension angle, perceived pain, stiffness, functional capacity and health-related quality of life after the cycle of mud therapy, thus confirming that it is effective in the management of OA. However, the biological mechanisms of action still remain unknown. Recently, a number of studies have focused on assessing the influence of mud therapy on different biomarkers [14,30–32], including



**Figure 2.** Correlation between serum inflammatory cytokine concentrations and clinical outcomes in patients with OA after the cycle of mud therapy: IL-8 and perceived pain (A) and TGF- $\beta$  and knee flexion angle ( $^{\circ}$ ) (B). VAS: visual analog scale.



**Figure 3.** Percentage of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> Treg cells (A) and CD8<sup>+</sup> CD28<sup>-</sup> Treg cells (B) in patients with OA before (pre-treatment) and after (post-treatment) the cycle of mud therapy. Columns represent the mean  $\pm$  SEM of independent assays performed in duplicate for each participant. \*\* $p < .01$ , \*\*\* $p < .001$  with respect to basal values.



**Figure 4.** Functional capacity of circulating neutrophils in patients with OA before (pretreatment) and after (posttreatment) the cycle of mud therapy: percentage of 'phagocytic neutrophils' (A) and phagocytic activity of circulating neutrophils (B). Columns represent the mean  $\pm$  SEM of independent assays performed in duplicate for each participant. \*\* $p < .01$ ; \*\*\* $p < .001$  with respect to basal values.

novel biomarkers such as microRNA [33], due to the importance of ascertaining the mechanisms of effectiveness of this therapy so it can be prescribed, carried out, funded and

regulated according to scientific evidence. Particularly, our group has shown that an immune-neuroendocrine regulation together with a systemic cytokine-mediated anti-inflammatory effect constitutes an immunophysiological mechanism of action that underlies the clinical benefits [14]. In the present work, we have assessed the circulating concentrations of two inflammatory cytokines: the pro-inflammatory IL-8 and the anti-inflammatory TGF- $\beta$ . Both have been reported to be elevated systemically in OA patients [8,34], contributing to the chronic low-grade inflammatory status present in this pathology [8]. This is particularly notable for IL-8 systemic concentrations, as they have been found to be dramatically elevated by 30-fold in OA patients compared with healthy individuals [8]. Results clearly showed that mud therapy induced a decline in the high systemic levels of both cytokines in OA patients, thus confirming a global anti-inflammatory effect of the intervention [14]. Furthermore, as the inflammatory response must be considered as a whole, in the context of a global anti-inflammatory response, a good balance between systemic pro- and anti-inflammatory cytokines is also important [35]. Thus, the inflammatory index (IL-8/TGF- $\beta$  ratio) decreased, revealing a greater decline in pro-inflammatory IL-8 levels than in those of TGF- $\beta$ . It is well known that IL-8 contributes to joint pain and hyperalgesia due to its pro-inflammatory and proalgesic effects [36]; and TGF- $\beta$ , despite being classically considered an anti-inflammatory cytokine, has a dual role in OA as a result of its anti-inflammatory/anabolic and pro-inflammatory/catabolic effects in the joint, the latter characterized by osteophyte formation

and cartilage damage [4,37]. Indeed, IL-8 levels were positively correlated with VAS pain score, and TGF- $\beta$  levels were negatively correlated with knee flexion angle after the intervention, showing that those patients with lower IL-8 and TGF- $\beta$  levels presented lower pain scores and greater knee flexion angles, respectively. Therefore, these results altogether confirmed that the reduction of systemic inflammation in OA after mud therapy constitutes a mechanism of effectiveness that contributes to the amelioration of pain and impairment.

To the best of our knowledge, the present work is the first to assess Treg-mediated inflammatory response in balneotherapy in general and, particularly, in mud therapy. Although their frequency and function in OA has not yet been clearly elucidated, the importance of Treg cells as immunoregulators in inflammatory pathologies has been highlighted in recent studies, suggesting the interest of developing new therapeutic approaches based on the manipulation of Treg cell responses [9,38]. Both CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> and CD8<sup>+</sup> CD28<sup>-</sup> Treg cells are essential in regulating immune reactions, preventing autoimmunity, moderating inflammation and maintaining immune homeostasis. They do so through several complex mechanisms, including suppression of aberrant or excessive immune responses by altering the functional status of immune cells [38–40]. Our results indicate that the increase in CD8<sup>+</sup> Treg cells in OA patients after mud therapy seems to be a mechanism contributing to reduce the inflammatory status. In fact, patients with different immune and inflammatory diseases have lower numbers of or less functional CD8<sup>+</sup> Treg cells [38]. In this context, induction of CD8<sup>+</sup> Treg cells has shown to have beneficial anti-inflammatory effects, effectively downregulating excessive inflammatory activity [38,41]. Surprisingly, CD4<sup>+</sup> Treg cells percentage decreased after the cycle of mud therapy in OA patients. There have been previous observations of raised levels of circulating CD4<sup>+</sup> Treg cells in OA [42–44], but paradoxically, despite elevated numbers of these cells, inflammation is still present in this pathology. This can be understood as an attempt of the immune system to control the inflammatory responses, but the persistence of inflammation could indicate that these cells present functional impairment that limits their capacity to suppress ongoing disease, perhaps due to an inhibition of their suppressive functions by pro-inflammatory cytokines or because of an increased number of activated effector T cells [44,45]. In this way, the decline in CD4<sup>+</sup> Treg cell numbers after mud therapy could be a reflection of the anti-inflammatory effects of this therapy, so the reduction in the inflammatory status could explain a lesser need of CD4<sup>+</sup> Treg cells to counterbalance the excessive inflammatory responses in OA. As far as we know, this report is the first in evaluating Treg cells in the context of the anti-inflammatory effects of balneotherapy, hence, unfortunately, we cannot discuss the results in relation to any other similar investigations.

Patients with OA present a reduced phagocytic activity of circulating neutrophils, and thus could be more susceptible to infection than healthy individuals [8]. In this context, the question arising now was: could the anti-inflammatory

effects of mud therapy induce further suppression of the immune system's defenses against pathogens? Results clearly showed that not only did the functional capacity of neutrophils not decrease any further, but instead it increased significantly, in accordance with previous results in mice macrophages after hydrotherapy [46]. This improved functional activity of neutrophils after the intervention reflects a greater defense capacity against pathogen challenge and thus a potential lower susceptibility to infections. Bearing in mind that stress-induced physiological concentrations of glucocorticoids mediate the stimulation of some of the innate immune system's functions, including phagocytosis [47,48], and that previous investigations from our research group found an increase in the systemic concentrations of cortisol after the same balneological intervention evaluated in the present study [14], it is plausible to hypothesize that this stress hormone could be mediating the stimulation of the phagocytic activity of neutrophils in OA patients after balneotherapy [12]. Further *in vitro* studies will be needed to confirm this hypothesis. This improvement in the neutrophil-mediated innate immune response together with the prominent anti-inflammatory effects observed in the present study and in our previous one [8] are suggestive of a 'bioregulatory effect of balneotherapy', a term introduced in other strategies, such as exercise, for treating low-grade inflammatory pathologies; consisting in a reduction in unhealthy inflammatory biomarkers together with optimal innate response such as phagocytic activity [49]. We believe this constitutes an interesting novel clinical aspect of the therapeutic effects of mud therapy.

It is noteworthy that although it has been reported that the main therapeutic effects are due to the elevated temperature of application, the chemical and biological components of mineral-medicinal water and mud could cross the skin barrier and play a part in these effects as well [12,13], so it is plausible to think that the mechanism of action probably results from a complex synergistic combination of both factors [12,30]. In this regard, further studies seem to be necessary to ascertain the ideal temperature to elicit optimal therapeutic responses without causing damaging effects. In addition, future studies looking deeper into the cellular and molecular mechanisms of action that mediate the role of IL-8 could also be relevant. In this context, results of the present investigation are in agreement with the increase in systemic concentrations of cortisol previously reported in OA patients undergoing balneotherapy [14], as increased physiological concentrations of cortisol induce a decrease in the release of inflammatory cytokines from immune cells [50] and thus may contribute to decrease the magnitude of the inflammatory response after balneotherapy interventions [12]. Thus, further *in vitro* studies evaluating the effect of cortisol on inflammatory cells from OA patients could clarify this interaction. Furthermore, studies focusing on the role of prostaglandins in mediating the hyperalgesic effects of IL-8 in OA patients (and its reduction after balneotherapy) may be, in our opinion, especially relevant.

In conclusion, results suggest that balneotherapy, particularly mud therapy, is an effective complementary approach

in the management of OA symptoms. Its anti-inflammatory effect, which is mediated by systemic inflammatory cytokines, contributed to the clinical improvement in pain and joint function; and changes in the percentage of circulating regulatory T cells seem to be also clearly involved in this anti-inflammatory effect. Moreover, neutrophil functional capacity increased after mud therapy, thus reflecting an optimal bioregulatory effect on the inflammatory and innate responses.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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