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RESEARCH SUBMISSIONS

# Reduced expression of inflammasome complex components in cluster headache

Erdi Şahin MD[1] | Zerrin Karaaslan MD[2,3] | Elif Şanlı MSc[2] | Özlem Timirci Kahraman PhD[4] | Canan Ulusoy PhD[2] | Elif Kocasoy Orhan MD[1] | Esme Ekizoğlu MD[1] | Cem İsmail Küçükali MD, PhD[2] | Erdem Tüzün MD[2] | Betül Baykan MD[1]

[1]Headache Unit, Department of Neurology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey

[2]Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey

[3]Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Turkey

[4]Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey

**Correspondence**
Betül Baykan, Department of Neurology, Istanbul Faculty of Medicine, Istanbul University, 34093 Fatih/Istanbul, Turkey.
Email: betul.baykan.baykal@gmail.com

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## Abstract

**Background:** The involvement of inflammation in the pathophysiology of cluster headache (CH) has been suggested, with a role implied for interleukin (IL)-1β. We aimed to measure peripheral blood expression levels of IL-1β–inducing systems, the inflammasome complex, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, and investigate their values as putative biomarkers in CH.

**Methods:** In this cross-sectional study conducted in the Headache Unit of Istanbul University, Turkey, blood mononuclear cells (PBMCs) and sera were collected from 30 patients with episodic migraine, 4 with chronic CH, and 47 healthy individuals. Levels of inflammasome complex components (NLRP1, NLRP3, caspase 1, and ASC), end products of inflammasome complex activity (IL-1β, IL-18, and nitric oxide synthase isoforms), neuron-specific enolase, other inflammatory factors (NF-κB, HMGB1, and s100b), and anti-inflammatory IL-4 were measured by real-time quantitative polymerase chain reaction and/or enzyme-linked immunosorbent assay.

**Results:** NLRP3 expression levels were significantly reduced in PBMC samples of patients with CH, obtained during CH attacks ($n = 24$) or headache-free (out of cycle) episodes ($n = 10$). CH-attack patients showed greater expression levels of IL-1β ($2^{-\Delta\Delta CT}$ median [25th–75th percentile], 0.96 [0.66–1.29 vs. 0.52 [0.43–0.73]) and NF-κB (1.06 [0.66–3.00] vs. 0.62 [0.43–1.19]) in PBMCs but not in sera compared with headache-free CH patients. However, these differences did not attain statistical significance ($p = 0.058$ and $p = 0.072$, respectively). Moreover, NLRP1 (52.52 [35.48–67.91] vs. 78.66 [54.92–213.25]; $p = 0.017$), HMGB1 (11.51 [5.20–15.50] vs. 13.33 [8.08–18.13]; $p = 0.038$), S100b (569.90 [524.10–783.80] vs. 763.40 [590.15–2713.00]; $p = 0.013$), NSE (11.15 [6.26–14.91] vs. 13.93 [10.82–19.04]; $p = 0.021$), nNOS (4.24 [3.34–12.85] vs. 12.82 [4.52–15.44]; $p = 0.028$), and eNOS (64.83 [54.59–91.14] vs. 89.42 [61.19–228.40]; $p = 0.034$) levels were lower in patients with three or more autonomic

**Abbreviations:** ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; CASP1, caspase 1; cDNA, complementary DNA; CH, cluster headache; CT, threshold cycle; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGB1, high mobility group box 1; IL, interleukin; NF-κB, nuclear factor-kappa B; NLRP1, NLR family pyrin domain containing 1; NLRP3, NLR family pyrin domain containing 3; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; NSE, neuron-specific enolase; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; qPCR, quantitative PCR; S100b, S100 calcium-binding protein B; TNF-α, tumor necrosis factor alfa.

Erdi Şahin and Zerrin Karaaslan contributed equally to this study.

  

manifestations ($n = 9$). No correlation was found between inflammation factors and clinical parameters of CH.

**Conclusion:** Our results support the involvement of the IL-1β system in attacks of CH. However, the components of the inflammasome complex are suppressed in the peripheral blood and do not appear to play a role in the pathophysiology of CH. These findings argue against a potential biomarker value of the inflammasome complex in CH.

**KEYWORDS**
cluster headache, IL-1β, inflammasome, inflammation, NF-κB, NLRP3

## INTRODUCTION

Cluster headache (CH), characterized by severe pain and cranial autonomic activation, can cause substantial burden.[1] The mechanisms underlying CH have not yet been elucidated. However, the well-known rapid and favorable response of episodic CH to prophylactic corticosteroid treatments may be a clue for the involvement of inflammation in the pathophysiology of CH. The circadian and seasonal periodic characteristics of this condition and the neuroimaging data indicate the participation of the hypothalamus, which is known to have complex interactions with the immune system.[2] The unique periodicity of CH may also be the result of an external immune trigger like seasonal allergic reactions or viral causes. In 1996, Martelletti and Giacovazzo published one of the most comprehensive hypotheses for CH pathophysiology, which included primarily the inflammatory pathway.[3] The earliest known study demonstrating the role of inflammation in CH dates back to 1993 and found that serum interleukin (IL)-1β levels were significantly higher in the patient group during the attack and attack-free periods compared with the control group.[4] In subsequent years, no significant infectious cause was found in a broad-based epidemiological study, which hypothesized that the condition might be primarily associated with viral infections.[5] New studies based on immunology and enzyme-linked immunosorbent assays during the 2000s found that serum-soluble IL-2 receptor levels were significantly higher in patients with CH, whereas there was no difference between the patient and control groups in IL-1, IL-6, soluble IL-6 receptor, and soluble glycoprotein 130 levels.[6,7] All of the aforementioned studies suggest that inflammation may play a role in the pathophysiology of CH and that more detailed further investigations are necessary to fully understand the inflammatory etiology.

Inflammasome, which recently began to be included in neurology literature, is a complex structure that develops because of various mechanisms induced by the receptor, adapter, and effector proteins responsible for the initiation of the inflammatory process of the natural immune system.[8] This complex includes several well-established inflammation factors, including IL-1, tumor necrosis factor alpha (TNF-α), NLR family pyrin domain containing 3 (NLRP3), caspase 1 (CASP1), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and IL-18. In recent years, several studies found significant involvement of the inflammasome complex in various neurological conditions, such as stroke, multiple sclerosis, and Rasmussen encephalitis.[9–11] Some evidence suggesting a role of NLR family pyrin domain containing 1 (NLRP1) and NLRP3 in headache disorders was demonstrated in recent studies.[12] One of the most important studies that showed the inflammasome complex mediated induction of cortical-spreading depression, which triggers migraine aura and pain, dates back to 2013.[13] The role of the inflammasome complex was reported in migraine pathophysiology, and several neuroinflammation factors were indicated as potential treatment targets and biomarkers for migraine.[14] Although IL-1β, which is produced by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and inflammasome activation, is found to be increased in sera of patients with CH,[4] comprehensive studies on the role of these inflammation pathways in CH pathophysiology are still lacking.

Enzyme-linked immunosorbent assay (ELISA) is a widely used method for the detection of cytokines and inflammasome molecules in body fluids.[15–17] The main principle of ELISA is quantification of a target molecule based on color change aroused by antigen–antibody interactions.[18] There are different types of ELISA that can be applied according to the experimental procedure used. The sandwich ELISA technique is widely used for measuring the levels of proteins in the circulation.[15,19]

The levels of inflammatory molecules released from immune cells correlate with the expression of specific mRNAs. Quantitative reverse transcription polymerase chain reaction (PCR) is a fast and sensitive method that simultaneously quantitates the expression levels of specific genes. Therefore, it is widely used to evaluate inflammatory markers.[20–22] The process includes conversion of RNA to complementary DNA (cDNA), amplification of cDNA by PCR, and quantification of the products. The assay measures the increase of fluorescent signal that corresponds to the produced amount of DNA at each PCR cycle. The PCR cycle at which fluorescence first reaches above threshold is named threshold cycle (CT). Lower CT values represent higher expression levels of specific mRNA.[23] ELISA and reverse transcription PCR methods may be used simultaneously to assess the levels of target molecules at protein and mRNA levels, respectively. This approach is particularly useful when mRNA and protein expression alterations of the same inflammation factor do not occur concurrently.

We hypothesized that inflammation participates in the pathophysiology of CH particularly through activation of the inflammasome complex. In this context, we measured levels of IL-1β, inflammasome complex components, and NF-κB to acquire information regarding the possible role of inflammation in the pathogenesis of CH and to discover novel potential treatment and putative biomarker modalities of CH.

## MATERIALS AND METHODS

### Patient and control selection

A total of 34 patients diagnosed with CH by headache specialists (B.B., E.K.O., and E.E.) according to the third edition of the International Classification of Headache Disorders[24] (ICHD-3) were included in this cross-sectional study. This study was conducted between 2018 and 2019 at the tertiary Headache Unit in the Neurology Department of Istanbul Faculty of Medicine/Istanbul University and molecular assays were done at the Neuroscience Department of Aziz Sancar Institute of Experimental Medicine in Istanbul University. Patients with other nervous system disorders and inflammatory diseases were excluded. None of the included patients were on medication during the study. The main study groups were preplanned as follows: (1) patients with current, in-cycle CH attacks (attack-CH group); (2) patients diagnosed with CH but who were out of cycle (headache-free CH group); and (3) normal control group.

Thirty patients with episodic CH and 4 with chronic CH diagnosed according to ICHD-3 were included in our study. In subgroup analyses, we divided these 34 patients into 2 groups: CH-attack group and headache-free CH group (patients with episodic CH that was out of cycle). Twenty patients with in-cycle episodic CH and 4 with chronic active CH were included in the CH-attack group, whereas 10 patients with out-of-cycle episodic CH were in the headache-free CH group. Patients with chronic CH were not in remission during our study; consequently the headache-free group consisted only of patients with out-of-cycle episodic CH (no active CH attack in the previous 6 weeks). Healthy individuals ($n = 47$) participated in the study as the control group (Table 1). Patients and controls with similar profiles were recruited specifically for this study and none of their data had been previously published. Control group enrollment took place in 2018–2019 during the same time as patients.

These healthy controls had neither a disease nor the use of prescription drugs. All participants underwent total blood count and blood chemistry analyses, and 6 patients with CH (none of the healthy controls) were diagnosed to have hyperlipidemia. Clinical and demographic findings such as sex, cigarette consumption, pain characteristics including the duration of pain, triggering factors, accompanying autonomic and nonautonomic symptoms, and other features, including cluster type (episodic or chronic), frequency and

**TABLE 1** Demographic and clinical features of patients with cluster headache and healthy controls

| | Cluster headache $n = 34$ | Healthy controls $n = 47$ | $p$ value |
|---|---|---|---|
| Age, mean ± SD, y | 37.3 ± 13.3 | 35.5 ± 10.9 | 0.800 |
| Sex, *n* (%) | | | |
| Female | 8 (23.5) | 18 (38.3) | |
| Male | 26 (76.5) | 29 (61.7) | 0.160 |
| Age of disease onset, mean ± SD, y | 33.1 ± 13.3 | – | |
| Disease duration, mean ± SD, y | 7.9 ± 7.3 | – | – |
| Cluster type, *n* (%) | | – | – |
| Episodic | 30 (88.2) | | |
| Chronic | 4 (11.8) | | |
| Cigarette smoking, *n* (%) | 15 (44.1) | – | – |
| Hyperlipidemia, *n* (%) | 6 (17.6) | – | – |

**TABLE 2** Characteristics of headache in patients with cluster headache ($n = 34$)

| Parameters | Mean |
|---|---|
| Headache duration, mean ± SD, min | 36.0 ± 53.2 |
| Episode duration, mean ± SD, d/y | 75.1 ± 94.0 |
| Headache duration, minimum–maximum, min | 15–180 |
| Headache severity, *n* (%) | |
| Very painful | 34 (100) |
| Episode frequency, *n* (%) | |
| Once a year | 20 (58.8) |
| Twice a year | 7 (20.6) |
| Biannual | 4 (11.7) |
| Continuous | 4 (11.7) |
| Side, *n* (%) | |
| Right | 16 (47.1) |
| Left | 18 (52.9) |
| Side switching, *n* (%) | 3 (8.8) |
| Accompanying autonomic symptoms, *n* (%) | |
| 0–2 symptoms | 25 (73.5) |
| ≥3 symptoms | 9 (26.5) |
| Conjunctival injection | 31 (88.5) |
| Rhinorrhea | 22 (64.7) |
| Eyelid edema | 14 (41.1) |
| Forehead and face sweating | 1 (2.9) |
| Forehead and face flushing | 4 (11.7) |
| Ear fullness | 3 (8.8) |
| Myosis/ptosis | 17 (50) |
| Agitation, *n* (%) | 34 (100) |

duration of episodes, and age of onset, were all noted (Tables 1 and 2). The ethics committee of Istanbul University, Istanbul Medical Faculty, approved the study protocol and all participants signed informed consent forms.

The primary objective was to establish the putative association between CH attacks and inflammasome complex components. The secondary objective was to establish the possible relationship between inflammasome complex component levels and clinical features of CH. The main motive of the secondary objective was to determine prognostic/diagnostic biomarkers for CH and to set forth novel targets for treatment of CH.

## Real-time quantitative PCR

IL-1β, NF-κB, NLRP1, and NLRP3 levels were analyzed within the scope of real-time quantitative PCR (qPCR) examinations. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll–Hypaque density gradient centrifugation that was resuspended in freezing solution and stored in liquid nitrogen ($1\times10^6$ cells in fetal bovine serum with 10% dimethyl sulfoxide). Frozen PBMCs were thawed and washed in complete medium enriched with 10% fetal calf serum, 1% minimum essential medium vitamin, 1% L-glutamine, 1% Na-pyruvate, 1% nonessential amino acids, and 1% penicillin-streptomycin at 1800 rpm at 4°C for 10 min. RNA was isolated from PBMCs by using an isolation kit (Jena Bioscience, Total RNA Purification Kit, PP-210) per manufacturer's recommendations and the quality was measured by the A260/A280 and 260/230 ratios (Thermo Scientific Nanodrop 2000 Spectrophotometer). RNA was then converted to cDNA by using the SCRIPT cDNA Synthesis Kit (Jena Bioscience, PCR511). Quantitative real-time PCR reactions were performed in Agilent Technologies Mx3005P QPCR System using SYBR green master mix (Jena Bioscience, qPCR GreenMaster UNG kit, PCR375) and primers obtained from DNA Technology (DN-10) (Table S1). The relative mRNA expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression using the simplified comparative threshold cycle delta, the CT method ($2^{-}$[ΔCT gene of interest − ΔCT GAPDH]).[25]

## ELISA

Sera were collected from blood samples obtained by cubital vein puncture from 24 patients with in-cycle CH. The remaining 10 patients with CH had not experienced headache for 6 weeks or more. Serum levels of inflammasome factors (NLRP1, NLRP3, ASC, high mobility group box 1 [HMGB1], CASP1, IL-1β, and IL-18), anti-inflammatory cytokine IL-4, NF-κB, neuron-specific enolase (NSE), S100 calcium-binding protein B (S100b), and nitric oxide synthase (NOS) isoforms (i.e., inducible NOS, endothelial NOS [eNOS], and neuronal NOS [nNOS]) were measured with commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA; Diaclone, Besancon Cedex, France; YHBioscience, Shanghai, China) according to the manufacturers' instructions.

## Statistics

All statistical analyses were performed using SPSS 21.0 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA) with preplanned comparisons. A 2-tailed test was used for hypothesis testing. The Kolmogorov–Smirnov test was used for testing the normality of the data. Categorical variables were reported as frequencies. Descriptive statistics were denoted as mean ± standard deviation for data that met parametric assumptions and median with 25th to 75th percentile (25p–75p) values for data that had not met parametric assumptions. These tests were applied since data of most groups did not show normal distribution. The Kruskal–Wallis test was applied to compare multiple study groups, and Dunn's test was performed for post hoc analysis. Mann–Whitney *U*-tests were performed for 2-group comparisons. Chi-square tests were used to compare categorical parameters between the groups. The Spearman correlation test was used for correlation analyses and $p<0.05$ was considered

**TABLE 3** Comparison of gene expression levels between HC and CH patients with (CH-attack) and without (headache-free CH) attack

| | | | | | *p* values for Dunn's post hoc test | | |
|---|---|---|---|---|---|---|---|
| | CH-attack ($n=15$) (median, 25th–75th percentile) | Headache-free CH ($n=9$) (median, 25th–75th percentile) | HC ($n=13$) (median, 25th–75th percentile) | K-W *p* value | CH-attack vs. headache-free CH | CH-attack vs. HC | Headache-free CH vs. HC |
| NLRP1, $2^{-\Delta\Delta CT}$ | 1.03 (0.64–1.38) | 1.40 (1.09–2.85) | 1.13 (0.67–1.64) | 0.155 | 0.180 | >0.999 | 0.404 |
| NLRP3, $2^{-\Delta\Delta CT}$ | 0.68 (0.51–0.84) | 0.54 (0.31–0.62) | 1.13 (0.81–1.2) | **0.001** | 0.419 | 0.041 | **0.001** |
| IL-1β, $2^{-\Delta\Delta CT}$ | 0.96 (0.66–1.29) | 0.52 (0.43–0.73) | 0.64 (0.36–1.8) | 0.063 | 0.058 | 0.941 | 0.785 |
| NF-κB, $2^{-\Delta\Delta CT}$ | 1.06 (0.66–3) | 0.62 (0.43–1.19) | 0.85 (0.59–1.57) | 0.077 | 0.072 | 0.833 | 0.641 |

*Note*: Significant *p* values are denoted in bold.

Abbreviations: CH, cluster headache; HC, healthy control; IL-1β, interleukin 1 beta; K-W, Kruskal–Wallis; NF-κB, nuclear factor-kappa B; NLRP1, NLR family pyrin domain containing 1; NLRP3, NLR family pyrin domain containing 3.

**TABLE 4** Comparison of serum levels of inflammation-related factors between HC and CH patients with (CH-attack) and without (headache-free CH) attack

| | CH-attack ($n = 24$) (median, 25th–75th percentile) | Headache-free CH ($n = 10$) (median, 25th–75th percentile) | HC ($n = 47$) (median, 25th–75th percentile) | K-W $p$ value | $p$ values for Dunn's post hoc test | | |
|---|---|---|---|---|---|---|---|
| | | | | | CH attack vs. headache-free CH | CH-attack vs. HC | Headache-free CH vs. HC |
| NLRP1, pg/ml | 59.65 (48.22–186.15) | 62.58 (48.65–171.98) | 91.74 (58.87–227.70) | 0.092 | >0.999 | 0.181 | 0.291 |
| NLRP3, pg/ml | 112.80 (100.25–266.50) | 122.55 (101.50–197.75) | 147.80 (107.10–241.10) | 0.514 | >0.999 | 0.951 | >0.999 |
| ASC, ng/ml | 2.77 (2.03–7.41) | 3.24 (2.29–5.21) | 4.44 (3.16–7.59 | 0.043 | >0.999 | 0.121 | 0.128 |
| HMGB1, ng/ml | 12.45 (5.75–16.94) | 12.08 (5.67–18.02) | 13.33 (5.97–22.45) | 0.696 | >0.999 | >0.999 | >0.999 |
| CASP1, ng/ml | 2.49 (0–11.27) | 5.24 (0–9.40) | 7.58 (3.92–12.84) | 0.099 | >0.999 | 0.322 | 0.188 |
| NF-κB, ng/ml | 2.64 (0.78–4.53) | 2.40 (0.86–4.12) | 2.98 (0.85–4.12) | 0.175 | >0.999 | 0.343 | 0.444 |
| IL-1β, pg/ml | 4.43 (0–8.31) | 5.79 (4.11–8.13) | 6.37 (0–10.27) | 0.684 | >0.999 | >0.999 | >0.999 |
| IL-18, ng/L | 43.28 (23.68–101.16) | 43.86 (20.20–66.85) | 45.94 (17.90–106.30) | 0.869 | >0.999 | >0.999 | >0.999 |
| IL-4, pg/ml | 0.91 (0.74–1.15) | 0.88 (0.61–1.12) | 0.89 (0.62–1.09) | 0.887 | >0.999 | >0.999 | >0.999 |
| S100b, ng/L | 713.10 (539.67–2575.50) | 718.85 (584.15–1457.25) | 1524 0.00 (682.50–2380.00) | 0.276 | >0.999 | 0.546 | 0.613 |
| NSE, ng/ml | 13.56 (7.78–18.53) | 14.03 (7.25–17.61) | 12.26 (6.93–24.80) | 0.928 | >0.999 | >0.999 | >0.999 |
| nNOS, ng/ml | 11.82 (3.77–13.93) | 13.75 (3.50–16.28) | 10.52 (3.67–20.03) | 0.898 | >0.999 | >0.999 | >0.999 |
| iNOS, U/L | 38.36 (22.92–102.8) | 38.72 (26.02–67.77) | 55.34 (37.20–107.20) | 0.075 | 0.999 | 0.171 | 0.224 |
| eNOS, U/ml | 66.99 (56.60–214.55) | 82.49 (56.42–104.30) | 82.17 (57.13–208.90) | 0.999 | >0.999 | >0.999 | >0.999 |

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; CASP1, caspase 1; CH, cluster headache; eNOS, endothelial nitric oxide synthase; HC, healthy control; HMGB1, high mobility group box 1; IL-1β, interleukin 1 beta; IL-4, interleukin 4; IL-18, interleukin 18; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; NLR 1, NLR family pyrin domain containing 1; NLRP3, NLR family pyrin domain containing 3; nNOS, neuronal nitric oxide synthase; NSE, neuron-specific enolase; S100b, S100 calcium-binding protein B.

to be statistically significant in all tests. The *p* values were adjusted with Bonferroni multiple correction testing. The threshold for significance was set to $p < 0.003$ ($n = 14$; we compared 14 inflammatory mediators between groups). No statistical power calculation was conducted prior to the study; the sample size was based on the available data.

## RESULTS

### Reduced NLRP3 mRNA expression levels in PBMCs of patients with CH

Thirty-four patients and 47 controls were enrolled in the Headache Unit and there were no missing data. To assess the significance of IL-1β in CH, we did a preliminary mRNA expression level analysis of IL-1β and intracellular inflammation factors NF-κB, NLRP1, and NLRP3 that are involved in production of IL-1β in PBMC samples obtained from healthy controls and patients with CH. These analyses showed that levels of inflammasome complex components are decreased while IL-1β level is increased. Patients with CH showed significantly reduced NLRP3 expression levels compared with healthy controls, in both the CH-attack (median [25p–75p] = 1.13 [0.81–1.20] vs. 0.68 [0.51–0.84], respectively; $p = 0.041$) and headache-free CH groups (median [25p–75p] = 1.13 [0.81–1.20] vs. 0.54 [0.31–0.62], respectively; $p = 0.001$) according to Dunn's post hoc test and Bonferroni correction. By contrast, the CH-attack group exhibited greater IL-1β ($2^{\Delta\Delta CT}$ median [25p–75p] = 0.96 [0.66–1.29] vs. 0.52 [0.43–0.73]) and NF-κB (median [25p–75p] = 1.06 [0.66–3.00] vs. 0.62 [0.43–1.19]) levels without attaining statistical significance ($p = 0.058$ and $p = 0.072$, respectively, according to Dunn's post hoc test). NLRP1 levels in PBMCs were comparable among groups (Table 3). The CH-attack group displayed higher IL-1β ($p = 0.004$) and NF-κB ($p = 0.029$) levels than those without attack (headache-free CH) during sample collection when the two groups were compared with the Mann–Whitney *U*-test.

### Reduced inflammasome complex component levels in sera of patients with CH

When levels of inflammation factors associated with inflammasome activity and IL-1β production were compared, patients with CH were found to have reduced ASC levels (median [25p–75p] = 4.44 [3.16–7.59] vs. 2.77 [2.03–7.41] vs. 3.24 [2.29–5.21]; $p = 0.043$). However, this significance did not persist after the Bonferroni correction. The patients with CH had lower levels of other inflammasome complex components, NLRP1 (median [25p–75p] = 91.74 [58.87–227.70] vs. 59.65 [48.22–186.15] vs. 62.58 [48.65–171.98]; $p = 0.092$), NLRP3 (median [25p–75p] = 147.80 [107.10–241.10] vs. 112.80 [100.25–266.50] vs. 122.55 [101.50–197.75]; $p = 0.514$), and CASP1 (median [25p–75p] = 7.58 [3.92–12.8] vs. 2.49 [0–11.27] vs. 5.24 [0–9.40]; $p = 0.099$), the inflammasome activator HMGB1 (median [25p–75p] = 13.33 [5.97–22.45] vs. 12.45 [5.75–16.94] vs. 12.08 [5.67–18.02]; $p = 0.696$), and the glial marker protein S100b (median [25p–75p] = 1524.00 [682.50–2380.00] vs. 713.10 [539.67–2575.50] vs. 718.85 [584.15–1457.25]; $p = 0.276$). However, these differences did not attain statistical significance. Respective levels among study groups (controls, CH-attack, headache-free CH) of NSE (median [25p–75p] = 12.26 [6.93–24.80] vs. 13.56 [7.78–18.53] vs. 14.03 [7.25–17.61]; $p = 0.928$), IL-4 (median [25p–75p] = 0.89 [0.62–1.09]

**TABLE 5** Results of Spearman's correlation analysis for inflammation parameters versus patient variables

| Parameters | Age | Age of onset | Disease duration | Headache duration |
|---|---|---|---|---|
| NLRP1 | *p*: 0.094 | *p*: 0.251 | *p*: 0.934 | *p*: 0.564 |
| | $r_s$: −0.29 | $r_s$: −0.08 | $r_s$: 0.01 | $r_s$: 0.10 |
| NLRP3 | *p*: 0.122 | *p*: 0.059 | *p*: 0.687 | *p*: 0.462 |
| | $r_s$: −0.27 | $r_s$: −0.17 | $r_s$: 0.07 | $r_s$: 0.13 |
| ASC | *p*: 0.057 | *p*: 0.710 | *p*: 0.541 | *p*: 0.833 |
| | $r_s$: −0.14 | $r_s$: −0.06 | $r_s$: 0.11 | $r_s$: 0.04 |
| HMGB1 | *p*: 0.051 | *p*: 0.057 | *p*: 0.849 | *p*: 0.126 |
| | $r_s$: −0.14 | $r_s$: −0.36 | $r_s$: 0.03 | $r_s$: 0.27 |
| CASP1 | *p*: 0.663 | *p*: 0.393 | *p*: 0.713 | *p*: 0.731 |
| | $r_s$: −0.08 | $r_s$: −0.15 | $r_s$: −0.06 | $r_s$: −0.06 |
| NF-κB | *p*: 0.564 | *p*: 0.257 | *p*: 0.763 | *p*: 0.945 |
| | $r_s$: −0.13 | $r_s$: −0.20 | $r_s$: −0.05 | $r_s$: 0.01 |
| IL-1β | *p*: 0.549 | *p*: 0.625 | *p*: 0.211 | *p*: 0.213 |
| | $r_s$: −0.11 | $r_s$: −0.09 | $r_s$: −0.22 | $r_s$: 0.22 |
| IL-18 | *p*: 0.581 | *p*: 0.210 | *p*: 0.790 | *p*: 0.285 |
| | $r_s$: −0.15 | $r_s$: −0.22 | $r_s$: 0.04 | $r_s$: 0.19 |
| IL-4 | *p*: 0.639 | *p*: 0.413 | *p*: 0.384 | *p*: 0.886 |
| | $r_s$: −0.08 | $r_s$: −0.14 | $r_s$: −0.15 | $r_s$: 0.02 |
| S100b | *p*: 0.133 | *p*: 0.058 | *p*: 0.623 | *p*: 0.265 |
| | $r_s$: −0.12 | $r_s$: −0.14 | $r_s$: 0.08 | $r_s$: 0.19 |
| NSE | *p*: 0.056 | *p*: 0.059 | *p*: 0.442 | *p*: 0.197 |
| | $r_s$: −0.15 | $r_s$: −0.17 | $r_s$: 0.14 | $r_s$: 0.22 |
| nNOS | *p*: 0.060 | *p*: 0.541 | *p*: 0.654 | *p*: 0.051 |
| | $r_s$: −0.19 | $r_s$: −0.11 | $r_s$: 0.08 | $r_s$: 0.34 |
| iNOS | *p*: 0.210 | *p*: 0.142 | *p*: 0.543 | *p*: 0.619 |
| | $r_s$: −0.22 | $r_s$: −0.26 | $r_s$: −0.11 | $r_s$: 0.09 |
| eNOS | *p*: 0.291 | *p*: 0.091 | *p*: 0.474 | *p*: 0.228 |
| | $r_s$: −0.37 | $r_s$: −0.27 | $r_s$: 0.13 | $r_s$: 0.21 |

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; CASP1, caspase 1; eNOS, endothelial nitric oxide synthase; HMGB1, high mobility group box 1; IL-1β, interleukin 1 beta; IL-4, interleukin 4; IL-18, interleukin 18; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; NLR 1, NLR family pyrin domain containing 1; NLRP3, NLR family pyrin domain containing 3; nNOS, neuronal nitric oxide synthase; NSE, neuron-specific enolase; S100b, S100 calcium-binding protein B.

vs. 0.91 [0.74–1.15] vs. 0.88 [0.61–1.12]; $p = 0.887$), and end products of inflammasome activity (IL-18, IL-1β, and NOS isoforms) were comparable (Table 4). When all patients with CH were compared with healthy controls, serum levels of NLRP1 (median [25p–75p] = 60.70 [48.65–183.10] vs. 91.74 [58.87–227.70]; $p = 0.029$), ASC (median [25p–75p] = 3.15 [2.07–5.71) vs. 4.44 [3.16–7.59]; $p = 0.012$), CASP1 (median [25p–75p] = 4.50 [0–9.74) vs. 7.58 [3.92–12.84)]; $p = 0.033$), and inducible NOS (median [25p–75p] = 38.58 [23.70–93.54] vs. 55.34 [37.20–107.20]; $p = 0.030$) were found to be reduced in patients with CH. However, these differences did not persist after Bonferroni correction. There were no significant differences between patients with and without CH attacks.

## Comparison of findings with regard to specific clinical features in CH

No significant correlation was found among mRNA and protein levels of investigated inflammation parameters versus age, age of onset, disease duration, and headache duration variables of patients with CH (Table 5). Also, there were no significant differences between male ($n = 26$) versus female ($n = 8$) and patients with chronic ($n = 4$) versus episodic ($n = 30$) CH. Remarkably, in patients with three or more autonomic manifestations ($n = 9$), when compared with those with fewer autonomic symptoms ($n = 25$), levels were lower for the medians (25p–75p) of NLRP1 (52.52 [35.48–67.91] vs. 78.66 [54.92–213.25]; $p = 0.017$), HMGB1 (11.51 [5.20–15.50] vs. 13.33 [8.08–18.13]; $p = 0.038$), S100b (569.90 [524.10–783.80] vs. 763.40 [590.15–2713.00]; $p = 0.013$), NSE (11.15 [6.26–14.91] vs. 13.93 [10.82–19.04]; $p = 0.021$), nNOS (4.24 [3.34–12.85] vs. 12.82 [4.52–15.44]; $p = 0.028$), and eNOS (64.83 [54.59–91.14] vs. 89.42 [61.19–228.40]; $p = 0.034$) (Figure 1). In smokers ($n = 20$) as compared with the nonsmoker group ($n = 14$), levels were significantly lower for NLRP3 (100.80 [94.30–186.60] vs. 141.40 [112.80–211.35]; $p = 0.014$), HMGB1 (11.95 [5.68–14.04] vs. 15.74 [6.86–19.87]; $p = 0.004$), IL-18 (41.54 [27.37–63.27] vs. 53.84 [24.61–110.15]; $p = 0.033$), S100b (581.70 [528.35–1607.60] vs. 874.50 [713.10–2379.50]; $p = 0.001$), NSE (12.73 [8.34–16.01] vs. 15.09 [8.19–18.86]; $p = 0.020$), nNOS (11.49 [3.61–14.36] vs. 12.39

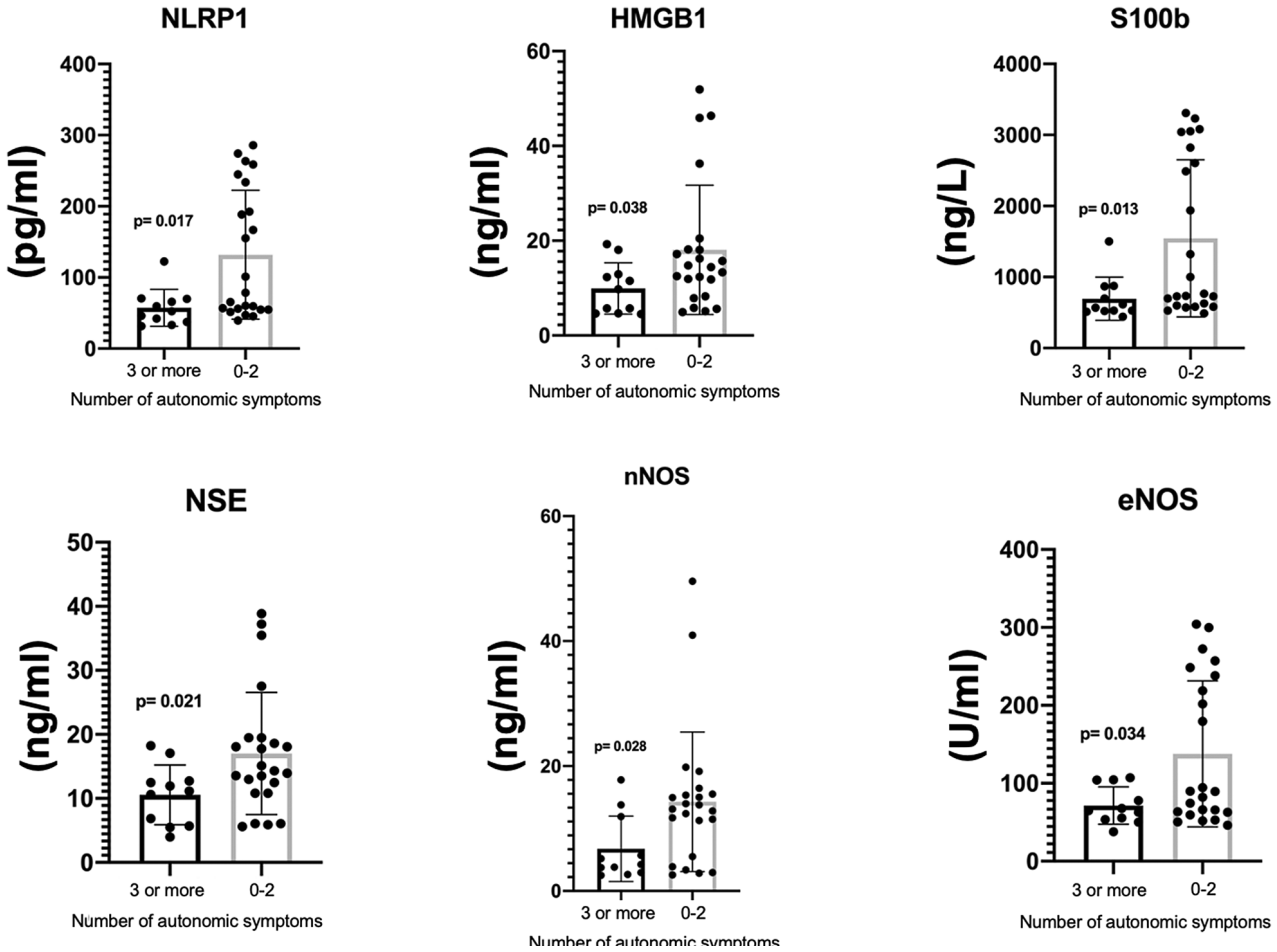


**FIGURE 1** Reduced serum levels of inflammasome-related factors, S100b (S100 calcium-binding protein B), and neuron-specific enolase (NSE) in patients with cluster headache with three or more than three autonomic symptoms. Vertical bars indicate standard deviations; *p* values denoted on each panel were obtained by Mann–Whitney *U*-test. eNOS, endothelial nitric oxide synthase; HMGB1, high mobility group box 1; NLRP1, NLR family pyrin domain containing 1; nNOS, neuronal nitric oxide synthase.

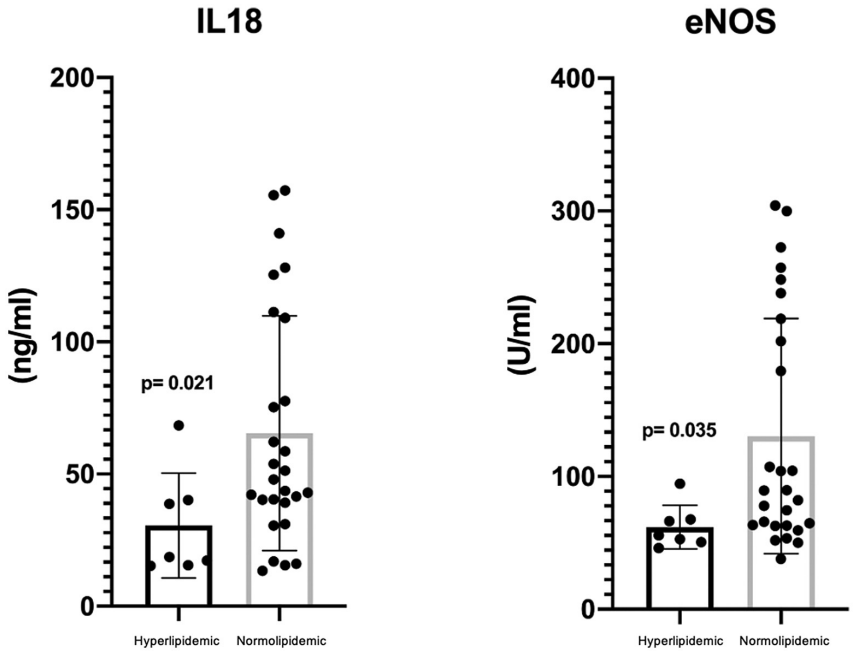


**FIGURE 2** Reduced serum levels of interleukin (IL)-18 and endothelial nitric oxide synthase (eNOS) in patients with cluster headache and hyperlipidemia. Vertical bars indicate standard deviations; *p* values denoted on the panels were obtained by Mann–Whitney *U*-test.

[4.45–16.65]; $p = 0.025$), and eNOS (64.83 [55.68–134.59] vs. 94.49 [60.80–225.10]; $p = 0.004$). Moreover, serum IL-18 (18.66 [15.52–40.14] vs. 47.92 [39.12–109.00]; $p = 0.021$) and eNOS (55.65 [50.62–67.68] vs. 89.42 [62.93–218.80]; $p = 0.035$) levels were lower in patients with hyperlipidemia ($n = 6$) (Figure 2).

## DISCUSSION

Our study showed greater IL-1β and NF-κB expression levels in PBMCs of patients with CH during the attack period. This finding supports a previous report demonstrating greater serum IL-1β levels in patients with CH.[4] Notably, several medications, including corticosteroids that are known to alleviate symptoms of CH, suppress IL-1β or the NF-κB complex, which is involved in IL-1β production.[26,27] A similar IL-1β elevation was not found in sera of patients with CH. This discrepancy may be owing to confounding effects of multiple cell types that contribute to serum IL-1β levels. The lack of correlation between IL-1β expression levels versus duration of pain, sex, cluster type, and frequency and duration of episodes suggests that the clinical features of the disease are not significantly associated with inflammation. Nevertheless, given the distinction from healthy controls, IL-1β might still be explored further as a potential biomarker or treatment target.

Activation of NF-κB induces transcription of the pro-form of IL-1β, whereas activation of the inflammasome complex is required for the release of the active form of the cytokine.[28] Thus, it is remarkable that levels of inflammasome complex components are decreased while IL-1β level is increased. A similar discrepancy was found in patients with focal epilepsy of unknown cause.[29] Putative causes of this discrepancy might be the overconsumption of the inflammasome pathways and/or suppression of proinflammatory cytokine production via negative feedback mechanisms. In this context, levels of S100b, a marker of glial activity,[30] was also suppressed in patients with CH. This finding further corroborates the notion of inhibited glial activity and subsequently suppressed glia-derived inflammasome expression in patients with CH.

Autonomic symptoms that accompany pain occur in different intensities and variety in each case. Inflammatory mechanisms have been implied to lead to the activation of sympathetic and parasympathetic systems that are involved in the occurrence of autonomic manifestations. Several studies have indicated that unilateral autonomic manifestations might be associated with excessive secretion of proinflammatory cytokines in the cavernous sinus.[31,32] We found reduced levels of inflammasome complex components and S100b in patients with multiple autonomic manifestations, suggesting that the activation of the cranial autonomic system might be one of the factors leading to compensatory suppression of glia-induced inflammatory responses.

Hyperlipidemia was the only systemic condition accompanying CH in the present study. Reduced IL-18 and eNOS levels in patients with CH and hyperlipidemia emphasize the previously established link between hyperlipidemia and inflammation. A study on IL-18

levels in peripheral arterial disease found that IL-18 had a positive correlation with low-density lipoprotein and triglyceride levels,[33] and animal experiments indicated that IL-18 levels were altered in hyperlipidemic mice.[34] Data obtained through the present study further support the notion that comorbid hyperlipidemia influences the levels of inflammasome activation end products in CH through unknown mechanisms.

Cigarette smoking and its ties to CH are complex. It has been hypothesized that various toxic metabolites, including those inhaled by cigarette smoking, may be associated with CH by activating the hypothalamic–pituitary axis.[35] On the other hand, numerous studies have shown that smoking is not a frequent attack trigger and its cessation does not affect attack burden.[36–40] It is tempting to speculate that inflammation activation may relate to unexplained association between smoking and CH, at least for some patients.

Suppression of levels of inflammasome complex components and S100b in cigarette smokers suggests that cigarette smoking induces inflammasome/glia-suppressing mediators that are pending characterization. Furthermore, the fact that patients frequently reduce smoking during the attack period because they recognize its relationship with headache attacks may have confounded the results of our study.

One of the limitations of our study was the unavailability of cerebrospinal fluid samples owing to ethical concerns, which could have yielded a more accurate representation of the cerebral inflammasome activity. Second, investigation of a broader panel of inflammatory cytokines induced by NF-κB activation (e.g., IL-6 and TNF-α) in future multicenter studies involving more patients with CH might provide more precise information on the complex balance between NF-κB and inflammasome complexes in CH pathogenesis.

## CONCLUSION

In conclusion, our results failed to establish an association between inflammasome complex activity and the pathogenesis of CH. However, greater PBMC expression levels of NF-κB and IL-1β in the CH-attack group suggest involvement of innate immunity in the development of bouts of CH attacks and potential biomarker value of these factors in differential diagnosis of CH. Also, suppression of inflammatory factors in patients with multiple autonomic symptoms sheds further light on the complex interaction between the autonomic system and inflammation. More comprehensive analyses of inflammation in CH can guide understanding the mechanisms that can lead to preventive treatment strategies and potential biomarkers.

### Data analysis

This report represents the primary analysis of these data, and it was registered to the Istanbul University Scientific Research Fund (IU-BAP TTU-2017-26478) and transferred to the database of the National Thesis Center of the Council of Higher Education (YOKSIS).

### AUTHOR CONTRIBUTIONS

*Study concept and design*: Cem İsmail Küçükali, Erdem Tüzün, Betül Baykan. *Acquisition of data*: Erdi Şahin, Zerrin Karaaslan, Elif Şanlı, Özlem Timirci Kahraman, Canan Ulusoy, Elif Kocasoy Orhan, Esme Ekizoğlu. *Analysis and interpretation of data*: Erdi Şahin, Zerrin Karaaslan, Özlem Timirci Kahraman, Cem İsmail Küçükali, Erdem Tüzün, Betül Baykan. *Drafting of the manuscript*: Erdi Şahin, Zerrin Karaaslan, Erdem Tüzün, Betül Baykan. *Revising it for intellectual content*: Cem İsmail Küçükali, Erdem Tüzün, Betül Baykan. *Final approval of the completed manuscript*: Erdi Şahin, Zerrin Karaaslan, Elif Şanlı, Özlem Timirci Kahraman, Canan Ulusoy, Elif Kocasoy Orhan, Esme Ekizoğlu, Cem İsmail Küçükali, Erdem Tüzün, Betül Baykan.

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### CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

### ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Istanbul University Istanbul Medical Faculty Clinical Research Ethics Committee (date: 21/04/2017 number: 2017/08). Written informed consent was obtained from all patients.

### DATA AVAILABILITY STATEMENT

All data used and analyzed in the current study are available from the corresponding author on reasonable request.

### ORCID

*Erdi Şahin* https://orcid.org/0000-0002-5792-2888

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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