pagepress

Confinia Cephalalgica 2025; 35:15795

Cytokines, BDNF, and CGRP levels in tear fluid of migraine patients assessed using a novel non-invasive approach

Marina Romozzi,[1,2*] Lucia Di Nardo,[3,4*] Vincenzo Trigila,[5] Giovanni Cuffaro,[6,7] Gustavo Savino,[6,7] Stanislao Rizzo,[6,7] Luigi Francesco Iannone,[8,9] Sonia Di Tella,[10] Antoinette MaassenVanDenBrink,[11] Catello Vollono,[1,2#] Paolo Calabresi[1,2#]

*These authors share first co-authorship
#These authors share senior co-authorship

[1]Department of Neurosciences, Università Cattolica del Sacro Cuore, Rome, Italy; [2]Neurology, Dipartimento di Neuroscienze, Organi di Senso e Torace, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy; [3]Immunology Facility, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy; [4]Department of Translational Medicine and Surgery, Università Cattolica del Sacro Cuore, Rome, Italy; [5]Department of Ophthalmology, University of Catania, Italy; [6]Ophthalmology, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy; [7]Ophthalmology, Dipartimento di Neuroscienze, Organi di Senso e Torace, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy; [8]Digital and Predictive Medicine, Pharmacology, Clinical Metabolic Toxicology – Headache Center and Drug Abuse, Laboratory of Clinical Pharmacology and Pharmacogenomics, Policlinico di Modena, Italy; [9]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy; [10]Department of Psychology, Università Cattolica del Sacro Cuore, Milan, Italy; [11]Department of Internal Medicine, Erasmus MC University Medical Center, Rotterdam, The Netherlands

ABSTRACT

Background: Recent research has focused on identifying innovative, non-invasive sources of migraine biomarkers, such as tear fluid and saliva, that may reflect underlying pathogenetic mechanisms, such as neurogenic inflammation. This study aimed to analyze the levels of interleukin (IL)-1β, IL-20, tumor necrosis factor-α (TNF-α), brain-derived neurotrophic factor (BDNF), and calcitonin gene-related peptide (CGRP) in the tear fluid of patients with migraine.

Methods: Consecutive patients with a diagnosis of migraine were included. Tear fluid was collected from migraine patients and healthy controls (HCs) through Schirmer test strips; cytokines (IL-10, IL-1β, and TNF-α) and BDNF were measured using the automated EllaTM (Bio-Techne) multiplex Enzyme-Linked Immunosorbent Assay (ELISA) platform, while CGRP concentrations were quantified using a CGRP sandwich ELISA kit. Clinical characteristics of migraine patients, severity, and disability scores were collected.

Results: Fifty-one patients with migraine (9 [17.6%] chronic, 16 with aura [31.4%]) and 17 age-matched HCs were included. Tear fluid CGRP concentrations were significantly elevated in migraine patients (7.4±7.6 pg/mL) compared to HCs (3.1±3.7 pg/mL; p=0.014). In the migraine group, tear CGRP levels were higher in the ictal phase (10.5±7.7 pg/mL) compared to the interictal phase (5.8±7.3 pg/mL) (p=0.021) and in patients with (10.4±9.2 pg/mL) *vs.* without (6.1±6.4 pg/mL) aura (p=0.042). BDNF tear levels did not differ between patients with migraine and HCs, but were higher in patients with chronic migraine (4.1±7.5 pg/mL) compared to episodic (0.6±2.5 pg/mL; p=0.018). Similarly, TNF-α tear levels did not differ between patients with migraine and HCs, but were higher in patients with chronic migraine (0.15±0.12 pg/mL) compared to episodic migraine (0.05±0.07 pg/mL; p=0.009). TNF-α levels were correlated with IL-10 (ρ=0.426, p<0.001), IL-1β (ρ=0.350, p=0.017), and BDNF (ρ=0.364, p=0.010). Additionally, IL-10 levels were correlated with BDNF levels (ρ=0.481, p<0.001). CGRP levels were negatively correlated with BDNF (ρ=−0.377, p=0.006).

Conclusions: Elevated CGRP levels were found in patients with migraine as compared to HCs. Correlations observed among changes in tear fluid IL-10, IL-1β, and BDNF levels suggest common regulatory mechanisms. Furthermore, the increase in both tear fluid BDNF and TNF-α in patients with chronic migraine suggests a role for these biomarkers in the chronicization of the disease. While measuring biomarkers in tear fluid represents a rapid and promising non-invasive approach, it requires supporting evidence from future larger studies.

Key words: CGRP, interleukins, biomarker, migraine, TNF-α.

Introduction

Migraine is one of the most common and debilitating neurological disorders, affecting more than one billion people worldwide. (1) Although its pathophysiological mechanisms are not yet fully understood, current evidence suggests that migraine involves the activation and sensitization of the trigeminovascular system *via* its main sensory neuropeptide, the calcitonin gene-related peptide (CGRP). (2) However, an additional regulatory peptide, the pituitary adenylate cyclase-activating peptide (PACAP), and various proinflammatory cytokines (3) have been implicated in migraine mechanisms. CGRP, released from peripheral terminals of trigeminal nerve fibers, induces two main responses: arteriolar vasodilatation (4) and mechanical hypersensitivity collectively referred to as neurogenic inflammation. (5) Activation of dural mast cells, which stimulates the production of some pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1, which may amplify sensitization of trigeminal fibers, has also been described. (6) Due to the proposed role of neurogenic inflammation in migraine mechanism, previous studies have investigated the relationships between this dis-

ease and various inflammatory markers in plasma and cerebrospinal fluid (CSF) of patients with migraine with inconsistent results across different conditions. (7-10)

Proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, have been found to be elevated in patients with migraine. Conversely, the anti-inflammatory cytokine, IL-10, was decreased in the peripheral blood during the interictal phase in patients with migraine compared to healthy controls (HCs) but increased during the ictal phase. (11) However, reported results widely varied across studies due to differences in the same sample source and detection methods (*e.g.*, enzyme-linked immunosorbent assay [ELISA] or radioimmunoassay). (12) Similarly, CGRP levels have been found elevated in the plasma of migraine patients compared to controls, and during the ictal phase compared to the interictal phase. (13) CGRP measurement in plasma is challenging due to its very low concentrations and short half-life, due to the presence of peptidases that may interfere with the assay. (14) Growing interest in other migraine-related mediators, including neurotrophins, particularly brain-derived neurotrophic factor (BDNF), which modulates pain signaling in trigeminal ganglion (TG) neurons. (15) Furthermore, CGRP and various cytokines regulate BDNF functioning, (16) and BDNF induces allodynia in experimental animals, although its role in migraine pathogenesis remains unclear. (17)

The growing interest in non-invasive approaches to detect soluble migraine biomarkers (18) led to exploring biological fluids other than blood and CSF. Based on the hypothesis that CGRP concentrations in tear fluids mirror local neuropeptide levels derived from activation of trigeminal nerve fibers of the ophthalmic (V1) branch, mainly of the cornea and lacrimal glands, or in saliva from the trigeminal innervation of the oral cavity and mucosa. (19-21) In particular, tear fluid biomarker analysis may offer valuable insights for several additional reasons, including a contribution of changes in systemic neuropeptide levels. (22) The lacrimal glands and tear fluid contain several pro- and anti-inflammatory cytokines, (23-25) whereas little information has been reported on BDNF levels in tear fluid. To date, no studies have analyzed cytokine levels and BDNF in the tear fluid of patients with migraine. Herein, we aimed to measure the concentration of CGRP, IL-1β, IL-10, TNF-α, and BDNF in the tear fluid of patients with migraine and to examine their correlations with some clinical migraine characteristics.

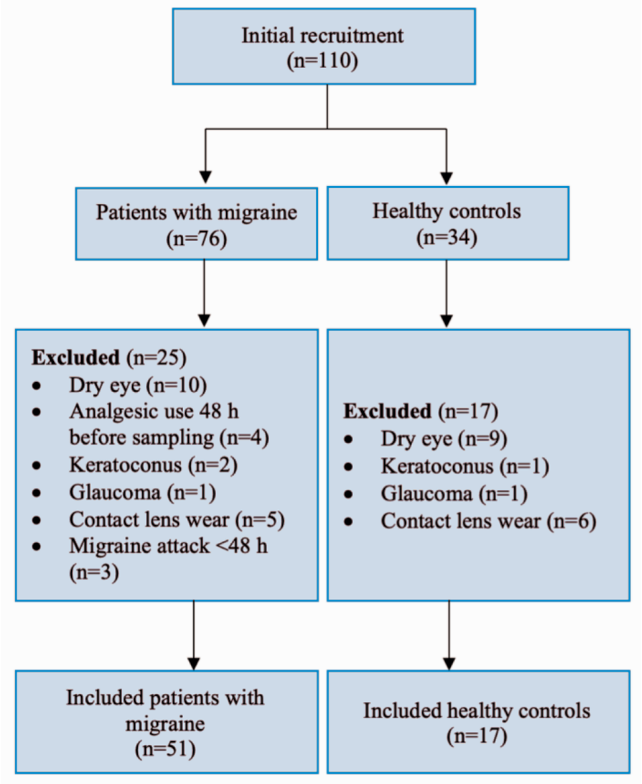


Figure 1. Flowchart of the study.

Results

We included 51 patients with migraine (mean age ± standard deviation [SD], 29.8±10.9, 80.4% females) and 17 age-matched HCs (24.6±2.2, 41.2% females). The study flowchart is reported in **Figure 1**. The 51 patients with migraine included 9 patients with chronic migraine (CM) (17.6%) and 2 of them with medication overuse headache (MOH) (3.9%). The remaining patients suffered from episodic migraine (EM). In 10 patients (19.6%), n=2 with CM and n=8 with EM, it was possible to sample CGRP during the attack (ictal phase). Compared to HCs, patients with migraine reported higher scores on the Hamilton Anxiety Scale (HAM-A) (HAM-A: 9.7±6.9 *vs.* 4.5±5.3; p=0.016) and the Hamilton Depression Scale (HAM-D) (HAM-D: 8.6±5.4 *vs.* 3.0±3.6; p=0.002). Based on HAM-D results, 13 patients (25.0%) were identified as having concomitant depression, while HAM-A results indicated that 19 patients (36.5%) had concomitant anxiety. Nine patients (17.6%) received preventive treatment (4 patients with CM and 5 with EM), including calcium antagonists (n=2), amitriptyline (n=4), and antiseizure medications (n=3). Detailed demographic and clinical characteristics of the cohort are reported in **Table 1**.

CGRP in tear fluid. Mean CGRP levels in tear fluid were significantly higher in patients with migraine (7.4±7.6 pg/mL) compared to HCs (3.1±3.7 pg/mL) in a sex-adjusted comparison (p=0.014) (**Figure 2** and **Table 2**). CGRP levels in patients during a migraine attack (ictal phase) were significantly higher (10.59±7.7 pg/mL; n=10 subjects) compared to levels measured during the interictal phase (5.8±7.3 pg/mL; n=41) (p=0.021). In addition, patients with aura (none of them sampled during the aura phase) had higher mean CGRP levels in tear fluid (10.4±9.2 pg/mL; n=16) than patients without aura (6.1±6.4 pg/mL; n=35) (p=0.042). CGRP levels were significantly higher in patients with EM (8.7±7.8 pg/mL; n=42) compared

Table 1. Patients' demographic and clinical features.

	Migraine cohort (n=51)
Demographics	
Age (years), mean±SD	29.8±10.9
Sex female, n (%)	41 (80.4)
Migraine features	
Age at onset, mean±SD	19.0±12.0
Chronic migraine, n (%)	9 (17.6)
Medication overuse headache, n (%)	2 (3.9)
Migraine with aura, n (%)	16 (31.4)
Monthly headache days, mean±SD	5.6±5.9
Absolute number of analgesics, mean±SD	4.8±3.9
NRS, mean±SD	7.3±1.6
Concomitant preventive treatment, n (%)	9 (17.6)
Questionnaires scores, mean±SD	
HIT-6	63.2±7.0
MIDAS	29.7±31.7
HAM-A	9.7±6.9
HAM-D	8.6±5.4

Percentages are calculated based on the column total; NRS score refers to the average intensity in the previous month; HAM-A, Hamilton Anxiety Rating Scale; HAM-D, Hamilton Depression Rating Scale; HIT-6, Headache Impact Test-6; MIDAS, Migraine Disability Assessment; NRS, Numeric Rating Scale; SD, standard deviation.

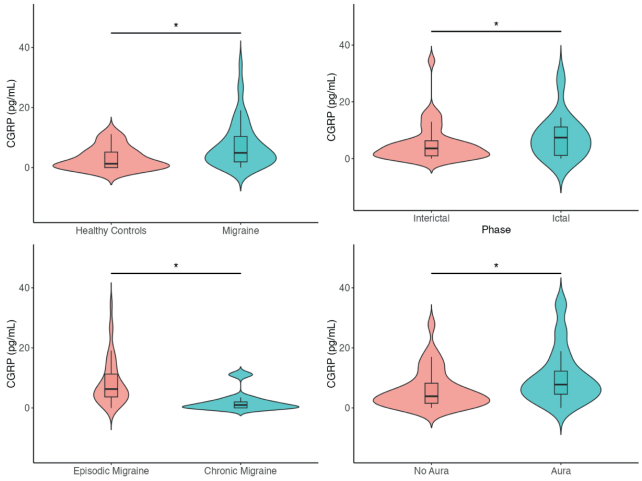
to CM (2.4±3.7 pg/mL; n=9). No significant differences were observed between patients with and without migraine concomitant preventive treatments (p=0.466) and between male and female subjects (p=0.623).

Finally, we found a significant moderate negative correlation between CGRP tear levels and the monthly migraine days (MHDs; ρ=−0.297, p=0.038), Migraine Disability Assessment (MIDAS; ρ=−0.439, p=0.005), and the Headache Impact Test (HIT-6) score (ρ=−0.317, p=0.036) (**Figure 3**). No significant correlation was found between CGRP levels and either HAM-A (ρ=0.089, p=0.630) or HAM-D (ρ=0.075, p=0.684) scores, suggesting no influence of depression and anxiety on CGRP tear fluid.

BDNF in tear fluid. Cytokines and BDNF were also evaluated in tear fluid. No significant differences were observed between the migraine group and HCs in IL-10, IL-1β, TNF-α, and BDNF levels in sex-adjusted comparisons (**Table 2**). However, BDNF levels were significantly higher in the CM (n=9) group (4.1±7.5 pg/mL) than in the EM (n=41) group (0.6±2.5 pg/mL; p=0.018). Similarly, TNF-α tear fluid levels were more elevated in the CM group (0.15±0.12 pg/mL) than in the EM group (0.05±0.07 pg/mL; p=0.009) (**Figure 4**). No significant differences were found in IL-10 (p=0.489) and IL-1β (p=0.143) levels of CM and EM.

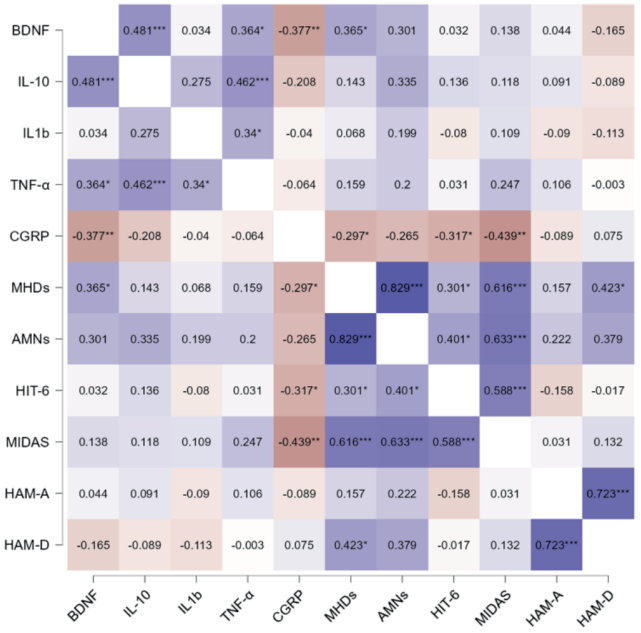
A moderately significant correlation between BDNF levels and MHDs was observed (ρ=0.365, p=0.010). However, no significant correlations were found among TNF-α, IL-10, or IL-1β levels and the HAM-A and HAM-D scales. No significant differences between patients with and without aura in the levels of BDNF (p=0.489), IL-10 (p=0.989), IL-1β (p=0.146), or TNF-α (p=0.551) were reported. No significant differences were found in BDNF (p=0.944), IL-10 (p=0.661), IL-1β (p=0.387), or TNF-α (p=0.205) levels in the ictal phase *vs.* the interictal phase. No significant associations were also found between HAM-A/HAM-D and cytokine levels (TNF-α, IL-10, IL-1β, and BDNF) (all p>0.1), and no differences emerged between patients with or without migraine preventive treatment for IL-10 (p=0.700), IL-1β (p=0.180), or TNF-α (p=0.582).

Correlations between cytokines, BDNF, and CGRP. Finally, we examined correlations among the different biomarkers within the migraine subgroup (n=51). TNF-α showed a moderate positive correlation with IL-10 (ρ=0.426, p<0.001), IL-1β (ρ=0.350,



CGRP, calcitonin gene-related peptide.

Figure 2. Violin plots with overlaid boxplots comparing tear fluid CGRP in patients with migraine and healthy controls, and comparison within the migraine group of tear fluid CGRP in ictal and interictal phases, migraine with and without aura, and episodic and chronic migraine. The violin shape represents the kernel density estimate of the data, while the boxplot inside shows the median and interquartile range.



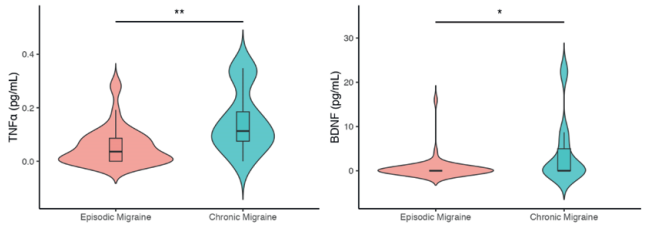
BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; HAM-A, Hamilton Anxiety Rating Scale; HAM-D, Hamilton Depression Rating Scale; HIT-6, Headache Impact Test-6; IL-1β, interleukin-1 beta; IL-10, interleukin-10; MIDAS, Migraine Disability Assessment; NRS, Numeric Rating Scale; TNF-α, tumor necrosis factor alpha.

Figure 3. Spearman's rho heatmap.

Table 2. Biomarkers assayed in tear fluid from patients with migraine compared with healthy controls.

Mean±SD (pg/mL)	Migraine cohort (n=51)	Healthy controls (n=17)	p-value
CGRP	7.41±7.58	3.12±3.71	0.014
TNF-α	0.07±0.09	0.11±0.07	0.073
IL-10	0.084±0.199	0.027±0.062	0.580
IL-1β	0.016±0.057	0.049±0.186	0.500
BDNF	1.2±4.0	1.2±3.6	0.500

Comparisons were performed using rank-based analysis of covariance (Rank ANCOVA), adjusting for sex as a covariate; values in bold are statistically significant; BDNF, brain-derived neurotrophic factor; CGRP, calcitonin gene-related peptide; IL-1β, interleukin-1 beta; IL-10, interleukin-10; SD, standard deviation; TNF-α, tumor necrosis factor-alpha.



BDNF, brain-derived neurotrophic factor; TNF-α, tumor necrosis factor alpha.

Figure 4. Violin plots with overlaid boxplots comparing tear fluid TNF-α and BDNF in patients with migraine (n=51), in the two groups of patients with chronic and episodic migraine. The violin shape represents the kernel density estimate of the data, while the boxplot inside shows the median and interquartile range.

p=0.017), and BDNF (ρ=0.364, p=0.010). Additionally, IL-10 was correlated with BDNF (ρ=0.481, p<0.001). CGRP was negatively correlated with BDNF (ρ=−0.377, p=0.006). Details on correlations are reported in **Figure 3**.

Discussion

Through a minimally invasive approach, this study presents findings on CGRP measurement in tears along with those of other potential biomarkers, such as cytokines and BDNF, in the tear fluid of patients with migraine, using a new non-invasive, validated method (Schirmer strips). We found that CGRP levels were more elevated in patients with migraine compared to HCs, during the ictal phase compared to the interictal period, and in patients with migraine aura compared to those without aura. These results align with our prior work. (18) The detection of CGRP in the tear fluid is feasible due to the presence of trigeminal nerve endings in the ocular surface, along with the dense innervation of the conjunctiva, cornea, and lacrimal glands by CGRP-positive sensory fibers. (23) This anatomical arrangement is comparable to that of the salivary gland and oral cavity, supporting the value of migraine biomarkers in the saliva. (13)

CGRP elevation in tear fluid of patients with migraine compared to HCs is consistent with two earlier studies that collected capillary tubes for tear fluid sampling, (20,21) while we used Schirmer strips for tear fluid collection. This technique is widely used and validated in the study of ocular conditions such as dry eye syndrome, offering a fast, non-invasive, and standardized approach for tear sampling. (18,26) While it remains uncertain whether capillary tube sampling might induce local ocular inflammation and thereby affect CGRP levels, the use of Schirmer strips avoids this potential confounder. (27) In a previous study, tear CGRP increased in the ictal phase prior to treatment with triptans or non-steroidal anti-inflammatory drugs (NSAIDs), and fell markedly post-treatment. (17) However, no changes were found in plasma CGRP levels, indicating that tear detection provides results superior to those obtainable with plasma assays. (20) Regarding CGRP's use as a potential biomarker for migraine, several studies have measured CGRP levels in patients with migraine in several other biological matrices, including plasma, serum, saliva, and CSF. Samples of saliva are easy to collect and show ictal elevations and treatment-linked reductions in CM, but the signal size is smaller than in tears, and preanalytical variability from flow rate, stimulation, and protein normalization can reduce between-study reproducibility. (28) Peripheral blood is scalable and standardized but suffers from dilution and site effects. Single-center studies and a meta-analysis report interictal CGRP elevations in EM and CM *vs.* controls, but heterogeneity is high and driven by specimen type, sampling site, storage, and other confounders; a prospective reappraisal found no diagnostic value for interictal serum CGRP, underscoring limited reproducibility without strict harmonization. (29) CSF may better reflect central mechanisms, and assays in this fluid report increased CGRP in CM, but its invasiveness, small sample sizes, and logistical constraints limit utility and reproducibility for longitudinal or multicenter studies. (30)

Interestingly, in our cohort, tear CGRP was higher in EM than in CM. This contrasts with a previous tear study, which found elevated tear CGRP *vs.* controls in both EM and CM and no interictal difference between them under a strict 48-72 h headache- and medication-free definition. (20) Possible explanations for the difference include rapid fluctuations dependent on the time of the sampling relative to the course of the ictal phase (episodic patients might be more often closer to attacks), sample size distribution (only 9 patients had CM, and the standard deviation of CGRP was high), and a shift toward central sensitization in CM that reduces peripheral ocular trigeminal release measurable in tears. (31)

Regarding our finding of significantly elevated concentrations of CGRP in the tear fluid of patients with migraine compared to patients without aura, this observation aligns with previous studies reporting increased CGRP levels in plasma and saliva in patients with migraine aura. (28,32) Indirect evidence supporting the involvement of CGRP in migraine with aura is provided by the effect of anti-CGRP mAbs in reducing the frequency and severity of aura episodes, as demonstrated in real-world studies and *post-hoc* analyses of clinical trials. (33,34) Furthermore, preclinical evidence showed a calcium-dependent CGRP release during potassium-induced cortical spreading depression (CSD, a possible trigger of migraine aura) and that CGRP antagonism reduces CSD. (35) Lastly, provocation experiments have demonstrated that intravenous infusion of CGRP can induce migraine with aura, suggesting a role of CGRP in mechanisms of CSD and aura. (35-37)

TNF-α in tear fluid was significantly elevated in patients with CM *vs.* EM, and its levels correlated with IL-1β and anti-inflammatory (IL-10) as well as BDNF. Studies on serum TNF-α levels in patients with migraine report conflicting results. (38) Several studies have reported increased serum TNF-α levels in patients with migraine and during migraine attacks. (4,35,36) However, this last finding was not confirmed in our cohort. Similar to our results in tear fluid, an increase in CSF TNF-α levels was found in patients with CM, suggesting an association between this pro-inflammatory cytokine and chronicization processes. (39) In our study, TNF-α levels in tear fluid were correlated with both IL-10 and IL-1β. These findings partly align with a previous study in which plasma TNF-α levels correlated with the pro-inflammatory cytokine IL-1β and negatively correlated with the anti-inflammatory cytokine IL-10. (38) In our study, the correlation between TNF-α and IL-10 may suggest a compensatory anti-inflammatory response to pro-inflammatory signaling related to migraine and its chronicization. However, we found no significant differences in cytokine levels between migraine patients and controls, probably due to the small sample size. Existing data on IL-10 and IL-1β levels in plasma or serum among migraine patients remain inconsistent. Most studies have reported decreased interictal IL-10 levels and increased levels during the ictal phase. (12) However, one study assessing IL-10 in the CSF found no differences in concentration during the ictal phase compared to controls. (40) Similarly, while a few studies reported elevated blood and salivary IL-1β levels in interictally assessed migraine patients, other studies found no significant differences in IL-1β levels between migraine patients and controls. (12)

The positive correlation between tear fluid TNF-α and BDNF in the migraine group is of particular interest, as preclinical data suggest that TNF-α upregulates BDNF expression in trigeminal ganglion neurons *via* activity-dependent mechanisms. (41) Furthermore, both BDNF and TNF-α were significantly higher in CM than in EM in our cohort. These findings suggest that cytokine-induced release of pain-modulating factors, such as BDNF, can contribute to neurogenic inflammation, trigeminal sensitization, and migraine chronification. (41) However, we observed a negative correlation between CGRP and BDNF levels in the migraine group, suggesting an inverse relationship between these two neuropeptides. Although CGRP and BDNF are thought to act

synergistically, CGRP is localized with BDNF in trigeminal ganglion neurons in rats and can enhance BDNF release *in vitro*, (42) our findings do not appear to confirm this regulatory pathway in humans *in vivo*. BDNF exerts complex and context-dependent functions within nociceptive pathways: while BDNF exerts antinociceptive effects in the central nervous system in animal models, (43) other studies indicate that BDNF contributes to allodynia and central sensitization, particularly in chronic neuropathic pain models.(16) In migraine, elevated BDNF and proinflammatory cytokine levels may be markers of disease chronification. Our findings of BDNF and TNF-α higher levels in patients with CM compared to those with EM, and the correlation between BDNF levels and the number of MHDs, align with this hypothesis.

Some studies have reported increased serum BDNF levels during migraine attacks, consistent with a central excitatory role, (17,44) while others have observed reduced BDNF levels in platelets of migraine patients compared to healthy controls. (45) Being BDNF released by corneal trigeminal nerve endings and also from the corneal epithelium and stroma, (46,47) it may represent a better biomarker when measured in the tear fluid. Resident glial cells, including Schwann cells, within the trigeminal system play a key role in migraine mechanism. (5,48) These cells express the CGRP receptors, and their activation triggers the release of pro-inflammatory cytokines, such as TNF-α and IL-1β, which in turn amplify trigeminal nociceptive transmission. (6) Moreover, CSD has been shown to activate microglia and astrocytes, triggering the expression of multiple pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, IFN-γ, and various adhesion molecules. (6)

Our study has several strengths. To our knowledge, it is the first to evaluate a comprehensive panel of biomarkers in tear fluid, including cytokines and BDNF, using a fully non-invasive collection method (the Schirmer test). It is also the first to correlate these biomarkers with tear CGRP levels, clinical variables, and migraine phenotypes. Furthermore, to minimize confounding from local or systemic inflammation, we applied strict exclusion criteria to our cohort, excluding patients with inflammatory or other painful comorbidities, as well as those with ocular pathologies, such as the common dry eye syndrome. Finally, we excluded patients with NSAID overuse to avoid additional confounders. Nevertheless, some limitations should be acknowledged. First, the sample size, although comparable to those reported in similar biomarker studies, is small. (17) The small sample size may have limited the statistical power, particularly for subgroup analyses within the migraine cohort (*i.e.*, comparisons between chronic and episodic migraine) and for correlation analyses (*e.g.*, between BDNF and CGRP levels). Notably, the finding of higher tear fluid levels in the episodic migraine group was somewhat unexpected and needs confirmation in larger studies. Moreover, migraine patients and HCs were not matched for sex, which may represent a potential confounding factor, even though a sex-adjusted comparison was performed between the groups of patients with migraine and healthy controls. Lastly, nine patients were on preventive treatments for migraine, which, although stable, may have altered the results of some biomarkers.

Conclusions

This is the first study to investigate cytokines and BDNF levels in the tear fluid and CGRP levels in patients with migraine, all collected with the Schirmer test strips. We observed correlations between TNF-α and other cytokines and BDNF, supporting the presence of inflammatory components in migraine pathophysiology. We also observed increased TNF-α and BDNF and reduced CGRP in patients with CM compared with those with EM. However, this latter finding should be confirmed in larger, longitudinal studies involving multiple biological fluids to better understand its significance in the context of migraine pathophysiology. Given its ease of use and patient-friendly nature, tear fluid collection with Schirmer strips is recommended for the highly needed larger, longitudinal studies.

Materials and Methods

Design and study population. This is a cross-sectional study conducted at the tertiary headache center of Fondazione Policlinico Universitario A. Gemelli IRCCS in Rome (Italy). We included consecutive outpatients with a diagnosis of migraine who met the International Classification of Headache Disorders-third edition (ICHD-3) criteria, and HCs who underwent tear fluid collection for analysis of cytokines (IL-1β, IL-10, TNF-α), BDNF, and CGRP. Healthy controls were volunteers recruited among staff members and faculty in the same timeframe. The study's inclusion criteria for migraine patients are: i) age ≥18 years; ii) diagnosis of migraine; iii) not taking any analgesics within 48 hours prior to the tears collection; and iv) signature of the informed consent.

Exclusion criteria include: i) patients with any ocular pathology (*e.g.*, glaucoma, dry eye syndrome, allergic conjunctivitis); ii) the use of contact lenses on the day of the examination; iii) affected by a clinically significant clinical condition evaluated by Investigators, including systemic inflammatory diseases; iv) other clinically significant painful conditions including fibromyalgia; v) treatment with steroids or other immune-modulator drugs or overuse for acute treatment of migraine of NSAIDs, combination drugs and use of opioids; and vi) ongoing treatment with an anti-CGRP drug (anti-CGRP mAbs or gepants).

Inclusion criteria for HCs were: i) age ≥18 years; ii) the absence of a previous or current history of primary or secondary headache disorders (confirmed through a comprehensive interview by a headache specialist); and iii) not taking any analgesics within 48 hours prior to the tears collection. The exclusion criteria were identical to those applied to the migraine patient group.

For patients with migraine, the following migraine-related variables were collected: socio-demographic data, general medical history, age at onset, duration and frequency of headaches (MHDs); average severity of the attacks in the month prior to the collection (Numerical Rating Scale [NRS]), presence of aura, acute and prior and/or concomitant preventive medication use, number of symptomatic drugs per month (analgesic monthly number [AMNs]). We assessed the severity and the headache-related disability through HIT-6 and MIDAS questionnaires. Psychiatric comorbidities were assessed through the HAM-A and HAM-D. Individuals scoring above the minimal thresholds (HAM-A >7; HAM-D >9) were classified as experiencing clinically relevant anxiety or depression, respectively. Finally, the ictal phase was defined as the presence of a headache with migraine features (according to ICHD-3) at the time of sample collection. The interictal phase was defined as a collection of samples in the absence of migraine attack, 72 hours before and 48 hours after the sampling.

Cytokines, BDNF and CGRP assay in tear fluid with the Schirmer test. Prior to collection, a systematic ophthalmological examination was performed by an ophthalmologist. Physical examination included the evaluation of any signs and symptoms of eye inflammation (eye pruritus, visual blurring), the assessment of subjective visual acuity, the tear film stability through the tear break-up time (TBUT), and the tear fluid production through the Schirmer test (in mm), considering TBUT <5 s and Schirmer ≤5 mm scores abnormal.

Tear fluid samples were collected through the Schirmer test blotting strips (35 mm Flo™ Tear Measurement strips). The filter papers were placed into the inferior eyelid and removed after 5 minutes. The strips were stored at −80°C until use. Absorbed proteins were allowed to solubilize from the strip through overnight incubation with 200 µL of 100 mM ammonium bicarbonate plus 20 µL of protease inhibitor cocktail. The supernatant was centrifuged for 20 min at 1000×g at 2-8°C and then transferred to a new tube for subsequent immunodetection. The ocular biomarker assay consists of a human CGRP sandwich ELISA kit (Novus Biologicals, Bio-Techne, Minneapolis, USA), following the manufacturer's instructions and in duplicate. Repeatability was characterized by a coefficient of variation (CV) <10%.

Detection limits and inter- and intra-assay variabilities for these assays are not available for tear fluid.

Multiple cytokines were measured using the automated ELISA platform Ella™ (Bio-Techne) for multiplex assays, including BDNF, IL-10, IL-1β, and TNF-α. Measurements were performed with the Simple Plex Cartridge Kit (four-analyte cartridge for use with human plasma/serum, containing BDNF/Free, IL-10, IL-1β/IL-1F2, and TNF-α 2nd).

Statistical analysis. An *a priori* sample size calculation was performed using G*Power software, based on previously published data on CGRP concentrations in tear fluid (18,20,21). Assuming a significance level of $p<0.05$ and a power of 0.80, the required total sample size was estimated to be 75 participants, with a 2:1 ratio of migraine patients to controls (50 patients with migraine and 25 HCs). Tear fluid levels of cytokines, BDNF, and CGRP from the left and right eyes were averaged for each participant.

Descriptive statistics were used to describe the demographic and clinical features of the sample. Numerical variables were reported as mean and SD. Categorical variables were presented as absolute numbers (n) and percentages (%). Normality of continuous variables was assessed using the Shapiro-Wilk test. Since BDNF, CGRP, and cytokine concentrations were not normally distributed, non-parametric tests were employed. Given that migraine and control groups were not matched for sex, comparisons between the two groups were performed using rank-based analysis of covariance (Rank ANCOVA), adjusting for sex as a covariate. Categorical variables were compared with the chi-squared (χ^2) test. Group comparisons within the intra-migraine group were performed using the Mann-Whitney U test. These comparisons included ictal *vs.* interictal phases, patients with *vs.* without aura, and CM *vs.* EM groups.

Correlations between the level of cytokines, BDNF, CGRP, and clinical variables were calculated using Spearman's rho correlation coefficients. The statistical significance was set at two-tailed $p<0.05$. Bonferroni's correction was applied to adjust for multiple comparisons. All data were analyzed using SPSS version 29.0 (IBM Corp., Armonk, NY, USA). Graphs were generated using R software (version 4.4.0; R Foundation for Statistical Computing, Vienna, Austria) and the ggplot2 package (version 3.5.1) with JASP (version 0.18.4; JASP Team, Amsterdam, The Netherlands).

References

1. Ashina M, Katsarava Z, Do TP, Buse DC, Pozo-Rosich P, Özge A, et al. Migraine: epidemiology and systems of care. Lancet 2021;397:1485-95.
2. Geppetti P, Capone JG, Trevisani M, Nicoletti P, Zagli G, Tola MR. CGRP and migraine: neurogenic inflammation revisited. J Headache Pain 2005;6:61-70.
3. Ashina M, Terwindt GM, Al-Karagholi MA-M, de Boer I, Lee MJ, Hay DL, et al. Migraine: disease characterisation, biomarkers, and precision medicine. Lancet 2021;397:1496-504.
4. Russell FA, King R, Smillie SJ, Kodji X, Brain SD. Calcitonin gene-related peptide: physiology and pathophysiology. Physiol Rev 2014;94:1099-142.
5. De Logu F, Nassini R, Hegron A, Landini L, Jensen DD, Latorre R, et al. Schwann cell endosome CGRP signals elicit periorbital mechanical allodynia in mice. Nature Communications 2022;13:646.
6. Biscetti L, Cresta E, Cupini LM, Calabresi P, Sarchielli P. The putative role of neuroinflammation in the complex pathophysiology of migraine: From bench to bedside. Neurobiol Dis 2023;180:106072.
7. Sarchielli P, Alberti A, Baldi A, Coppola F, Rossi C, Pierguidi L, et al. Proinflammatory cytokines, adhesion molecules, and lymphocyte integrin expression in the internal jugular blood of migraine patients without aura assessed ictally. Headache 2006;46:200-7.
8. Sarchielli P, Alberti A, Vaianella L, Pierguidi L, Floridi A, Mazzotta G, et al. Chemokine levels in the jugular venous blood of migraine without aura patients during attacks. Headache 2004;44:961-8.
9. Martelletti P, Stirparo G, Morrone S, Rinaldi C, Giacovazzo M. Inhibition of intercellular adhesion molecule-1 (ICAM-1), soluble ICAM-1 and interleukin-4 by nitric oxide expression in migraine patients. J Mol Med (Berl) 1997;75:448-53.
10. Acarsoy C, Ruiter R, Bos D, Ikram MK. No association between blood-based markers of immune system and migraine status: a population-based cohort study. BMC Neurology 2023; 23:445.
11. Munno I, Marinaro M, Bassi A, Cassiano MA, Causarano V, Centonze V. Immunological aspects in migraine: increase of IL-10 plasma levels during attack. Headache 2001;41:764-7.
12. Thuraiaiyah J, Erritzøe-Jervild M, Al-Khazali HM, Schytz HW, Younis S. The role of cytokines in migraine: A systematic review. Cephalalgia 2022;42:1565-88.
13. Alpuente A, Gallardo VJ, Asskour L, Caronna E, Torres-Ferrus M, Pozo-Rosich P. Salivary CGRP and Erenumab Treatment Response: Towards Precision Medicine in Migraine. Ann Neurol 2022;92:846-59.
14. Tesfay B, Karlsson WK, Moreno RD, Hay DL, Hougaard A. Is calcitonin gene-related peptide a reliable biochemical marker of migraine? Curr Opin Neurol 2022;35:343-52.
15. Ichikawa H, Yabuuchi T, Jin HW, Terayama R, Yamaai T, Deguchi T, et al. Brain-derived neurotrophic factor-immunoreactive primary sensory neurons in the rat trigeminal ganglion and trigeminal sensory nuclei. Brain Res 2006;1081:113-8.
16. Zhang X, Xu Y, Wang J, Zhou Q, Pu S, Jiang W, et al. The effect of intrathecal administration of glial activation inhibitors on dorsal horn BDNF overexpression and hind paw mechanical allodynia in spinal nerve ligated rats. J Neural Transm (Vienna) 2012;119:329-36.
17. Fischer M, Wille G, Klien S, Shanib H, Holle D, Gaul C, et al. Brain-derived neurotrophic factor in primary headaches. J Headache Pain 2012;13:469-75.
18. Romozzi M, Di Nardo L, Trigila V, Cuffaro G, Savino G, Iannone LF, et al. CGRP increase in tear fluid of migraine patients is reversed by anti-CGRP monoclonal antibodies. J Neurol Neurosurg Psychiatry 2025;96:914-6.
19. Alpuente A, Gallardo VJ, Asskour L, Caronna E, Torres-Ferrus M, Pozo-Rosich P. Salivary CGRP can monitor the different migraine phases: CGRP (in)dependent attacks. Cephalalgia 2022;42:186-96.
20. Kamm K, Straube A, Ruscheweyh R. Calcitonin gene-related peptide levels in tear fluid are elevated in migraine patients compared to healthy controls. Cephalalgia 2019;39:1535-43.
21. Raffaelli B, Storch E, Overeem LH, Terhart M, Fitzek MP, Lange KS, et al. Sex Hormones and Calcitonin Gene-Related Peptide in Women With Migraine: A Cross-sectional, Matched Cohort Study. Neurology 2023;100:e1825-35.
22. Barmada A, Shippy SA. Tear analysis as the next routine body fluid test. Eye (Lond) 2020;34:1731-3.
23. Ma L, Yang L, Wang X, Zhao L, Bai X, Qi X, et al. CGRP Released by Corneal Sensory Nerve Maintains Tear Secretion of the Lacrimal Gland. Invest Ophthalmol Vis Sci 2024;65:30.
24. Williams RM, Bauce L, Lea RW, Singh J, Sharkey KA. Secretion and serotonin release in the isolated rat lacrimal gland: the effects of substance P and calcitonin gene-related peptide. J Auton Nerv Syst 1996;61:37-42.
25. Czerwinski S, Mostafa S, Rowan VS, Azzarolo AM. Time course of cytokine upregulation in the lacrimal gland and presence of autoantibodies in a predisposed mouse model of Sjögren's Syndrome: the influence of sex hormones and genetic background. Exp Eye Res 2014;128:15-22.
26. Kumar NR, Praveen M, Narasimhan R, Khamar P, D'Souza S, Sinha-Roy A, et al. Tear biomarkers in dry eye disease: Progress in the last decade. Indian J Ophthalmol 2023;71:1190-202.
27. Tran MT, Ritchie MH, Lausch RN, Oakes JE. Calcitonin gene-related peptide induces IL-8 synthesis in human corneal epithelial cells. J Immunol 2000;164:4307-12.

28. Alpuente A, Gallardo VJ, Asskour L, Caronna E, Torres-Ferrus M, Pozo-Rosich P. Dynamic fluctuations of salivary CGRP levels during migraine attacks: association with clinical variables and phenotypic characterization. J Headache Pain 2024; 25:58.
29. Garelja ML, Rees TA, Hay DL. Calcitonin gene-related peptide and headache: Comparison of two commonly used assay kits highlights the perils of measuring neuropeptides with enzyme-linked immunosorbent assays. Headache 2025.
30. Kamm K. CGRP and Migraine: What Have We Learned From Measuring CGRP in Migraine Patients So Far? Front Neurol 2022;13:930383.
31. Martami F, Holton KF. Unmasking the relationship between CGRP and glutamate: from peripheral excitation to central sensitization in migraine. J Headache Pain 2025;26:101.
32. Cernuda-Morollón E, Larrosa D, Ramón C, Vega J, Martínez-Camblor P, Pascual J. Interictal increase of CGRP levels in peripheral blood as a biomarker for chronic migraine. Neurology 2013;81:1191-6.
33. Romozzi M, Burgalassi A, Vollono C, Albanese M, Vigani G, De Cesaris F, et al. Prospective evaluation of aura during anti-calcitonin gene-related peptide monoclonal antibody therapy after 52 weeks of treatment. Confinia Cephalalgica 2024;34.
34. Ashina M, Goadsby PJ, Dodick DW, Tepper SJ, Xue F, Zhang F, et al. Assessment of Erenumab Safety and Efficacy in Patients With Migraine With and Without Aura: A Secondary Analysis of Randomized Clinical Trials. JAMA Neurol 2022;79: 159-68.
35. Tozzi A, de Iure A, Di Filippo M, Costa C, Caproni S, Pisani A, et al. Critical role of calcitonin gene-related peptide receptors in cortical spreading depression. Proc Natl Acad Sci USA 2012; 109:18985-90.
36. Hansen JM, Hauge AW, Olesen J, Ashina M. Calcitonin gene-related peptide triggers migraine-like attacks in patients with migraine with aura. Cephalalgia 2010;30:1179-86.
37. Romozzi M, Calabresi P. Is there a role of calcitonin gene-related peptide in cortical spreading depression mechanisms?- Argument pro. J Headache Pain 2025;26:90.
38. Oliveira AB, Bachi ALL, Ribeiro RT, Mello MT, Tufik S, Peres MFP. Unbalanced plasma TNF-α and IL-12/IL-10 profile in women with migraine is associated with psychological and physiological outcomes. J Neuroimmunol 2017;313:138-44.
39. Rozen T, Swidan SZ. Elevation of CSF tumor necrosis factor alpha levels in new daily persistent headache and treatment refractory chronic migraine. Headache 2007;47:1050-5.
40. Bø SH, Davidsen EM, Gulbrandsen P, Dietrichs E, Bovim G, Stovner LJ, et al. Cerebrospinal fluid cytokine levels in migraine, tension-type headache and cervicogenic headache. Cephalalgia 2009;29:365-72.
41. Bałkowiec-Iskra E, Vermehren-Schmaedick A, Balkowiec A. Tumor necrosis factor-α increases brain-derived neurotrophic factor expression in trigeminal ganglion neurons in an activity-dependent manner. Neuroscience 2011;180: 322-33.
42. Buldyrev I, Tanner NM, Hsieh HY, Dodd EG, Nguyen LT, Balkowiec A. Calcitonin gene-related peptide enhances release of native brain-derived neurotrophic factor from trigeminal ganglion neurons. J Neurochem 2006;99:1338-50.
43. Cirulli F, Berry A, Alleva E. Intracerebroventricular administration of brain-derived neurotrophic factor in adult rats affects analgesia and spontaneous behaviour but not memory retention in a Morris Water Maze task. Neurosci Lett 2000;287:207-10.
44. Tanure MT, Gomez RS, Hurtado RC, Teixeira AL, Domingues RB. Increased serum levels of brain-derived neurotropic factor during migraine attacks: a pilot study. J Headache Pain 2010; 11:427-30.
45. Blandini F, Rinaldi L, Tassorelli C, Sances G, Motta M, Samuele A, et al. Peripheral levels of BDNF and NGF in primary headaches. Cephalalgia 2006;26:136-42.
46. Yang AY, Chow J, Liu J. Corneal Innervation and Sensation: The Eye and Beyond. Yale J Biol Med 2018;91:13-21.
47. Asiedu K, Markoulli M, Bonini S, Bron AJ, Dogru M, Kwai N, et al. Tear film and ocular surface neuropeptides: Characteristics, synthesis, signaling and implications for ocular surface and systemic diseases. Exp Eye Res 2022; 218:108973.
48. Lennerz JK, Rühle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, Messlinger K. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. J Comp Neurol 2008;507:1277-99.

Correspondence: Luigi Francesco Iannone, University of Modena and Reggio Emilia, Modena; Italy.
E-mail: luigifrancesco.iannone@unimore.it

Conflict of interest: CV has received research support, speaker honoraria, and support to attend national and international conferences from Lundbeck, Pfizer, and AbbVie, Jazz Pharmaceutical, and Angelini Pharma; LFI received financial support, honoraria for scientific lectures and presentations, consulting fees for the participation in advisory boards and support for attending meetings from: Teva, Eli Lilly, Lundbeck, Pfizer, Organon and AbbVie; he is Associate Editor for Frontiers in Neurology Headache and Neurogenic Pain section and Review Editor for Therapeutic Advances in Neurological Disorders; MR has received research support and support to attend national and international conferences from Angelini Pharma, Lundbeck, Jazz Pharmaceutical, and AbbVie; PC has received research support, speaker honoraria, and support to attend national and international conferences from AbbVie, Bayer Schering, Bial, Biogen-Dompè, Biogen-Idec, Eisai, Genzyme, Lundbeck, Lusofarmaco, Merck-Serono, Novartis, Prexton, Teva, UCB Pharma, and Zambon. The remaining authors have no relevant financial or non-financial interests to disclose regarding this paper.

Ethics approval and consent to participate: institutional review board approval was not required. All participants provided informed consent to participate in the study.

Availability of data and materials: data supporting the findings of the present study are reported in the article. The data collected and analyzed for the current study are available from the corresponding author on reasonable request.

Acknowledgments: we would like to acknowledge the support received by the Immunology Core Facility, Gemelli Science and Technology Park (GSTeP), Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome, Italy.

Received: 29 July 2025. Accepted: 23 October 2025.

Confinia Cephalalgica 2025; 2:15795 doi:10.4081/cc.2025.15795