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Biomarkers in migraine beyond diagnosis

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Biomarkers in migraine beyond diagnosis

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Editorial: Biomarkers in migraine beyond diagnosis

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Editorial on the Research Topic
Biomarkers in migraine beyond diagnosis

Migraine is a disabling type of primary headache that directly affects more than one billion people worldwide. Recent studies have provided important new insights into its genetic causes, anatomical and physiological features, and pharmacological mechanisms. In current clinical practice, migraine was diagnosed according to the International Classification of Headache Disorders (ICHD-3 criteria). Although the evolution of this classification system reflects an increasing understanding of the heterogeneity and variable clinical features of migraine, the diagnosis and treatment remain inadequate.

One of the main barriers to the precision diagnosis and treatment of migraine is the lack of reliable biomarkers. Biomarkers can have a wide range of clinical applications, including diagnosis, subtype classification, prognosis, and treatment effect assessment. The specific, individualized, and multi-perspective biomarkers of migraine can significantly promote the accurate diagnosis of migraine, and promote the exploration of pathophysiology and new treatment strategies for migraine.

To improve clinical decision-making for migraine, this Research Topic aimed to identify the potential biomarkers for migraine and to further investigate the association of biomarkers with diagnosis, stratification, prognosis, and therapy.

Nine articles had been finally included in this Research Topic, containing seven pieces of original research, one opinion, and one review.

Genetic, environmental, metabolic, and neuropeptides may all be involved in the pathogenesis of migraine. Some substances can be detected in serum and may thus serve as corresponding biomarkers. Four studies explored changes in serum concentrations of substances in migraine patients, respectively paying close attention to potential cation channel subfamily V member 1 (TRPV1), vasoacive intestinal peptide (VIP), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Togha et al.), urate (Hong et al.), immunoglobulin G Glycosylation (Xu et al.), and Calcitonin gene-related peptide (CGRP) (Frank et al.).

Besides serum studies, previous neuroimaging studies have explored structural and functional changes in the brain of migraine patients, but few studies have explored biological markers associated with drug efficacy and predicting refractory migraine attacks. Three articles are related to diagnostic methods using neuroimaging: predicting

sumatriptan treatment response in persons with migraine disease through neuroimaging (Wu et al.) and volume or diffusion abnormalities (Santoro et al.).

One study focused on the alteration of gut microbiota in migraine patients and investigated migraine combined with irritable bowel syndrome (Liu et al.).

Migraine has a certain genetic predisposition, and genes may play a role in its diagnosis. One opinion discussed the use of gene prioritization to score and rank suggestive candidate genes in migraine (Frederiksen).

Studies in recent years have proven that multi-functional neuropeptide CGRP plays a major role in the pathophysiology of migraine. The article (Kamm) on this topic reviewed the current understanding of CGRP in migraine pathophysiology and presented the possible applications of CGRP as a migraine biomarker.

In conclusion, published articles confirmed the complexity of migraine pathogenesis. Therefore, objective diagnostic biomarkers and personalized treatment strategies were needed. Furthermore, the clinical evaluation of patients should be comprehensive, based on large sample clinical studies and extensive evidence-based studies.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Evaluation of Serum Levels of Transient Receptor Potential Cation Channel Subfamily V Member 1, Vasoactive Intestinal Polypeptide, and Pituitary Adenylate Cyclase-Activating Polypeptide in Chronic and Episodic Migraine: The Possible Role in Migraine Transformation

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Objectives: This study aimed to investigate the role of serum levels of transient receptor potential cation channel subfamily V member 1 (TRPV1), vasoacive intestinal peptide (VIP), and pituitary adenylate cyclase-activating polypeptide (PACAP) in the development and also the transformation of migraine in patients suffering from migraine.

Methods: Eighty-nine participants with a mean age of 39 years were divided into 23 episodic migraine (EM), 36 chronic migraine (CM), and 30 healthy control groups. Demographic, anthropometric, and headache characteristic information, and also blood samples, was collected. Serum levels of TRPV1, VIP, and PACAP were measured using the enzyme-linked immunosorbent assay (ELISA) technique.

Results: Based on our findings, the serum level of TRPV1 was significantly higher in CM compared to the control group ($p < 0.05$), whereas serum levels of VIP ($p < 0.01$) and PACAP ($p < 0.05$) in the EM group were significantly more than the control group. There was no significant difference between EM and CM groups.

Conclusions: An elevation in the serum levels of TRVP1 among chronic migraineurs and increments in the levels of VIP and PACAP were observed among EM patients compared to healthy subjects. However, our data failed to demonstrate the probable role of these biomarkers in migraine progression, and more studies are needed to clarify the molecular mechanisms involved in migraine progression.

Keywords: TRPV1, VIP, PACAP, migraine, migraine transformation

INTRODUCTION

Migraine is a prevalent debilitating neurological disorder with moderate to severe headache which lasts 4 or 72 h. Headache is often unilateral with associating symptoms of photophobia, phonophobia, nausea, or vomiting (1). In one-third of patients, the aura (transient focal neurological symptoms) precedes the headache. Migraine is divided into two types of chronic (CM) and episodic (EM) based on the number of headaches occurring monthly. EM can change to CM, which is much more severe and characterized by headaches that exceed more than 14 days per month with at least 3 months of repetition (2, 3).

Migraine imposes a heavy socioeconomic burden on society and this is while its exact mechanism is not yet fully known (2, 4–9). Genetics, environmental factors, metabolic changes, and hormones, may all contribute to the onset of migraines. A number of well-studied mechanisms that are probably involved in migraine pathogenesis are as follows: trigeminovascular pain pathway, proinflammatory cytokines, and neuroinflammation, and also the activity of some factors such as nitric oxide and neuropeptides including calcitonin gene-related peptide (CGRP), substance P, neurokinin A, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating polypeptide (PACAP) (5–9).

Among these neuropeptides, VIP, a 28-amino acid peptide, is a vasodilator that releases from the cranial parasympathetic preganglionic and cerebral perivascular nerves. Although VIP plasma levels were shown to increase after migraine attacks and/or interictal period in both EM or CM, its infusion did not induce migraine attack. It has been proposed that VIP may play a role in triggering migraine chronification through vasodilation and nociceptor sensitization (10, 11). Besides, PACAP is a peptide that is mainly made up of 38 amino acids and is also found in the sensory ganglion, parasympathetic ganglion, and secondary neurons of the trigeminal nucleus. This neuropeptide seems to be similar to VIP structurally and could cause vasodilation. Moreover, PACAP could increase trigeminal nociceptor's excitability by increasing cAMP. The infusion of this neuropeptide was shown to stimulate headache in migraineurs. In this regard, targeting the inhibition of PACAP receptors has been investigated for migraine treatment (12–15). PACAP levels were observed to be elevated during migraine headaches and found to be decreased by sumatriptan, a medication used in the treatment of migraine (16, 17). Additionally, transient receptor potential vanilloid 1 (TRPV1), a Na^{+} and Ca^{2+} permeable channel, is among the other factors which are assumed to play a role in migraine pathogenesis. TRPV1 could be activated by capsaicin, severe heat, low pH, inflammatory factors, and some lipid-derived substances such as anandamide (7, 8, 18–20). This channel is found in trigeminal ganglions and its stimulation might cause the release of other neuropeptides such as CGRP. Therefore, the role of TRPV1 in the pathophysiology of migraine has attracted much attention in the recent years. It should be noted that the agonists and antagonists of this channel are under investigation for therapeutic aspects in migraine disease (7, 8, 19, 20).

Considering the unknown molecular mechanism involved in migraine pathogenesis and the possible role of various agents in the progression of EM to CM, this study aimed to investigate the plasma levels of some less-studied factors including VIP, PACAP, and TRPV1 in patients suffering from EM, CM, and healthy controls.

MATERIALS AND METHODS

Study Population

In this case-control study, the population was comprised of 89 subjects (71 women and 18 men) with an average age of 39 years who were divided into three groups including subjects with chronic migraine (CM group, $n = 36$ patients), individuals with episodic migraine (EM group, $n = 23$ patients), and healthy subjects (control group, $n = 30$ headache-free volunteers). Based on the convenience sampling method, the sampling process was performed from September 2017 to June 2020 at Sina University Hospital Headache Clinic, Tehran University of Medical Sciences, Tehran, Iran. Following an advertisement using posters describing the study aims placed all over the hospital (primarily in headache clinic), the patients with migraine and the age- and sex-matched non-headache controls, who were healthy subjects from the hospital staff or patient companions, were included in this study. Diagnosis of EM and CM was performed by a neurologist based on the third edition of International Headache Society criteria (ICHD-III) (21).

The inclusion criteria considered for enrolling in this study were as follows: age range between 18 and 65, having a body mass index (BMI) between 18.5 and 35 kg/m^2, not being pregnant or breastfeeding, and not having a positive medical history for any of the following disorders: cardiovascular, infectious, or endocrinological diseases, renal, hepatic, immunological, and allergic disorders, and also other chronic neurological diseases such as Alzheimer's disease, multiple sclerosis, epilepsy, or Parkinson's disease. Besides, having migraine headaches (with or without aura) for at least 6 months prior to the study and excluding the diagnosis of medication overuse headache (MOH) were the specific inclusion criteria for the case group. Subjects who did not meet the mentioned conditions or were unwilling to fill out a questionnaire were excluded. The study protocol was approved by National Institute for Medical Research Development (NIMAD) (grant number 957537) and confirmed by the ethical committee of NIMAD with ID: IR.NIMAD.REC.1396.054. After a complete explanation of the research process, all participants filled out the consent forms.

Demographic, Anthropometric, and Clinical Information of Patients

After the initial interview and demographic data collection, anthropometric measurements were performed based on the method provided by the World Health Organization. Height and weight were measured to calculate BMI that was obtained as weight (kg) divided by height squared (m^2). For the purpose of body weight measurement, Seca Clara 803 digital scale (accuracy of 0.01 gr; Seca GmbH & Co. KG., Hamburg, Germany) was used. Height was also measured using a Seca 216 wall-mount

stadiometer (accurate to 0.1 cm without shoes; Seca GmbH & Co. KG., Hamburg, Germany) in bare feet. The patients were also questioned about the number and type of abortive or analgesic medication use 30 days after the first visit.

Headache Diaries and Visual Analog Scale

In the next step, participants were visited by a neurologist or headache subspecialist (M.T.) and migraine and its type were determined based on ICHD-III criteria (2). Patients were also guided on how to fill out the headache diary form designed by senior researcher Prof. M.T. (22). These diaries were included information about the severity, duration (time elapsed from headache onset to cease of headache by itself or through abortive medications, whichever is sooner), frequency (i.e., number of headache days) and time of discontinuation of the migraine attacks, number and type of analgesics used, and the stimulating factors of headache such as menstruation and light during 30 days. Head pain severity scores were rated through the visual analog scale (VAS), a 10-cm measurement instrument; the left side (number 0) indicates the absence of pain and its right side (number 10) indicates the most severe pain.

Blood Sample Collection and Biochemical Assessments

A 10-ml blood sample was collected from each EM participant at the second visit, about 30 days after the first visit and at least 72 h after his/her last headache attack to be more indicative of the interictal phase of migraine. For CM cases, since the headaches lasted more than 15 days (between 15 and 30 days) per month, it was not possible to collect blood samples in the interictal phase. Blood samples were divided into 18 microtubes that were stored in −80°C freezers and 10 microtubes that were kept in −20°C freezers. All serum samples were sent to the laboratory of Sina Hospital for biochemical studies. Serum levels of target factors (TRPV1, PACAP, and VIP) were then measured using commercial enzyme-linked immunosorbent assay (ELISA) kits from Bioassay Technology Laboratory (Shanghai Korain Biotech Co., Ltd, Shanghai, China) and Crystal day Biotech Co. (Shanghai Crystal day Biotech Co., Ltd., Shanghai, China). Serum levels of these biomarkers were measured as per instructions of the manufacturers of the ELISA kits. All assays were carried out in triplicate. The intraassay and interassay coefficient of variation (CV) was <8 and <10%, respectively.

Statistical Analysis

SPSS software version 24 was used for data analysis. The normality of the data was evaluated using the Shapiro–Wilk test. All quantitative data were reported as mean [standard deviation (SD)] or median (interquartile range, IQR) and all qualitative data as percentage and frequency. Chi-squared test, independent-sample *t*-test, or Mann–Whitney *U*-test was applied for analyzing the categorical or continuous variables between the studied groups. Kruskal–Wallis and its related *post hoc t*-test were used for making comparisons between the groups. In all statistical tests, a *p*-value <0.05 was considered significant.

TABLE 1 | Comparison of gender, age, and BMI between the studied groups [data are shown as means (SD)].

Variable	Control ($n = 30$)	Chronic migraine ($n = 36$)	Episodic migraine ($n = 23$)	*p*-value
Percentage of women	73.3	75.0	95.6	0.087
Age (years)	41 (8)	39 (8)	38 (9)	0.509
BMI	24.88 (3.70)	26.65 (4.37)	25.24 (4.38)	0.203

TABLE 2 | Comparison of headache characteristics between chronic and EM groups [data are shown as means (SD)].

	Chronic migraine ($n = 36$)	Episodic migraine ($n = 23$)	*p*-value
Headache days per month	25.74 (5.03)	8.78 (3.26)	<0.001
Headache severity (VAS)	7.42 (2.41)	7.37 (1.82)	0.936
Attack duration (hours per month)	19.33 (12.30)	15.48 (15.65)	0.296
Using abortive medication (days per month)	14.42 (10.25)	6.00 (4.17)	<0.001

RESULTS

Basic Characteristics of the Studied Groups

Eighty-nine participants (71 women and 18 men) with a mean age of 39 years were divided into three groups including control ($n = 30$), CM ($n = 36$), and EM ($n = 23$). The mean (SD) of age and BMI of participants are presented in **Table 1**. There was no significant difference in age, gender, or BMI between the studied groups.

Headache Characteristics

The mean of headache characteristics including frequency, duration, and severity of headache and also the use of abortive drugs were compared between episodic and CM groups. As presented in **Table 2**, headache frequency in the CM group was significantly higher than EM group [25.74 (5.03) vs. 8.78 (3.26), p-value < 0.001]. However, there was no significant difference in the duration and severity of the headache between the two groups. Moreover, the mean of abortive drug use in the CM group was significantly higher compared to the EM group [14.42 (10.25) vs. 6.00 (4.17), p-value < 0.001].

Medication Use

The medication consumption of studied subjects at baseline and after the intervention consisted of abortive [including triptans, ergotamine derivative, and non-steroidal antiinflammatory drugs (NSAIDs)] and prophylactic drugs [including propranolol, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin–norepinephrine reuptake inhibitors (SNRIs)] were also compared between chronic and episodic migraineurs. Based on the results, there was a significant

TABLE 3 | Comparison of serum levels of TRPV1 (ng/ml), VIP (ng/l), and PACAP (ng/ml) according to the studied groups [data are shown as median (interquartile range)].

Variable / Groups Median (IQR)	Control ($n = 30$)	Chronic migraine ($n = 36$)	Episodic migraine ($n = 23$)	*p*-value
TRPV1 (ng/mL)	1.61 (1.83)[#]	2.91 (2.72)[#]	1.84 (1.93)	0.034
VIP (ng/L)	284.50 (90.40)[#]	286.44 (46.83)	303.24 (50.38)[#]	0.027
PACAP (ng/mL)	2.57 (.64)[#]	2.72 (1.06)	2.73 (0.46)[#]	0.043

[#]*show statistically significant differences between groups.*

increase in NSAID intake in the EM group [$n = 16$ (69.6%)] compared to the CM [$n = 14$ (38.9%)] group. No significant differences were observed between EM and CM groups on the use of other mentioned drugs.

Serum Concentration of TRPV1, VIP, and PACAP

The serum levels of TRPV1, VIP, and PACAP in the control, CM, and EM groups are presented in **Table 3** and **Figures 1A–C**. The median (IQR) value of TRPV1 was higher in the CM compared to the control group [2.91 (2.72) vs. 1.61 (1.83) ng/mL, p-value $= 0.034$], but no significant differences were observed in the comparison of the EM and the control groups. Also, a comparison of serum levels of TRPV1 between the EM and the CM showed an insignificant difference. On the other hand, there was a significant increase in the median (IQR) value of VIP in the EM group when compared to the control group [303.24 (50.38) vs. 284.50 (90.40) ng/L, p-value $= 0.027$]. However, no significant differences in the serum level of VIP were found in the CM group as compared to the EM or control subjects. In addition, it was demonstrated that the median (IQR) value of PACAP in the EM group was significantly greater than that of the control group [2.72 (1.06) vs. 2.57 (0.64) ng/mL, p-value $= 0.043$]. However, PACAP elevation in the EM group was not significant compared to the CM group. Furthermore, the increment in PACAP levels in the CM group was insignificant when compared to the control group.

DISCUSSION

In this study, to investigate some molecular alterations involved in migraine chronification, serum levels of TRPV1, VIP, and PACAP were evaluated in patients with episodic and CM, and also healthy individuals. Based on our findings, the elevation of serum levels of VIP and PACAP was observed in patients with EMs but TRPV1 levels were higher in the serum samples of patients suffering from CMs when compared to the healthy subjects. The current findings might suggest a possible role for these factors in migraine pathogenesis, though more research is required in this area.

Unfortunately, migraine headache in 14% of episodic migraineurs can change to chronic type, which are much more severe and prolonged (23–25). So far, researchers have identified some risk factors for EM progression to a chronic form such as age, gender, obesity, and stressful life events. However, finding molecular biomarkers of the migraine patients' serum can be innovative in preventing migraine chronification (26, 27).

During the recent decades, the activation of sensory neurons in trigeminal ganglion has received more attention in the pathophysiology of migraine. TRPV1, a non-selective cation channel, is abundantly expressed in the trigeminal ganglion and its activation might lead to release of several neuropeptides involved in central sensitization including CGRP, VIP, PACAP, and substance P. These molecules are peripherally secreted from trigeminal afferents and induced intracellular elevation of cAMP or cGMP with consequent vasodilation and inflammatory events within both the dura mater and trigeminal ganglion, which is important in triggering and amplification of pain (28, 29). TRPV1 is known to exacerbate the excitability of nociceptors in response to noxious stimuli such as mechanical and thermal stimuli and proalgesic substances and therefore promote hyperalgesia. Ictal and interictal hyperalgesia, assessed using the standardized quantitative sensory testing (QST) protocol, is observed in and around the trigeminocervical region in migraine patients (30–34). These regions' sensitization seems to play a crucial role in migraine chronification, as a higher frequency of cutaneous allodynia has been observed in CM patients. A wicked circle of TRPV1 high expression and subsequently related neuropeptides overrelease could account for this phenomenon (35). TRPV1 has been suggested to have an important role in dural vasodilation, which is one of the proposed basic mechanisms in migraine pathophysiology (36, 37). Proalgesic agents can upregulate TRPV1 expression and channel activity. Ethanol has shown that may be able to induce migraines through TRPV1 stimulation followed by CGRP elevation in the trigeminovascular system (38, 39). Nitroglycerin induces CMs by increasing the mRNA expression of TRPV1 in the trigeminal ganglion (40). Capsaicin has shown that causes headache by stimulating TRPV1 and activating the extracellular signal-regulated kinase (ERK) pathway (41). Experimental studies have shown that TRPV1 antagonists could decrease the sensitization of second-order trigeminal neurons or could prevent dural vasodilation (42, 43). Sumatriptan is a migraine abortive substance through vasoconstriction and inhibition of CGRP secretion from trigeminal ganglion (44) and may also act as TRPV1 desensitizer (45, 46).

Recent research has revealed that TRPV1 single-nucleotide polymorphism may be considered as a risk biomarker of episodic to CM transformation (47). CM patients were found to have a significant TRPV1 increase in nerve fibers (mainly in C fibers) in the scalp arteries wall compared with healthy controls (48). The results of this study also showed that serum level of TRPV1 was higher in patients with CM than healthy subjects, whereas this increase was not observed in the EM group. Based on these data, it seems that serum levels of TRPV1 may have a role in migraine progression but more evaluations are needed.

Vasoactive intestinal peptide is one of the most important neuropeptides secreted from parasympathetic perivascular nerve fibers in the trigeminovascular system and acts as a potent vasodilator (49). Parasympathetic activation could be able to

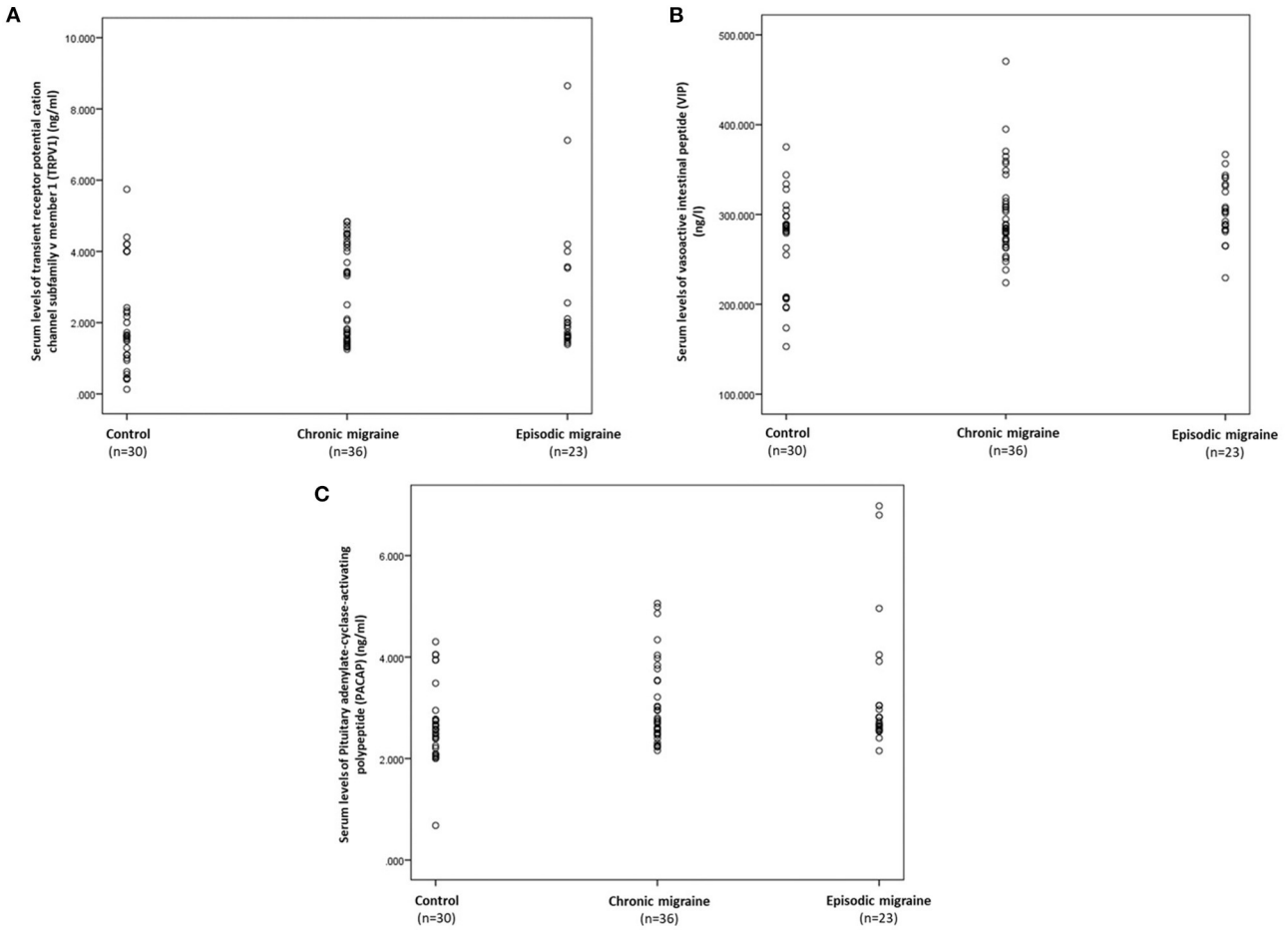

FIGURE 1 | Scatter plots displaying the individual's distribution of serum concentrations of TRPV1 **(A)**, VIP **(B)**, and PACAP **(C)** according to the control, chronic, and EM groups. TRPV1, transient receptor potential vanilloid type-1 receptor; VIP, vasoactive intestinal polypeptide; PACAP, pituitary adenylate cyclase-activating polypeptide.

sensitization of afferent nociceptors, this oversensitization and repeated stimulation might have a role in the transformation of EM to the chronic one, and VIP is assumed to have a role in migraine chronification (50). Studies that conducted on people with migraine indicated that serum VIP level was elevated in CM patients with increased cranial parasympathetic system activity during migraine attacks (51) and also in the interictal period in both episodic and CM (11, 52). Cernuda-Morollón et al. have shown that interictal CGRP and VIP increased in peripheral blood in CM patients compared to healthy controls (52). Their next study showed that interictal serum VIP level was higher in CM and EM compared to healthy controls without any meaningful difference between CM and EM patients (11). Partly consistent with these prior studies, our obtained results also showed elevated serum VIP level in EM patients between headache attacks compared to the control group, but this elevation not observed in the CM patients.

Vasoactive intestinal peptide and PACAP share two common G protein-coupled receptors, $VPAC_1$ and $VPAC_2$, with similar affinity. PACAP has an additional specific receptor, PAC_1, which has a higher affinity for PACAP than for VIP (53). In other words, although activation of all three receptors increases cAMP, PACAP *via* the PAC_1 can induce adenylate cyclase activation about 100-fold more than VIP (54). So, PACAP/PAC_1 signaling could notably elevate cAMP in peripheral trigeminal nociceptors, leading to nociception. Indeed, human and animal studies have shown that trigeminal neurons are sensitized through the elevation of cAMP (55). PACAP is a parasympathetic neuropeptide that is released from the efferent arm of the trigeminal-facial arch and has a VIP-like vasodilation property. PACAP has been proposed to have roles in mast cell degranulation, neurogenic inflammation, and migraine headaches whereas parasympathetic blocking reduces this pain (50, 56, 57). Cranial autonomic symptoms are prevalent in up to 50% of migraine patients. Likewise, these symptoms have been observed after PACAP administration (13, 58). Intravenous administration of PACAP could induce the release of CGRP in the trigeminal nucleus caudalis and lead to migraine attacks, and sumatriptan could be able to inhibit PACAP elevation (13, 16, 59). Electrical and chemical stimulation of the trigeminovascular system causes plasma PACAP elevation in rats, so it was assumed that PACAP could be considered as a biomarker in migraine

pathogenesis (60). Human concordant data have also been achieved in this field. As mentioned by past results, plasma levels of PACAP were higher in both cubital and jugular veins during migraine attacks but were lower in interictal periods compared to healthy subjects (16, 61). PAC_1 receptor blockade seems to have antimigraine effects, but more clinical trials are required to consider whether the long-term PACAP receptor blockade will have adverse side effects or not (14). Our findings showed that interictal serum PACAP levels were higher in EM patients than in the control group and PACAP increase in the CM was not enough to be significant. Our obtained results were opposite of the findings of Sara Pérez-Pereda and her colleague's research in 2020 as they showed that PACAP increases the risk of CM and not EM (10). In this regard, three points may be considered for variety of results: first, medications use, second, the time of collecting blood samples (during a migraine attack or in the interictal period), and third, parasympathetic system activity or inactivity. Mentioned factors can affect VIP and PACAP level, and also other possibly involved factors in migraine pathogenesis at the time of sampling. Therefore, considering the different results of previous studies, it seems that the role of these factors should be appraised while introducing TRPV1, VIP, and PACAP as risk biomarkers for migraine progression.

In this study, peripheral TRVP1, VIP, and PACAP were evaluated in EM patients in the interictal and in CM patients in the ictal phase. Due to the persistence of headaches more than 15 days per month (from 15 to 30 days) in CM patients, they do not have a true interictal phase of migraine and it was not possible to assess their serum biomarkers between attacks. A number of limitations can be mentioned for this study; first, as the peripheral levels of TRPV1, VIP, and PACAP were assessed merely in the interictal phase of migraine in EM patients, to achieve more comprehensive results, it is necessary to measure the CSF and serum levels of these biomarkers both between and during attacks in EM patients. Another limitation of this study was the lack of a prior sample size estimation. Moreover, applying a powered longitudinal study design, especially for exploring intraindividual longitudinal changes in these biomarkers and also the confounding factors (including medications use and comorbidities), could further clarify the associations between levels of TRPV1, VIP, and PACAP and migraine progression or reversion, which needs additional studies in the future.

CONCLUSION

In conclusion, compared to healthy controls, a significant elevation in the serum levels of TRVP1 was noted among chronic migraineurs. Besides, significant increments in the levels of VIP and PACAP were observed among EM patients. These findings might be a point to investigate new strategies for antimigraine drugs. However, our data failed to demonstrate the probable role of these biomarkers in migraine progression, and more studies are needed to clarify the molecular mechanisms involved in migraine progression.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Institute for Medical Research Development (NIMAD) (grant no. 957537) and confirmed by the ethical committee of NIMAD with ID: IR.NIMAD.REC.1396.054. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Content preparation, study design, acquisition, and analysis of data were all done by MT and ZG. SR, ZG, and MT drafted the manuscript. The manuscript was critically revised by MT, ZG, FK, and FZ. All authors contributed to the article and approved the submitted version.

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The Use of Neuroimaging for Predicting Sumatriptan Treatment Response in Patients With Migraine

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Objectives: To identify the neuroimaging predictors for the responsiveness of patients to sumatriptan and use an independent cohort for external validation.

Methods: Structuralized headache questionnaire and 3-Tesla brain magnetic resonance imaging were performed in migraine patients. Regional brain volumes were automatically calculated using FreeSurfer version 6.0, including bilateral amygdala, anterior cingulated cortex, caudate, putamen, precuneus, orbitofrontal cortex, superior frontal gyri, middle frontal gyri, hippocampus, and parahippocampus. A sumatriptan-responder was defined as headache relief within 2 h after the intake of sumatriptan in at least two out of three treated attacks. We constructed a prediction model for sumatriptan response using the regional brain volume and validated it with an independent cohort of migraine patients.

Results: A total of 105 migraine patients were recruited, including 73 sumatriptan responders (69.5%) and 32 (30.5%) non-responders. We divided the migraine patients into derivation ($n = 73$) and validation cohorts ($n = 32$). In the derivation cohort, left hippocampal volume was larger in sumatriptan responders (responders vs. non-responders: 3,929.5 ± 403.1 vs. 3,611.0 ± 389.9 mm^3, $p = 0.002$), and patients with a larger left hippocampal volume had a higher response rate to sumatriptan (>4,036.2 vs. ≤4,036.2 mm^3: 92.0 vs. 56.3%, $p = 0.001$). Based on the findings, we constructed a prediction model using the cutoff value of 4,036.2 mm^3, and we found that patients with a left hippocampal volume >4,032.6 mm^3 had a higher response rate to sumatriptan than those with a left hippocampal volume ≤4,032.6 mm^3 (84.6 vs. 42.1%, odds ratio [OR] = 7.6 [95% confidence interval = 1.3–44.0], $p = 0.013$) in the validation cohort.

Conclusion: Our study showed that left hippocampal volume is helpful to identify sumatriptan non-responders. This proof-of-concept study shows that left hippocampal volume could be used to predict the treatment response to sumatriptan in migraine patients.

Keywords: migraine, sumatriptan, hippocampus, prediction model, brain MRI

INTRODUCTION

Migraine is a common and disabling neurological disorder that affects 9–15% of the general population (1–3). Currently, migraine treatment can be classified into acute and preventive therapies, and acute treatments can be categorized as migraine-specific and non-specific (4, 5). Triptans, which are 5-$HT_{1B/1D}$ receptor agonists, are widely used migraine-specific medications to abort acute migraine attacks (6). Even though generic products have emerged, sumatriptan is still the most widely prescribed acute treatment medication for migraine (7, 8). Additionally, clinical trials and post-marketing experience have shown its efficacy and tolerability since the introduction of sumatriptan in the 1990s (9, 10). According to current evidence and real-world experiences, ∼30% of migraine patients are non-responders to triptans, and individual responsiveness to triptans is variable (11). To date, the variability in the treatment response is not fully understood (12), and only a few studies have identified the predictors for triptan response in migraine. Current evidence showed that a lower pretreatment pain severity and a higher polygenic risk score were associated with a better response to triptans (7, 8). An early study suggested that triptans' efficacy is less optimal after a patient develops allodynia, but new controlled studies have shown conflicting results (9, 10). Regarding the neuroimaging predictors, no study directly identified structural or functional neuroimaging predictors for sumatriptan response in migraine. On the other hand, the neuroimaging predictor for preventive therapies for migraine has been identified. In chronic migraine, the iron deposition in the periaqueductal gray matter could be used for outcome prediction for onabotulinumtoxinA injection (11). Also, another study found responders to onabotulinumtoxinA injection have cortical thickening in the right primary somatosensory cortex, anterior insula, left superior temporal gyrus, and pars opercularis than non-responders (12). Currently, neuroimaging can be used to differentiate migraine from other primary headache disorders and certain brain regions associated with headache frequency, severity, and long-term outcomes after preventive therapies (13, 14). Hence, this proof-of-concept study hypothesized that neuroimaging could help predict the treatment outcomes of sumatriptan in migraine patients.

METHODS

We retrospectively analyzed the medical records, headache questionnaires, and neuroimaging of patients with migraine who visited the headache clinics of Taipei Veterans General Hospital (TVGH) between January 1, 2015, and December 27, 2017. The included patients should be able to complete the headache questionnaire, and the patient's medical records should be done by board-certificated neurologist specialized in headache medicine.

Inclusion and Exclusion Criteria of Migraine Patients

The inclusion criteria were as follows: (1). The patient's headache fulfilled the International Classification of Headache Disorders (ICHD-3) criteria for migraine with or without aura, and the headache diagnosis was made by headache specialists; (2). Patients aged between 20 and 49 years; (3). Patients who completed the headache questionnaire; (4). Patients who had used sumatriptan to treat their migraine; (5). Patients who were able to report their treatment response to sumatriptan; and (6). Patients who were able to undergo magnetic resonance imaging (MRI) examinations without contraindications. The exclusion criteria were as follows: (1). Patients with underlying hypertension, diabetes mellitus, cerebrovascular diseases, epilepsy, or other neurodegenerative disorders; (2). Patients who had a history of traumatic brain injury or concussion; and (3). Patients who had been diagnosed with psychiatric disorders were excluded, including major depressive disorders, bipolar disorders, anxiety disorders, or schizophrenia.

Measures of Sumatriptan Response

All migraine patients participated in a semistructured interview at subsequent visits, which included questions about their response to sumatriptan, the timing of sumatriptan use, and usage of concomitant medications with sumatriptan. A sumatriptan responder was defined as patients with a decrease in headache intensity from moderate or severe to none or mild within 2 h after the intake of sumatriptan, in at least two out of three treated attacks (15–17). Patients with concomitant usage of acute medications other than sumatriptan were excluded from this study to ensure that the treatment responses came purely from sumatriptan.

Headache Frequency and Severity

In this study, we retrospectively analyzed items of the headache questionnaire, including headache frequency (headache days per month) and the Migraine Disability Assessment Score (MIDAS) questionnaire. The MIDAS questionnaire is widely used in clinical studies and controlled trials in headache medicine for analyzing migraine-related disability in a 3-months period (18, 19). The total score of the MIDAS questionnaire is the sum of five items, including the number of days of missed work/school, reduced productivity at work/school, missed household work, reduced productivity in household work, and missed family and/or social activities.

Brain Neuroimaging

All participants underwent whole-brain MRI using the same 3.0 T magnetic scanner (Discovery MR750 scanner, GE Healthcare, United States). Acquisition of T1-weighted images was based on 3D-FSPGR and AX-BRAVO sequences with the following parameters: repetition time = 9.384 ms, echo time = 4.036 ms, slice thickness = 1 mm, flip angle = 12°, and matrix size = 256 × 256 × 172 mm^2 using 3D-FSPGR protocol; repetition time = 9.184 ms, echo time = 3.68 ms, slice thickness = 1 mm, flip angle = 12°, and matrix size = 256 × 256 mm^2 using AX-BRAVO protocol. Both 3D-FSPGR and AX-BRAVO were gradient-echo imaging sequences from GE Healthcare suitable for brain volume calculation, and regional brain volumes calculated from automated segmentation of

T1-weighted structural images are reliable measures within the same scanner platform, even after upgrades (20).

Structural Data Processing

After imaging acquisition, preprocessing steps were conducted for better quality and creditability for subsequent analysis to measure the cortical morphological features. The first approach was to correct the head orientation to avoid any motion artifacts by making the AC-PC line congruent with the y-axis by using ART (the acpcdetect program in automatic registration toolbox, https://www.nitrc.org/projects/art). All the images were resized to 1 mm^3 isotropic voxel with a size of 256 × 256 × 256. Second, bias field correction was performed to remove the inhomogeneity of images by using N4 Bias Field Correction in Advanced Normalization Tools (ANTs). Finally, skull stripping was performed by using HD-BET, which applies artificial neural networks as processing algorithms. Automated brain volume measurements were subsequently conducted using FreeSurfer version 6.0, which is open-source software for processing and analyzing human brain MRI images. The cortical volumes (mm^3) of the region of interest (ROIs) associated with migraine and analgesic effects were calculated, including the bilateral amygdala, anterior cingulate cortex, caudate, putamen, precuneus, orbitofrontal cortex, superior frontal gyri, middle frontal gyri, hippocampus, and parahippocampus (13, 21, 22).

Statistics

Comparisons of demographics and clinical profiles between derivation and validation cohort were analyzed by using chi-square or *t*-tests as appropriate. Also, the differences in demographics and clinical profiles in responders and non-responders were analyzed by using chi-square or *t*-tests as appropriate. In the derivation cohort, Bonferroni's correction for multiple comparisons was applied in the comparison of the 20 ROIs between responders and non-responders (corrected for 20 pairwise comparisons: $p < 0.05/20 = 0.0025$). The significant variables were examined by using a classification and regression tree in order to obtain bivariate cutoff values for maximal sensitivity and specificity (23). A chi-square has been applied to compare response rates to sumatriptan between two sides of the cutoff value in the derivation and validation cohorts. The validation of the prediction model in both derivation and validation cohorts was considered exploratory; hence, we used $p < 0.05$ as the significance threshold. All statistical analyses were conducted with IBM SPSS (version 22.0).

Ethics

The study protocol was approved by the Institutional Review Board of Taipei Veterans General Hospital (2020-03-005AC).

RESULTS

Demographics

A total of 105 individuals with migraine (77 female and 28 male) were included in this study. Among them, 73 were sumatriptan responders (69.5%) and 32 (30.5%) were non-responders (**Figure 1**). The mean age of the study population was 33.2 (standard deviation [SD] = 8.3]) years. The prevalence of aura was 30.5%, and chronic migraine (CM) accounted for 25.7% of the participants. The derivation and validation sets were randomly divided into at a ratio of 7:3, and there was no difference in demographics between the derivation and validation cohorts, as shown in **Table 1**.

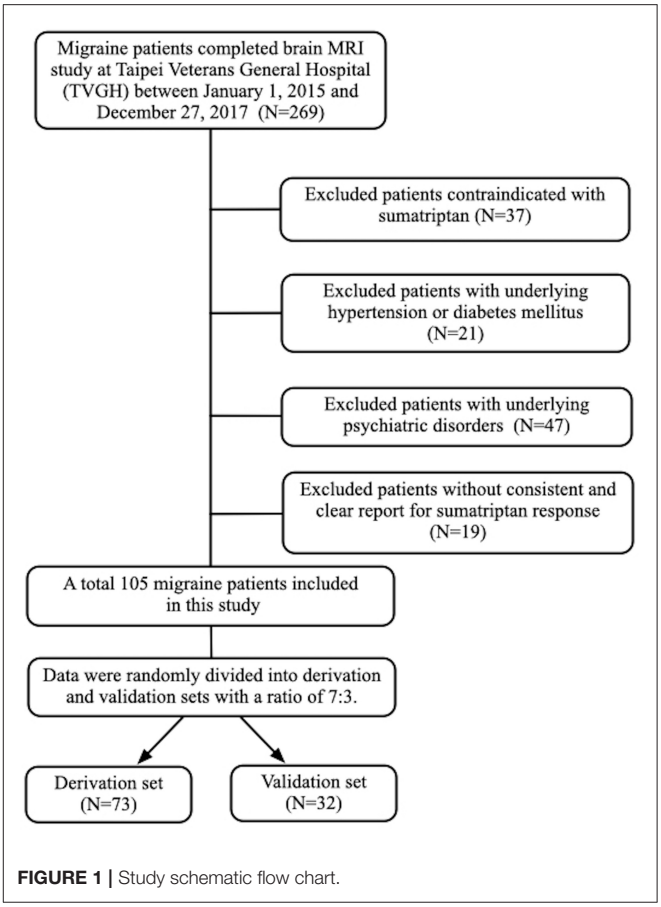


FIGURE 1 | Study schematic flow chart.

Potential Confounding Factors of Responders and Non-Responders

Regarding the demographic factors, there are no differences between responders and non-responder in age (mean [SD] years for responders vs. non-responders: 33.4 [9.7] vs. 33.5 [8.7], $p = 0.960$) or sex (responders: 12 males and 38 females; non-responders: 4 males and 19 females, $p = 0.529$). Also, there were no differences in the clinical profiles between responders and non-responders, including prevalence of aura (responders vs. non-responders: 30.4 vs. 32.0%, $p = 0.894$), chronic migraine (CM) (responders vs. non-responders: 26.1 vs. 30.0%, $p = 0.732$), MIDAS (responders vs. non-responders: 29.4 [32.3] vs. 27.8 [26.6], $p = 0.839$), or headache frequencies (mean [SD] headache days per month for responders vs. non-responders: 7.7 [7.1] vs. 8.4 [7.9], $p = 0.678$) (**Table 2**).

TABLE 1 | Demographics and descriptive statistics of potential confounding factors between the derivation and validation groups.

Variables	Derivation group (*N* = 73)	Validation group (*N* = 32)	*p value**
Age, mean (SD), years	33.4 (9.3)	32.6 (7.8)	0.669
Sex, No. (%)			
Men	16 (21.9%)	12 (37.5%)	0.098
Women	57 (78.1%)	20 (62.5%)	
Prevalence of migraine with aura	23 (31.5%)	9 (28.1%)	0.732
Prevalence of chronic migraine	21 (28.8%)	6 (18.8%)	0.284
Headache frequency	7.9 (7.3)	8.4 (5.3)	0.736
MIDAS	28.9 (30.4)	20.1 (17.0)	0.130
Sumatriptan responder	68.5%	59.4%	0.370
Total intracranial volume	1,537,205.7 (149,894.7)	1,529,646.7 (158,269.3)	0.816
Sequence of brain MRI			
3D-FSPGR	8 (11.0%)	3 (9.4%)	0.806
AX-BRAVO	65 (89.0%)	29 (90.6%)	

**Results were considered significant by $p < 0.05$.*

TABLE 2 | Demographics and clinical profiles of responders and non-responders in the derivation and validation group.

Variables	Derivation group (*N* = 73)			Validation group (*N* = 32)		
	Responders (*N* = 50)	Non-responders (*N* = 23)	*p value**	Responders (*N* = 19)	Non-responders (*N* = 13)	*p value**
Age, mean (SD), years	33.4 (9.7)	33.5 (8.7)	0.960	32.2 (8.4)	33.2 (7.1)	0.719
Sex, No. (%)						
Men	12 (24.0%)	4 (17.4%)	0.529	9 (47.4%)	3 (23.1%)	0.170**
Women	38 (76.0%)	19 (82.6%)		10 (52.6%)	10 (76.9%)	
Prevalence of migraine with aura	16 (32.0%)	7 (30.4%)	0.894	6 (31.6%)	3 (23.1%)	0.605**
Prevalence of chronic migraine	15 (30.0%)	6 (26.1%)	0.732	4 (21.1%)	3 (23.1%)	0.893**
Headache frequency	7.7 (7.1)	8.4 (7.9)	0.678	6.9 (4.0)	9.2 (6.2)	0.166
MIDAS	29.4 (32.3)	27.8 (26.6)	0.839	17.8 (12.2)	23.4 (13.7)	0.23
Total intracranial volume, mm^3	1,554,551.5 (158,291.6)	1,499,497.4 (124,722.1)	0.115	1,567,413.4 (179,437.5)	1,474,449.3 (104,185.8)	0.104
Sequence of brain MRI						
3D-FSPGR	5 (10.0%)	3 (13.0%)	0.703	2 (10.5%)	1 (7.7%)	0.790
AX-BRAVO	45 (90.0%)	20 (87.0%)		17 (89.5)	12 (92.3%)	

**Results were considered significant by $p < 0.05$.*
***p-value calculated by linear-by-linear association.*

Regional Brain Volume and Sumatriptan Response (in the Derivation Cohort)

Among the 20 ROIs, the left hippocampal volume was larger in the sumatriptan responders (responders vs. non-responders: 3,929.5 [403.1] vs. 3,611.0 [389.9] mm^3, $p = 0.002$) (**Table 3**). Using the classification and regression trees (CRT), we obtained a cutoff value of 4,036.2 mm^3. By using the chi-square test, we found patients with a larger left hippocampal volume (> 4,036.2 vs. ≤4,036.2 mm^3) had a higher response rate to sumatriptan (92.0 vs. 56.3%, $p = 0.001$) in the derivation cohort ($n = 73$). We further explored the possible confounding effects on hippocampal values, and we found that hippocampal volume on both sides did not correlate with headache frequency (Left: Pearson's $r = 0.069$, $p = 0.561$; Right: Pearson's $r = 0.107$, $p = 0.368$) or MIDAS (Left: Pearson's $r = 0.052$, $p = 0.664$; Right: Pearson's $r = 0.189$, $p = 0.110$).

Predicting Sumatriptan Response by Regional Brain Volume

Based on the results from the derivation cohort ($n = 73$), we used a cutoff value of 4,032.6 mm^3 to construct a prediction model by using a classification and regression tree (**Figure 2**). The validation cohort ($n = 32$), which had no differences in demographics or clinical profiles between the derivation and validation, has been used to examine the prediction model (**Table 1**). In the validation cohort (**Figure 2**), patients with a left hippocampal volume >4,032.6 mm^3 had a higher responder rate than those with a left hippocampal volume ≤ 4,032.6 mm^3

TABLE 3 | GMV of ROIs (mm^3) of responders and non-responders in the derivation group.

Variables	Derivation group (*N* = 73)			Validation group (*N* = 32)		
	Responders (*N* = 50)	Non-responders (*N* = 23)	*p value**	Responders (*N* = 19)	Non-responders (*N* = 13)	*p value**
Left side						
Amygdala	1,512.8 (240.0)	1,470.8 (217.4)	0.477	1,541.7 (204.0)	1,468.5 (177.8)	0.303
Anterior cingulated cortex	4,534.4 (652.5)	4,673.7 (645.3)	0.398	4,627.2 (798.7)	4,536.8 (328.8)	0.703
Caudate	3,373.6 (822.2)	3,164.9 (773.3)	0.308	3,312.0 (964.1)	3,012.9 (508.5)	0.264
Putamen	4,980.6 (675.5)	4,786.3 (446.9)	0.213	5,649.2 (818.3)	3,167.8 (374.4)	0.390
Precuneus	6,158.4 (894.6)	5,906.1 (585.7)	0.221	6,412.9 (1,108.4)	5,174.9 (1,112.0)	0.383
Orbitofrontal cortex	1,114.2 (214.1)	1,038.5 (157.8)	0.124	1,164.1 (221.2)	1,084 (166.9)	0.283
Superior frontal gyri	18,456.4 (2,417.7)	18,337.7 (1,787.0)	0.834	18,835.8 (2,574.1)	18,576 (1,666.4)	0.751
Middle frontal gyri	11,064.5 (1,547.8)	11,056.4 (1,271.4)	0.583	11,270.3 (1,882.9)	16,876.2 (1,749.2)	0.644
Hippocampus	3,929.5 (403.1)	3,611.0 (390.0)	0.002**	4,134.4 (401.4)	3,946.3 (370.3)	0.190
Parahippocampus	3,527.4 (555.7)	3,289.1 (473.8)	0.079	3,584.7 (555.8)	3,411.5 (313.9)	0.271
Right side						
Amygdala	1,692.9 (264.2)	1,674.0 (243.7)	0.772	1,758.9 (142.5)	1,724.1 (192.6)	0.561
Anterior cingulated cortex	5,842.0 (833.8)	5,826.5 (744.0)	0.959	5,980.2 (761.8)	5,999.5 (725.0)	0.943
Caudate	3,466.4 (762.0)	3,237.2 (737.2)	0.232	3,451.6 (838.0)	3,167.8 (374.4)	0.205
Putamen	5,095.9 (660.1)	4,914.9 (482.7)	0.242	5,360.8 (685.4)	5,174.9 (1,112.0)	0.562
Precuneus	5,722.0 (831.1)	5,462.5 (600.0)	0.184	6,067.2 (966.7)	5,810.8 (885.6)	0.452
Orbitofrontal cortex	976.1 (225.6)	1,030.2 (1,787.0)	0.344	1,055.8 (250.0)	1,056.5 (289.8)	0.994
Superior frontal gyri	16,990.3 (2,243.9)	16,563.1 (1,695.2)	0.426	17,558.6 (2,273.9)	18,576.1 (1,712.5)	0.369
Middle frontal gyri	9,630.2 (1,595.3)	9,483.2 (1,321.4)	0.701	9,663.0 (1,531.8)	9,820.5 (692.3)	0.697
Hippocampus	4,036.1 (434.4)	3,820.2 (430.5)	0.052	4,136.9 (396.7)	4,073.7 (356.1)	0.648
Parahippocampus	3,469.0 (564.2)	3,510.4 (672.9)	0.785	3,298.9 (561.3)	3,375.3 (366.9)	0.670

*p-value calculated by t-test. **The results were considered significant by $p < 0.05$.

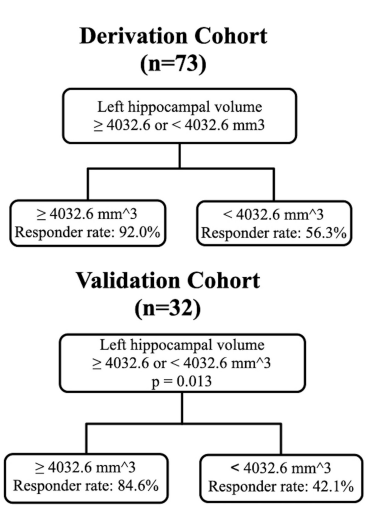
(>4,032.6 vs. ≤4,032.6 mm^3: 84.6 vs. 42.1%, odds ratio [OR] = 7.6 [95% confidence interval = 1.3–44.0], $p = 0.013$), with a high specificity and lower optimal sensitivity (specificity = 84.6%, sensitivity = 57.9%, accuracy = 68.8%).

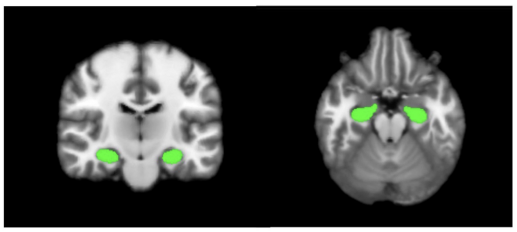
DISCUSSION

This study found that sumatriptan responders have a larger left hippocampal volume than non-responders. When applying the prediction model to the independent validation cohort, patients with a left hippocampal volume >4,032.6 mm^3 had a higher responder rate than those with a left hippocampal volume ≤ 4,032.6 mm^3 (OR = 7.6). The prediction model has a high specificity (84.6%) but a lower optimal sensitivity (57.9%). Instead of identifying good responders, the left hippocampal volume seems to be more suitable for identifying the "poor responders" to sumatriptan.

There are some studies that have aimed to identify predictors for the treatment response to triptans in migraine patients. One early study in 2004 found that pretreatment pain severity is a reliable predictor for the response to sumatriptan (7). Another recent study used genome-wide association studies and found a higher polygenic risk score for migraine associated with the sumatriptan response, which implies that a higher genetic burden of migraine is associated with a better response to migraine-specific treatment (8). To our knowledge, the present study identified left hippocampal volume as a new predictor for the response to triptans in migraine. However, the exact underlying mechanisms are unknown. One possible explanation is the direct effect of sumatriptan on the hippocampus. Although small amounts of triptan may cross the blood–brain barrier (BBB), sumatriptan has lower lipophilicity than other newer triptans (24). The relatively low brain penetration of sumatriptan is less likely to produce direct effects on the hippocampus (25). Additionally, a human postmortem brain study found that the distribution of sumatriptan-binding sites ($5HT_{1D}$ receptor) is higher in the visual cortex, globus pallidus, and frontal cortex than in the hippocampus (26). Therefore, the association between the hippocampus and sumatriptan response seems unlikely to be attributed to the direct effect on the hippocampus.

The second explanation for our study findings is the "maladaptive theory." This hypothesis is supported by studies that found that patients with smaller hippocampal volumes may be more vulnerable or have maladaptation to stressful events (27). One brain perfusion study found the activation of the amygdala, brainstem, and hippocampus was associated with the analgesic effect of ibuprofen in tooth extraction, and these regions belong to the descending modulatory pathway (22). Another prospective study combined structural and functional MRI to analyze





Confusion matrix of validation cohort

	Actual Responder	Actual Non-responder
Predicted Responder	True positive=11	False positive=2
Predicted Non-responder	False negative=8	True negative=11

FIGURE 2 | The prediction model in the derivation cohort and validation cohort with the visualization of hippocampus.

patients with subacute back pain, which found that patients resistant to treatment have smaller amygdala and hippocampal volumes than those responsive to treatment (28). In menstrual pain, one study found that patients with a hippocampal volume associated with BDNF Val66Met polymorphisms and a smaller hippocampal volume had higher severity of menstrual pain (29). Additionally, studies found that patients with chronic pain conditions, (i.e., fibromyalgia, complex regional pain, and chronic low back pain) had smaller hippocampal volumes (30, 31). Regarding migraine, one study from our group found that a smaller hippocampal volume was associated with poor outcomes, indicating that the "maladaptive theory" could be applied to migraine patients (14). These findings suggest that there are reciprocal interactions between the hippocampus and pain; that is, individuals with an underlying smaller hippocampus may be more maladaptive to headaches or other pain conditions, less responsive to analgesics, and more vulnerable to developing chronic pain disorders. In this study, the "non-responders to sumatriptan" might be considered a maladaptive response to pain from a more vulnerable brain (32). The third explanation for the association between sumatriptan response and left hippocampal volume is the pain memory bias. One study could support this explanation, which found exaggerated remembered pain is not uncommon in patients with chronic low back pain. This phenomenon could be attributed to the shape displacement of the left posterior hippocampus (33). However, whether the biased pain memory could be analogized to the memory of analgesic response warrants further research and is beyond the scope of the present study.

The current study has limitations. First, the smaller hippocampal volume may be due to the aging process. Also, our study protocols did not include tests for cognitive function. Hence, the responsiveness to sumatriptan may have memory or recall bias. Nevertheless, the mean age of the present study population was ∼30 years, which is an unlikely population to have cognitive deficits. Second, not responsive to one triptan, (i.e., sumatriptan) is not able to predict the response to other triptans (34), and further study is warranted to analyze the neuroimaging predictors of more than one acute medication for migraine. Third, our study excluded patients more than 50-year-old. Hence, our research findings could not represent the pediatric or elder population. The reason for selecting patients between 20 to 49-year-old is to avoid the measurement of brain volume being confounded by the aging process. Also, migraine prevalence peaks from the age 20s to 50s. The prediction model derived from this age range could represent most migraine patients in clinical settings (35). Fourth, our study design did not adjust for confounding factors, such as age, gender, intracranial volume, or ethnicity. The reason for not adjusting these factors is that our proof-of-concept study aimed to construct a prediction model easily applicable to the general population. Also, a recently-published review article addressed that there is no consensus for which and how many covariates should be adjusted for structural imaging studies and stated that "The current results highlight that the use of covariates has statistical and interpretative ramifications (36)." Fifth, the number of responders and non-responders is different in the derivation cohorts, and the imbalanced training dataset may cause overrepresentation of the majority class. On the other hand, our derivation and validation groups were based on data of consecutive patients, and the proportion of sumatriptan responders is usually higher than non-responders in the migraine population. The consecutive patients could prevent the possible confounding effect from the patient selection process.

CONCLUSION

This study found left hippocampal volume associated with the response to sumatriptan in migraine patients, and non-responders tend to have smaller left hippocampal volume.

According to the prediction model, patients with left hippocampal volume >4,032.6 mm^3 had a two-fold higher response rate than those ≤4,032.6 mm^3 in an independent validation cohort.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Taipei Veterans General Hospital (2020-03-005AC). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

J-WW, Y-TW, and S-JW had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. J-WW, P-YL, Y-TW, and S-JW: concept and design. J-WW, P-YL, S-TC, Y-LC, Y-TW, and J-FL: interpretation and analysis of neuroimaging. J-WW, Y-FW, K-LL, W-TC, and S-JW: treatment of all patients. J-WW and S-JW: drafting of the manuscript. J-WW: statistical analysis. J-WW, S-TC, Y-TW, and S-JW: obtained funding. Y-TW, J-FL, and S-JW: administrative, technical, or material support. Y-TW and S-JW: supervision. All authors critical revision of the manuscript for important intellectual content and acquisition, analysis, or interpretation of data. All authors contributed to the article and approved the submitted version.

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An Exponential Curve Relationship Between Serum Urate and Migraine: A Cross-Section Study From NHANES

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Background: Migraine is a common neurological disease and an important cause of disability worldwide. Serum urate is the end product of purine metabolism in Homo sapiens and other hominoids. Previous studies about the serum urate level in migraine were contradictory. Hence, we present a cross-section study to clarify the association between serum urate and migraine and explore the dose effect of serum urate on migraine.

Materials and Methods: The data for this cross-section study were acquired from the National Health and Nutrition Examination Survey (NHANES). A diagnosis of migraine was made through patient the self-reported and prescription medication. For data analysis, the weighted linear regression model, weighted chi-square test, logistic regression models, smooth curve fittings, and the two-piecewise linear regression model were utilized for data analysis. All data analysis was conducted on Empower software.

Results: Totally, 18,637 participants were enrolled in this study, of which 208 were migraineurs. The rest were set as control. There existed a statistically significant difference in mean age ($p = 0.0389$), gender ($p < 0.0001$), race ($p < 0.0001$), data release cycle ($p = 0.048$), drug usage, blood albumin ($p < 0.0001$), blood total protein ($p < 0.0001$), hemoglobin ($p < 0.0001$), serum iron ($p < 0.0001$), and serum urate ($p < 0.0001$) between the two groups. According to logistic regression models, there existed no consistent linear relationship between serum urate and migraine before (model 1: odd ratio (OR) = 0.83, $p = 0.0004$) or after adjusting for confounders (model 2: $OR = 0.96$, $p = 0.5198$; model 3: $OR = 0.84$, $p = 0.0184$). However, smooth curve fittings found an exponential curve relationship between serum urate and migraine. Furthermore, when serum urate was more than 7.8 mg/dl, higher serum urate was correlated with higher migraine occurrence (model 1: $OR = 1.54$, $p = 0.0022$; model 2: $OR = 1.51$, $p = 0.0050$; model 3: $OR = 1.77$, $p = 0.0348$). Besides, 8 out of the 208 migraineurs had a serum urate higher than 7.8 mg/dl.

Conclusions: In conclusion, there existed an exponential curve relationship between serum urate and migraine, with an infliction point of 7.8 mg/dl. When serum urate was more than 7.8 mg/dl, increased serum urate was correlated with higher migraine occurrence.

Keywords: migraine, headache, serum urate, NHANES, cross-section study

INTRODUCTION

Migraine is a common neurological disease and an important cause of disability worldwide, whose years of life lived with disability is 45.1 million and disability-adjusted life-years is 1.9% (1, 2). It is characterized by a recurrent, unilateral, moderate or severe, pulsating headache. The headache attack may last 4–72 h and be associated with nausea and/or phonophobia/photophobia (2).

Currently, migraine is considered an energy deficit syndrome partially due to mitochondrial dysfunctions (3). Besides, it is a complex neuroinflammatory disorder involving predominant activation of the trigeminovascular system with unclear molecular mechanisms (3, 4). These metabolic factors include behavioral factors, environmental factors, dietary triggers, hormonal changes, and genetic changes (3, 5). Oxidative stress is the imbalance between oxidation and antioxidation, which might be influenced by metabolic factors. Some migraineurs showed lower activity of catalase, non-oxidized thiol concentration, and total antioxidant capacity in serum (6, 7). Meanwhile, migraineurs showed the decreased activity of superoxide dismutase in their platelets and erythrocytes (7, 8).

Serum urate is the end product of exogenous and endogenous purine metabolism in Homo sapiens and other hominoids, which acts as an antioxidant *in vivo* and is associated with oxidative stress (9). It had been found that serum urate could lead to an increase in oxidative stress levels in a manner independent of xanthine oxidoreductase activity, especially in female (9). The gender-specific relationship of serum urate with oxidative stress might be due to the difference of serum estrogen level (10). Furthermore, serum urate showed lower levels and was posited as a neuroprotective agent in some neurological disorder (9, 11–13). On the contrary, some studies found that serum urate might act as a pro-oxidant, which might promote the oxidation stress (13). In addition, the contradiction of anti-oxidant effects and pro-oxidant effects might be due to the dose effect of serum urate (13). A previous study showed that a lower serum urate level was found in migraine, which was not influenced by the subtypes of migraine (14). However, another study found that serum urate in migraine might be more than control group, which had not shown the results of statistical hypothesis testing (15). Moreover, the reports had never controlled the confounders, which were mainly iron and ferritin, acted as pro-oxidants or anti-oxidants (16). Here, we take the advantage of data from the National Health and Nutrition Examination Survey (NHANES) to clarify the association between serum urate and migraine and explore the dose effect of serum urate in migraine.

MATERIALS AND METHODS

Study Population

Data analyzed in this study were acquired from NHANES (1999–2018), which was designed to assess the nutritional status and health of children and adults in the United States. The program of NHANES was reviewed and approved by the Prevention National Center for Health Statistics Research (NCHS) and the Centers for Disease Control (CDC) Research Ethics Review Board (17), and all participants signed written informed consent (17).

The data release cycles with diseases which were encoded with the International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM) were enrolled. The participants enrolled without data on serum uric acid (sUA), serum iron, and hemoglobin were excluded.

Variables

Migraine Definition

The identification of migraine cases was based on the self-reported questionnaire (18–20). The procedures were as followed. First, the survey participants were asked about the usage of prescription medications according to the prescription medication questionnaire, which was summarized in the supplementary material. Then, the interviewers used Lexicon Plus® to organize the prescription medicine and used ICD-10-CM to encode the diseases, which were the reasons for the usage of prescription medicine. Finally, we identified the migraine cases on the open information of prescription medication of NHANES, whose ICD-10-CM encode was G43 or G43.P.

Exposure Variable

Serum urate was the exposure variable of this cross-section study. Besides, the serum urate was measured by the Beckman Synchron LX20. The covariates included in the study were as follows: age, gender, race, data release cycle, drug usage, blood albumin, blood globulin, blood total protein, hemoglobin, blood creatinine, blood urea nitrogen, ferritin, serum iron, urine albumin, urine creatinine, and urine albumin/creatinine ratio.

Statistical Methods

Empower software (www.empowerstats.com; X&Y solutions, Inc., Boston MA) was utilized for data analysis. The NHANES sample weights had been applied to all estimates of the study. Continuous variables were expressed as mean ± standard deviation (SD), and the difference test between groups was

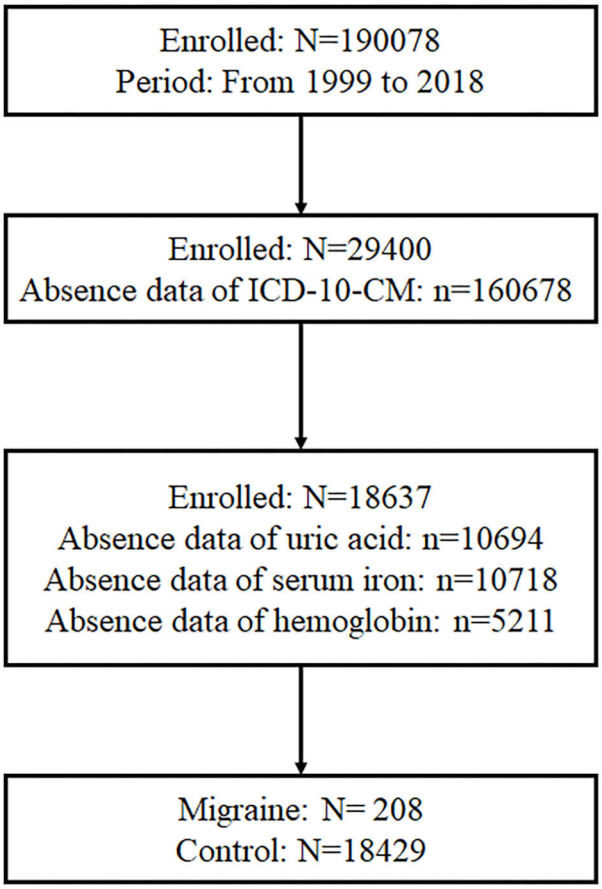

FIGURE 1 | Participants screening procedure. In total, 190,078 participants participated in the NHANES from 1999 to 2018, and 29,400 participants had information about ICD-10-CM. Of the 29,400 participants, 18,637 participants had data of serum urate, serum iron, and hemoglobin. Besides, 208 participants suffered from migraine and had prescription medications for migraine.

calculated by a weighted linear regression model. Meanwhile, categorical variables were expressed as percentage, and the difference test of groups was calculated by weighted chi-square test. Logistic regression models were applied to estimate the independent correlation between migraine and serum urate before or after adjustment of confounders. Moreover, when a non-linear relationship between serum urate and the risk of migraine existed, smooth curve fittings were used to examine whether the independent variables were partitioned into intervals (21). When partitioned intervals existed, the inflection point was calculated according to the two-piecewise linear regression model (21, 22). A log-likelihood ratio test, which compared a standard linear regression model to a two-piecewise linear regression model, was used to examine whether a threshold existed (22). In addition, the value of p not more than 0.05 was set as a significant level.

RESULTS

Description of Study Participants

As displayed in **Figure 1**, there were 190,078 participants who participated in the NHANES from 1999 to 2018, and 29,400 participants had information of ICD-10-CM. Of the 29,400 participants, 18,637 participants had data on serum urate, serum iron, and hemoglobin. Meanwhile, 208 participants suffered from migraine and had prescription medications for migraine, and the rest of participants without migraine were set as control.

Baseline Characteristic of Study Participants

In the study, the mean age of the migraine group and the control group was 46.54 ± 15.01 and 44.15 ± 19.20 years, respectively. Meanwhile, there were 22.73% of men in the migraine group and 48.81% of men in the control group. There were statistically significant differences in the mean age and gender between the two groups. Besides, statistically significant differences were found in the distribution of races ($p < 0.0001$) and data release cycles ($p < 0.048$) between the two groups. Meanwhile, the migraine group had a higher rate of usage of non-steroidal anti-inflammatory drugs (NSAIDs) (36.14 vs. 5.84%, $p < 0.0001$), opioid (6.71 vs. 2.35%, $p < 0.0001$), triptans (34.32 vs. 0%, $p < 0.0001$), antiepileptic drugs (48.21 vs. 3.27%, $p < 0.0001$), antidepressants (35.88 vs. 6.17%, $p < 0.0001$), β-blockers (20.42 vs. 7.50%, $p < 0.0001$), Ca^{2+} blockers (8.83 vs. 5.41%, $p = 0.013$), and antihistamines (7.62 vs. 0.28%, $p < 0.0001$), as compared with the control group. Furthermore, the migraine group had the lower values of blood albumin (4.17 vs. 4.26, $p < 0.0001$), blood total protein (6.95 vs. 7.10, $p < 0.0001$), hemoglobin (13.73 vs. 14.15, $p < 0.0001$), serum iron (77.39 vs. 86.26, $p < 0.0001$), and serum urate (4.86 vs. 5.36, $p < 0.0001$), as compared with the control group. However, there were no differences in blood globulin, blood creatinine, blood urea nitrogen, ferritin, urine albumin, urine creatinine, or urine albumin/creatinine ratio. The data are shown in **Table 1**.

The Relationship Between Serum Urate and Migraine

The results of logistic regression models are shown in **Table 2**. Model 1 was a non-adjusted model, and model 2 was adjusted for age, gender, race, and data release cycle. Meanwhile, model 3 was adjusted for age, gender, race, data release cycle, blood albumin, blood globulin, blood total protein, hemoglobin, blood urea nitrogen, ferritin, serum iron, usage of NSAIDs, usage of opioid, usage of triptans, usage of antiepileptic drugs, usage of antidepressants, usage of β-blockers, usage of Ca^{2+} blockers, and usage of antihistamines. Although we found that lower serum urate was correlated to higher migraine occurrence in model 1 (odds ratio (OR) = 0.832, 95% confidence interval (CI) = (0.751, 0.922), $p = 0.00044$) and model 3 ($OR = 0.84$, 95% CI = (0.72, 0.97), $p = 0.0184$), the results were not consistent in model 2 ($OR = 0.96$, 95% CI = (0.86, 1.08), $p = 0.5198$). Furthermore, in the subgroup analysis, which was stratified by gender, we found

TABLE 1 | Characteristic of participants enrolled.

	Control (n = 18,429)	Migraine (n = 208)	*P*
Age	44.15 ± 19.20	46.54 ± 15.01	*0.0389*
Gender			*<0.0001*
Male	48.81%	22.73%	
Female	51.19%	77.27%	
Race			*<0.0001*
Mexican American	9.89%	5.72%	
Other Hispanic	6.49%	2.41%	
Non-Hispanic White	63.06%	78.73%	
Non-Hispanic Black	11.20%	6.56%	
Other Race	9.36%	6.58%	
Data release cycle			*0.048*
8	33.41%	36.08%	
9	33.31%	26.34%	
10	33.28%	37.58%	
Drug usage			
NSAIDs*	5.84%	36.14%	*<0.0001*
Opioid	2.35%	6.71%	*<0.0001*
Triptans	0.00%	34.32%	*<0.0001*
Antiepileptic drugs	3.27%	48.21%	*<0.0001*
Antidepressants	6.17%	35.88%	*<0.0001*
Beta-blockers	7.50%	20.42%	*<0.0001*
Ca^{2+} blockers	5.41%	8.83%	*0.013*
Antihistamines	0.28%	7.62%	*<0.0001*
Blood albumin (g/dL)	4.26 ± 0.36	4.17 ± 0.33	*<0.0001*
Blood globulin(g/dL)	2.84 ± 0.43	2.79 ± 0.44	0.0659
Blood total protein (g/dL)	7.10 ± 0.44	6.95 ± 0.45	*<0.0001*
Hemoglobin(g/dL)	14.15 ± 1.46	13.73 ± 1.35	*<0.0001*
Blood creatinine (mg/dL)	0.87 ± 0.37	0.83 ± 0.21	0.1257
Blood urea nitrogen (mg/dL)	14.01 ± 5.43	13.38 ± 4.66	0.0553
Ferritin (ng/ml)	119.64 ± 97.97	118.77 ± 84.57	0.883
Serum iron (ug/dl)	86.26 ± 36.22	77.39 ± 30.97	*<0.0001*
Urine albumin (ug/ml)	37.05 ± 259.07	23.09 ± 59.56	0.371
Urine creatinine (mg/dL)	124.51 ± 81.42	122.56 ± 82.02	0.6935
Urine albumin/creatinine ratio(mg/g)	35.26 ± 286.76	19.98 ± 64.26	0.3764
Serum urate (mg/dL)	5.36 ± 1.40	4.86 ± 1.45	*<0.0001*

Continuous variables were shown as mean ± standard deviation (SD), and the value of p was calculated by the weighted linear regression model. Categorical variables were shown as percentage, and the p was calculated by weighted chi-square test.

**NSAIDs: non-steroidal anti-inflammatory drugs.*

that there was no correlation between migraine and serum urate, except for model 3 ($OR = 0.79$, 95% $CI = (0.67, 0.94)$, $p = 0.0092$) in women. Meanwhile, when stratified by race, we found that a negative correlation between migraine and serum urate existed in non-Hispanic White. However, a positive correlation existed in other races. These results demonstrated that there was no relationship or the relationship was non-linear between migraine and serum urate.

A bell curve found that the data on serum urate in migraineurs fit a normal distribution (**Figure 2A**). Furthermore, smooth curve fittings were utilized to characterize the non-linear relationship between migraine and serum urate, and found an exponential curve relationship between serum urate and migraine, which is shown in **Figure 2**. The inflection point was 7.8 mg/dl according to the two-piecewise linear regression model. When serum urate was <7.8 mg/dl, there existed a negative correlation between serum urate and migraine in model 1 ($OR = 0.77$, 95% $CI = (0.69, 0.86)$, $p < 0.0001$) or model 3 ($OR = 0.77$, 95% $CI = (0.65, 0.90)$, $p = 0.0015$). However, there was no correlation between serum urate and migraine in model 2 ($OR = 0.90$, 95% $CI = (0.80, 1.02)$, $p = 0.0872$). Furthermore, when serum urate was more than 7.8 mg/dl, higher serum urate was correlated with higher migraine occurrence in model 1($OR = 1.54$, 95% $CI = (1.17, 2.04)$, $p = 0.0022$), model 2 ($OR = 1.51$, 95% $CI = (1.13, 2.02)$, $p = 0.0050$), and model 3 ($OR = 1.77$, 95% $CI = (1.04, 3.02)$, $p = 0.0348$). In addition, 200 of the 208 (96.1%) migraineurs have a serum urate not more than 7.8 mg/dl, and 17,493 of 18,429 (94.9%) control have a serum urate not more than 7.8 mg/dl. The data are shown in **Table 3**.

DISCUSSION

According to the nationally representative cross-section study of the United States, there existed no consistent linear relationship between serum urate and migraine according to logistic regression models, without or with stratified by gender or race, except in non-Hispanic White. However, we found an exponential curve relationship between serum urate and migraine, with an infliction point of 7.8 mg/dl. When serum urate was more than 7.8 mg/dl, increased serum urate was correlated with higher migraine occurrence. However, when serum urate was <7.8 mg/dl, there was no consistent relationship between serum urate and migraine without or with confounders adjusted. To the best of our knowledge, our study demonstrates that serum urate is a risk factor for migraine for the first time. Our study provides a target and rationale for serum urate control in migraineurs.

Usually, serum urate levels higher than 6 mg/dl lead to the deposition of monosodium urate (MSU) crystal in tendons, joints, or other unusual tissues at physiological pH (∼7.4) (23). Meanwhile, previous studies have found that inflammation plays an important role in the pathophysiology of migraine, and the levels of serum inflammatory cytokines, such as CRP, IL-1β, IL-6, and TNF-α are higher in migraineurs compared with healthy controls (24). Moreover, NSAIDs and corticosteroids

TABLE 2 | Association between serum urate and migraine.

	Model 1 OR* (95%CI#) *P*	Model 2 OR (95%CI) *P*	Model 3 OR (95%CI) *P*
Serum urate (mg/dL)	0.83 (0.75, 0.92) *0.0004*	0.96 (0.86, 1.08) 0.5198	0.84 (0.72, 0.97) *0.0184*
Stratified by gender			
Male	0.99 (0.79, 1.24) 0.9442	0.98 (0.78, 1.23) 0.8494	1.02 (0.74, 1.40) 0.8982
Female	0.99 (0.88, 1.12) 0.8518	0.96 (0.85, 1.09) 0.5307	0.79 (0.67, 0.94) *0.0092*
Stratified by race			
Mexican American	0.85 (0.63, 1.15) 0.2940	1.05 (0.76, 1.43) 0.7854	0.86 (0.55, 1.34) 0.5030
Other Hispanic	0.71 (0.46, 1.10) 0.1274	0.97 (0.60, 1.55) 0.8830	0.62 (0.31, 1.24) 0.1743
Non-Hispanic White	0.72 (0.62, 0.84) *<0.0001*	0.83 (0.71, 0.97) *0.0216*	0.79 (0.64, 0.97) *0.0279*
Non-Hispanic Black	0.88 (0.69, 1.13) 0.3135	1.00 (0.77, 1.31) 0.9805	0.89 (0.61, 1.29) 0.5242
Other races	1.21 (0.94, 1.56) 0.1313	1.48 (1.13, 1.94) *0.0050*	1.02 (0.60, 1.73) 0.9415

Model 1 was adjusted for nothing.

Model 2 was adjusted for age, gender, race, and data cycle release.

Model 3 was adjusted for age, gender, race, data release cycle, blood albumin, blood globulin, blood total protein, hemoglobin, blood urea nitrogen, ferritin, serum iron, usage of NSAIDs&, usage of opioid, usage of triptans, usage of antiepileptic drugs, usage of antidepressants, usage of β-blockers, usage of Ca^{2+} blockers, and usage of antihistamines.

**OR: odds ratio. #CI: confidence interval. &NSAIDs: non-steroidal anti-inflammatory drugs.*

are used for shortening a migraine attack (25, 26). Serum urate has been proposed as a neuroprotective agent in stroke, Parkinson's disease, multiple sclerosis, and Alzheimer's disease, in which the high serum urate levels have been linked to the lower severity of neurological injury (13). However, studies in animal or human had failed to prove the neuroprotective effect of serum urate by regulating the serum urate levels (13). A previous study found that migraineurs had the lower levels of serum urate and ferritin, as compared with healthy controls (14). Meanwhile, there existed no statistically significant differences between the different subtypes of migraine, which compared migraine with/without aura or episodic/chronic migraine (14). Another study, which was aimed at assessing the change of serum urate in migraineurs receiving topiramate, found that the serum urate of migraine pretreatment with topiramate and the well-matched control group were 3.61 ± 0.89 and 3.09 ± 1.86, respectively (15). Besides, topiramate would increase the serum urate level in migraine (15). The normal range of serum urate in humans is 2.5–7.0 mg/dl in men and 1.5–6.0 mg/dl in women (13). Hyperuricemia is defined as a serum urate more than 6.8 mg/dl, which mostly individuals would suffer from gout (13). Furthermore, hyperuricemia is a risk factor for hypercholesterolemia, diabetes, hypertension, cardiovascular and cerebrovascular events, and so on (13). In the present study, the migraine group had lower serum urate before adjusting for confounders. However, the linear relationship was not consistent after adjusting for confounders. Furthermore, an exponential curve relationship, with an infliction point of 7.8 mg/dl, was found in serum urate and the risk of migraine. Our findings demonstrated that serum urate, when more than 7.8 mg/dl, might be a risk factor for migraine. Furthermore, we also suggest that there is a threshold effect on the neuroprotective effect and that the infliction point might be 7.8 mg/dl. Besides, the underlining mechanism might be that MSU crystal formed might trigger inflammation *via* IL-1b, TNF-a, IL-6, IL-8, and oxidative stress (27–31).

Currently, there are a lot of risk factors for migraine identified by researchers. It had been found that 38 genetic loci, which were enriched in vascular biology, were associated with migraine (4, 32). Meanwhile, another study had found that the genetically mediated hypercalcemia might increase the risk of migraine (32, 33). In addition, migraine with aura was associated with the increasing risk of other comorbidities, such as perioperative stroke, patent foramen ovale, and restless legs syndrome (32). Risk factors associated with the progression from episodic migraine to chronic migraine were summarized in a previous review (34, 35). Besides the fair and non-modifiable risk factors included female gender, low family socioeconomic status, and major life events (34, 35). Furthermore, the moderate and modifiable risk factors were obesity, persistent-frequent nausea associated with migraine, asthma, non-cephalic pain, snoring, and the efficacy of abortive migraine treatments (34, 35). Moreover, strong and modifiable risk factors were the frequency of headache day, depression, and acute medication use/overuse (34, 35). In our analysis, we found that the serum urate levels of more than 7.8 mg/dl might be a risk factor for migraine, which is a modifiable risk factor.

The limitations of this study were as follows: first, the present study could not distinguish the acute attack of migraine, the frequency and intensity of migraine attack, and migraine with/without aura, because of the missing information of NHANES. Second, the present study was a cross-section study, whose follow-up data were absent. Besides, the conclusions needed to be proven according to a prospective longitudinal study in the future, which should control the confounders, such as age, race, gender, intensity and frequency of migraine attack, and drug usage. Finally, the medication usage was analyzed in our analysis, but the dosages of drugs were unknown. Further studies

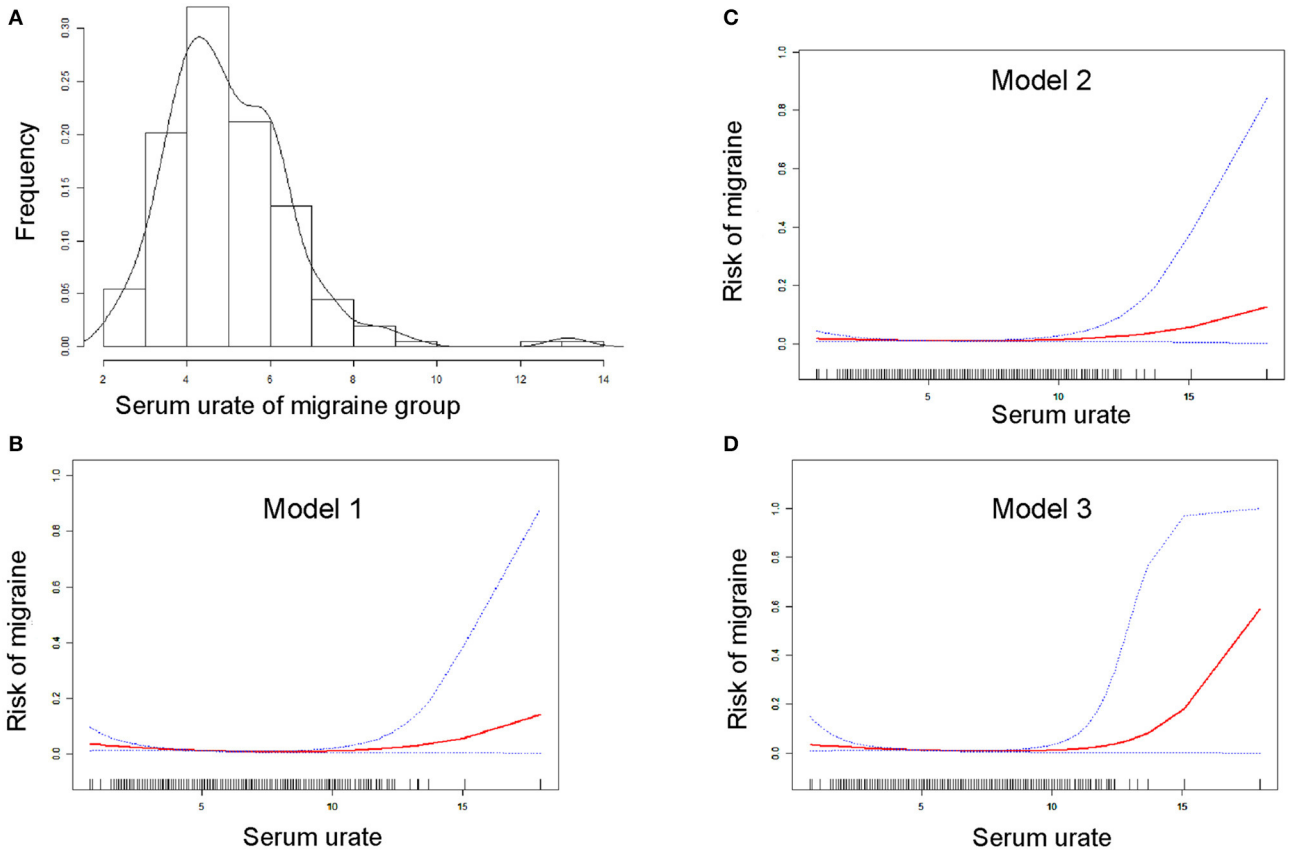

FIGURE 2 | Smooth curve fitting for the relationship between serum urate and the risk of migraine. **(A)** Shows the bell curve of serum urate of migraine group. **(B–D)** Show the smooth curve fitting for the relationship between serum urate and the risk of migraine. The horizontal axis presents serum urate (continuous variable), and the ordinate presents the risk of migraine (0 = non-migraine, 1 = migraine). Red line means smooth curve fitting between serum urate and migraine. Blue bands mean 95% of confidence interval (*CI*) from the fit. Model 1 **(B)** was adjusted for nothing. Model 2 **(C)** was adjusted for age, gender, race, and data release cycle. Model 3 **(D)** was adjusted for age, gender, race, data release cycle, blood albumin, blood globulin, blood total protein, hemoglobin, blood urea nitrogen, ferritin, serum iron, usage of NSAIDs, usage of opioid, usage of triptans, usage of antiepileptic drugs, usage of antidepressants, usage of β-blockers, usage of Ca^{2+} blockers, and usage of antihistamines.

TABLE 3 | Threshold effect analysis of serum urate on migraine using the two-piecewise linear regression model.

	Model 1 OR* (95%CI#) *P*	**Model 2** OR (95%CI) *P*	**Model 3** OR (95%CI) *P*
Fitting by the standard linear model	0.83 (0.75, 0.92) *0.0004*	0.96 (0.86, 1.08) 0.5198	0.99 (0.88, 1.11) 0.8620
Fitting by two-piecewise linear model			
Inflection point	7.8	7.8	7.8
Serum urate <7.8 mg/dL	0.77 (0.69, 0.86) *<0.0001*	0.90 (0.80, 1.02) *0.0872*	0.77 (0.65, 0.90) *0.0015*
Serum urate >7.8 mg/dL	1.54 (1.17, 2.04) *0.0022*	1.51 (1.13, 2.02) *0.0050*	1.77 (1.04, 3.02) *0.0348*
Log likelihood ratio	*0.002*	*0.014*	*0.005*

Model 1 was adjusted for nothing.
Model 2 was adjusted for age, gender, race, and data cycle release.
Model 3 was adjusted for age, gender, race, data release cycle, blood albumin, blood globulin, blood total protein, hemoglobin, blood urea nitrogen, ferritin, serum iron, usage of NSAIDs&, usage of opioid, usage of triptans, usage of antiepileptic drugs, usage of antidepressants, usage of β-blockers, usage of Ca^{2+} blockers, and usage of antihistamines.
**OR: odds ratio. #CI: confidence interval. &NSAIDs: non-steroidal anti-inflammatory drugs.*

could be conducted to investigate the alteration of serum urate levels in migraineurs with different drugs in real world studies.

In conclusion, there existed an exponential curve relationship between serum urate and migraine, with an infliction point of 7.8 mg/dl. When the serum urate was more than 7.8 mg/dl, increased serum urate was correlated with higher migraine occurrence.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Centers for Disease Control and Prevention (CDC) National Health and Nutrition Examination Survey (NHANES), https://wwwn.cdc.gov/nchs/nhanes/Default.aspx, NHANES 1999-2018.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Prevention National Center for Health Statistics Research (NCHS) and Centers for Disease Control (CDC) Research Ethics Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YX, HX, and YW proposed the idea. PH and YL acquired the data. PH analyzed the data. YX wrote the first draft. HX, YW, and PH revised the draft. All authors have approved the final article.

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A Preliminary Study on Change of Serum Immunoglobulin G Glycosylation in Patients With Migraine

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Background and Objective: Migraine is a common neurological disease, but its pathogenesis is still unclear. Previous studies suggested that migraine was related to immunoglobulin G (IgG). We intended to analyze the immune characteristics of migraine from the perspective of IgG glycosylation and provide theoretical assistance for exploring its pathogenesis.

Methods: The differences in the serum level of IgG glycosylation and glycopeptides between patients with episodic migraine and healthy controls were analyzed by applying the poly(glycerol methacrylate)@chitosan (PGMA@CS) nanomaterial in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). We constructed a binary classification model with a feedforward neural network using PyTorch 1.6.0 in Python 3.8.3 to classify the episodic migraine and healthy control groups.

Results: Twenty patients with migraine and 20 healthy controls were enrolled and the blood samples and clinical information were collected. Forty-nine IgG N-glycopeptides were detected in the serum of the subjects. The serum level of N-glycopeptide IgG1 G0-NF ($p = 0.012$) was increased in patients with migraine. The serum level of N-glycopeptide IgG3/4 G2FS ($p = 0.041$) was decreased in patients with migraine with family history of headache. It was found that the serum level of the IgG1 G1 ($p = 0.004$) and IgG2 G0 ($p = 0.045$) was increased in patients with migraine with aura, while the serum level of IgG2 G0N ($p = 0.043$) in patients with migraine with aura was significantly lower than that in patients with migraine without aura. In addition, a linear feedforward neural network (FFNN) was used to construct a binary classification model by detected IgG N-glycopeptides. The area under the curve (AUC) value of the binary classification model, which was constructed with 7 IgG N-glycopeptides, was 0.857, suggesting a good prediction performance. Among these IgG N-glycopeptides that were constructed the model, IgG1 G0-NF was overlapped with the differential IgG N-glycopeptide between patients with migraine and healthy controls detected with MALDI-TOF-MS.

Conclusion: Our results indicated that the serum level of N-glycopeptides IgG1 G0-NF might be one of the important biomarkers for the diagnosis of migraine. To the best of our knowledge, this is the first study about the changes of IgG N-glycosylation in patients with migraine by the method of MALDI-TOF-MS. The results indicated a relationship between the migraine and immune response.

Keywords: migraine, IgG N-glycosylation, N-glycopeptide, MALDI-TOF-MS, machine learning

INTRODUCTION

Migraine is a primary headache, characterized by recurrent pulsating moderate or severe headache, which is typically unilateral but sometimes bilateral. It is often accompanied by nausea, vomiting, photophobia, and phonophobia. About one-third of patients have visual, somatosensory, or other kinds of aura (1). The prevalence of migraine is 14.7% globally and 9.3% in China (2). Migraine is considered to be the seventh most disabling disease in the world, seriously affecting the quality of life of patients (3). Several hypotheses have been proposed about the mechanism of migraine, such as trigeminal nerve vascular theory (4) and cortical spreading depression theory (5). However, these theories still cannot fully explain the pathogenesis of migraine. Currently, the diagnosis of migraine mainly depends on the symptoms of patients, lacking specific laboratory biomarkers.

Although the exact pathophysiology has not yet been determined, it has been reported that restricting the intake of sensitized or intolerant food in patients with migraine complicated with irritable bowel syndrome (IBS) can reduce the frequency of migraine and abdominal pain (6). Aydinlar et al. proposed that the inflammatory response caused by the increase of immunoglobulin G (IgG) antibodies played a certain role in migraine attacks (7). Li et al. and Lu et al. reported that the IgG content of patients with migraine was significantly higher than that of the control group (8, 9) and negatively correlated with the ictal phase (8). It was long thought that the effects of calcitonin gene-related peptide and substance P release from peripheral terminals only have correlation with vasodilation and increased capillary permeability (10, 11). However, the nervous system also has the potential means to modify immune function by the neuropeptide-mediated signaling from sensory neurons to immune cells (12–14). Michoud et al. revealed a link between nociceptors and immune cells in 2020 (15). All of these findings suggest that there is a certain correlation between migraine and IgG antibodies.

Immunoglobulin G is the core component of antibodies and an important serum glycoprotein with glycosylation modification. The fragment crystallizable (Fc) N-glycans of IgG undergoes specific changes in abnormal physiological or pathological conditions (16). It has been found that the sialic acid residue reduced in rheumatoid arthritis and immune-mediated osteoporosis (17, 18), the galactose and N-acetylglucosamine decreased in osteoarthritis (19), and the core fucose increased in liver cancer (20). Therefore, the detection of IgG N-glycosylation variation can be used to monitor the immune status, thereby assisting the early diagnosis and prognosis assessment of related diseases. However, the studies on IgG N-glycosylation were mainly focused on immune and tumor diseases. Currently, with the maturity of detection technology, studies have been conducted on nonimmune and nonneoplastic diseases. Freidin et al. investigated the correlation between IgG N-glycosylation and lower back pain and found multiple-related glycan modules (21). Lundström et al. found that the fucosylation of IgG1 increased and the levels of galactose and N-acetylneuraminic acid decreased in patients with Alzheimer's disease (AD) (22). The relationship between the glycosylation and diseases is attracting an increasing attention, but the change of serum IgG glycosylation in patients with migraine has not been reported.

Mass spectrometry (MS)-based glycoproteomics is a high-throughput and powerful approach for system-wide screening of glycosylation-based biomarkers (23). It has already been demonstrated that the use of MS can investigate aberrations in glycosylation associated with several diseases (24). In this study, the serum IgG N-glycosylation profile and N-glycopeptides were studied in patients with migraine and healthy controls by MS and the difference between the two groups was compared. Meanwhile, the difference in IgG glycosylation between subgroups of migraine, including the different subtypes, phases, and family history, was also analyzed. This study aims to explore the potential pathogenesis and biological markers of migraine.

MATERIALS AND METHODS

Study Cohort

In this study, 40 subjects were enrolled, including 20 patients with migraine (episodic migraine) and 20 healthy individuals (**Table 1**). The baseline data was collected to determine the subtypes of migraine (with or without aura) and whether the patients had a family history of headache. The phases of migraine (ictal or interictal) were inquired when the blood samples were collected. Patients with migraine who met the migraine diagnostic criteria of the International Classification of Headache Disorders 3rd Edition (ICHD-3) (1) and had a headache history of more than 1 year were selected as patients with migraine. The enrolled healthy individuals were age and sex matched and did not have any headache history. In addition, the subjects with the following characteristic were excluded: (1) Patients with severe infection, blood system disease, liver disease, malignant tumor, severe mental disease, or immune system disease; (2) Subjects who have received blood product transfusions in the past 6 months; and (3) Pregnant and lactating women.

TABLE 1 | Characteristics of patients with migraine and controls.

	Migraine group ($n = 20$)	Control group ($n = 20$)	t/χ^2	*P* value
Gender (male/female)	7/13	9/11	0.417	0.519
Age(years) (mean±SD)	40.1 ± 11.2	39.5 ± 11.0	0.194	0.847
BMI(kg/m^2) (mean±SD)	22.15 ± 2.03	24.58 ± 3.05	−2.911	0.006*
Ictal phase	7 (35%)	/	/	/
Migraine with aura	5 (25%)	/	/	/
Family history of headache	11 (55%)	/	/	/
Comorbidities		/	/	/
Hypertension	1 (5%)	1 (5%)	0.000	1.000
Hyperlipidemia	2 (10%)	2 (10%)	0.000	1.000
Allergic diseases	9 (45%)	3 (15%)	4.286	0.038*

*Statistical method: Student's t-test, chi-square test; *p < 0.05.*

This study was approved by the Medical Ethics Committee of the Second Clinical Hospital of Peking University Health Science Center. All the subjects provided a written informed consent before this study.

Blood Sample Collection and Serum Preparation

Blood samples (4 ml) were taken from the antecubital vein in citrated serum tubes (BD Biosciences), after an overnight fast. After centrifuged at 2,000–3,000 g for 10 min, the supernatant was collected into a test tube and the protease inhibitors were added at a ratio of 1:500. The mixture was aliquoted into 200 μl and stored in a refrigerator at −80°C.

Immunoglobulin G Isolation and Digestion

The isolation of IgG from human serum was the same as the previous study (25) with protein G beads (Kirgen Biosciences, Shanghai, China). After measuring the concentration of protein IgG from human serum by a bicinchoninic acid (BCA) protein assay, the IgG was treated with a tube-gel digestion.

Immunoglobulin G proteins (10.0 μg) were heated at 95°C for 15 min for degeneration. After recovering to room temperature, the samples were mixed with 10.0 μl acrylamide/bis solution (30%, w/v; Bio-Rad, Hercules, California, USA), 6.5 μl Tris-HCl solution (1.5 M, pH 8.8; Macgene), 0.5 μl sodium dodecyl sulfate (SDS) solution (10%, w/v; Beijing Biotechnology Corporation Ltd.), and 2.0 μl ammonium persulfate solution (10%, w/v; Bioss Antibodies, Woburn, Massachusetts, USA). Finally, 1.0 μl N,N,N',N'-tetramethylethylenediamine (TEMED) (Bioss Antibodies) were mixed immediately and the polymerization reaction was conducted for at least 30 min at room temperature.

After the gel was formed in the tube, it was washed with ddH_2O for 2 h. Then, the gel pieces were washed and dehydrated successively with acetonitrile (ACN) (Thermo Fisher Scientific) and 100 mM NH_4HCO_3/ACN (1:1, v/v). Proteolytic digestion was performed with trypsin (1:50, mass ratio) dissolved in NH_4HCO_3 (50 mM) at 37°C overnight. After digestion, gel pieces were eluted with 5% formic acid/ACN (1:2, v/v). Finally, the digested peptides were vacuum-dried.

Enrichment of N-Glycopeptides

N-glycopeptides were enriched from the digested samples with poly(glycerol methacrylate)@chitosan (PGMA@CS) nanomaterial according to the previous report by Jie et al. (26). Briefly, digested samples were redissolved with loading buffer [89% ACN-3% trifluoroacetic acid (TFA) solution, v/v] and added to PGMA@CS incubating on an Eppendorf shaker. After incubation, PGMA@CS was washed with 89% ACN-3% TFA solution twice times and dehydrated with ACN. Finally, IgG N-glycopeptides were eluted with 30 μl of 20 mg/ml dihydroxybenzoic acid solution (70% ACN−1% H_3PO_4, v/v) for 20 min at 30°C.

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry Analysis

A volume of 0.6 μl prepared sample was loaded onto 384 polished stainless steel matrix-assisted laser desorption/ionization (MALDI) target plates. MALDI-time-of-flight (MALDI-TOF) mass spectra were acquired on the AXIMA-CFP Plus Mass Spectrometer (Kratos Analytical Ltd., Shimadzu Corporation, Kyoto, Japan), equipped with a nitrogen laser (337.1 nm). Mass spectra were obtained in the positive ion and linear mode with an acceleration voltage of 20 kV. The power was set to 105 kV and the ions between m/z 1,000 and 4,500 were acquired. Each mass spectrum was automatically generated by averaging 200 laser shots and the optimal acquisition point was m/z 2,602. Each spectrum was internally calibrated with the theoretical mass of the IgG N-glycopeptides (m/z 2602.0, 2796.1, 2958.2, and 3217.3).

Data Extraction and Statistical Analysis

Mass spectrometry (MS) data were processed using Launchpad V2.4 Kompact MALDI software with a threshold of 0.050 mV and a top-hat baseline subtraction. The peaks between m/z 2,450 and 3,500 cm^{-1} were exported for statistical analysis. Missing values were replaced by zero. We used the ratio value of each N-glycopeptide peak height to the sum of heights of all the Fc N-glycopeptides in the same profile as its relative intensity. Four parallel experiments were performed for each sample and the average value was the final percentage. N-glycopeptides intensities between patients and controls were compared with the two-tailed Student's *t*-test in IBM SPSS statistics version 26 and the box plots were made by Origin 2021.

Given that many of these structures have the same glycan features such as bisecting GlcNAc, fucosylation, and galactosylation, which are closely related to IgG activity, additional derived glycan traits were calculated according to the following formulas: the abundance of bisecting GlcNAc (bi-N): G0N + G0NF + G1N + G1NF + G2N + G2NF; the abundance of fucosylation (F): G0F + G0NF + G0-NF + G1F + G1FS + G1NF + G1-NF + G2F + G2FS + G2NF; the abundance of

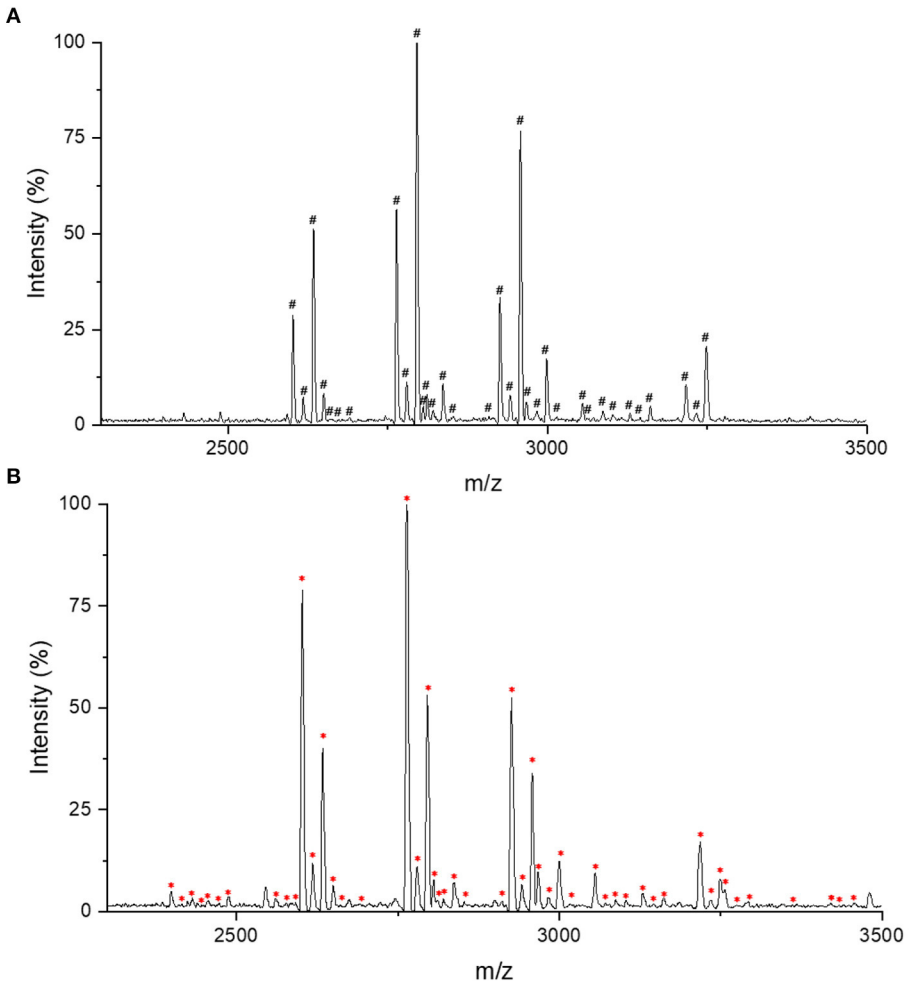

FIGURE 1 | Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) profiling in the positive-ion linear mode of enriched immunoglobulin G (IgG) N-glycopeptides. **(A)** The IgG N-glycopeptides enriched with chitosan@poly(glycidyl methacrylate)@iminodiacetic acid (CS@PGMA@IDA). **(B)** The IgG N-glycopeptides enriched with poly(glycerol methacrylate)@chitosan (PGMA@CS). #Represents the detected 33 IgG N-glycopeptides by CS@PGMA@IDA. *Represents the detected 49 IgG N-glycopeptides by PGMA@CS.

fucosylation of neutral glycans (F neutral, FN): G0F + G0NF + G0-NF + G1F + G1NF + G1-NF + G2F + G2NF; the abundance of fucosylation of sialylated glycans (F sialo, FS): G1FS + G2FS; and the abundance of sialylation (S): G1S + G1FS + G2FS + G2S. Relative quantification of IgG galactosylation was measured by a Gal-ratio formula: G0/(G1 + G2 × 2); the abundance of agalactosylation (G0): G0 + G0F + G0N + G0NF + G0-NF; the abundance of monogalactosylation (G1): G1 + G1F + G1FS + G1-N +G1N + G1NF + G1-NF + G1NFS + G1S; and the abundance of digalactosylation (G2): G2 + G2F + G2FS +G2N + G2NF + G2NFS + G2S + G2S2.

Model Construction

We used F-test to select features that had significant difference between the healthy and migraine groups using Pandas 1.0.5 (27) and scikit-learn 0.23.1 (28) in Python 3.8.3. The top seven features were chosen. To optimize the model parameters efficiently, all the values of the features (N-glycopeptide abundances) were normalized by min-max scaling. Then, we constructed a binary classification model with feedforward neural network using PyTorch 1.6.0 (29) in Python 3.8.3 to classify the migraine and healthy groups. The network consisted of one input layer of 7 features (IgG1 G0-NF, IgG1 G2NF, IgG2 G0N, IgG2 G1N, IgG2 G2N, IgG2 G2NF, and IgG3/4 G0), two hidden layers of 6 and 4 neurons both using Sigmoid as activation function, and finally one output layer of 2 neurons followed by function Softmax, representing two results (having migraine or not). When predicting, the network will calculate from the input abundances of featured N-glycopeptides and give two results representing possibilities of each status. The higher one will be considered as the predicted result. The network can express as follows:

$$\mathbf{Y} = Softmax(\mathbf{W_3}^{\mathrm{T}} Sigmoid(\mathbf{W_2}^{\mathrm{T}} Sigmoid(\mathbf{W_1}^{\mathrm{T}}\mathbf{X} + \mathbf{B_1}) + \mathbf{B_2}) + \mathbf{B_3})$$

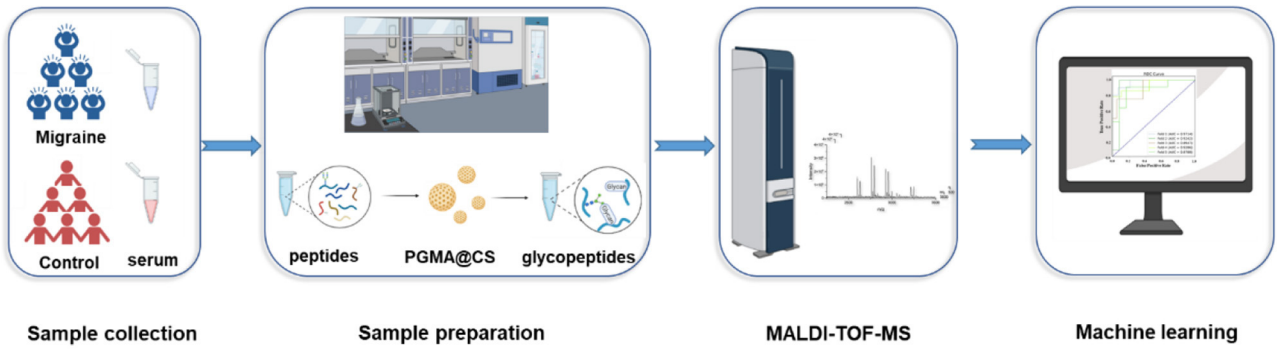

FIGURE 2 | The high-throughput and effective strategies for obtaining and analyzing IgG intact N-glycopeptides for patients with migraine and healthy control subjects.

Where, $\mathbf{X} = [x_1, x_2, \ldots, x_n]$ represents the n input features, $\mathbf{W_1}^T$[shape: (7,6)], $\mathbf{W_2}^T$[shape: (6,4)], and $\mathbf{W_3}^T$[shape: (4,2)] are three weight matrices and $\mathbf{B_1}$[shape: (6,1)], $\mathbf{B_2}$[shape: (4,1)], and $\mathbf{B_3}$[shape: (2,1)] are three intercept matrices. $\mathbf{Y} = [y_1, y_2]$ is the predicted possibilities of each status. The expressions of Sigmoid and Softmax are as follows:

$$Sigmoid\,(x) = \frac{1}{1 + e^{-x}}$$

$$Softmax\,(x_i) = \frac{e^{x_i}}{\sum_{i=1}^{n} e^{x_i}}$$

5-fold cross-validation was applied to verify the reliability of the model due to the limitation of the amount of data. The model's performance was evaluated by the area under the curve (AUC) values (scikit-learn Python module) (30). The dataset was randomly partitioned into five equal-sized subsets, in which four subsets were used as training sets and the other one subset was used as a test set. The training process was executed five times, with each of the divided training set and test set. The receiver operating characteristic (ROC) curves and the AUC values were given by each fold that can represent the generalization ability of the model. The model is completely random (has no prediction ability at all) at an AUC of 0.5 and the higher the average AUC value is, the higher the performance of the model. The model performs perfectly with no mistake in prediction when the average AUC value equals one. The charts were plotted using Python-Matplotlib (31). Finally, a model of the same features, network form, and hyperparameters were applied to the whole dataset as the training set to give the final prediction model.

RESULTS

Baseline Clinical Information of the Patients and Control Groups

Twenty patients with migraine and 20 healthy controls were enrolled. In the migraine group, there were 5 patients with aura and 15 patients without aura. Seven patients were in the ictal phase and 13 patients were in the interictal phase. Nine patients with migraine were comorbid with allergic diseases. It has been reported that gender and age have a greater impact on glycosylation (32, 33). Our data indicated that there were no significant differences between the two groups of subjects in the age, gender, family history of headache, and comorbidity of allergic diseases (**Table 1**).

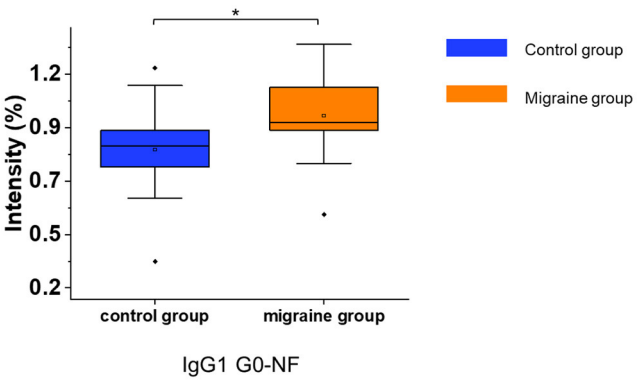

FIGURE 3 | The serum level of IgG N-glycopeptides in the migraine ($n = 20$) and healthy control groups ($n = 20$). The level of IgG N-glycopeptides was presented as box plots showing median values, upper and lower quartiles, as well as the whole range for patients and controls. The glycan species were named as G0, G1, and G2 according to the numbers of galactoses, F, fucose; N, N-acetylglucosamine. *$p < 0.05$.

Strategy for Immunoglobulin G N-Glycopeptide Analysis With Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry

For IgG intact N-glycopeptides analysis, it is essential to purify N-glycopeptides before MS analysis. On one hand, the presence of nine glycoproteins from the purification process of IgG by protein G will confuse the analyses (25).

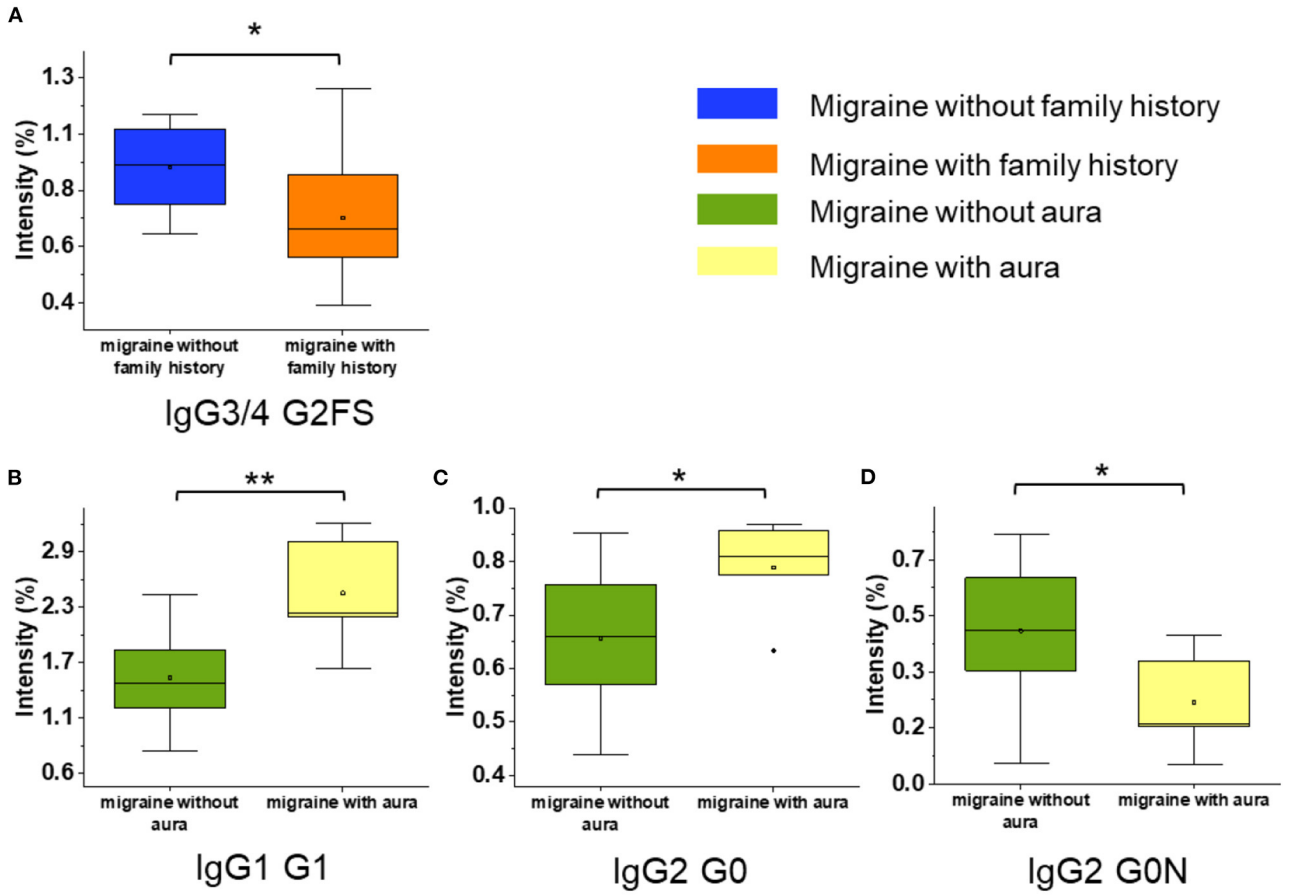

FIGURE 4 | The serum level of IgG glycopeptides in the different migraine subgroups and phases. **(A)** The serum level of IgG3/4 G2FS of migraine with and without family history ($n = 11/9$). **(B–D)** The serum level of IgG glycopeptides of migraine with and without aura ($n = 5/15$). The level of IgG glycopeptides was presented as box plots showing median values, upper and lower quartiles, as well as the whole range for patients and controls. The glycan species were named as G0, G1, and G2 according to the numbers of galactoses, F, fucose; S, sialic acid; N, N-acetylglucosamine. $^{*}p < 0.05$, $^{**}p < 0.01$.

On the other hand, compared with unmodified peptides, glycopeptides have a lower abundance and relatively lower ionization efficiency, which can also affect the detection (26). In previous reports, we established a high-throughput and effective strategies for obtaining and analyzing IgG intact N-glycopeptides with MALDI-TOF-MS, which used chitosan@poly(glycidyl methacrylate)@iminodiacetic acid (CS@PGMA@IDA) to enrich the N-glycopeptides (25). After comparing the CS@PGMA@IDA with the novel nanosphere PGMA@CS (**Figures 1A,B**), it can be seen that more IgG N-glycopeptides were detected with PGMA@CS, which shows the high purification and enrichment ability.

In this study, we chose to use PGMA@CS instead of CS@PGMA@IDA to improve the IgG N-glycopeptides enrichment ability, optimizing the strategy for migraine cohorts (**Figure 2**). Overall, after collecting the human serum, the serum preparation was proceeded, including the IgG isolation, tube-gel digestion, and the IgG N-glycopeptides enrichment by PGMA@CS. Then, the purified IgG N-glycopeptides were detected by MALDI-TOF-MS and the data were analyzed by machine learning to explore the potential biomarkers of migraine.

It should be noted that IgG subclasses have a high various abundances and high similar peptide moieties: IgG1, 60%, EEQYNSTYR; IgG2, 32%, EEQFNSTFR; IgG3, 4%, EEQYNSTFR; and IgG4, 4%, EEQFNSTYR (25). All of the N-glycopeptides IgG3 and IgG4 are the isomers, thus the signals of IgG3 and IgG4 N-glycopeptides were considered together. In addition, due to the low abundance of IgG3 and IgG4 and isomers existence, the signal of IgG3/4 N-glycopeptides might overlap with that of other subclasses of IgG N-glycopeptides. Therefore, the IgG3/4 Fc N-glycopeptide was ignored when the signals overlapped (34, 35). A total of 49 N-glycopeptides were detected and considered in this study, as shown in **Supplementary Table 1**.

Repeatability

Three standard intravenous immunoglobulins (IVIGs) (5 μg) were used to determine the precision of the analytical workflow. The precision was determined by calculating the relative SDs (RSDs) of intensities of the six minor components (m/z = 2,602.1, 2,634.0, 2,764.1, 2,796.1, 2,926.2, and 2,958.2). It was found that the relative SD was < 15.0% over each sample plate (**Supplementary Table 2**). This error range was in an acceptable

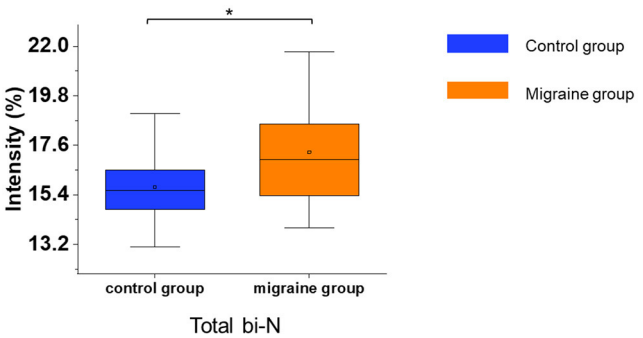

FIGURE 5 | The serum level of IgG N-glycosylation in the migraine ($n = 20$) and control groups ($n = 20$). The serum level of IgG N-glycosylation was presented as box plots showing median values, upper and lower quartiles, as well as the whole range for patients and controls. bi-N, bisecting N-acetylglucosamine. *$p < 0.05$.

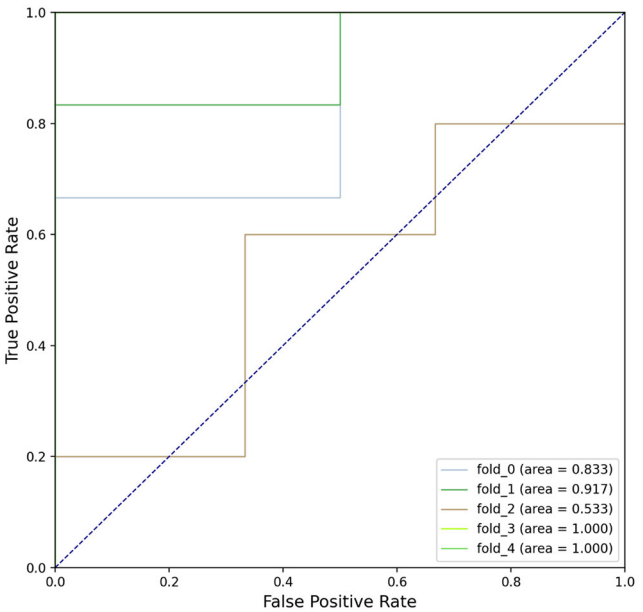

FIGURE 6 | The receiver operating characteristic (ROC) curve of the feedforward neural network (FFNN) model performing in 5-fold cross-validation's test sets; each line represents the prediction of a fold of test samples in the validation.

range for complex biological sample analysis (36), so no batch correction was performed (25).

Serum Immunoglobulin G N-Glycopeptides Profiling in Patients With Migraine

After the consistent stability of our strategy was confirmed, the serum IgG N-glycopeptides were analyzed in patients with migraine and healthy controls by this workflow (**Supplementary Table 3**). The glycopeptide IgG1 G0-NF was increased in patients with migraine compared with the healthy control group ($p = 0.012$, **Figure 3**) with the two-tailed Student's *t*-test.

Patients with migraine were further grouped based on the phase of migraine, aura, and family history of headache. There was no statistical difference in the gender, age, family history of headache, and comorbidity with allergic diseases between subgroups. Then, we profiled the N-glycopeptides in different subgroups of migraine (**Supplementary Table 4**). In patients with migraine with family history of headache, the IgG3/4 G2FS ($p = 0.041$, **Figure 4A**) was significantly decreased. It was found that the serum level of the IgG1 G1 ($p = 0.004$, **Figure 4B**) and IgG2 G0 ($p = 0.045$, **Figure 4C**) was increased in patients with migraine with aura, while the serum level of IgG2 G0N ($p = 0.043$, **Figure 4D**) in patients with migraine with aura was significantly lower than that of patients with migraine without aura. No significant difference in IgG glycopeptides was found between the ictal phase and interictal phase of patients with migraine.

N-Glycosylation Profiling in Patients With Migraine

The derived N-glycosylation characteristics were also used to distinguish patients with migraine from healthy controls. Considering the isomers exist, the glycosylation of IgG3/4 was not considered to avoid the information mistakes due to neglect. The distribution of the total IgG galactosylation was referred to as Gal-ratio. The higher Gal-ratio reflects the lower content of IgG galactosylation. Other glycan features were annotated in Methods. The summary of changes in the glycan features among the controls, patients with migraine, and patients with migraine subgroup in the discovery set is given in **Supplementary Tables 5, 6**.

In the serum of patients with migraine, the median of the total bisecting N-acetylglucosamine was significantly increased ($p = 0.017$, **Figure 5**). Other glycosylations such as fucosylation and sialylation had no significant difference (**Supplementary Table 5**). In addition, the migraine subgroups were also analyzed for N-glycosylation, but there were no significant differences in N-glycosylation among the migraine subgroups.

Construction of the Predictive Model to Identify Migraine

At present, the diagnosis of migraine still depends on medical history and there is no gold standard for diagnosis. Thus, we constructed a feedforward neural network (FFNN) to distinguish the migraine group from the healthy group. We selected seven N-glycopeptides (IgG1 G0-NF, IgG1 G2NF, IgG2 G0N, IgG2 G1N, IgG2 G2N, IgG2 G2NF, and IgG3/4 G0) as features, constructed a two hidden layer network, and tuned hyperparameters for better performance. Then, 5-fold cross-validation was used to verify the reliability of the model; the ROC curves of validation are shown in **Figure 6**. The average AUC value of the model was 0.857 (0.833, 0.917, 0.533, 1.000, and 1.000), which indicated that the model had good accuracy in predicting both the migraine and healthy individuals and its performance was stable when training sets and test sets change between different folds. These results indicated that the model had a good performance in

identifying migraine individuals. IgG1 G0-NF was overlapped with the differential glycopeptides between the migraine and healthy control groups. The parameters of the model are given in the **Supplementary Information** "Model Parameters."

DISCUSSION

Immunoglobulin G may be involved in the occurrence of migraine, though the specific pathogenesis of migraine is still unclear. IgG N-glycosylation can affect complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and inflammatory processes (37). Therefore, we focused on the IgG N-glycosylation and N-glycopeptides of migraine with the method of MS, in order to find the biomarkers of migraine, and provide clues for the exploration of the pathophysiological mechanism of migraine.

Mass spectrometry-based approaches are often used for glycoprotein characterization. But, due to the low abundance and relatively low ionization efficiency of glycopeptides compared with unmodified peptides, it is necessary to purify N-glycopeptides before MS analysis. Therefore, we used PGMA@CS to enrich the IgG N-glycopeptides in patients' serum with migraine. The more precise measurements of N-glycopeptides can assist us in better understanding the situation of IgG N-glycosylation in patients with migraine.

Statistical analysis showed that total bisecting N-acetylglucosamine was increased in patients with migraine, while there was no significant change in galactosylation, sialylation, and fucosylation. Bisecting N-acetylglucosamine can enhance antibody FC that binds to the Fcγ receptor IIIa (FcγRIIIa) with higher affinity (38) and enhances the ADCC efficacy of the antibody. Maurice et al. reported that patients with Lambert–Eaton myasthenic syndrome (LEMS) below 50 years showed elevated levels of bisecting N-acetylglucosamine on both the IgG1 and IgG2 (39). LEMS is an immune-related disease with consistent glycosylation changes. We speculated that migraine might also be immune related.

Subgroup analyses were conducted in order to explore the relationship between the migraine aura, phase of migraine, family history, and IgG glycopeptide. In the migraine group, IgG1 G0-NF was higher than that in the healthy control group; IgG1 G1 and IgG2 G0 were higher and IgG2 G0N was lower in patients with migraine with aura than those without aura. IgG3/4 G2FS was lower in patients with migraine with family history of headache than those without family history of headache.

A prediction model of migraine was established. It was found that a group of IgG N-glycopeptides had good prediction performance (IgG1 G0-NF, IgG1 G2NF, IgG2 G0N, IgG2 G1N, IgG2 G2N, IgG2 G2NF, and IgG3/4 G0). The average AUC was 0.857, which indicated that our application of IgG N-glycopeptide model to predict migraine might be reliable. Moreover, we speculated that the serum N-glycopeptide IgG1 G0-NF might be one of the potential biomarkers for the diagnosis of migraine based on our results, but it needs to be tested in a larger sample.

CONCLUSION

In summary, the IgG N-glycosylation was analyzed in patients with migraine and healthy controls in this study. The change of serum N-glycosylation profiling in patients with migraine and the different migraine subgroups was explored. Besides, a predictive model of migraine was established, showing a good prediction performance. The relationship between IgG N-glycosylation and migraine has not been reported previously; our data show that it was a possible exploration direction. There is also a major limitation in this study that the sample size was inadequacy and the conclusion needs to be tested in a larger sample. But, this preliminary study suggested that the comprehensive IgG N-glycosylation analysis has the potential to identify useful biomarkers and new therapeutic targets for migraine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Review Committee of Peking University People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HG, YH, JX, and XZ designed the study. YH collected the clinical information and blood samples, and performed the pretreatment of blood samples. XZ and YW performed the MALDI-TOF-MS and data analysis. NL and ZL performed the binary classification model construction. JZ given advises for the study design. JX, HG, YH, YW, NL, and XZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.860555/full#supplementary-material

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Early Onset Diffusion Abnormalities in Refractory Headache Disorders

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Objective: This study sought to determine if individuals with medically refractory migraine headache have volume or diffusion abnormalities on neuroimaging compared to neurotypical individuals.

Background: Neuroimaging biomarkers in headache medicine continue to be limited. Early prediction of medically refractory headache and migraine disorders could result in earlier administration of high efficacy therapeutics.

Methods: A single-center, retrospective, case control study was performed. All patients were evaluated clinically between 2014 and 2018. Individuals with medically refractory migraine headache (defined by ICDH-3 criteria) without any other chronic medical diseases were enrolled. Patients had to have failed more than two therapeutics and aura was not exclusionary. The initial MRI study for each patient was reviewed. Multiple brain regions were analyzed for volume and apparent diffusion coefficient values. These were compared to 81 neurotypical control patients.

Results: A total of 79 patients with medically refractory migraine headache were included and compared to 74 neurotypical controls without headache disorders. Time between clinical diagnosis and neuroimaging was a median of 24 months (IQR: 12.0–37.0). Comparison of individuals with medically refractory migraine headache to controls revealed statistically significant differences in median apparent diffusion coefficient (ADC) in multiple brain subregions ($p < 0.001$). *Post-hoc* pair-wise analysis comparing individuals with medically refractory migraine headache to control patients revealed significantly decreased median ADC values for the thalamus, caudate, putamen, pallidum, amygdala, brainstem, and cerebral white matter. No volumetric differences were observed between groups.

Conclusions: In individuals with medically refractory MH, ADC changes are measurable in multiple brain structures at an early age, prior to the failure of multiple pharmacologic interventions and the diagnosis of medically refractory MH. This data supports the hypothesis that structural connectivity issues may predispose some patients toward more medically refractory pain disorders such as MH.

Keywords: migraine, MRI, diffusion imaging, chronic pain, biomarker

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INTRODUCTION

Migraine headache (MH) is a common and chronic condition with multi-factorial neurovascular etiologies characterized by recurrent paroxysmal attacks of throbbing headaches with or without autonomic nervous system dysfunction (1). Along with tension type-headaches (TH), migraines are one of the most frequently occurring neurologic phenomena in children and young adults (2, 3). Beyond pain, these disorders can have dramatic impacts on performance at school and work, causing marked burden of disease in patients suffering from them (4, 5).

Currently, no biomarker is available for predicting which individuals are likely to suffer from medically refractory MH. Previously, patients with MH were identified to have early cerebral diffusion abnormalities on magnetic resonance imaging (MRI) in pain sensitization regions compared to controls (6). However, that study was cross-sectional in nature and did not follow patients longitudinally to determine the outcome or severity of their headache disorders. Furthermore, there was great heterogeneity with respect to when these neuroimaging studies were acquired. Although other studies have identified late micro-structural and connectivity differences amongst individuals with vs. without MH disorders (7–10), no studies have assessed differences in severity in this population or the possibility of early prediction of refractory headache disorders based on imaging.

The aim of this study was to investigate if individuals with medically refractory MH have diffusion or volumetric abnormalities on their earliest neuroimaging study with the goal of identifying if headache disorder disease severity is associated with early neuroimaging abnormalities.

MATERIALS AND METHODS

Standard Protocol Approvals, Registrations, and Patient Consents

All data collection, review and analysis were conducted after approval by the Stanford University institutional review board (No. 36206).

Data Availability

All data is available in an anonymized format to qualified investigators following release approval by the institutional review board.

Study Design

Retrospective, cross-sectional. Patients who had been diagnosed with medically refractory headache disorders were retrospectively assessed for imaging abnormalities on their first lifetime neuroimaging (MRI) study. Imaging data was extracted from the first lifetime neuroimaging study but clinical data was only extracted from the last clinical encounter to ensure capture of most recent headache-related diagnosis.

Abbreviations: ADC, Apparent diffusion coefficient; CMH, Chronic migraine headache; DWI, Diffusion weighted imaging; EMH, Episodic migraine headache; ICHD-3, International Classification of Headache Disorders Version 3.0; MRI, Magnetic resonance imaging; MANCOVA, Multivariate analysis of covariance; MH, Migraine headache; TH, Tension headache.

Inclusion Criteria

Inclusion for the study cohort were: a diagnosis of MH as defined by the International Classification of Headache Disorders Version 3.0 (ICHD-3). (1) patients were sub-grouped into episodic MH (EMH) and chronic (CMH) and MH with or without neurologic aura per ICHD-3 criteria. All types of aura were included for the purposes of this study. All patients required neuroimaging within 18 months of the MH diagnosis, which was acquired on a 3T MRI scanner at the institutions mentioned above for consistency purposes. Prior neuroimaging findings must have been clinically interpreted as normal, which excluded any patients with incidental findings or abnormalities (e.g., T2 signal prolongation of unknown significance—also known as unidentified bright objects), developmental venous anomaly, Chiari I abnormality, etc.).

Control subjects obtained brain MRI at 3T as part of standard of care for evaluation and interpreted by board-certified neuroradiologists to have normal exam. A comprehensive manual chart review was performed to ensure no prior history MH or underlying neurologic, cognitive, or neuropsychiatric disorders, as well as cancer history, or other clinical diseases requiring chronic medical therapies, chemotherapy, or radiation. Clinical reasons for imaging included syncope, nausea, scalp nevus, cholesteatoma, sinus disease, orbital strabismus, and family history of aneurysm, vascular malformation, or cancers. All included cases were reviewed by two authors. In cases where inclusion was discrepant, the senior author served as an arbiter for inclusion/exclusion.

Exclusion Criteria

Strict exclusion criteria were applied and comprised the following: inadequate data or image-registration quality, any concern for co-morbid secondary headache (e.g., use of non-headache related pharmacotherapy with side effect of headache), current or prior history of developmental delay or intellectual disability, history of or active medication-overuse headache, tension-type headache, underlying cardiac disease, underlying pulmonary disease, epilepsy, prior or current hemorrhage, vascular lesions (aneurysm, AVM, fistula, or steno-occlusive disease), or prior strokes, given their potential impact on regional diffusion properties in the brain. Patients were permitted to have failed no greater than one preventative headache therapies prior to neuroimaging. Additionally, any patient with a previously diagnosed genetic, metabolic, or chronic medical disease was excluded from this study. Patients with any focal neurologic findings, even if incidental, were excluded. Patients with incomplete or inconsistent data were also excluded. Patients who had an active headache or migraine at the time of neuroimaging (as documented on the day of encounter on a screening form administered by the radiology technician) were excluded. All included cases were reviewed by two authors. In cases where inclusion was discrepant, the senior author served as an arbiter for inclusion/exclusion.

Clinical Data Collection

All data were collected retrospectively by manual chart review and medication ordering summaries. Clinically related headache

data included family history of headache in a first degree relative, age of onset, the number and types of therapies failed, if opioids were utilized at any time point, ICDH-3 diagnosis type (including status of aura), and medications used at the time of imaging. Medically refractory was determined as having failed at least two preventative headache therapies of three different classes, consistent with the American Headache Society criteria (11). In patients with serial neuroimaging, only the first neuroimaging study was examined for the purposes of this study. Time to last headache was not collected as this was not feasible for the retrospective review, but patients' MR imaging intake records were reviewed to exclude patients with active headache or migraine.

MR Imaging Acquisition

All subjects underwent brain MRI at 3T (Discovery 750W; GE Healthcare, Milwaukee, Wisconsin) with an 8-channel head coil on a single MR imaging scanner. Echo-planar whole-brain diffusion-weighted MRI (DWI) was acquired in all cases with repetition time (TR) = 1,500 ms, echo time (TE) = 37 ms, flip angle = 90°, FOV = 24 cm^2, acceleration factor = 2, in-plane resolution = 0.94 mm^2, acquisition matrix = 128 × 128 interpolated to a 256 × 256 matrix, 44 sections with 4-mm slice thickness, no skip, two diffusion-weightings of b = 0 s/mm^2 and b = 1,000 s/mm^2, with diffusion gradients acquired in 3 directions averaged for the latter. Apparent diffusion coefficient (ADC), derived from DWI, has demonstrated high reproducibility and was performed as part of routine institutional neuroimaging (12). Documentation of imaging encounters were reviewed to ensure patients had no active headache or migraine at the time of scan.

Image Processing

A custom image-processing pipeline was used in this work to extract quantitative values of regional brain volume and ADC values, previously described in more detail by Forkert et al. (13) Briefly described, after motion correction of the DWI dataset acquired with and without diffusion-weighting using rigid registration, the quantitative apparent diffusion coefficient parameter map was calculated by applying the Stejskal-Tanner equation. For regional diffusion and volumetric analysis, the Montreal Neurological Institute-152 brain atlas was non-linearly registered to the DWI dataset and the resulting transformation was used to warp the Harvard-Oxford subcortical atlas brain regions to the subject-specific brain anatomy (14). The Harvard-Oxford brain regions warped to the DWI datasets were used directly for volume assessment and calculation of median ADC values to ensure that the volume and ADC measurements are based on exactly the same brain regions. Gray matter images were unmodulated. Brain regions included in this brain atlas are the cerebral cortex, cerebral white matter, thalamus, caudate, putamen, globus pallidus, amygdala, hippocampus, brain stem, and nucleus accumbens. Two experienced observers (NDF and KWY) checked all registration results to ensure suitable data and registration quality. The aligned brain atlas regions were then used to measure the corresponding regional brain volumes and median ADC values combined for corresponding brain structures in the left and right hemispheres, whereas the lateral ventricles were only used for volumetric assessment.

Statistical Analysis

Multivariate analysis of covariance (MANCOVA) was used for group comparison of the control group and individuals with MH using the volumetric and median ADC values as dependent variables, age as a covariate, duration of symptoms (episodic/chronic) and the class (MH) as the fixed factor. To assess whether diffusion metrics are predictive of migraine status, simple and multiple logistic regression models were constructed. SPSS (Version 24.0, IBM, Armonk, NY) was used for MANCOVA statistical analyses. Graphpad Prism (Version 9.1.1) was used for regression analysis. A P-value < 0.05 (Bonferroni-corrected) was considered significant. To minimize risk of Type 1 error that can occur in the setting of multiple comparisons, we employed the most conservative Bonferroni correction for all of our analyses.

For analyses involving volumetric data, we corrected for 11 tests given that we ran tests for 11 brain regions. For analyses involving diffusion, we corrected for 10 tests given that we ran tests for 10 brain regions.

RESULTS

In total, 112 patients met inclusion criteria. Thirty-three of these patients met at least one exclusion criteria, leading to 79 patients for analysis (70%). Of those excluded, the most frequent reasons were incomplete data ($n = 15$), diagnosis of an alternative exclusionary type of headache ($n = 8$), incidental imaging finding ($n = 4$), and corrupted imaging sequences ($n = 4$). Cohen's kappa

TABLE 1 | Clinical and Demographic Data.

Characteristics	Control ($n = 74$)	Migraine ($n = 79$)
Median age (IQR: 25th– 75th)[a]	20.5 (16.0–26.0)	22.3 (17.5–26.5)
Median age at onset (IQR: 25th–75th)		13.0 (10.5–15.0)
Sex (n, %)[b]	24 (32.4)	18 (22.8)
M		
F	50 (67.6)	61 (77.2)
First degree relative with migraine		34 (43.0)
Migraine Type—no. (%)		27 (34.2)
Episodic without Aura		
Episodic with Aura		10 (12.7)
Chronic without Aura		28 (35.4)
Chronic with Aura		14 (17.7)
Median number of medications tried (IQR: 25th–75th)		2.0 (0.5–3.5)
Episodic without Aura		
Episodic with Aura		3.5 (0.5–7.3)
Chronic without Aura		12.0 (7.5–14.0)
Chronic with Aura		15.5 (8.3–19.5)

[a] *T-value = 1.175, P-value = 0.242.*
[b] *Fisher's exact test P-value = 0.207.*

for inter-rate agreement for application of inclusion/exclusion was 0.99 (one disagreement). A total of 84 patients were identified for the control arm of this study with 74 (88%) meeting no exclusionary criteria. Demographic data is presented in **Table 1**. The median age of the MH cohort was 22.3 (IQR: 17.5–26.5) compared to the control cohort with a median age of 20.5 years (IQR: 16.0–26.0). Seventy-seven percent ($n = 61$) of patients in the MH group were female compared to 67% ($n = 50$) in the control group. Patients with MH were classified as either episodic or chronic per ICDH-3 criteria (1). Thirty-seven patients had episodic MH and 42 patients had chronic MH. Ten patients with episodic MH had associated aura compared with 14 chronic MH patients. The median age of onset for patients was 13 years (IQR: 10.0–13.0) with the median time between diagnosis and first MRI being 24 months (IQR: 12.0–37.0).

There was a statistically significant difference between the average number of first-degree relatives with MH who had EMH (0.43) and CMH (0.76) after controlling for the covariate effects of age and sex ($p = 0.034$, 95th% CI: 10.6–13.5, **Figure 1A**). Neither age nor sex was significantly related to the number of relatives with migraine history.

The median number of medication failures for all patients was 6 (IQR: 3.0–12.0). Patients with EMH without aura tried a median of 2.0 medications, while those with aura tried a median of 3.5 medications. Patients with CMH without aura tried a median of 12.0 medications while those with aura tried a median of 15.5 medications (**Figure 1B**). There was a statistically significant difference between the number of medications tried by individuals with EMH compared to CMH after controlling for the covariate effects of age and sex ($p < 0.001$, 95% CI: 0.17–0.74). Neither age nor sex were found to be significant covariates regarding to the number of medications tried.

Comparison of individuals with MH to controls revealed statistically significant differences in median ADC in multiple brain subregions ($p < 0.001$, **Figures 2**, **3**). *Post-hoc* pair-wise analysis comparing the migraine to control patients revealed significantly decreased median ADC values for the thalamus, caudate, putamen, pallidum, amygdala, brainstem, and cerebral white matter (**Table 2**). The nucleus accumbens, cerebral cortex and hippocampus did not display statistically significant differences in ADC although similar trends in lower ADC were present in individuals with migraine. Simple and multiple logistic regression models were constructed to assess predictive ability of diffusion in the seven significant brain regions, though area under the ROC curve (AUC) values associated with these models were suggestive of poor discrimination (range: 0.55–0.70) (**Supplementary Figure S1**). No volumetric differences were observed between groups.

Secondary analyses revealed differences between MH sub-groups. Compared to controls, patients with CMH had lower median ADC values in the thalamus, putamen, pallidum, amygdala, brainstem, and cerebral white matter. Compared to controls, individuals with EMH showed higher median ADC in the thalamus (**Table 3**). There were no statistically significant ADC differences between individuals with CMH and EMH. No volumetric differences were observed between MH sub-groups and controls. Individuals with or without aura did not show any statistically significant diffusion differences, either when compared to each other or sub-type (e.g., EMH with aura vs. EMH without aura).

DISCUSSION

This study builds on prior work identifying early ADC diffusion changes in several areas of the limbic and pain systems of the central nervous system in patients with migraine (**Figure 4**) (6). The main contribution of this study is the finding that in individuals with medically refractory MH, these changes are measurable in additional brain structures at an early age, *prior to* the failure of multiple pharmacologic interventions and the diagnosis of medically refractory MH. This data supports the hypothesis that structural connectivity issues may predispose some patients toward more medically refractory pain disorders such as MH.

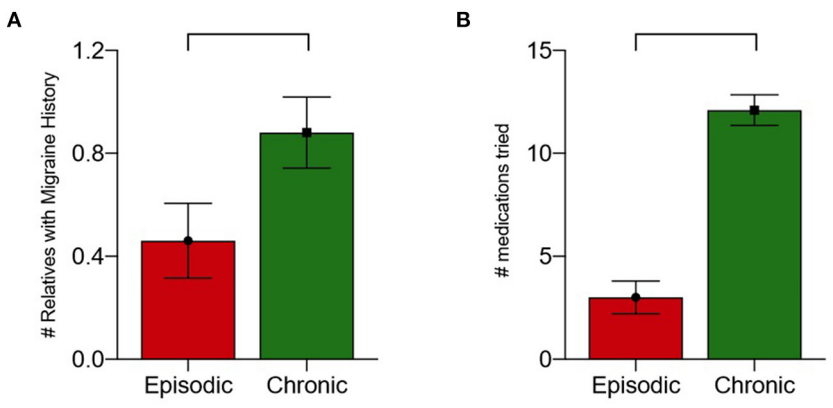

FIGURE 1 | (A) Number of first-degree relatives with migraine history by migraine type with correction for age and sex ($p = 0.035$). **(B)** Number of medications tried by migraine type ($p < 0.001$).

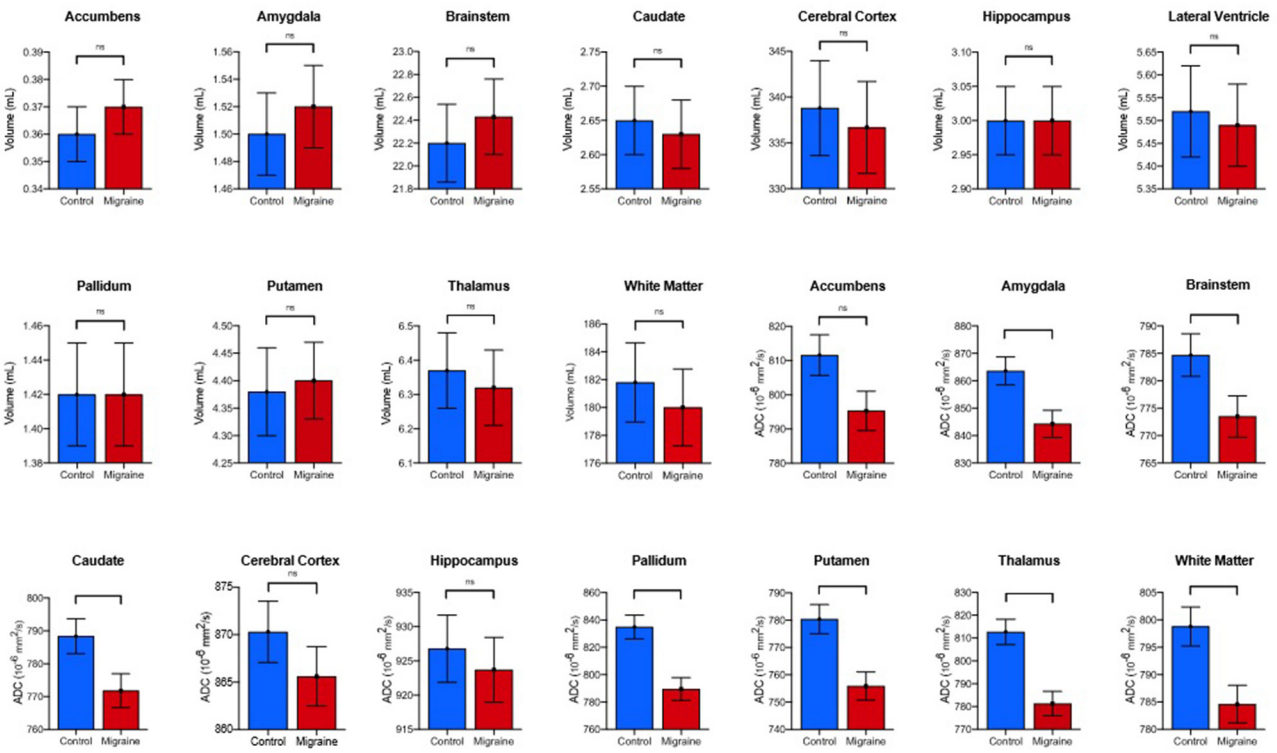

FIGURE 2 | Comparison of estimated marginal means and standard error means of volume and diffusion in individuals with MTH vs. controls. *P*-values and significance generated from *post-hoc* pair-wise analysis as denoted as follows: ns, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Bonferroni-corrected).

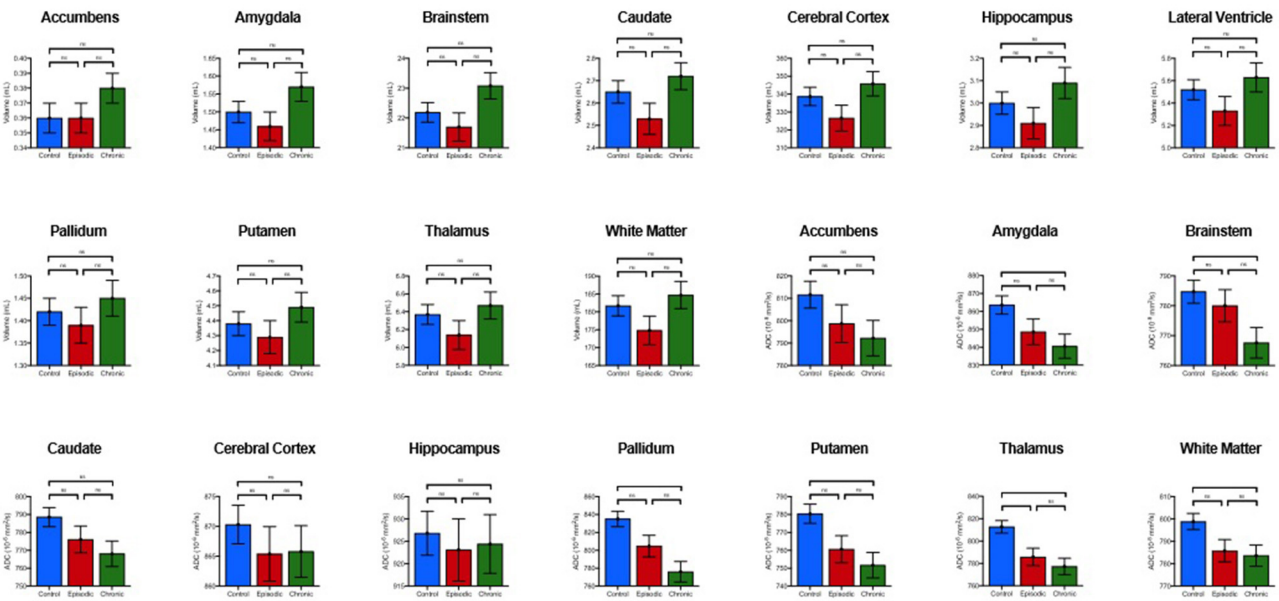

FIGURE 3 | Comparison of estimated marginal means and standard en-or means of volume and diffusion amongst chronic MTH, episodic MTH and controls. *P*-values and significance generated from *post-hoc* pair-wise analysis as denoted as follows: ns, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Bonferroni-corrected).

Diffusion abnormalities of the limbic and pain sensitization structures (thalamus, caudate, putamen, pallidum, amygdala, brainstem, and cerebral white matter) were observed in individuals with MH. These structures have been previously

TABLE 2 | Volumetric and apparent diffusion coefficient values by brain region.

	Control ($N = 74$)				Migraine ($N = 79$)				Univariate test	
	Mean[a]	SE	Lower 95% CI	Upper 95% CI	Mean[a]	SE	Lower 95% CI	Upper 95% CI	Mean Diff.	*p*-value[b]
Volume (mL)										
Cerebral white matter	181.8	2.85	176.1	187.4	180.0	2.76	174.5	185.4	−1.78	0.656
Cerebral cortex	338.8	5.17	328.6	349.0	336.7	5.00	326.9	346.6	−2.05	0.777
Lateral ventricle	5.52	0.10	5.33	5.70	5.49	0.09	5.31	5.67	−0.03	0.833
Thalamus	6.37	0.11	6.15	6.59	6.32	0.11	6.10	6.53	−0.06	0.728
Caudate	2.65	0.05	2.55	2.75	2.63	0.05	2.53	2.72	−0.02	0.731
Putamen	4.38	0.08	4.23	4.53	4.40	0.07	4.25	4.54	0.02	0.873
Pallidum	1.42	0.03	1.36	1.47	1.42	0.03	1.37	1.47	0.00	0.943
Hippocampus	3.00	0.05	2.90	3.10	3.00	0.05	2.91	3.10	0.00	0.955
Amygdala	1.50	0.03	1.44	1.55	1.52	0.03	1.46	1.57	0.02	0.641
Accumbens	0.36	0.01	0.35	0.38	0.37	0.01	0.35	0.39	0.00	0.760
Brainstem	22.20	0.34	21.53	22.86	22.43	0.33	21.79	23.08	0.23	0.622
Median ADC (10^{-6} mm^2/s)										
Cerebral white matter	798.8	3.57	791.7	805.8	784.6	3.45	777.8	791.5	−14.15	**0.005**[c]
Cerebral cortex	870.3	3.23	863.9	876.7	865.6	3.13	859.5	871.8	−4.64	0.307
Thalamus	812.7	5.51	801.8	823.6	781.3	5.33	770.8	791.8	−31.41	**<0.001**[c]
Caudate	788.4	5.33	777.9	799.0	771.8	5.15	761.6	782.0	−16.63	**0.027**[c]
Putamen	780.4	5.34	769.9	791.0	755.9	5.17	745.7	766.1	−24.53	**0.001**[c]
Pallidum	834.9	8.68	817.7	852.1	789.5	8.40	772.9	806.1	−45.40	**<0.001**[c]
Hippocampus	926.8	4.90	917.1	936.5	923.7	4.74	914.4	933.1	−3.03	0.659
Amygdala	863.6	5.09	853.5	873.7	844.3	4.93	834.6	854.1	−19.26	**0.008**[c]
Accumbens	811.6	5.94	800.0	823.3	795.3	5.75	783.9	806.6	−16.33	0.051
Brainstem	784.7	3.88	777.0	792.4	773.5	3.76	766.1	780.9	−11.22	**0.041**[c]

[a] *Covariates appearing in the model are evaluated at the following values: age (years) at time of MRI = 21.71, sex (1 as female) = 0.73.*

[b] *Based on the linearly independent pairwise comparisons among the estimated marginal means.*

[c] *$P < 0.05$ for statistical significance, Bonferroni-corrected. Bolded items correspond to a p values < 0.05.*

implicated in resting-state functional MRI studies in individuals with CMH, each with a unique role in pain processing (15). However, functional neuroimaging is not a clinical standard of care, highlighting the importance of easy-to-use methods of assessment reported in this study which can be added on to routine neuroimaging sequences with minimal cost. Regression analysis did not reveal a predictive model for type of headache or migraine diagnosis later in life although sub-group analysis limited the power to assess these findings. Ultimately, greater patient volume in each group will be needed to power a clinical predictive measure that could inform clinical decision making and is an area under evaluation by the research team.

The neuroanatomic regions identified as abnormal in this study each have independent yet interlinked roles in pain processing. The amygdala has been implicated in pro-nociceptive functionality in addition to perpetuating the cortex-driven pain association (16–19). It has been reported that prolonged potentiation of the nociceptive information can be caused by aberrant activation of the amygdala (20), potentially providing insight into those at risk for more medically refractory pain disorders. Thalamic activation in pain is widely recognized. Multiple studies have identified abnormal connectivity of the thalamus to limbic and cortical structures in individuals with MH (19, 21–23). It has also been hypothesized that the neurocognitive impact of migraines (extreme fatigue, poor concentration, and sensitivity to external stimuli) may also be implicated through dysfunctional thalamic circuitry (19, 23, 24). Due to their high connectivity, thalamic circuits are tightly interwoven with the structures of the basal ganglia and brainstem, which have all also been implicated in chronic pain disorders (19, 25–29). Although the significance of diffusion abnormalities in the cerebral white matter is more difficult to ascertain, there is emerging data that insular white matter may be implicated in increased nociceptive perception as well (19, 30, 31). Although this study was underpowered to evaluate differences between patients with and without aura, individuals with MH have been hypothesized to have white matter insult caused by microvascular ischemic changes, which occur during migraines (32). Although the complexities of pain and nociception in the CNS are extraordinarily complex, this study identifies abnormalities in nearly all of these structures in individuals with the most severe MH, highlighting the possible utility of diffusion neuroimaging to identify patients at risk for medically refractory courses at an early stage. Although further study would be needed, it could be hypothesized that this group of individuals may benefit from more early and aggressive therapeutic interventions.

TABLE 3 | Volumetric and apparent diffusion coefficient values by migraine type and brain region.

	Control ($N = 74$)		Episodic migraine ($N = 37$)		Chronic migraine ($N = 42$)		Univariate test[b]		
	Mean[a]	SE	Mean[a]	SE	Mean[a]	SE	*p*-value[c]	*p*-value[d]	*p*-value[e]
Volume (mL)									
Cerebral white matter	181.7	2.83	174.8	3.98	184.7	3.77	0.469	1.000	0.218
Cerebral cortex	338.7	5.12	326.6	7.21	345.8	6.81	0.520	1.000	0.164
Lateral ventricle	5.52	0.09	5.33	0.13	5.63	0.13	0.746	1.000	0.286
Thalamus	6.37	0.11	6.14	0.16	6.47	0.15	0.712	1.000	0.379
Caudate	2.65	0.05	2.53	0.07	2.72	0.06	0.441	1.000	0.141
Putamen	4.38	0.08	4.29	0.11	4.49	0.10	1.000	1.000	0.488
Pallidum	1.42	0.03	1.39	0.04	1.45	0.04	1.000	1.000	0.864
Hippocampus	3.00	0.05	2.91	0.07	3.09	0.07	0.864	0.834	0.180
Amygdala	1.50	0.03	1.46	0.04	1.57	0.04	1.000	0.425	0.152
Accumbens	0.36	0.01	0.36	0.01	0.38	0.01	1.000	1.000	0.698
Brainstem	22.19	0.33	21.70	0.47	23.08	0.44	1.000	0.338	0.101
Median ADC (10^{-6} mm^2/s)									
Cerebral white matter	798.8	3.58	785.8	5.03	783.6	4.76	0.113	**0.036**[f]	1.000
Cerebral cortex	870.3	3.23	865.4	4.56	865.8	4.31	1.000	1.000	1.000
Thalamus	812.7	5.52	785.8	7.76	777.3	7.34	**0.016**[f]	**<0.001**[f]	1.000
Caudate	788.5	5.33	776.0	7.50	768.0	7.09	0.537	0.071	1.000
Putamen	780.4	5.35	760.6	7.53	751.7	7.11	0.099	**0.005**[f]	1.000
Pallidum	835.0	8.62	804.7	12.13	776.0	11.47	0.131	**<0.001**[f]	0.262
Hippocampus	926.8	4.91	923.1	6.92	924.4	6.54	1.000	1.000	1.000
Amygdala	863.6	5.10	848.6	7.18	840.6	6.78	0.269	**0.023**[f]	1.000
Accumbens	811.6	5.96	798.7	8.38	792.2	7.92	0.637	0.159	1.000
Brainstem	784.7	3.86	780.0	5.43	767.6	5.14	1.000	**0.027**[f]	0.296

[a] *Covariates appearing in the model are evaluated at the following values: age (years) at time of MRI = 21.71, sex (1 as female) = 0.73.*
[b] *Based on the linearly independent pairwise comparisons among the estimated marginal means.*
[c] *Episodic—Control.*
[d] *Chronic—Control.*
[e] *Chronic—Episodic.*
[f] *$P < 0.05$ for statistical significance, Bonferroni-corrected. Bolded items correspond to a p values < 0.05.*

Individuals with CMH had lower median ADC values in the thalamus, putamen, pallidum, amygdala, brainstem, and cerebral white matter compared to EMH and controls. These findings are clinically relevant for two reasons. First, it indicates that the neuroimaging abnormalities found in patients with MH may be mostly driven by individuals with CMH. Second, it highlights that pain sensitization centers such as the thalamus, amygdala, and brainstem show micro-structural changes as measured by DWI in individuals with severe and chronic disease even at an early age. It is possible that these areas may also show atrophy (macro-structural changes) over time (33) as well although given the infrequency of clinically indicated repeat neuroimaging in patients with well-established CMH, this analysis was not feasible for this study.

This study is not without limitations. First, this is a retrospective study analyzing patients for imaging abnormalities after their diagnosis has been made, introducing the possibility of observer bias. This was mitigated by having the clinical data extraction and analysis be performed independently by the authors. This study was performed using single-center data, which may limit its generalizability. As a retrospective chart-based review, there is the risk of diagnostic inaccuracy with regards to type of MH although this was mitigated by ensuring all patients met ICHD-3 criteria. In addition, patients had been seen by multiple providers who had different mechanisms of reporting clinical features of their patients and as such, data on clinical phenotypes was far too incomplete for analysis. As previous studies have indicated that diffusion findings may be transient in adults, whether the patient was having a headache at the time of scanning or near the time of scanning may affect the quantitative imaging findings (34–36). The authors did note that total number of failed pharmacotherapeutics was higher in the CMH group. This is logical given the greater disease burden although the authors cannot rule out an impact on total pharmacotherapy exposure and longstanding diffusion changes. It is impossible to rule out the effect of active or previously attempted pharmacotherapy on neuroimaging findings in this study. The authors attempted to sub-analyze individuals with similar active and historical treatments but given the heterogeneity in headache management, total patients in each group was well below any

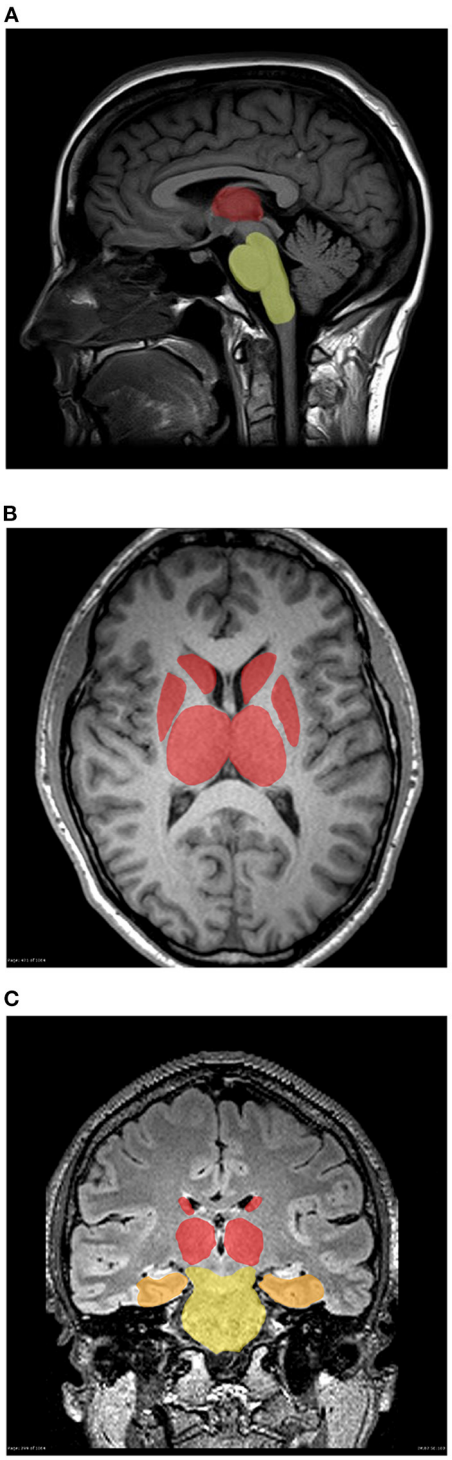

FIGURE 4 | (A–C) Three-dimensional mapping of deep brain structure demonstrating gradient of smallest to greatest diffusion abnormalities in individuals with migraine vs. controls **(A)** Sagital, **(B)** Axial, and **(C)** Coronal. Brainstem (yellow), amygdala (orange), thalamus, putamen and pallidum (red).

ability to statistically analyze. Prospective studies will be needed to address this variable. Although an ideal comparator group for patients with medically refractory headaches would have been patients with medication responsive headaches, there was insignificant numbers of individuals with early neuroimaging in this latter group. In addition, severity bias in these individuals would have interfered with interpretation of data. Future, prospective studies, by the authorship group will focus having a dedicated "non-refractory" group to serve as an additional comparator arm to neurotypical controls. Further, this study did not evaluate neuroimaging findings in other individuals with chronic, non-headache, pain disorders. While the findings in this study are presumed to be specific to headache and migraine, it is possible that other chronic pain disorders (e.g., fibromyalgia) could produce similar findings and is a logical next step for this study group to investigate. For this analysis, we combined data from both the left and right hemispheres to reduce the number of hypotheses tested, which omitted trends in laterality. In addition, we recognize that segmentation of small cerebral structures can be imperfect, but an automated approach (with visual quality control) was used to ensure reproducibility, given that manual segmentation is prone to observer bias. Another limitation of this study is that we did not compare this data of individuals with MH to patients with TH. Given the lower acuity, infrequency of utilization of preventative pharmacotherapy, infrequency of neuroimaging, and higher likelihood of mixed or secondary headaches, such an analysis of this population would be inferior for the purposes of identifying imaging abnormalities in the most medically refractory headache disorders. Finally, we excluded four patients for having incidental neuroimaging findings (all punctate T2 signal prolongations of unclear significance), which may have skewed our severity toward less impacted individuals.

CONCLUSIONS

This study identifies early cerebral diffusion changes in individuals with medically refractory MH compared to healthy controls years before therapeutic failures. The hypothesized underlying pathophysiologic mechanisms of nociception and pain sensitization in MH are probable explanations for the observed neuroimaging abnormalities. Further study is needed to investigate the predictive value of these identified diffusion abnormalities.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Stanford University: 0456218. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JS and PM were responsible for drafting and revision of the manuscript for content, including medical writing for content. He had a major role in the acquisition of data. He was responsible for study concept and design and analysis and interpretation of data. MH was responsible for acquisition of the data, analysis, and interpretation of the data. EM played a major role in the acquisition of the data and assisted with the interpretation of data. She also revised and edited the manuscript for intellectual content. ET was responsible for drafting and revising the manuscript for intellectual content and supervised data collection. She also assisted with analysis and interpretation of the data. SM was responsible for study concept and design. She assisted with analysis and interpretation of the data and assisted with revision and editing of the manuscript for intellectual content. NF was responsible for drafting and revision of the manuscript for content, including medical writing for content. He had a major role in the acquisition of data and supervised data collection. He was responsible for study concept and design and analysis and interpretation of data. KY was responsible for drafting and revision of the manuscript for content, including medical writing for content. She had a major role in the acquisition of data and supervised data collection. She was responsible for study concept and design and analysis and interpretation of data. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.898219/full#supplementary-material

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OPINION
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Prioritizing Suggestive Candidate Genes in Migraine: An Opinion

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Keywords: GWAS, bioinformatics, gene prioritization, headache, migraine, causation, pathophysiology, membrane potential

INTRODUCTION

The search for the underlying causes of migraine has been ongoing for decades, with genome-wide association studies (GWASs) enabling the discovery of common single nucleotide polymorphisms (SNPs) associated with this disorder, along with suggestive candidate genes (examples include PHACTR1, TRPM8 and PRDM16) (1–3). Suggestive candidate genes have predominantly been selected based on their genomic location and on expert knowledge. The term "*suggestive candidate gene*" reveals the level of evidence of the finding (i.e., suggesting a link between a gene and condition) thereby indicating that validation is necessary.

Gene prioritization, especially relying on computationally-intensive multi-omics analyses [e.g., weighing score based on evidence source (4)], has been used to help identify candidate genes truly associated with a condition (5–7). Gene prioritization is conducted to rank *"genes according to their likelihood of being associated with the disease"* and thus researchers can distinguish between credible and non-credible suggestive candidate genes, and thus select the most credible genes to further study (8). Meta-analyses of GWASs have also helped to confirm findings (2, 3). Even though some SNP-condition associations are non-reproducible, it is not enough evidence to rule out those findings. As we know, many common conditions are multifactorial in nature and the genetic architecture and regulatory networks differ between individual patients (9, 10).

Despite of the initial methodologies used to identify suggestive candidate genes being similar across migraine GWASs, the use of downstream gene prioritization varies. This may in part be explained by the continuous advancements of bioinformatics tools over time, but may also be explained by a lack of defined systematic gene prioritization efforts, particularly focused on causality. Adding an additional gene prioritization step in future GWASs to further prioritize identified suggestive candidate genes may (i) reduce the reporting of false positives, and (ii) enhance our etiological understanding of the disorder. As the number of suggestive candidate genes increases together with the number of publications, there is a growing need for valid gene prioritization (11). Here, the current and potential future state of gene prioritization in migraine GWASs will be discussed.

GENE PRIORITIZATION IN MIGRAINE

Depending on the study objective, gene prioritization might help to answer the question: *"What is the likelihood of the suggestive candidate genes truly causing common migraine?"* Yet, here it is important to keep in mind that evidence points to a multifactorial etiology of common migraine (12), and that the causes of common migraine are largely unknown.

Further Validation of Suggestive Candidate Genes Needed

GWASs have provided some clues about the migraine etiology, particularly at the SNP level. One limitation of this approach is the uncertainty of causality. For instance, the genotyped SNPs found

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to be significantly associated with migraine might be in linkage disequilibrium (LD) with the causal variants, rather than being causal themselves, and the LD structure might contain numerous genes (13, 14). Researchers have sought to find out how these SNPs may be associated with the disorder, and have frequently looked into whether those SNPs are located in coding or non-coding regions. If located in a non-coding region, suggestive candidate genes have primarily been identified focusing on the genes located nearest to the SNPs or on functionally-relevant genes in the proximate genomic region of the SNPs. It has however been found that *"only about one-third of causal genes are the nearest gene to the GWAS hit"* (13), and the implication of non-coding variants is rarely studied. So, even though the GWAS methodology itself is hypothesis-free, the identification of suggestive candidate genes has predominantly been hypothesis-driven. Findings from GWAS in migraine have been discussed by van den Maagdenberg and colleagues (15).

When examining existing migraine GWASs, suggestive candidate genes have primarily been identified by (i) examining genes in proximate genomic region (2, 3, 16–21), (ii) reporting the genes for coding SNPs (2, 3, 19, 20, 22, 23), (iii) reporting nearest gene (21, 22), and (iv) using LD analysis outputs for guidance (2, 16–19, 24). These methodologies cannot be used to infer causality, and selection of suggestive candidate genes in the proximate genomic regions of SNPs of interest is generally based on expert knowledge (and today's knowledge). Therefore, GWAS findings may be biased toward the perspectives held by those experts. For example, in addition to migraine being described as a neurovascular disorder, several other theories have been proposed throughout the years. Recently, researchers have started to describe migraine as a purely neurological condition (e.g., with *"primarily neuronal origin with the vascular manifestations"*) (25). Other theories have arisen throughout the past decade where researchers present migraine as a neuro-glio-vascular disorder (26) or dysfunctional neurolimbic pain network (27).

To account for some of these limitations, additional gene prioritization has been conducted in some migraine GWASs. Examples of applied downstream gene prioritization methodologies include tissue-based gene expression analysis (3), and expression quantitative trait locus (eQTL) analysis using human control tissues [e.g., umbilical cords (16), cerebellum and frontal cortex (3), thyroid and brain (17)]. Despite of the use of some advanced tools to prioritize suggestive candidate genes in migraine GWASs, there is still a gap in gene prioritization efforts that need to be addressed (e.g., causality is not thoroughly examined). The existence of this gap can in part be explained by the difficulty in obtaining relevant omics data of diseased tissue, especially for neurological conditions.

Additional Gene Prioritization Step in Future GWASs

Due to the emergence of advanced bioinformatics tools, gene prioritization in GWASs can be taken a step further. This opportunity is important to consider as the combination of GWAS and eQTL does not inform us about whether *"gene expression and the trait are affected by the same underlying causal variant"* as stated by Zhu and colleagues (28). Causality cannot be inferred. Referring to the disease-associated loci, Cano-Gamez and Trynka state that *"it is unclear which genes they regulate"* (29).

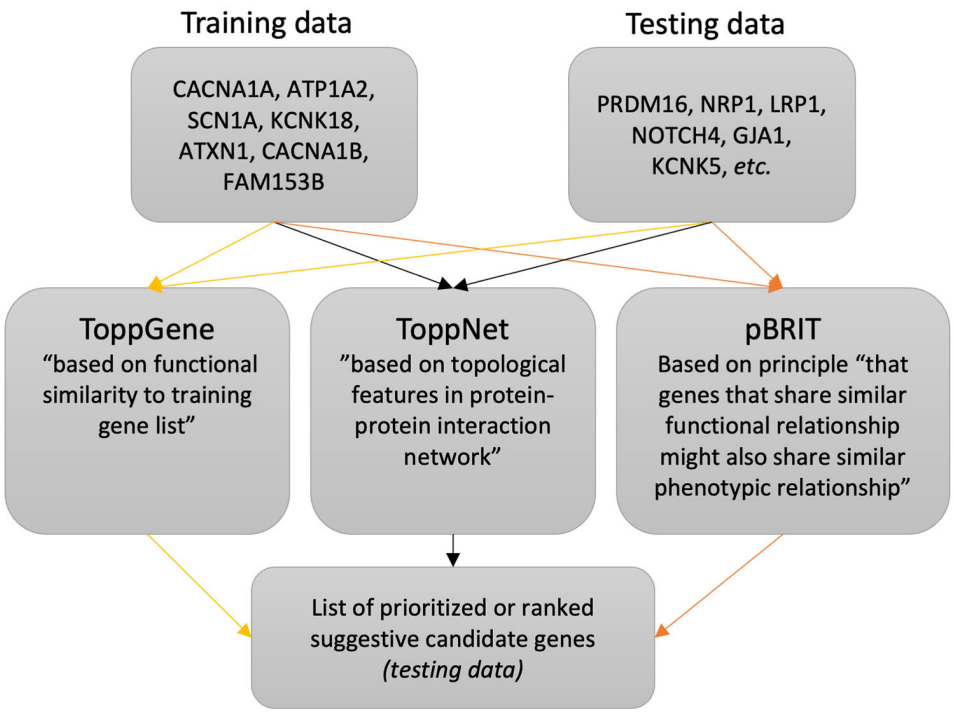
There are several other reasons why filtering of the list of suggestive candidate genes is important, including (i) evidence sources such as the GWAS catalog (30, 31) are used in downstream bioinformatics analysis to examine potential involvement of genes in disease and (ii) researchers want to reveal how the genetic background of an individual influences their biological functions and disease susceptibility. If the cause(s) of a disorder is known, health professionals can provide more targeted treatment instead of just trying to manage symptoms. Importantly, applying our knowledge about genetic causes of familial/monogenic migraine may help us separate signal from noise among the GWAS findings (as causality in these cases have been established), and thus examine the clinical relevance of suggestive candidate genes in common migraine.

Currently, we know of the following monogenic forms of migraine: Familial hemiplegic migraine type 1 (FHM1; mutations in the calcium channel gene CACNA1A), type 2 (FHM2; mutations in the sodium/potassium-transporting ATPase gene ATP1A2) and type 3 (FHM3; mutations in the sodium channel gene SCN1A) (32, 33). Those genes all seem to affect neurotransmission, susceptibility for cortical spreading depression and cognitive function (34–42). Among families with migraine, mutations in several other genes, such as KCNK18 (potassium channel gene), ATXN1 (chromatin-binding factor gene) and CACNA1B (calcium channel gene), have been found (32, 43, 44). These six genes are involved in regulation of membrane potential (GO:0042391), based on ToppGene [a candidate gene prioritization tool] phenotype and functional annotations (45).

The first step toward conducting additional gene prioritization in future GWASs is to understand each component of a gene prioritization tool. A gene prioritization tool *"represents a unique combination of evidence sources, prioritization strategy and input requirements"*, as defined by Zolotareva and Kleine (46). Testing data, training data and evidence sources are used as inputs. Training data (genes used to prioritize) has previously been created based on established genes underlying familial forms of a disease, for example for Alzheimer's disease (46). To obtain training data, a list of genes previously linked to migraine (e.g., causative rather than susceptible) can be created based on the biomedical literature (46–48). Information stored in databases such as ClinVar (focus on genomic variation in human health) (49) and OMIM (based on reviews of the biomedical literature by experts) (50, 51) can also be utilized to enhance the decision process.

When using gene prioritization tools like ToppGene (45), ToppNet (45) or pBRIT (52), the user also needs to define the testing data (genes to prioritize). To obtain testing data, identified suggestive candidate genes in migraine GWAS can be used. Alternatively, the complete list of suggestive candidate genes can be identified through the NHGRI-EBI GWAS catalog (i.e., a curated collection of all published human GWAS) and corresponding R package gwasrapidd (30, 31). For some tools, the user can adjust training parameters (or use default settings). If using ToppGene, this includes choosing evidence

A



B

	ToppGene	ToppNet	pBRIT
GJA1	**2**	6	2
LRP1	**4**	5	9
YAP1	**8**	4	15
TGFBR2	**6**	1	22
NRP1	**14**	10	6
REST	**15**	12	5
JPH3	**17**	14	4
TRPM8	**5**	35	3
PRDM16	**18**	16	10
KCNK5	**1**	43	1
MTDH	**23**	8	19
USP9X	**12**	9	29
JAG1	**9**	33	11
NGF	**3**	45	7
NOTCH4	**21**	30	8
SLC24A3	**10**	47	17
UFL1	**31**	3	47
FGF23	**7**	58	33
SDR9C7	**48**	2	61
MOAP1	**61**	7	56

FIGURE 1 | Proposed gene prioritization for future GWASs. **(A)** Suggested gene prioritization workflow. According to the described approach (i.e., creating training data focused on known familial migraine genes), the gene prioritization strategy will seek to prioritize suggestive candidate genes in relation to the genes forming the training data. This prioritization may be based on features such as similarity (e.g., focused on genetic sequence, involvement in biological pathways, or accompanying

(Continued)

FIGURE 1 | phenotypes) and/or proximity (e.g., in PPI network or focused on gene location and linkage) (46). Other tools then those described here exist (46, 53, 54). **(B)** Example of ToppGene (45), ToppNet (45), and pBRIT (52) outputs showing a selection of ranked suggestive candidate genes from migraine GWASs. Online resources: https://toppgene.cchmc.org/; http://143.169.238.105/pbrit/.

sources/features (e.g., the Gene Ontology (GO) resource to explore gene functions (53), and PubMed to explore the biomedical literature). Overall, evidence sources (together with computational approaches) have been used to estimate gene similarity/proximity focused on the testing and training data (46).

This proposed additional gene prioritization step is visualized in **Figure 1A**.

Gene Prioritization Efforts in Existing GWASs

Use of established gene prioritization tools can help us to more confidently predict whether a suggestive candidate gene is credible or not or, more likely, to uncover how credible a suggestive candidate gene might be (i.e., ranking by score). This may help us to facilitate the selection of genes that are most likely to be associated with the migraine, beyond the capabilities of expression data.

The suggestive candidate genes GJA1 and KCNK5 (2) (rarely reported in migraine GWASs) ranked in the top 2 based on ToppGene and pBRIT outputs (**Figure 1B**; being mindful that databases continuously get updated). Both genes are involved in regulation of membrane potential (GO:0042391) as are the majority of genes known to cause familial/monogenic migraine. Yet, Gormley and colleagues stated that *"loci identified to date do not support the idea of common variants in ion channel genes being strong susceptibility components in prevalent forms of migraine"* (2). However, recent migraine GWAS findings point in another direction. Hautakangas and colleagues found a risk variant in CACNA1A that seemed to be specific for migraine with aura, and stated that *"CACNA1A seems involved in both monogenic and polygenic forms of migraine"* (55).

This indicates that the proposed gene prioritization step (**Figure 1A**) is likely to be beneficial for future migraine GWASs.

DISCUSSION

Here, use of gene prioritization to score and rank suggestive candidate genes in migraine was discussed. In some migraine GWASs, expression data from control human tissues (difficulty in obtaining diseased human brain tissues) have been used to prioritize suggestive candidate genes. Even if diseased human brain tissues were used, such analysis is not able to infer causality of the genetic variants. Hence, the overall goal with this opinion piece is to advance the conversation about gene prioritization in GWAS, presented from the perspective of migraine.

As we already know of genes implicated in the causation of familial/monogenic migraine, this information may have a role to play when prioritizing suggestive candidate genes in future migraine GWASs. Our knowledge about familial/monogenic migraine (e.g., hallmarks of less prevalent migraine types) can potentially help us to better understand underlying causes of common migraine. One question worth answering is *"does common migraine share genetic risk factors with familial/monogenic migraine?"*. Recent evidence points to some degree of shared genetic risk factors (55).

When using gene prioritization approaches, one needs to pay attention to limitations. For example, there may be a difference in prioritization performance between monogenic and polygenic disorders (focusing on predicting novel disease genes) (56), potentially due to *"the assumption of functional coherence among genes contributing to the same disease"* and the fact that *"complex diseases tend to perturb multiple biological processes"*, as stated by Linghu et al. (56). Moreover, the choice of gene prioritization tool(s) and the combination of gene prioritization components (evidence sources, prioritization strategy and input requirements) is key to enhance accuracy and precision. So, how do you best separate signal from noise?

The proposed gene prioritization approach is likely to be relevant for other fields, and could be used beyond that of causation. For example, the gene prioritization could be conducted from the perspective of disorder chronification or treatment effectiveness which then will guide the creation of training data (i.e., genes used to prioritize).

AUTHOR CONTRIBUTIONS

SF conceptualized the opinion piece and wrote the manuscript.

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REVIEW
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CGRP and Migraine: What Have We Learned From Measuring CGRP in Migraine Patients So Far?

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The multi-functional neuropeptide calcitonin gene-related peptide (CGRP) plays a major role in the pathophysiology of migraine. The detection of elevated CGRP levels during acute migraine headache was the first evidence of the importance of the peptide. Since then, elevated CGRP levels have been detected not only during spontaneous and experimentally induced migraine attacks but also interictally. However, the detection of CGRP in peripheral blood shows conflicting results. In this respect, alternative detection methods are needed and have been already proposed. This article summarizes what we have learned from studies investigating CGRP in jugular and peripheral blood and reviews the latest state of research concerning the detection of CGRP in saliva and tear fluid as well as their contribution to our understanding of migraine pathophysiology.

Keywords: migraine, headache, calcitonin gene-related peptide, neuropeptide, trigeminal system

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INTRODUCTION

Migraine is a highly prevalent disorder with a complex pathophysiology involving the peripheral and central nervous system (1–4). Although many aspects of the pathophysiology remain elusive the importance of the trigemino-vascular system (TVS) with its peripheral and central afferents connecting intracranial vasculature and meninges to the brainstem plays a key role in the generation of migraine pain (5–7). Activation of the trigeminal system leads to release of vasoactive neuropeptides, in particular calcitonin gene-related peptide (CGRP), followed by neurogenic inflammation, nociceptive modulation and peripheral and central sensitization (4, 8). The importance of CGRP in migraine pathophysiology is highly supported by different research results:

(1) CGRP levels are elevated in ictal (during the migraine attack) and interictal (48–72 h headache and medication-free) migraine patients (2),
(2) CGRP levels are reduced after abortive and prophylactic treatment (2),
(3) CGRP can induce migraine-like headaches in migraine patients (9, 10),
(4) CGRP antagonists and CGRP antibodies are effective abortive and prophylactic migraine treatments, respectively (11, 12).

This article reviews our current understanding of CGRP in migraine pathophysiology. It focuses on studies investigating CGRP in different migraine states and discusses what we have learned from measuring CGRP as a marker for migraine.

CALCITONIN GENE-RELATED PEPTIDE AND THE CGRP RECEPTOR IN THE NERVOUS SYSTEM

Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid regulatory neuropeptide and potent microvascular vasodilator that was first described in 1982 (13). It belongs to the calcitonin family comprising calcitonin, adrenomedullin, adrenomedullin 2 (intermedin) and amylin (14). In humans, two forms, α-CGRP and β-CGRP, are described (15). They show structural similarity and share 94% homology as well as identical binding affinity and intensity (13, 16). Here, the term CGRP will be used, unless otherwise essential.

α-CGRP is located in the central and peripheral nervous system and is primarily produced and stored in Aδ- and C-fiber sensory neurons in the trigeminal ganglion (TG) and dorsal root ganglia (DRG) (16, 17). α-CGRP is produced *via* tissue-specific alternative splicing from the Calcitonin I gene on chromosome 11, that also gives rise to calcitonin (18). First, a pre-mRNA transcript of the Calcitonin I gene is produced, then exon 4 is spliced out and the transcript is translated in a 121 amino acid pro-hormone. Finally, it is cleaved in the mature 37 amino acid polypeptide and stored in dense-core vesicles for transport to axon terminals (19, 20). β-CGRP is found primarily in the enteric nervous system and pituitary gland. It stems from the Calcitonin II gene, also located on chromosome 11 (16, 21).

CGRP can be subdivided into four sections (16, 22, 23): the N-terminus end, consisting of seven amino acids, is a ring-like structure formed by a disulfide bond at amino acid 2 and 7 (16). It is responsible for receptor activation and affinity (23). Amino acids 8–18 form an α-helix, which is responsible for orientation of CGRP and efficient receptor binding (23, 24). Amino acids 19–27 are present as β- or γ-twist (22). Although little is known, this part seems to be involved in receptor binding. The C-terminus (amino acids 28–37) builds the binding epitope and interacts with the N-terminus of the CGRP receptor (16, 25).

The CGRP Receptor

The Calcitonin family members bind to G-protein coupled receptors (GPCRs) to exert their actions (26).

The CGRP receptor is a membrane-bound heterodimer comprising the calcitonin receptor-like receptor (CLR) and the receptor activity modifying protein 1 (RAMP1) (16, 27, 28). Further, the two cytosolic proteins, receptor component protein (RCP) and the α-subunit of the GS protein (GαS) belong to the receptor complex. All components are needed to form a functional receptor which is distributed within the peripheral and central nervous system as well as the cardiovascular system (29).

The CLR is a member of the class B "secretin-like" family of G protein-coupled receptors (GPCR). It is structured in seven transmembrane-spanning domains with an extracellular N-terminus and a cytosolic C-terminus. By binding of CGRP to the N-terminus the signaling cascade is initiated (16).

RAMP1 belongs to the RAMP family including RAMP1, RAMP2, and RAMP3. It is specific to the CGRP receptor and consists of one transmembrane-spanning domain with a long extracellular N-terminal domain and a short intracellular C-terminus. It is responsible for high affinity binding of CGRP and receptor trafficking (30, 31).

If the CLR is combined with RAMP2 or RAMP3, respectively, receptors for adrenomedullin or adrenomedullin 2 are formed (32).

RCP is needed for signal transduction, in detail it connects the CLR and the cytosolic G protein-mediated signaling pathway leading to the production of cyclic adenosine monophosphate (cAMP) (2).

After CGRP binding, the receptor is phosphorylated and internalized (2, 26).

The AMY_1 receptor formed by the calcitonin receptor (CTR) interacting with RAMP1 is another CGRP receptor (26, 32), however its physiological relevance needs to be determined (28). *In vitro*, both CGRP and amylin bind to the AMY1 receptor. Due to high potency of CGRP at this receptor and its widespread distribution a physiological role has been hypothesized (27).

THE ROLE OF CGRP IN MIGRAINE PATHOPHYSIOLOGY

The trigeminal nerve, the trigemino-vascular system (TVS) and the trigemino-cervical complex (TCC) play a pivotal role in the generation of migraine pain (5, 33). However, the origin of migraine attacks - whether peripheral or central - remains unclear (3, 34). Recent data suggest a central origin (35, 36), although this is beyond the scope of this review.

The Trigeminal Nerve

Together with the ophthalmic, maxillary and mandibular branches (V1-3), the trigeminal nerve is the largest cranial nerve (V1: 26.000 fibers; V2: 50.000 fibers; V3: 78.000 fibers), responsible for tactile and pain perception of the face and the meninges as well as for motor control of masticatory muscles (37–39).

The ophthalmic division (V1) innervates the upper part of the face, most of the dura mater and cerebral vasculature (see **Figure 1**) (37, 39, 41). It terminates in the lacrimal, frontal and nasociliary nerve which again give rise to small sensory terminal afferents. Due to the major innervation of intracranial structures by the ophthalmic division, most nociceptive stimuli are conveyed by this branch to the trigeminal ganglion (TG) (39).

The maxillary division (V2) innervates sensitively the mid-part of the face including the upper lip and cheek. Sensitivity of the lower face and motor innervation of the chewing muscles is provided by the mandibular branch (V3) (37, 39).

The trigeminal ganglion (TG) consists of 20.000–150.000 pseudo-unipolar neurons with distal axonal branches forming the abovementioned divisions and a proximal axonal branch reaching the TCC in the brainstem (39, 42). Fifty percent of small- and medium-sized neurons show CGRP immunoreactivity (30, 43) primarily found in sensory neurons and their unmyelinated C-fibers or thinly myelinated Aδ-fibers, not in glial cells. CGRP is commonly colocalized with substance P (SP).

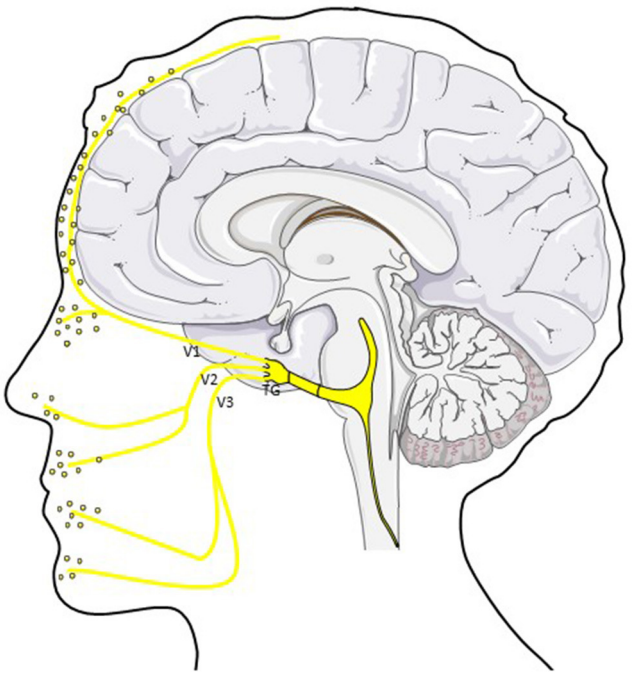

FIGURE 1 | CGRP in the trigemino-vascular system. CGRP is released from peripheral afferents of the ophthalmic (V1), mandibular (V2) and maxillary (V3) division of the trigeminal nerve. Different studies showed elevated CGRP levels in saliva and tear fluid in ictal and interictal migraine patients (40). TG; trigeminal ganglion.

The TG also contains the CGRP receptor, however CGRP and CGRP receptor components are rarely co-expressed (30). CLR and RAMP1 are expressed in 40% of large neurons, satellite glial cells and in the wall of vessels of the TG (30, 44, 45).

TG neurons innervating intracranial vessels store several other neuropeptides like SP, neurokinin A/B, pituitary adenylate cyclase-activating peptide (PACAP), dynorphins, serotonin, amylin and glutamate which are also thought to be involved in migraine pathophysiology (37).

The Trigemino-Vascular System

The trigemino-vascular system comprises the trigeminal nerve and its afferents to the intracranial vasculature and the meninges (46, 47). Nociceptive nerve fibers innervate pial, subarachnoid and dural blood vessels. Highest density of trigeminal fibers is found along proximal arteries and decreases in distal vessels, however, it was suggested that small cerebral vessels are also involved in pain (48–50). CGRP is also present in veins, although to a lesser degree. Due to low CGRP levels in blood, it was concluded that the peptide rather acts locally in the vessel wall (16).

Upon activation of the trigeminal system, CGRP and other neuropeptides like SP or PACAP are released from peripheral afferents and subsequently neurogenic inflammation occurs.

Neurogenic Inflammation

Neurogenic inflammation is a neural-driven inflammatory process caused by the release of vasoactive neuropeptides. It is hypothesized to be a key mechanism of migraine pathophysiology (51–54) comprising plasma extravasation and vasodilation leading to nociceptor activation and sensitization (47, 55); Also, activated meningeal nociceptors lead to a release of vasoactive and proinflammatory peptides (55, 56). Nevertheless, the initiation of meningeal inflammation remains unclear. For example, activation by cortical spreading depolarization (CSD) or through the release of inflammatory mediators by mast cells is discussed (3, 4).

The Trigemino-Cervical Complex

Nociceptive signals from the meninges and intracranial vessels are transmitted mainly *via* the ophthalmic branch (V1) to first-order sensory neurons in the TG. From there, pain signals are conveyed to second-order neurons of the trigemino-cervical complex (TCC) in the brainstem consisting of neurons of the trigeminal nucleus caudalis (TNC) and C1 and C2 dorsal horns of the cervical spinal cord (see **Figure 2**) (5, 33).

The TCC projects to different areas in the brain stem and the thalamus where nociceptive signals are further processed (4, 5, 33, 57).

Central Pain Pathways

From the thalamus, nociceptive signals are projected from third-order neurons to cortical and subcortical structures involved in pain perception (58), but thalamic nuclei are also involved in non-headache symptoms like photo- or phonophobia (33, 59).

In the CNS, pain signals are processed in the so-called pain matrix consisting of the primary and secondary somatosensory cortex, insula, anterior cingulate cortex and prefrontal cortex (4, 58).

INVESTIGATIONS OF CGRP IN MIGRAINE PATIENTS

Ictal CGRP—Investigations of CGRP During Spontaneous and Experimentally-Induced Migraine Attacks in Blood

In the late 1980s and early 1990s the neurovascular aspect of migraine pathophysiology was brought into focus (60, 61). Further, it became possible to investigate neuropeptides and their role in the innervation of cerebral vasculature and in migraine (62).

First, ictal migraine patients—referring to patients with a migraine headache at the time of study participation- and subsequently other migraine states like interictal or chronic migraine were investigated. These studies established and confirmed the importance of the trigeminal system and the neuropeptide CGRP in the pathophysiology of migraine. To date, only CGRP was reliably detected in migraine patients (6). An overview of studies investigating CGRP in blood, saliva and tear fluid gives **Table 1**.

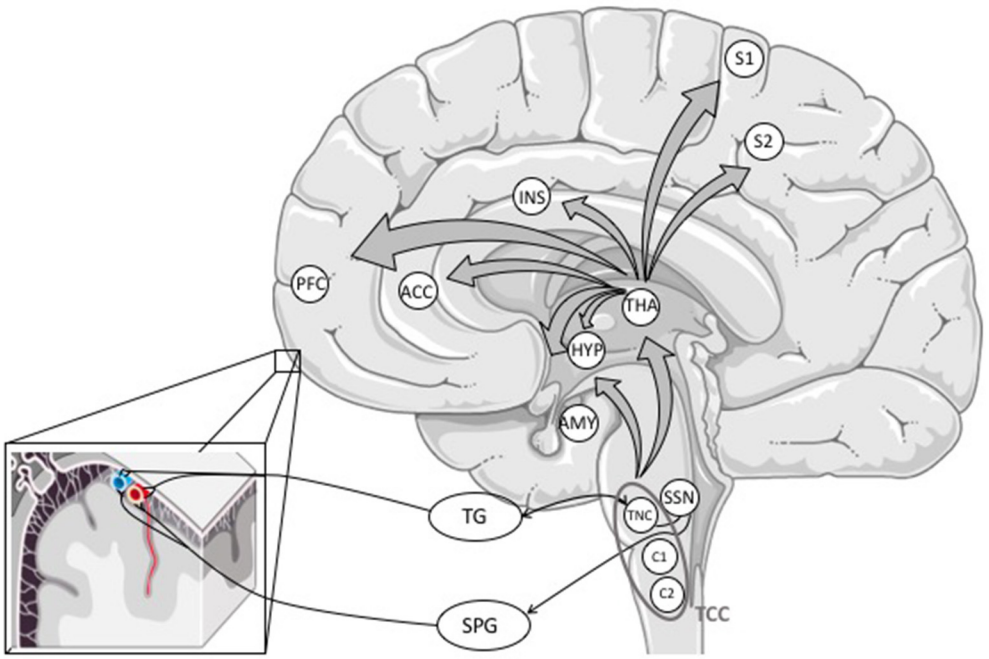

FIGURE 2 | Primary afferents from the meninges and cerebral blood vessels reach the trigeminal ganglion, mostly through the ophthalmic branch (V1). The information is processed *via* first-order neurons in the TG to second-order neurons in the trigeminal nucleus caudalis forming the trigemino-cervical complex with C1 and C2 dorsal horns of the cervical spinal cord. The TCC projects to different areas in the brainstem (not outlined in this figure) and the thalamus. The activation of the TCC might also activate the trigeminal autonomic reflex. From the thalamus, nociceptive signals are conveyed to (sub-)cortical structures involved in pain perception (16). ACC, anterior cingulate cortex; AMY, amygdala; HYP, hypothalamus; INS, insula; PFC, prefrontal cortex; S1 and S2, somatosensory cortex; SPG, sphenopalatine ganglion; SSN, superior salivary nucleus; TCC, trigemino-cervical complex; TG, trigeminal ganglion; THA, thalamus; TNC, trigeminal nucleus caudalis.

In 22 patients with migraine with or without aura blood was collected during acute headache (with a median duration of 3 h) from the external jugular and cubital vein (62). CGRP levels were significantly elevated in migraine patients compared to healthy controls. Interestingly, elevated CGRP levels were only shown in blood drawn from the cranial circulation, but not in peripheral blood. Also, no changes in blood levels of SP, NPY, and VIP were detected.

The importance of the TG was further confirmed in 9 patients undergoing thermocoagulation—a therapeutic procedure to destroy tissue by heat produced by high-frequency electric currents—of the trigeminal ganglion for tic douloureux or atypical facial pain (63).

CGRP levels in the external jugular vein were elevated in patients with facial flushing, but otherwise not. The authors concluded that the activated trigeminal ganglion leads to elevated peptide levels (63).

If CGRP is intended to be used as a biomarker, it needs to serve as an objective disease measurement or indicator of a (patho-)physiological state (64–66). Several studies—especially studies measuring CGRP in the jugular vein- have shown increased CGRP levels during acute migraine and decreased CGRP levels after headache resolution. From these studies, it can be concluded that CGRP is a marker of the migraine attack. However, due to inconsistent findings in the overall studies, standardization of study procedures is needed to draw further conclusions (67–69).

Eight migraine patients treated a migraine attack with up to two doses of subcutaneous sumatriptan 3 mg (70). Blood was drawn from the external jugular vein during headache, before abortive treatment, immediately and 2 h after headache resolution. Six patients completely responded to the treatment and CGRP levels were significantly decreased after resolution of headache (70).

A further study investigated not only ictal, but also interictal CGRP levels in juvenile migraineurs compared to healthy controls (71). For interictal measurements migraine patients had to be headache-free for at least 48 h. During migraine attacks blood was drawn within 2–4 h after onset. Ictal CGRP levels were significantly elevated with maximum CGRP levels 2 h after onset. CGRP levels returned to baseline 2 h after spontaneous resolution, as shown in a subset of patients. Interestingly, no difference in CGRP levels was found in interictal migraine patients compared to controls (71).

CGRP levels were also investigated in experimentally-induced migraine attacks (72, 73). The application of nitroglycerin (NTG) is a common model to evoke a migraine-like headache in migraineurs (74, 75). Fifteen female migraineurs and eight healthy controls received nitroglycerin 0.5 mg sublingual. Cubital blood was drawn before NTG application and 60 and 120 min after beginning of a migraine-like headache. If no headache occurred, blood was drawn 5 and 6 h after drug administration (73).

TABLE 1 | Overview of studies investigating CGRP in blood, saliva, and tear fluid.

	Participants	Samples	Examination	Method	CGRP concentration
Plasma and serum					
Goadsby et al. (62)	22 MWA/MOA patients ($f = 16$; 36 ± 13 y), HC	Plasma (EJV, CV)	Ictal	RIA Detection limit: 10 pmol/l	MWA EJV: 92 ± 11 pmol/l (vs. HC, $p < 0.001$) CV: 40 ± 6 pmol/l MOA EJV: 86 ± 4 pmol/l (vs. HC, $p < 0.001$) CV: 43 ± 6 pmol/l HC: < 40 pmol/l
Goadsby et al. (70)	8 migraine patients ($f = 7$; 34 ± 6 y)	Plasma (EJV)	Ictal, post-sumatriptan s.c. 3 mg	RIA Detection limit: 10 pmol/l	CGRP pre-treatment: 60 ± 8 pmol/l ($p < 0.05$) CGRP responder ($n = 6$): 40 ± 8 pmol/l
Gallai et al. (71)	45 MOA patients ($f = 20$; 16.3 ± 2.6 y), 30 MWA patients ($f = 12$; 15.4 ± 2.3 y), 20 HC ($f = 15$; 15.1 ± 2.1 y)	Plasma (CV)	Interictal (headache-free 48 h prior to blood sampling), ictal (2–4 h after migraine onset)	RIA Detection limit: 1 pmol/l	Interictal MOA: 34.7 ± 7.2 pmol/l (vs. HC, n.s.) MWA: 39.3 ± 8.6 pmol/l (vs. HC, n.s.) HC: 38.2 ± 6.5 pmol/l Ictal MOA: 51.4 ± 7.8 pmol/l (vs. interictal, $p < 0.03$) MWA: 50.3 ± 6.7 pmol/l (vs. interictal, $p < 0.05$)
Ashina et al. (78)	20 EM patients ($f = 16$; 40 ± 9 y), 20 HC ($f = 12$; 41 ± 14 y)	Plasma (CV)	Interictal (72 h medication- and headache-free prior to blood sampling)	RIA Detection limit: <1 pmol/l	EM: 75 ± 8 pmol/l (vs. HC, $p = 0.005$) HC: 49 ± 3 pmol/l
Juhasz et al. (73)	15 migraine patients ($f = 15$, 41.9 ± 2.3 y), 8 HC ($f = 8$, 38.5 ± 4.4 y)	Plasma (CV)	NTG-induced headache attack, blood sampling before and after headache	RIA Detection limit: n.a.	Migraine patients Basal, with headache: 20.2 ± 1.9 pmol/l (vs. without headache, $p = 0.018$) Basal, without headache: 14.0 ± 1.3 pmol/l Basal: 18.4 ± 1.7 pmol/l (vs. HC, $p = 0.24$), 1 h-post-headache onset: 22.2 ± 2.6 (vs. basal, $p < 0.05$) HC: 15.1 ± 2.0 pmol/l
Juhász et al. (72)	19 migraine patients ($f = 19$; 45 ± 1.4 y)	Plasma (CV)	NTG-induced headache attack, blood sampling before and after sumatriptan nasal spray	RIA Detection limit: n.a.	Sumatriptan responder ($n = 6$) Ictal: 16.9 ± 2.8 pmol/l (vs. 1 h-post suma, $p = 0.034$) 1 h post-sumatriptan: 14.7 ± 2.2 pmol/l Sumatriptan non-responder ($n = 6$) Ictal: 24.3 ± 2.5 pmol/l 1 h post-sumatriptan: 23.8 ± 2.4 pmol/l
Sarchielli et al. (76)	20 EM patients (n.a.)	Plasma (EJV)	Ictal, pre- and posttreatment with rizatriptan	RIA Detection limit: <1 pmol/l	Responder ($n = 10$) Pre-treatment: 12.2 ± 3.2 pmol/l Post-treament (2 h): 3.4 ± 1.1 pmol/l (vs. pre-treatment, $p < 0.0001$) Post-treatment (12 h): 2.1 ± 0.8 pmol/l (vs. pre-treatment, $p < 0.0001$) Non-responder ($n = 10$) Pre-treatment: 7.4 ± 2.4 pmol/l Post-treament (2 h): 7.9 ± 3.1 pmol/l (vs. pretreatment, n.s.) Post-treatment (12 h): 7.2 ± 3.1 pmol/l (vs. pretreatment, n.s.)

(Continued)

TABLE 1 | Continued

	Participants	Samples	Examination	Method	CGRP concentration
Tvedskov et al. (77)	21 EM patients ($f = 17$; 39 y)	Plasma (EJV, CV)	Interictal (headache- and medication-free 72 h), ictal	RIA Assay I: Detection limit: n.a. Assay II: Detection limit: <1 pmol/l	CGRP Assay I (EJV, $n = 17$) Ictal: 17.18 pmol/l (vs. interictal, $p = 0.44$) Interictal: 15.88 pmol/l CGRP Assay I (CV, $n = 21$) Ictal : 16.86 pmol/l (vs. interictal, $p = 0.69$) Interictal: 17.57 pmol/l CGRP Assay II (EJV, $n = 17$) Ictal: 32.59 pmol/l (vs. interictal, $p = 0.42$) Interictal: 30.59 pmol/l CGRP Assay II (CV, $n = 21$) Ictal: 33.37 pmol/l (vs. interictal, $p = 0.43$) Interictal: 31.84 pmol/l
Fusayasu et al. (79)	95 migraine patients ($f = 77$; 30.0 ± 10.4 y), 52 HC ($f = 39$; 29.2 ± 9.7 y)	Plasma (CV)	Interictal (headache-free 72 h)	EIA Detection limit: <4 pg/ml	Migraine patients: 19.0 ± 9.1 pg/ml (vs. HC, $p < 0.01$) HC: 13.4 ± 4.4 pg/ml
Rodríguez-Osorio et al. (80)	47 EM patients ($f = 46$; 37.8 ± 10.4 y), 23 HC ($f = 22$; 31.8 ± 11.0 y)	Serum (CV)	Interictal (Headache- and medication-free 72 h prior to blood sampling), ictal	ELISA Detection limit: n.a.	EM Interictal: 164.2 ± 139.1 pg/ml (vs. HC, $p < 0.0001$) Ictal ($n = 19$): 298.2 ± 100.3 pg/ml (vs. interictal $p < 0.0001$) HC: 37.1 ± 38.5 pg/ml
Cernuda-Morollón et al. (83)	103 CM patients ($f = 103$; 43.1 ± 11.7 y), 43 EM patients ($f = 43$; 44.4 ± 11.6 y), 31 HC ($f = 31$; 38.6 ± 12.8 y)	Serum (CV)	No medication 24 h prior and no headache at blood sampling	ELISA Detection limit: <4.3 pg/ml	CM: 74.90 ± 28.29 pg/ml (vs. HC, $p < 0.001$) EM: 46.37 ± 15.21 pg/ml (vs. HC, $p < 0.005$) HC: 33.74 ± 16.10 pg/ml
Cernuda-Morollón et al. (92)	81 CM patients ($f = 77$; 46.2 ± 11.0 y), 33 HC ($f = 33$; 39.4 ± 13.2 y)	Serum (CV)	Medication- 24 h prior and headache-free at blood sampling, treatment with OnabotulinumtoxinA	ELISA Detection limit: <4.3 pg/ml	CM 64.9 ± 31.0 pg/ml (vs. HC, $p < 10^{-10}$) Responder ($n = 61$): 70.4 ± 31.9 pg/ml (vs. non-responder, $p < 0.005$) Non-responder ($n = 20$): 48.3 ± 21.2 pg/ml HC: 33.3 ± 15.7 pg/ml
Cernuda-Morollón et al. (85)	83 CM patients ($f = 79$; 44.2 ± 12.0 y)	Serum (CV)	Medication- 24 h prior and headache-free at blood sampling before and 1 month after OnabotulinumtoxinA treatment	ELISA Detection limit: <4.3 pg/ml	Responder ($n = 64$) Pre-treatment: 76.85 pg/ml (vs. non-res., $p < 0.001$) Post-treatment: 52.48 pg/ml (vs. pre-tr., $p = 0.003$) Non-responder ($n = 19$) Pre-treatment: 50.45 pg/ml Post-treatment: 51.89 pg/ml (vs. pre-treatment, n.s.)
Domínguez et al. (93)	62 CM patients ($f = 60$; n.a.), 24 HC (n.a.)	Serum (CV)	Medication- 48 h prior to and headache-free at blood sampling, treatment response to OnabotulinumtoxinA	ELISA Detection limit: n.a.	CM Responder ($n = 47$): 133.1 ± 86.6 ng/ml (vs. non-responder, $p = 0.004$) Non-responder ($n = 15$): 58.2 ± 91.7 ng/ml (vs. HC, $p < 0.001$) HC: 26.9 ± 12.5 ng/ml
Lee et al. (86)	99 EM patients [$f = 78$; 44 y (31–49)], 44 CM patients [$f = 36$; 39.5y (31–54)], 27 HC [$f = 25$; 34 y (27–42)]	Serum (CV)	EM: headache- and medication-free 24 h prior to blood sampling, CM: medication-free 24 h, headache-free at day of blood sampling	ELISA Detection range: 12.35–1,000 pg/ml	CM: 64.9 ± 15.32 pg/ml (vs. HC, $p = 0.104$) EM: 67.0 ± 20.70 pg/ml (vs. HC, $p = 0.133$) HC: 75.7 ± 20.07 pg/ml

(Continued)

TABLE 1 | Continued

	Participants	Samples	Examination	Method	CGRP concentration
Pérez-Pereda et al. (84)	101 CM patients ($f = 89$, 41 ± 10 y), 98 EM patients ($f = 89$, 41 ± 10 y), 97 HC ($f = 88$, 41 ± 10 y)	Serum (CV)	Interictal (medication- and headache-free 72 h prior to blood sampling)	ELISA Detection range: 12.35–1,000 pg/ml	CM: 18.02 pg/ml (14.4–24.7, vs. HC, $p < 0.001$) EM: 14.66 pg/ml (10.29–17.45, vs. HC, n.s.) HC: 13.99 pg/ml (10.10–17.87)
Saliva					
Nicolodi and Bianco (87)	15 migraine patients ($f = 8$; 43 ± 3.5 y), 34 HC ($f = 18$; 43.7 ± 4 y)	Saliva	Interictal (medication-free 72 h prior to blood sampling), ictal	RIA Detection limit: n.a.	Migraine patients Ictal: 27.3 ± 2.9 pmol/l (vs. interictal, $p < 0.01$) Interictal: 14.3 ± 2.5 pmol/l (vs. HC, $p < 0.05$) HC: 22.02 ± 1.7 pmol/l
Bellamy et al. (88)	5 migraine patients (n.a.), 5 HC (n.a.)	Stimulated saliva	Interictal (headache-free 72 h prior to blood sampling), ictal	RIA Detection limit: n.a.	Interictal: 53 pmol/mg total protein (vs. HC, $p < 0.01$) Ictal: 65 pmol/mg total protein 2 h-post-sumatriptan: 25 pmol/mg total protein (vs. ictal, $p < 0.01$)
Cady et al. (89)	22 EM patients ($f = 20$; 38.9 ± 2.7 y)	Stimulated saliva	Ictal, pre- and post-treatment with rizatriptan	RIA Detection limit: n.a.	Rizatriptan responder ($n = 14$) Basal: 51.1 ± 3.8 pmol/l total protein Rizatriptan non-responder ($n = 8$) Basal: 42.5 ± 4.0 pmol/l total protein
Jang et al. (82)	33 CM patients ($f = 21$; 43.7 ± 18.1 y), 36 HC ($f = 19$; 44.3 ± 14.2 y)	Saliva, plasma (CV)	n/a	EIA Detection limit: n.a.	CM Saliva: 431.6 ± 272.8 pg/ml (vs. HC, $p = 0.026$) Plasma: 253.6 ± 195.2 pg/ml (vs. HC, $p = 0.003$) HC Saliva: 301.5 ± 188.9 pg/ml Plasma: 136.2 ± 92.5 pg/ml
Cady et al. (90)	20 CM patients ($f = 15$; 48.5 ± 12.87 y)	Stimulated saliva	Interictal, pre- and 1 month post-Onabotulinumtoxin A	RIA Detection limit: n.a.	Pre-treatment: 39.4 ± 7.5 pg/mg total protein (vs. post-treatment, n.s.) Post-treatment: 25.5 ± 4.1 pg/mg total protein
Alpuente et al. (81)	22 EM patients ($f = 22$; 30.4 ± 9.4 y), 22 HC ($f = 22$; 31.2 ± 11.1 y)	Saliva, plasma (CV)	Interictal (headache-free 72 h prior to sampling), ictal	ELISA Detection limit: 0.39 pg/ml	EM Interictal: 98.0 (80.3) pg/ml (vs. HC, $p = 0.034$) Ictal: 247.0 (181.9–312.0) pg/ml HC: 54.3 (44.0) pg/ml
Tear fluid					
Kamm et al. (91)	48 EM patients ($f = 42$; 37.3 ± 12.0 y), 45 CM patients ($f = 37$; 34.4 ± 12.1 y), 48 HC ($f = 33$; 33.2 ± 9.6 y)	Tear fluid, plasma (CV)	Interictal (headache- and medication-free 72 h prior to sampling), ictal	ELISA Detection limit: 0.39 pg/ml	Migraine patients Interictal TF: 1.10 ± 1.27 ng/ml (vs. HC, $p = 0.022$) Interictal plasma: 6.32 ± 3.08 pg/ml (vs. HC, $p = 0.528$) Ictal, unmedicated TF: 1.92 ± 1.84 ng/ml (vs. interictal, $p = 0.102$) Ictal, medicated TF: 0.56 ± 0.47 ng/ml (vs. interictal, $p = 0.011$) HC TF: 0.75 ± 0.80 ng/ml Plasma: 6.57 ± 4.25 pg/ml

CM, chronic migraine; CV, cubital vein; EIA, enzyme immunoassay; EJV, external jugular vein; ELISA, enzyme-linked immunosorbent assay; EM, episodic migraine; HC, healthy control; MOA, migraine without aura; MWA, migraine with aura; NTG, nitroglycerin; RIA, radioimmunoassay.

As described before, an immediate headache occurred in a subset of migraineurs and controls and disappeared spontaneously, but no change in CGRP levels was seen during this headache. A migraine-like headache occurred in 2 of 8 controls and 10 of 15 migraine patients with a mean latency of ∼6.5 h and a median intensity of 3.5 on the numerical rating scale (NRS). Migraineurs developing a headache showed significantly higher CGRP levels compared to patients without headache. Again, basal CGRP levels didn't show differences between the study groups.

In a following study, this research group investigated the influence of sumatriptan nasal spray 20 mg on CGRP levels in peripheral blood during an experimental migraine attack (72).

Nineteen female migraine patients developed a migraine-like headache attack after the application of sublingual nitroglycerin 0.5 mg. Cubital blood was drawn 120 min after migraine onset, immediately before and 60 min after sumatriptan application.

Based on the sumatriptan response, two groups were divided: patients ($n = 6$) who improved at least 30% showed significantly decreased CGRP levels, whereas patients ($n = 13$) who didn't improve accordingly showed no decrease in CGRP levels.

These study results were confirmed and extended by another study monitoring treatment response after rizatriptan in 20 EM patients (76). During six consecutive migraine attacks, the efficacy of rizatriptan was clinically screened. Ten responder (significant pain reduction within 2 h after rizatriptan intake and no headache recurrence within the next 48 h) and 10 non-responder (no significant reduction in pain intensity within 24 h after rizatriptan intake) were chosen.

During a spontaneous migraine attack, patients reached the headache center within 2 h and the external jugular vein was immediately catheterized. CGRP levels were analyzed at the time of catheterization and 1, 2, 4, 6, and 12 h after triptan administration. Ictal CGRP levels were significantly higher in treatment responder compared to non-responder. Further, CGRP levels were significantly reduced in responder, already after 1 h, but even more after 2 h and stayed at this level during the 12 h observation period. CGRP levels in non-responder didn't change significantly over the course of the migraine attack (76).

In spite of these study results, one study didn't find any CGRP level differences in external jugular or antecubital blood ictally and interictally (77). Patients enrolled in this study called the study team at the beginning of a migraine attack, restrained from taking acute medication and blood was drawn within 60 min after initial contact.

Interictal CGRP levels were investigated when patients were headache and abortive medication-free for 72 h. The study group used 2 CGRP assays, however no ictal and interictal differences in CGRP levels could have been detected.

Interictal CGRP—Investigation of CGRP During Headache-Free Periods

The aforementioned study results highlighted CGRP as a potential marker of trigeminal activation as the neuropeptide is elevated during migraine attacks and reduced after headache resolution (2). From these study results it can be concluded that the neuropeptide represents different (patho-)physiological states of a migraine attack and can be used as biomarker. Further studies investigated interictal CGRP levels and the possible role of CGRP as a biomarker for migraine itself. Moreover, other and less invasive methods were examined and patient groups were chosen due to headache frequency and abortive medication intake.

Interictal CGRP in Episodic Migraine Patients

In interictal episodic migraine (EM) patients elevated (78–80) as well as unchanged CGRP levels were found in peripheral blood compared to healthy controls (71, 73, 77, 81).

The majority of the studies included migraine patients being headache- and abortive medication-free 72 h prior to blood drawing. No correlation between migraine attack frequency and CGRP levels was found (78).

Interictal CGRP in Chronic Migraine Patients

Different studies detected elevated CGRP levels in peripheral blood of chronic migraine (CM) patients compared to healthy controls (82), but also compared to EM patients (83, 84). The studies used different headache- and medication free periods which makes a comparison of the study results difficult (see **Table 1**).

However, another study investigating serum CGRP levels didn't find differences in CM patients and healthy controls. Migraine patients were headache-free 24 h prior to investigation (86).

Interictal CGRP as Treatment Response Marker

CGRP was analyzed as a potential marker for treatment response in CM patients (85, 92, 93). Eighty-three and, respectively, eighty-one CM patients received at least two injections of OnabotulinumtoxinA (155–195 units) following the PREEMPT protocol (94); treatment responder were defined as patients with a $\geq$50% reduction of headache episodes and a $\geq$50% subjective benefit (85) or as moderate (reduction of headache episodes and subjective benefit between 33 and 66%), respectively, excellent responder (reduction of headache episodes and subjective benefit $>$ 66%) (92). CGRP levels were determined before and 1 month after OnabotulinumtoxinA administration.

77%, respectively, 75% of CM patients were considered responder. In both studies, pretreatment CGRP levels were significantly higher in responder compared to non-responder. CGRP levels decreased significantly after 1 month in the responder group, whereas this reduction could not have been detected in non-responder (85).

Other Sources of CGRP for the Investigation in Migraine Patients

Due to the innervation of the trigeminal nerve and its ophthalmic, maxillary and mandibular branches other sources for detecting CGRP have been investigated in migraine patients (38, 39).

In general, saliva and tear fluid receive increasing attention as diagnostic fluids and to date, few studies have investigated CGRP in saliva and tear fluid (see **Table 1**) (95).

Former studies detected CGRP in human tears and changes in these peptide levels are hypothesized to represent (patho-)physiological alterations (96). The eye -more precisely the cornea, conjunctiva, meibomian and lacrimal glands- is innervated by sensory, sympathetic and parasympathetic nerves originating in the TG, the superior cervical and pterygopalatine ganglion, respectively (97–99). The cornea is highly innervated by CGRP-positive fibers from the ophthalmic branch (V1) (98), whereas the CGRP-positive innervation of the meibomian and lacrimal glands seems to be scarce (97, 99). Saliva is mainly produced by the parotid, submandibular and sublingual glands as well as numerous minor glands located in the submucosa of the mouth (100). Salivary glands are controlled by the autonomic nervous system, and to a lesser degree innervated by CGRP-positive fibers that evoke salivary secretion (101–103).

Advantages of the measurement of CGRP in these compartments might be higher neuropeptide concentrations due to direct innervation and a non-invasive and easy sample collection which enables repetitive measurements.

CGRP in Saliva

Salivary CGRP Levels in Episodic Migraine Patients

Salivary CGRP levels were first investigated in 15 migraineurs compared to 34 healthy subjects in 1990 (87). Saliva was obtained ictally and interictally when patients had restrained from taking abortive medication for 72 h.

Significantly elevated CGRP levels were detected ictally, whereas lower CGRP levels were detected in interictal migraine patients compared to healthy controls (87).

Stimulated salivary CGRP levels were shown interictally and ictally in five EM patients compared to five healthy controls (88). Patients had to be headache-free 72 h prior to interictal investigation. After rinsing the mouth, saliva production was stimulated using 2% citric acid applied to the tongue. Initial saliva was discarded in order to avoid mixing unstimulated and stimulated saliva and 5 mL saliva was sampled. Saliva collection has been trained in the clinic and was performed independently by patients at home.

As shown before, the intake of sumatriptan 100 mg reduced ictal CGRP levels compared to unmedicated patients. In contrast to the above mentioned study results, interictal CGRP levels were significantly elevated in migraine patients compared to healthy controls. Importantly, no changes in peptide levels were detected between sampling in the clinic and at home (88).

These study results were extended by monitoring CGRP levels over the course of a spontaneous migraine attack in 22 EM patients by the same study group (89).

Compared to baseline CGRP levels, no change in CGRP levels during premonitory phase could have been detected, but during the occurrence of a mild or moderate headache. After intake of rizatriptan and headache resolution salivary CGRP levels were found to be near baseline levels. As shown before, triptan responder showed a significant increase of ictal CGRP levels, whereas non-responder didn't show significant changes in salivary CGRP levels during the migraine attack.

The authors further differentiated two groups: one group ($n = 6$) showed already elevated CGRP levels during the premonitory phase, sustained during the headache phase. In contrast, the other group ($n = 8$) showed highest CGRP levels during a moderate headache.

In a recent study salivary CGRP levels were continuously monitored and investigated interictally and ictally (81). Twenty-two EM patients and twenty-two healthy controls were included. For interictal sampling patients had to be headache-free for 72 h, in every participant peripheral blood was drawn interictally once. Saliva was independently sampled by patients and stored at home. Interictal saliva levels were significantly elevated in EM patients compared to healthy controls, whereas no significant difference was detected in CGRP plasma levels. Forty-nine migraine attacks were monitored by taking saliva samples at headache onset, after 2 and 8 h.

Again, ictal CGRP levels were elevated. Dependent on CGRP levels, the authors stated CGRP-independent and CGRP-dependent migraine attacks with significantly higher CGRP levels. Eighty percent of migraine attacks were CGRP-dependent and 20% were CGRP-independent. Relating to patients, 13 of 22 migraine patients showed only CGRP-dependent, 3 of 22 patients showed exclusively CGRP-independent attacks and 6 patients showed both types of migraine attacks. These study results support the above mentioned results as they indicate that several neuropeptides might be involved to different degrees in a migraine attack.

Salivary CGRP Levels in CM Patients

Salivary CGRP levels in chronic migraine is less investigated. One study showed significantly elevated CGRP levels in 33 CM patients compared to 36 healthy controls in resting whole saliva and periperhal blood (82).

Salivary CGRP as a Treatment Response Marker

To date, one study investigated salivary CGRP levels in 20 CM patients receiving OnabotulinumtoxinA compared to placebo (90). At inclusion, baseline salivary CGRP levels were determined and patients were divided in two study groups: group A received OnabotulinumtoxinA as described in the PREEMPT protocol (94), group B received saline. After 4 months treatment regimens were switched. Patients were instructed to obtain monthly saliva samples. In both study groups, headache days were significantly reduced after treatment, whereas the reduction of headache days was greater after OnabotulinumtoxinA treatment.

In the OnabotulinumtoxinA group, CGRP levels decreased at month 2 and 3, although this change didn't reach significance which the authors ascribe to little patient number. A respective decrease of CGRP levels was not detected after saline.

Tear Fluid CGRP in EM and CM Patients

In our study group, tear fluid CGRP levels were investigated in 48 EM, 45 CM and 48 healthy controls (91). Interictal (no headache and abortive medication in the last 48 h) and ictal migraineurs visiting our outpatient headache center were continuously included. Tear fluid was sampled using a plastic capillary located at the lateral canthus of both eyes. Blood was drawn from the cubital vein.

In general, we found CGRP levels to be about 140× higher in tear fluid compared to plasma levels. Further, tear fluid CGRP levels were significantly elevated in interictal migraine patients compared to healthy controls. No differences in tear fluid CGRP levels could have been detected in episodic and chronic migraine patients. One explanation for this finding might be the high frequency of migraine days in EM patients.

As shown before, ictal migraine patients who had restrained from taking abortive medication 48 h prior to investigation showed highest CGRP levels, although this only showed to be a trend which is likely due to little patient number ($n = 13$).

Ictal migraine patients with intake of acute medication 48 h prior to investigation showed significantly reduced CGRP levels in tear fluid compared to interictal and unmedicated ictal patients.

DISCUSSION

Measuring CGRP in migraine patients has led to a better understanding of pain in migraine pathophysiology as well as it laid the foundation of the development of new abortive and prophylactic treatments (40). Further, the neuropeptide is recognized as a marker for the acute migraine attack and it might be a marker for migraine itself which could help to objectify the diagnosis in the future (67).

However, results of CGRP measurement in peripheral blood remain conflicting as well as comparability and reproducibility is often limited (69, 104).

Most probably, the differences in study results are caused by using distinct methods and inhomogeneous study groups (69).

Recent studies used more controlled inclusion and exclusion criteria, e.g., concerning ictal or interictal migraine or monthly headache frequency. Differences between interictal and ictal migraine patients have been shown in several studies and reduced CGRP levels were detected after intake of abortive medication up to 12 h in blood and 48 h in tear fluid (76, 91).

To date, little is known concerning the influence of monthly migraine frequency. There are studies showing increased CGRP levels in CM compared to EM patients (83, 84), however other studies didn't find significant differences (86, 91) or a correlation with number of the headache days (78).

In this respect, the analysis of CGRP levels in chronic migraine might be especially challenging since headache- and medication-free periods are scarce due to ≥15 headache days/month (105).

However, the investigation of rigorous subgroups concerning headache days, associated symptoms or distinct clinical factors, but also comorbidities, age and gender will contribute to our understanding of CGRP. Further, interferences of the above mentioned factors with the peptide could be investigated which might also explain missing comparability of study results (67, 68).

Almost all studies used different study methods concerning blood drawing, processing and analysis as well as many study protocols haven't been sufficiently described. Thus, direct comparison of the studies is not possible and might be one of the most important explanations of different research results as well as limited reproducibility (69). CGRP is rapidly degraded with a short half-life of 7–9 min (106). This rapid degradation was proposed to cause negative study results in studies with longer processing times (104). Preparation of pre-chilled vials, the application of peptidase inhibitors [although the effect of peptidase inhibitors was also questioned (104)] and storage on ice until immediate processing needs to be carefully considered (69, 104). In this respect, the analysis of CGRP in plasma might be beneficial compared to serum.

Also, various analysis methods like radioimmunoassay (RIA), enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) as well as the implementation of these procedures will affect reproducibility (68). This question was recently addressed and suggestions were proposed for standardization of study protocols (69).

After release, the neuropeptide is thought to be taken up by post-capillary veins and can subsequently be detected in the circulation (69). However, CGRP levels are low in blood and dilution needs to be considered (16). This is especially important if blood is taken peripherally with a wide distance from the location of release.

Alternative approaches like CGRP measurement in saliva or tear fluid has been proposed and their potential role in determination of the neuropeptide has been shown (81, 87, 88, 91). Advantages of these methods are higher CGRP concentrations due to direct innervation which might allow to detect even subtle differences in CGRP levels. CGRP-dependent and CGRP-independent migraine attacks as well as different CGRP levels over the course of a migraine attack have been detected in saliva. In the future, this might lead to a better understanding of the contributing neuropeptides or different expression patterns of these in different patient subgroups.

In this respect, the identification of molecule profiles might be an interesting approach for the future (67).

Further, the proposed sampling techniques are easy applicable and even self-administered sampling is possible. As it has already been shown this gives the opportunity to conduct longitudinal studies in real-life conditions and larger patient number might be easier to recruit since these sampling methods are well accepted by participants.

CONCLUSION

Taken together, the detection of CGRP in migraine patients has enormously enhanced our understanding of migraine pathophysiology and provided new treatments. To enhance this knowledge, higher standardization of study protocols is needed in order to provide better comparability and reproducibility.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Short Report of Longitudinal CGRP-Measurements in Migraineurs During a Hypoxic Challenge

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Background: Calcitonin gene related peptide (CGRP) plays a key role in the pathophysiology of migraine and is therefore considered a potential biomarker for primary headache disorders. The challenge remaining is establishing standardized protocols for its assessment in various extracellular compartments and identifying pathological situations associated with an increase in CGRP.

Methods: We performed longitudinal measurements of CGRP plasma levels in 30 volunteers with the diagnosis of episodic migraine with and without aura under controlled circumstances during an induced migraine attack under a hypoxic challenge. Blood samples were collected from a cubital vein and CGRP plasma levels measured using ELISA.

Results: CGRP levels varied significantly between the subjects at baseline (15.48–1,889.31 pg/ml) but were neither associated with socio-demographic data nor with headache/migraine frequency or intensity collected before hypoxic exposure. CGRP levels during hypoxia fluctuated around baseline and increased with prolonged hypoxia but did not differ significantly in subjects with migraine or headache compared to those without. However, subjects experiencing migraine without aura showed significantly higher levels than those with aura. Ictal CGRP levels were increased in females, in subjects with a negative family history regarding headaches, in those older than 30 years of age or with a recent headache attack before the experiment ($p < 0.05$).

Conclusion: CGRP plasma levels seem to be highly variable even at baseline in migraine patients and increased during hypoxic challenge and migraine attacks. This is the first in human longitudinal measurement of peripheral CGRP levels during induced migraine attacks using a highly standardized protocol.

Keywords: CGRP, longitudinal measurement, plasma levels, migraine, headache, hypoxia

INTRODUCTION

The debilitating nature and socio-economic consequences of migraine have increased the interest in developing therapies directly targeting the pathophysiology of migraine generation. Hence, patients now have access to drugs interfering with the calcitonin gene-related peptide (CGRP) pathway. This 37 amino-acid neuropeptide was shown to play a major role in the pathogenesis of migraine (1). Besides its potent vasodilatory effect, CGRP contributes to neurogenic inflammatory responses and possibly to sensitization of trigeminal nociceptors when it is released in cranial tissues. Together with glutamate, CGRP is also released, in parallel with substance P, in the spinal trigeminal nucleus, where it contributes to central sensitization and enhancement of nociceptive transmission (2). After its release, CGRP is degraded within minutes by peptidases resulting in significantly reduced concentrations in the peripheral circulatory system.

Since CGRP has been attributed a pivotal function in the trigeminovascular system, research has focused on its pathophysiologic significance in pain disorders such as migraine and other primary and secondary headaches. Migraine and many of its mimics are diagnosed solely through patient reported medical history utilizing the diagnostic criteria of the International Classification of Headache Disorders, version 3 (ICHD-III) (3).

Whereas other disease entities can be confirmed through standardized laboratory tests, a well-established surrogate marker for primary headache disorders has yet to be developed. In line with several previous approaches, it appears consequent to assess easy-to-measure peripheral CGRP levels as biomarker in headache disorders (4, 5). In addition, from a clinical point of view, it would be extremely desirable to have an easily accessible parameter that objectifies or even predicts the response to costly therapies such as monoclonal antibodies targeting CGRP or its receptor.

There are some inherent caveats in the development of CGRP as a biomarker. First and foremost, the short half-life of about 10 mins as well as the still not entirely understood abundance of possible sites of production and elimination as well as cross-interaction between CGRP subtypes and CGRP-receptor subtypes and associated receptors add to the complexity of establishing such a marker (6, 7).

Another limitation is the heterogenous approach to CGRP extraction and measurement across different research groups. This has recently been addressed by a profound methodological study on CGRP measurement in peripheral human blood (8). Based on this study, we analysed ictal CGRP concentrations of migraineurs that were exposed to a hypoxic challenge that triggers migraine headaches.

The rationale to use hypoxia as a trigger for migraine headaches derives from multiple observations. These include higher prevalence of migraines in elevated regions (9, 10) oxidative stress as mechanism for migraine triggers (11), detection of tissue hypoxia during cortical spreading depression (12) and, foremost, safe and successful induction of migraine headache in experimental settings utilizing hypoxia (13). The hypoxic challenge performed in this study has been described in an earlier publication of our group (14).

METHODS

Sample Characteristics

Thirty volunteers were recruited from our tertiary headache outpatient clinic and *via* advertisement at the Medical University of Innsbruck. The study was approved by the local ethics community (AN2016-0126 363/4.14).

Participants met the following inclusion criteria: diagnosis of migraine with or without aura according to the ICHD-3 diagnostic criteria, history of migraine for over 12 months and migraine frequency of at least 1 day per month over the last 3 months prior to screening. Participants with chronic migraine and/or medication overuse were excluded, as well as patients who received preventative migraine treatment (e.g., betablockers, antiepileptics, tricyclic antidepressants etc.) during 12 months prior to the screening. If a participant reported headaches or used acute medication within 24 h before the experiment, it was postponed. Eligible patients had to complete a headache diary 10 days prior to and 10 days after the experiment. The trial was conducted in a normobaric hypoxic chamber (NHC) located on the campus of the University of Innsbruck's Department of Sport Science (590 m). The inspiratory fraction of oxygen (FiO_2) in the NHC was lowered to 12.6% to simulate a stay at 4,500 m above sea level. A detailed description of the methods and the experimental design have been published elsewhere (14). All volunteers entered the NHC at ∼9.00 am to avoid possible circadian fluctuations of CGRP. The use of acute medication to alleviate the headaches was not permitted at any time during the experiment.

Sample Collection

First blood samples were drawn at the beginning of the experiment prior to the hypoxic challenge (T0). Consecutive blood samples were taken under hypoxia at hourly intervals after entering the NHC (T1-Toff). After 6 h, the experiment in the NHC was terminated and 2 follow-up blood samples after 1 and 2 h post hypoxic exposure were taken. Blood was drawn from a cubital vein using EDTA-K tubes (S-Monovetten, Sarstedt, Nümbrecht, Germany) and centrifuged at 4°C for ∼4 min with 4,000 rpm. The supernatant plasma was taken off with an Eppendorff pipette, transferred to cryovials (Nunc CryoTubes, Merck, Darmstadt, Germany), and frozen at −80°C within 10–12 min.

Sample Processing

For detailed information on the processing and analysis of the samples, please refer to our previous article (8). In short, samples were processed with a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA; CGRP Enzyme Immunoassay #A05481, shortly named CGRP EIA, Bertin Bioreagent, Montigny-le-Bretonneux, France) for α- and β-CGRP, with a cross-reactivity with amylin, calcitonin and substance *P* of <0.01%. For this purpose, a synthetic interstitial solution was prepared, and protease inhibitors were added to create a standard buffer, which was fitted with human CGRP and

TABLE 1 | Baseline characteristics of participants allocated to headache-type group under hypoxia.

Characteristic	Headache			Migraine			Aura			Total
	Headache *n* =24 (80.0%)	**No headache *n* = 6 (20.0%)**	***p*-value**	**Migraine *n* = 19 (63.3%)**	**No migraine n=11 (36.6%)**	***p*-value**	**Aura *n* = 5 (16.6%)**	**No aura *n* = 25 (83.3%)**	***p*-value**	***n* = 30**
Female, *n* (%)	19 (63.3%)	3 (10.0%)	0.175	14 (46.6%)	8 (26.6%)	0.637	3 (10.0%)	19 (63.3%)	0.405	22 (73.3%)
Male, *n* (%)	5 (16.6%)	3 (10.0%)		5 (16.6%)	3 (10.0%)		2 (6.6%)	6 (20.0%)		8 (26.6%)
Age, years ± SD	26.5 ± 6.8	31.8 ± 3.8	0.125	27.1 ± 7.5	28.3 ± 7.9	0.684	32.2 ± 9.6	26.6 ± 6.9	0.135	27.6 ± 7.5
BMI, kg/m² ± SD	21.3 ± 2.4	23.4 ± 2.4	0.077	21.2 ± 2.5	22.7 ± 2.7	0.140	22.4 ± 2.7	21.6 ± 2.6	0.015	21.7 ± 2.6
Monthly migraine attack frequency, days ± SD	3.6 ± 3.2	1.7 ± 1.7	0.171	4.1 ± 3.5	1.8 ± 1.4	0.053	6.4 ± 5.3	2.6 ± 2.0	0.580	3.2 ± 3.0
Monthly intake of acute medication, days ± SD	3.6 ± 6.4	2.3 ± 1.9	0.680	3.8 ± 6.9	2.3 ± 1.4	0.565	9.5 ± 13.7	2.2 ± 1.7	0.363	3.4 ± 5.9
Prior use of migraine prophylaxis, *n* (%)	7 (100%)	0 (0%)	NA	7 (100%)	0 (0%)	NA	2 (28.6%)	5 (71.4%)	NA	7 (23.3%)
Family history of migraine, *n* (%)	17 (85.0%)	3 (15.0%)	0.372	14 (70.0%)	6 (30.0%)	0.425	4 (20.0%)	16 (80.0%)	0.640	20 (66.7%)

Table modified from "Migraine and aura triggered by normobaric hypoxia" by Frank et al. (14).

Two-sided Fisher's exact-test, with Yate's correction when appropriate, was carried out to compare continuous means between groups. Categorial data was analysed for correlation using Chi^2-test. Level of statistical significance was defined as $\alpha = 5\%$ (p-value = 0.05).

NA, not applicable.

diluted to create serial dilutions of CGRP. Furthermore, human blood plasma was used as an alternative to the standard buffer. With these preparations a reference curve was fitted to later determine the individual CGRP concentrations of each sample.

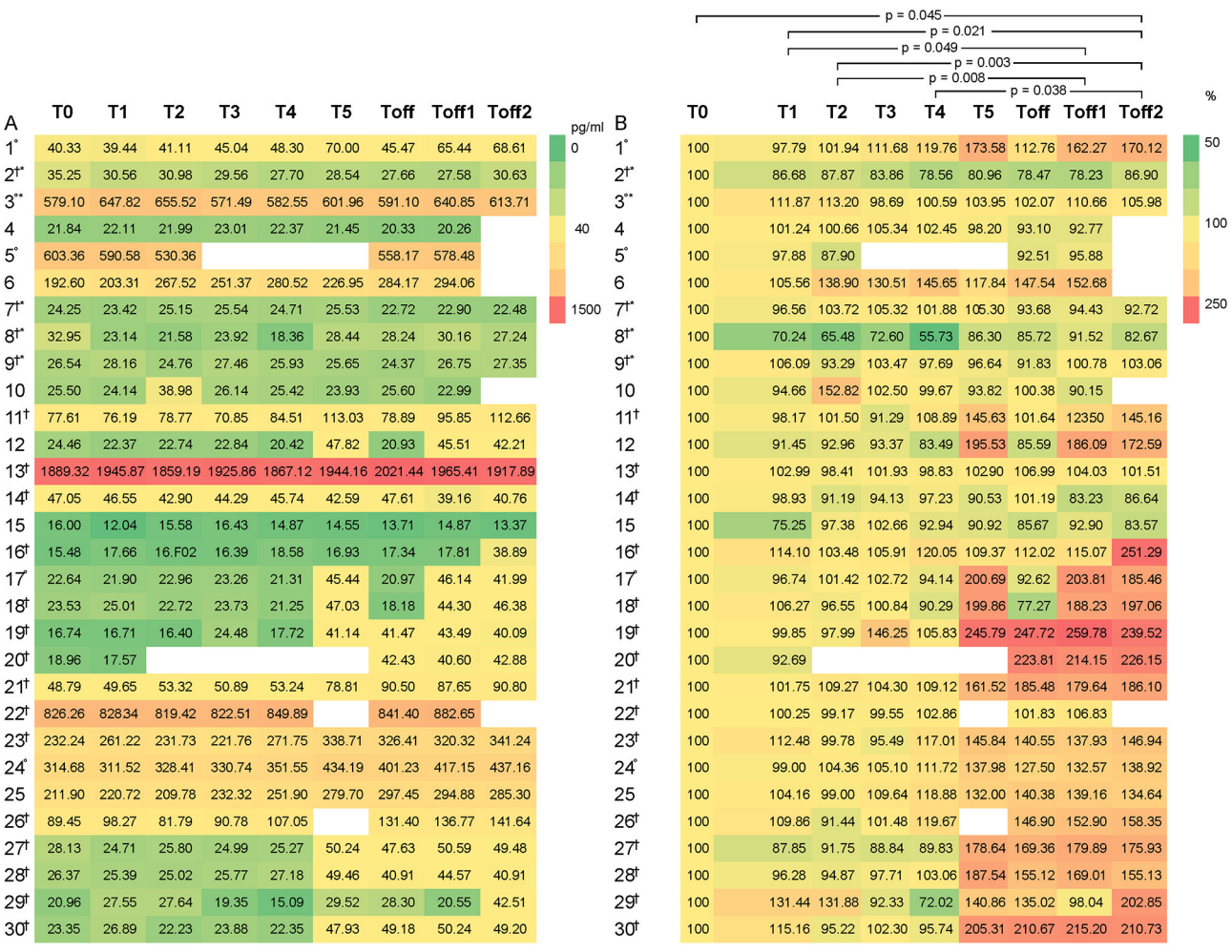
Data Analysis

The Shapiro-Wilk-Test was used to test for normality. To reduce skewness, we applied the log-transformation χ (15). We performed a repeated measures ANOVA to determine the changes of the CGRP levels over the time points. The Greenhouse–Geisser adjustment was used to correct for violations of sphericity. To test for possible vulnerability, the Pearson's correlation coefficient was used to associate CGRP levels with periinterventional headache/migraine days as well as hours since the last headache/migraine attack before the experiment. A p value of 0.05 was considered significant. Values are given as mean ± standard error of the mean (SEM) or as median with confidence intervals (box plots). Missing data on follow-up observation was addressed by using the last observation carried forward (LOCF) principle. The analyses were performed with SPSS version 26.0 (IBM Corporation, Armonk, NY, US).

RESULTS

Sample Characteristics

We assessed plasma samples of a total of 30 patients (22 female, 8 male) with episodic migraine according to ICHD-3 diagnostic criteria, of which 16 patients (11 women) had migraine with aura and 14 patients (11 women) had migraine without aura. The examinations were carried out for 26 patients per protocol, enduring 6 h under hypoxia. Three participants left the NHC prematurely, due to severe migraine headache, and one examination was discontinued for safety reasons due



A

	T0	T1	T2	T3	T4	T5	Toff	Toff1	Toff2
1°	40.33	39.44	41.11	45.04	48.30	70.00	45.47	65.44	68.61
2†*	35.25	30.56	30.98	29.56	27.70	28.54	27.66	27.58	30.63
3°*	579.10	647.82	655.52	571.49	582.55	601.96	591.10	640.85	613.71
4	21.84	22.11	21.99	23.01	22.37	21.45	20.33	20.26	
5°	603.36	590.58	530.36				558.17	578.48	
6	192.60	203.31	267.52	251.37	280.52	226.95	284.17	294.06	
7†*	24.25	23.42	25.15	25.54	24.71	25.53	22.72	22.90	22.48
8†*	32.95	23.14	21.58	23.92	18.36	28.44	28.24	30.16	27.24
9†*	26.54	28.16	24.76	27.46	25.93	25.65	24.37	26.75	27.35
10	25.50	24.14	38.98	26.14	25.42	23.93	25.60	22.99	
11†	77.61	76.19	78.77	70.85	84.51	113.03	78.89	95.85	112.66
12	24.46	22.37	22.74	22.84	20.42	47.82	20.93	45.51	42.21
13†	1889.32	1945.87	1859.19	1925.86	1867.12	1944.16	2021.44	1965.41	1917.89
14†	47.05	46.55	42.90	44.29	45.74	42.59	47.61	39.16	40.76
15	16.00	12.04	15.58	16.43	14.87	14.55	13.71	14.87	13.37
16†	15.48	17.66	16.F02	16.39	18.58	16.93	17.34	17.81	38.89
17°	22.64	21.90	22.96	23.26	21.31	45.44	20.97	46.14	41.99
18†	23.53	25.01	22.72	23.73	21.25	47.03	18.18	44.30	46.38
19†	16.74	16.71	16.40	24.48	17.72	41.14	41.47	43.49	40.09
20†	18.96	17.57					42.43	40.60	42.88
21†	48.79	49.65	53.32	50.89	53.24	78.81	90.50	87.65	90.80
22†	826.26	828.34	819.42	822.51	849.89		841.40	882.65	
23†	232.24	261.22	231.73	221.76	271.75	338.71	326.41	320.32	341.24
24°	314.68	311.52	328.41	330.74	351.55	434.19	401.23	417.15	437.16
25	211.90	220.72	209.78	232.32	251.90	279.70	297.45	294.88	285.30
26†	89.45	98.27	81.79	90.78	107.05		131.40	136.77	141.64
27†	28.13	24.71	25.80	24.99	25.27	50.24	47.63	50.59	49.48
28†	26.37	25.39	25.02	25.77	27.18	49.46	40.91	44.57	40.91
29†	20.96	27.55	27.64	19.35	15.09	29.52	28.30	20.55	42.51
30†	23.35	26.89	22.23	23.88	22.35	47.93	49.18	50.24	49.20

B

	T0	T1	T2	T3	T4	T5	Toff	Toff1	Toff2
1°	100	97.79	101.94	111.68	119.76	173.58	112.76	162.27	170.12
2†*	100	86.68	87.87	83.86	78.56	80.96	78.47	78.23	86.90
3°*	100	111.87	113.20	98.69	100.59	103.95	102.07	110.66	105.98
4	100	101.24	100.66	105.34	102.45	98.20	93.10	92.77	
5°	100	97.88	87.90				92.51	95.88	
6	100	105.56	138.90	130.51	145.65	117.84	147.54	152.68	
7†*	100	96.56	103.72	105.32	101.88	105.30	93.68	94.43	92.72
8†*	100	70.24	65.48	72.60	55.73	86.30	85.72	91.52	82.67
9†*	100	106.09	93.29	103.47	97.69	96.64	91.83	100.78	103.06
10	100	94.66	152.82	102.50	99.67	93.82	100.38	90.15	
11†	100	98.17	101.50	91.29	108.89	145.63	101.64	12350	145.16
12	100	91.45	92.96	93.37	83.49	195.53	85.59	186.09	172.59
13†	100	102.99	98.41	101.93	98.83	102.90	106.99	104.03	101.51
14†	100	98.93	91.19	94.13	97.23	90.53	101.19	83.23	86.64
15	100	75.25	97.38	102.66	92.94	90.92	85.67	92.90	83.57
16†	100	114.10	103.48	105.91	120.05	109.37	112.02	115.07	251.29
17°	100	96.74	101.42	102.72	94.14	200.69	92.62	203.81	185.46
18†	100	106.27	96.55	100.84	90.29	199.86	77.27	188.23	197.06
19†	100	99.85	97.99	146.25	105.83	245.79	247.72	259.78	239.52
20†	100	92.69					223.81	214.15	226.15
21†	100	101.75	109.27	104.30	109.12	161.52	185.48	179.64	186.10
22†	100	100.25	99.17	99.55	102.86		101.83	106.83	
23†	100	112.48	99.78	95.49	117.01	145.84	140.55	137.93	146.94
24°	100	99.00	104.36	105.10	111.72	137.98	127.50	132.57	138.92
25	100	104.16	99.00	109.64	118.88	132.00	140.38	139.16	134.64
26†	100	109.86	91.44	101.48	119.67		146.90	152.90	158.35
27†	100	87.85	91.75	88.84	89.83	178.64	169.36	179.89	175.93
28†	100	96.28	94.87	97.71	103.06	187.54	155.12	169.01	155.13
29†	100	131.44	131.88	92.33	72.02	140.86	135.02	98.04	202.85
30†	100	115.16	95.22	102.30	95.74	205.31	210.67	215.20	210.73

FIGURE 1 | Heat maps to illustrate the distribution of CGRP levels, given as pg/ml, among the participants (1–30). CGRP levels are displayed as colors ranging from green to red as shown in the key. The X-axis shows the different times points of the experiment with T0 as baseline, T1 as first blood sample 1 h after entering the HAC and consecutive hourly blood samples. Toff represents the first blood sample immediately after leaving the HAC. On the left side **(A)**, the absolute values of the CGRP-levels are indicated and on the right side **(B)** the percentage change compared to baseline. Subject experiencing °headache; †migraine; *aura during the experiment. Participant 3 experienced headache that did not fulfill the ICHD criteria for migraine headache due to a lack of nausea or photophobia and phonophobia but had aura symptoms.

to a pronounced decrease in systolic blood pressure in one asymptomatic patient. All participants were followed up 24 h after leaving the NHC. Mean age was 27.56 years (SD ± 7.54), 24 (80%) were younger than 30 years. Mean body mass index was 21.74 kg/m^2 (SD ± 2.63). Mean monthly migraine attack frequency, as reported by the patients, was 3.25 attacks (SD ± 3.05). Mean monthly intake of abortive migraine medication was 3.39 days (SD ± 5.88). Twenty patients (66.7%) had a positive family history for migraine (**Table 1**).

Headache and Migraine

A total of 24 patients (80.0%) reported headaches during the experiment. Nineteen patients (63.3%) developed migraine headache accompanied by autonomic features such as nausea, photophobia and phonophobia, and five (16.7%) developed migraine aura. Incidence of total headache and migraine was increasing throughout the experiment and peaked at Toff, which entailed volunteers completing 6 h of exposition to hypoxia as well as those terminating prematurely. The mean onset of headache was between T4/T5 and that of migraine at T5 during the experiment.

CGRP

CGRP at Baseline

CGRP levels differed significantly between the subjects, ranging from 15.48 to 1,889.31 pg/ml at baseline (**Figure 1**). High CGRP plasma levels at baseline were not associated with age, monthly migraine or headache days, attack frequency, attack duration, attack intensity, sex, family history of migraine, the use of abortive medication, years lived with migraine or headache, BMI, level of physical activity or any other of the collected data. There were two outliers regarding baseline and consecutive CGRP levels, subject 13 (female, 42 years, mean CGRP 1,926 pg/ml) and subject 22 (female, 22 years, mean CGRP 838.64 pg/ml). However, no differences in categorial or metric variables were found for these two participants.

TABLE 2 | Overview of mean CGRP concentration throughout the experiment.

Time	Mean (CGRP) pg/ml	SD
T0	185.19	380.01
T1	190.29	391.33
T2	192.43	390.31
T3	179.81	390.22
T4	183.67	383.77
T5	179.75	388.92
Toff	206.84	400.88
Toff1	212.93	395.67
Toff2	184.21	390.78

Throughout all individual time points the mean CGRP concentration and standard deviation (SD) did not vary significantly ($p > 0.05$). The repeated measures ANOVA was corrected for sphericity using the Greenhouse-Geisser correction.

CGRP During Hypoxic Challenge

The mean absolute CGRP concentration over all time points showed no significant variation regarding absolute values and standard deviation between the subjects (**Table 2**).

In **Figure 1B**, the relative change of CGRP plasma levels in percent at the different sample time points is compared to the baseline values. An apparent difference was found from baseline compared to T5 onwards. We used the Friedman's test and corrected for multiple testing to analyse each individual CGRP concentration for any given time point. We found a significant difference, with an increase of CGRP levels in line with prolonged hypoxia, between T2–Toff1 ($p = 0.008$), T2–Toff2 ($p = 0.003$), T1–Toff1 ($p = 0.049$), T1–Toff2 ($p = 0.021$), T4–Toff2 ($p = 0.038$) and T0–Toff2 ($p = 0.045$), respectively.

CGRP levels of ictal migraine and headache patients compared to participants with no migraine or headache during the experiment are illustrated in **Figures 2A,B**. A repeated measures ANOVA with Greenhouse-Geisser correction determined that mean CGRP levels did not show a statistically significant difference between participants experiencing migraine [$F_{(2.73;\ 76.53)} = 1.02$; $p = 0.385$] or headache [$F_{(2.81;\ 78.74)} = 1.70$; $p = 0.176$] and subjects with absence of headache or migraine. However, we found a significant difference in CGRP levels between subjects with and without aura during the experiment [$F_{(2.99;\ 83.69)} = 3.08$; $p = 0.032$] (**Figure 2C**). Patients experiencing migraine attacks without aura in the course of the hypoxic challenge had significantly higher CGRP concentrations compared to participants with aura symptoms. Higher CGRP levels during the experiment were significantly associated with female sex ($p = 0.001$; **Figure 3A**), with age (>30 years or <30 years; $p = 0.021$; **Figure 3B**) and a negative family history of migraine ($p = 0.009$; **Figure 3C**). No other parameters showed a significant correlation with CGRP concentration (monthly migraine or headache days, headache or migraine intensity, years lived with headache or migraine, BMI, O_2 saturation, blood pressure, pulse rate).

Using the headache diaries, the time between the last migraine or headache attack and the hypoxic challenge was assessed. Mean temporal lag between the last headache or migraine attack was 96 hours (range 24–240 h) and 132 h (range 24–240 h), respectively. A bivariate Pearson correlation showed a medium correlation between higher CGRP and a shorter lag from the last headache attack ($r = -0.41$; $p < 0.05$), but none for the last migraine attack ($r = -0.16$; $p = 0.963$) or the number of peri-interventional headache days ($r = 0.282$; $p = 0.139$).

DISCUSSION

Since the 1980s, attempts have been made to quantify CGRP concentration in plasma, saliva, cerebrospinal fluid, or tear fluid (4, 5, 16–20). Due to its short half-life of ∼10 mins, lack of standardized procedures for measuring and analysing CGRP as well as the variable sample materials, the study results gathered so far are conflicting. Thus, even 40 years after the discovery of this neuropeptide, still no reference values of ictal and interictal CGRP concentration are determined.

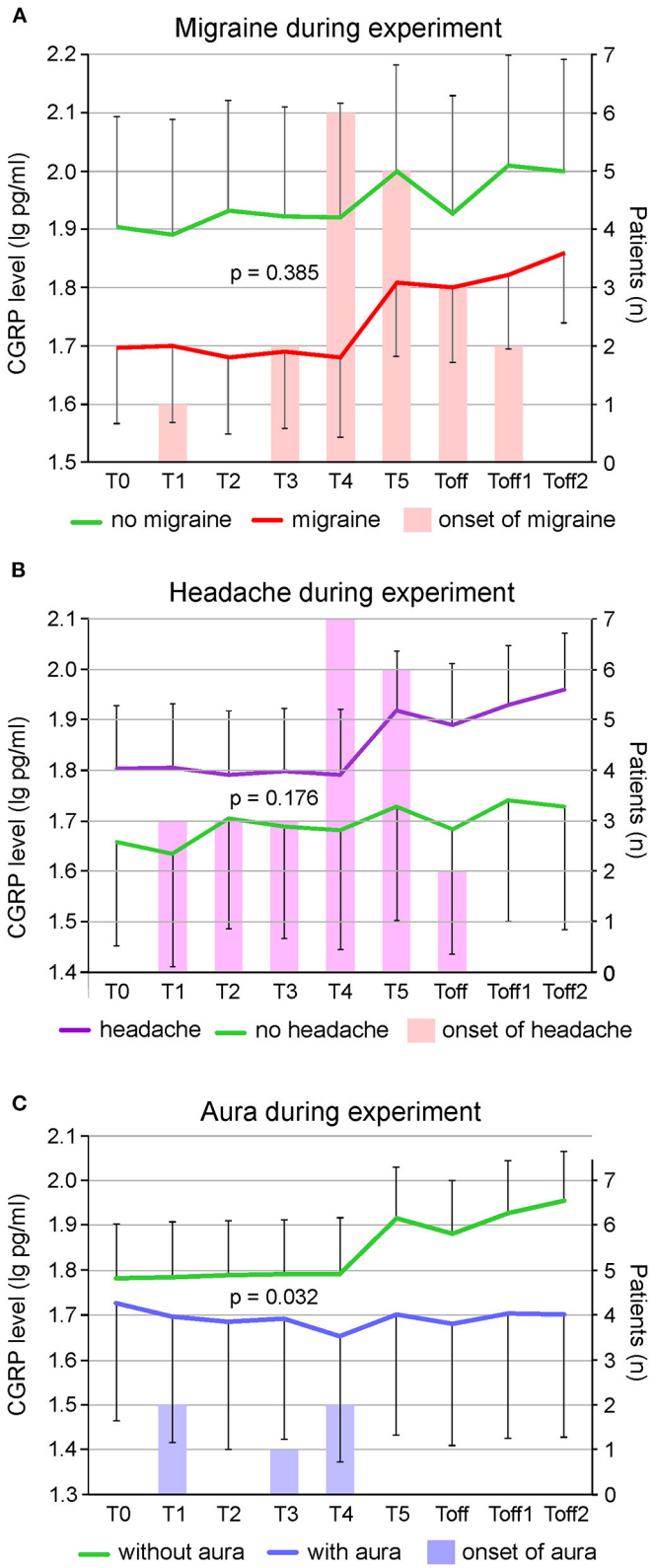


FIGURE 2 | Changes in logarithmized CGRP levels (pg/ml) over the time course of the experiment in participants with migraine ($n = 24$) vs. no migraine ($n = 6$) **(A)**, in subjects with (including migraine, $n = 19$) vs. without headache ($n = 11$) **(B)** and subjects experiencing aura symptoms ($n = 5$) vs. no aura symptoms

(Continued)

FIGURE 2 | ($n = 25$) **(C)**. The right Y-axis depicts the number of participants with the onset of migraine **(A)**, headache **(B)** and aura symptoms **(C)** at the different timepoints during the experiment. The lower and upper limits of standard error of the mean are given. There was a significant difference in plasma CGRP levels between subjects reporting aura or not during the experiment ($p = 0.027$) but there were no significant differences in participants with and without migraine ($p = 0.385$) or headache ($p = 0.176$). Mean onset of migraine peaked at T5, of headache between T4 and T5 and for aura at T3.

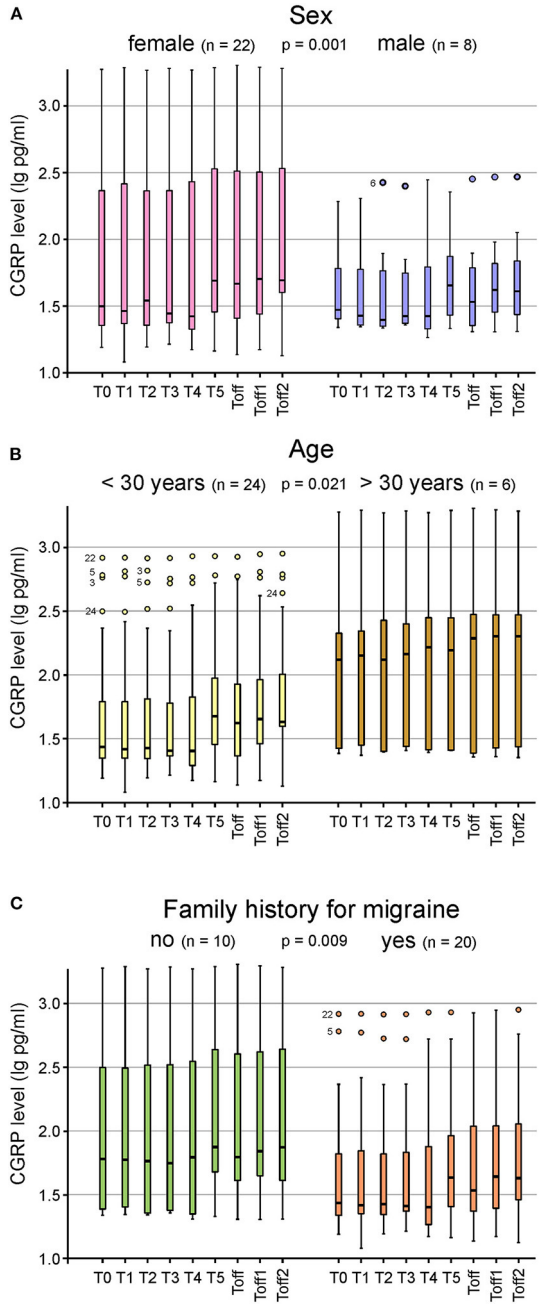


FIGURE 3 | Box plots for the CGRP plasma levels at the different timepoints shown for sex **(A)**, age **(B)** and family history for migraine **(C)**. Significantly higher CGRP levels were found in female subjects ($n = 22$; $p = 0.001$) **(A)**, in participants older than 30 years ($n = 24$; $p = 0.021$) **(B)** and in those with a negative family history ($n = 10$; $p = 0.009$) **(C)**. Mild outliers are marked with a circle (O), the number next to it represents the corresponding patient.

Herein, we present a longitudinal study of a total of 30 participants with episodic migraine with or without aura who were observed under controlled and highly standardized conditions in a normobaric hypoxic chamber. Besides migraine, the volunteers did not suffer from any neurological, psychiatric, cardiovascular, or respiratory disorder. The participants did not overuse acute medication and were not taking any preventative medication for their migraine. The hourly blood samples were taken during a hypoxic challenge. 24 (80%) of the subjects developed a headache, migraine was triggered in 19 (63.3%) and 5 (16.7%) developed a migraine aura. Several studies have identified hypoxia as potent trigger of migraine attacks (14, 21) and therefore hypoxia was used to induce migraine in this study.

Main findings of the study are (1) absolute plasma CGRP concentration differs significantly at baseline without a verifiable explanation. (2) CGRP levels increased significantly in line with hypoxic challenge. (3) A negative family history for migraine, age >30 and female sex were associated with higher CGRP levels during the experiment. (4) Ictal CGRP plasma levels were significantly higher in subjects experiencing migraine without aura. (5) Higher CGRP levels were temporally associated with a recent headache attack.

At baseline CGRP plasma concentration ranged from 15.48 to 1,889.31 pg/ml. We found no explanation for this variation in the variables collected, as there were no significant differences regarding demographic or headache specific features in our subjects. The CGRP concentration remained robust during the experiment intra-individually, indicating stable and standardized testing conditions for each sample, as has been demonstrated before by our group. Therefore, it may be speculated, that CGRP is produced, released and/or degraded at different rates individually.

Hypocapnic hypoxia leads to vasodilatation—a possible involvement of CGRP in the pathophysiology of vasoactive adaptation could be suspected. However, a study did not find altered CGRP levels during hypoxia (22).

In recent years, literature has emerged that offers contradictory results about CGRP measurements in plasma interictally as well as during an attack. Cernuda-Morollón et al. (5) found significantly increased CGRP levels interictally in women with chronic migraine compared to healthy controls or women with a diagnosis of episodic migraine or cluster headache. Fekrazad et al. (23) corroborated these results with their study and consequently proposed the use of CGRP as a biomarker in chronic migraine. Contrary to our findings, both studies found a weak association between age and baseline CGRP concentration. However, we must point out that our population consisted mostly of subjects below 30 years of age with only episodic migraine.

Lee et al. (24) found no elevated CGRP levels in patients with chronic migraine and no association with number of headache days, severity of attacks or headache on the day of blood sampling. Our result support the assumption that CGRP is neither associated with the number of monthly headache days nor monthly migraine days.

There is only limited data on CGRP measurements in human regarding migraine with and migraine without aura. As cortical spreading depression results in a significant depolarization, one can expect an influence on numerous neuronal interactions directly influencing the release of neurotransmitters and neuromodulators. Exemplarily, the expression of cortical CGRP mRNA was induced by repetitive CSD in mice 24 h following stimulation (25, 26). Taking this into account, it is conceivable that CGRP levels differ mainly in the postictal phase between patients with and without aura. This might explain the inconsistent results of studies investigating CGRP levels between those two groups.

Correct sampling of blood including the exact time point of sampling is also important when considering sex and gender influences on CGRP. An association of sex hormones (particularly estrogen, progesterone, and their interaction) with migraine is largely known. Recent studies provided evidence that CGRP levels are also modulated by these hormones (27). Therefore, to provide reliable data on CGRP differences between female and male subjects, plasma levels of sex hormones would be required.

To summarize, there is far too few data and too many heterogenous sampling and analysing methods regarding CGRP levels in human to compare results from different studies. Our study, however, bypasses the individual factors by longitudinal measurements of CGRP levels mainly depending on hypoxic stimulation. Hence, changes within groups and subgroups can be interpreted to be associated with the hypoxic challenge.

Limitations

The findings in this report are subject to at least four limitations. First, the sample size with 30 subjects is small but comparable to other studies (23). However, we are aware that some subgroup analyses are based on a few participants only—and therefore cannot be examined for possible confounders such as gender or age. Second, a headache or migraine attack was "artificially" triggered in subjects using a hypoxic challenge. Utilizing a well-established migraine model minimizes confounders like hormones, nutrition, prophylactic, acute medication, or vague onset of the migraine attack. However, hypoxia cannot be fully ruled out as a confounder. Therefore, our results may not be applicable to unprovoked attacks. Third, our study is missing a control group. Initially, the study was designed using an active control, since we only expected inducing a migraine attack in 50% of the subjects. As mentioned elsewhere (21), a blinded control group is not feasible in a high-altitude chamber trial. Fourth, as this is a pilot study, we have chosen a very conservative statistical approach using Greenhouse-Geisser correction for violation of sphericity in repeated measures ANOVA. Still, our results are significant, which gives strength to our data.

A positive aspect of the study is the harmonized and standardized implementation of the experiment and the examinations in a homogenous patient population. As all subjects entered the NHC at the same time of the day, possible cycling variations of CGRP were minimized.

Taken together we could show significant different baseline levels in migraineurs, with reliable fluctuations during a provoked migraine attack. Since our measurements were done with a commercially available ELISA following a strict published protocol, we believe that our study could serve as a benchmark for future investigations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The study was reviewed and approved by the Ethics Committee of the Medical University of Innsbruck, Austria. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FF: investigation, methodology, conceptualization, data curation, formal analysis, validation, and writing—original draft preparation. KK: investigation, conceptualization, data curation, formal analysis, and original draft preparation. KM: data analysis, methodology, supervision, and writing—review and editing. GB: conceptualization, methodology, validation, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Alteration of gut microbiota in migraine patients with irritable bowel syndrome in a Chinese Han population

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Objective: Migraine is frequently reported in patients with irritable bowel syndrome (IBS), and emerging evidence suggests that gut microbiota plays a role in migraine and IBS. However, alterations in the gut microbiome in migraine patients with IBS remain unknown. This study aimed to explore the compositions of gut microbiota in migraine patients with IBS in a Chinese Han population.

Methods: Sixteen migraine patients with IBS and thirteen age- and gender-matched IBS patients with similar dietary and lifestyle habits were enrolled in this pilot study. Demographic data, clinical data, eating habits, lifestyle habits, comorbidities, and medications were recorded using a unified case registration form. Questionnaires for the Migraine Disability Assessment (MIDAS), Pittsburgh Sleep Quality Index (PSQI), Hamilton Anxiety Scale (HAMA), and Hamilton Depression Scale (HAMD) were completed. Fecal samples were collected, and microbial DNA was extracted. Gut microbiota 16S ribosomal RNA (16S rRNA) gene sequencing targeting the V4 region was performed using the Illumina HiSeq 2500 high-throughput sequencing platform. The relationships between gut microbiota and clinical characteristics of migraine were analyzed.

Results: The structure of gut microbiota differed between migraine patients with IBS and patients with IBS, while the richness and diversity of gut microbiota in migraine patients with IBS showed no significant difference from that of patients with IBS. We found a higher relative abundance of the genus Parabacteroides and a lower relative abundance of the genera Paraprevotella, Lachnospiraceae_UCG-010, Lactococcus, Collinsella, and Comamonas in migraine patients with IBS than in patients with IBS. According to random forest predictive models, the phylum Bacteroidota shows the most important role in migraine patients with IBS. Furthermore, no statistical correlation was found between significantly different taxa at the genus level and migraine clinical data.

Conclusion: This study identified that altered gut microbiota occurred in Chinese Han migraine patients with IBS, but no correlation was found between

gut microbiota and the clinical characteristics of migraine. Further study is needed to better understand the role of gut microbiota in the pathogenesis of migraine in IBS.

KEYWORDS

migraine, irritable bowel syndrome, gut microbiota, 16S rRNA, gut-brain axis

Introduction

Migraine is a common functional disorder characterized by recurrent headache accompanied by various autonomic, affective, and cognitive symptoms (1). Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder characterized by abdominal pain and altered bowel habits without the presence of organic lesions (2). Migraine and IBS share many similarities (3), such as incidence, female predominance, characterized by chronic and recurrent pain, lack of organic damage, similar trigger factors, benign course, and central hypersensitization. Additionally, both disorders are often associated with comorbidities such as somatic and psychiatric diseases. The mechanisms underlying this association are not entirely clear. Migraine and IBS can alter gut microbiota composition and thereby may affect the gut-brain axis and inflammatory status (3). In addition, hereditary and genetic polymorphism, serotonin, and sexual hormones are also believed to play a role (3).

However, the prognosis of IBS is fairly good, whereas that of migraine is worse since suicide and stroke are risk factors associated with migraine (4). According to the Global Burden of Disease (GBD) Study 2018 (5), migraine has become the leading cause of disability in those aged less than 50 years. Previous studies found that migraine is frequently reported in patients with IBS. A study found that approximately 17% of patients with IBS had migraine, while only 8% of the control group suffered from migraine (6). A meta-analysis of six studies showed that the risk of migraine in patients with IBS was 25–50%, while that in the control group was 4–19%, and individuals who suffered from IBS had a coexisting headache with an estimated odds ratio of approximately 2.66 (4). Migraine in patients with IBS worsens the prognosis of IBS. However, biomarkers for migraine in patients with IBS have not yet been discovered.

Previous studies have found that gut microbiota dysbiosis plays an important role in IBS (7, 8). Emerging evidence suggests that the gut microbiota also plays a role in migraine. Animal experiments by our team verified that the gut microbiome was involved in normal mechanical pain sensation and the pathogenesis of migraine (9). Another study showed that gut microbiota dysbiosis contributed to the chronicity of migraine-like pain by upregulating TNFα levels in the trigeminal nociceptive system (10). A clinical study showed that probiotics could be an effective and beneficial supplement to improve migraine headaches in those with both chronic and episodic migraines (11). Another clinical study indicated that food elimination based on IgG antibodies in migraine patients with IBS may effectively reduce symptoms associated with both disorders and has a positive impact on the quality of life in patients and on the healthcare system (12). It is currently believed that the gut microbiota may act through the microbiota–gut–brain axis, which refers to bidirectional interactions between the gut microbiome and brain *via* the vagus nerve, enteroendocrine signaling, immune system crosstalk, and neurotransmitters (13).

Recent evidence from bacterial cultures suggests that migraine patients with IBS present a higher incidence and severity of fecal dysbiosis than patients with IBS (14). However, the precise characteristics of the gut microbiota in migraine patients with IBS have not been fully elucidated. The aim of this study was to explore the composition of gut microbiota in migraine patients with IBS in a Chinese Han population.

Materials and methods

Subjects

Migraine patients with IBS were recruited at the International Headache Center of Chinese PLA General Hospital from April to August 2016. Age- and gender-matched patients with IBS were recruited from visitors coming to the Medical Examination Center for routine exams. The sample size was calculated by G*Power (ver. 3.1.9.7) based on the *t*-test design (15). In accordance with the Ethics Committee of PLA General Hospital, all participants were eligible for inclusion if they were aged 18–60 years and provided informed consent. This study was conducted in accordance with the guidelines set forth by the Declaration of Helsinki. The migraine diagnosis was made by experienced neurologists at the headache center, and the IBS diagnosis was made by experienced gastroenterologists. Thus, the study population comprised migraine patients with IBS (M_IBS group) and patients with IBS (IBS group) (Figure 1).

All participants met the Rome IV criteria for the diagnosis of IBS (2), and migraine was diagnosed according to the third edition of the International Headache Society classification (ICHD-3) (16).

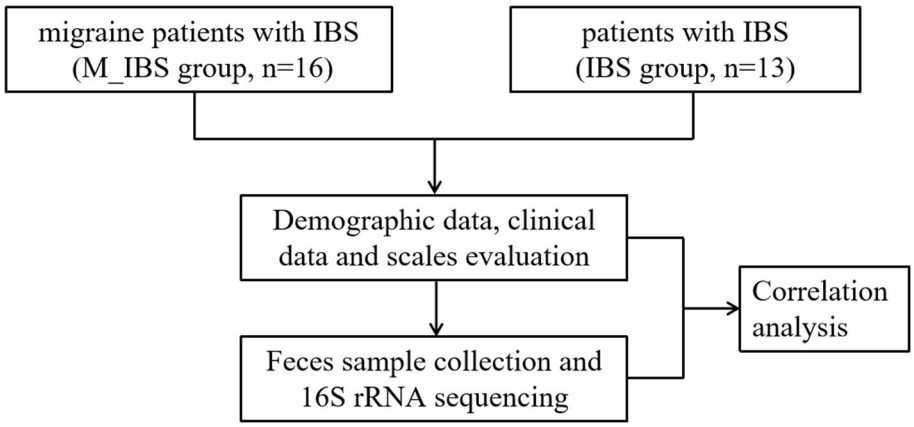

FIGURE 1
Flowchart illustrating the recruitment of migraine patients with IBS and patients with IBS and the research implementation plan.

Potential subjects with any of the following were excluded from this study: any other type of headache defined by the ICHD-3; antibiotic therapy at least 3 months before enrollment into the cohort; diarrhea on the day of fecal sampling; the score assessed using the Hamilton Rating Scale for Anxiety (HAMA) was over 21, and the score assessed using the Hamilton Rating Scale for Depression (HAMD) was over 20; any previous serious medical condition, including both somatic and psychiatric dysfunctions; drug misuse, overuse, or daily intake of medication; and pregnant or nursing females.

Clinical data collection

Patients were interviewed for medical history. Each patient underwent a detailed physical and neurological examination and either magnetic resonance imaging or computed tomography of the head to rule out organic diseases of the brain. The following detailed information was recorded for each participant: demographic and headache data; eating habits; lifestyle habits; and comorbidities and medications. Information regarding headaches included disease duration (DD), attack frequency (AF), visual analog scale (VAS) score, and MIDAS score, which were evaluated by the migraine disability assessment (MIDAS) questionnaire (17). Sleep condition was evaluated using the Pittsburgh Sleep Quality Index (PSQI) (18), and mood condition was assessed using the HAMA (19) and HAMD (20) (Figure 1).

Fecal sample collection and DNA extraction

The disposable sterile collection container and tubes were distributed to the participants in advance. After the feces were discharged into the sterile container, the middle part of the feces was placed in the tube using a sterile stick. Fecal samples were immediately stored in liquid nitrogen and later transferred into a −80°C freezer for preservation.

Genomic DNA in the stool samples (approximately 100 mg per sample) was extracted using a Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit (P11496, Invitrogen^{TM}, Thermo Fisher Scientific). The concentration of genomic DNA in each fecal sample was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). DNA integrity and sizes were assessed using 1% agarose gel electrophoresis (AGE).

16S rRNA sequencing and data processing

The gene located in the 16S rRNA V4 region was detected by specific primers, namely, 515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT. The NEBNext® Ultra^{TM} RNA Library Prep Kit for Illumina® (E7530 L, NEB) was used to generate sequenced libraries on the Illumina HiSeq platform (Allwegene Technologies Inc., Beijing, China). The raw data were mainly processed using QIIME 2.0, USEARCH (Version 10.0.240), and other R packages mentioned below (21, 22). Trimmomatic was used to filter the nucleotides of poor quality, and reads < 50 nt were removed (parameters: LEADING: 20, TRAILING: 20, MINLEN: 50) (23). FLASH and Pear were used to assemble overlapping read pairs (24, 25). Chimeras were filtered out by UCHIME (26). The clean tags were left after the screening flow above, and they were clustered into operational taxonomic units (OTUs) by the UPARSE algorithm with a sequence similarity no less than 97% (27). Finally, an OTU table was obtained by quantifying the frequency

of the OTUs in each sample. Simultaneously, the OTUs were aligned to the SILVA 132 database and assigned taxonomy at the kingdom, phylum, class, order, family, genus, and species levels (28).

Statistical analyses

IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, N.Y., USA) and R software (ver. 3.6.1, the R Project for Statistical Computing) were used for the statistical analysis. Comparisons between groups were performed using Pearson's chi-square test for categorical variables and the Wilcoxon rank-sum test and Student's *t*-test for quantitative variables. To control the false discovery rate (FDR) for multiple testing, the q-value (corrected *p*-value) was calculated using the Benjamini–Hochberg method. Alpha diversity and beta diversity measures were calculated using the QIIME program based on the rarefied OTU counts. Differential abundance analysis was performed using the Wilcoxon rank-sum test at the phylum and genus levels. Distinguishment of the gut microbiota specific to migraine patients with IBS was identified using the linear discriminant analysis (LDA) effect size (LEfSe) method (LEfSe, https://huttenhower.sph.harvard.edu/galaxy/) (29), which is part of the QIIME package. Random forest (RF) models were used to predict disease status based on gut microbiota and the clinical data profile (significantly different taxa at each level and OTUs assessed using the Wilcoxon rank-sum test) using the default parameters of the R implementation of the algorithm (Boruta algorithm, "randomForest" package) (30). Correlations between migraine clinical data and significantly different taxa at the genus level with a prevalence ≥10% for 16 migraine patients with IBS were calculated using Spearman's rank correlation analysis with the R package "cor.test". $P < 0.05$ was considered to be statistically significant.

Results

Clinical characteristics

The demographic characteristics of the M_IBS group and IBS group are shown in Table 1. The study population consisted of 29 Chinese Han people with IBS, including 16 migraine patients with IBS patients (5 men and 11 women) and 13 patients with IBS (3 men and 10 women). The age range of the participants was from 23 to 58 years. The average age of migraine patients with IBS patients was 39.69 ± 11.57 years, while that of patients with IBS was 37.00 ± 8.70 years. There was no significant difference between the two groups in sex ($\chi^2 = 0.240$, $P = 0.697$), age ($t = 0.693$, $P = 0.494$), BMI ($t = 0.971$, $P = 0.340$), education ($\chi^2 = 1.203$, $P = 0.273$), or region ($\chi^2 = 1.745$, $P = 0.488$). No significant difference was found in PSQI ($Z = -0.773$, $P = 0.439$) and HAMD ($t = 2.028$, $P = 0.053$) scores between the two groups, while HAMA scores were higher in the M_IBS group than in the IBS group ($t = 2.988$, $P = 0.006$). However, the HAMA and HAMD scores of all subjects did not meet the diagnostic criteria for anxiety and depression; that is, anxiety or depression was not observed in any of the subjects included in this study.

TABLE 1 Demographic characteristics in the M_IBS and IBS groups.

Variable	M_IBS	IBS	*p*-value
Number, n	16	13	
Gender, n Male/n Female	5/11	3/10	0.697
Age, y	39.69 ± 11.57	37.00 ± 8.70	0.494
BMI, kg/m^2	23.42 ± 4.09	22.07 ± 3.23	0.340
Education, $n \geqq 9$y/$n > 9$y	5/11	1/12	0.273
Regions, n North of China /n South of China	14/2	13/0	0.488
PSQI, median (IQR)	5 (4.75)	4 (7)	0.439
HAMA	9.88 ± 4.49	4.77 ± 4.40	0.006**
HAMD	6.25 ± 4.49	3.31 ± 2.95	0.053

**$p < 0.01$; BMI, body mass index; PSQI, Pittsburgh Sleep Quality Index; IQR, interquartile range; HAMA, Hamilton Anxiety Scale; HAMD, Hamilton Depression Scale.

The eating and lifestyle habits of the M_IBS group and IBS group are shown in Table 2. There was no significant difference between the two groups in eating habits, including smoking ($\chi^2 = 0.050$, $P = 1.000$), alcohol ($\chi^2 = 0.562$, $P = 0.632$), tea ($\chi^2 = 0.082$, $P = 1.000$), coffee ($\chi^2 = 0.738$, $P = 0.606$), breakfast ($\chi^2 = 0.738$, $P = 0.606$), refined grain ($Z = -0.839$, $P = 0.401$), coarse grain ($Z = -0.923$, $P = 0.356$), takeaway food ($\chi^2 = 0.057$, $P = 1.000$), beans ($\chi^2 = 0.014$, $P = 1.000$), yogurt ($\chi^2 = 2.644$, $P = 0.192$), meat ($\chi^2 = 0.842$, $P = 1.000$), vegetables ($\chi^2 = 0.842$, $P = 1.000$), fruits ($\chi^2 = 0.842$, $P = 1.000$), and fermented food ($\chi^2 = 0.562$, $P = 0.632$), and lifestyle habits, including bowel movements (bowel movements per day $\chi^2 = 1.756$, $P = 0.238$; bowel movement quality $\chi^2 = 4.253$, $P = 0.119$), exercise ($\chi^2 = 0.293$, $P = 0.588$), staying up late ($\chi^2 = 0.566$, $P = 0.667$), pressure ($\chi^2 = 0.042$, $P = 0.837$), and mood ($\chi^2 = 0.404$, $P = 0.663$).

Comorbidities and medications of the M_IBS group and IBS group are shown in Table 3. There was no significant difference between the two groups in comorbidities, including hypertension ($\chi^2 = 0.023$, $P = 1.000$), hyperlipidemia ($\chi^2 = 2.719$, $P = 0.232$), diabetes ($\chi^2 = 0.842$, $P = 1.000$), allergies ($\chi^2 = 0.240$, $P = 0.697$), asthma ($\chi^2 = 0.842$, $P = 1.000$), and gastric ulcer ($\chi^2 = 0.023$, $P = 1.000$), and medications, including antihypertensives ($\chi^2 = 0.023$, $P = 1.000$), statins ($\chi^2 = 0.842$, $P = 1.000$), antidiabetic drugs ($\chi^2 = 0.842$, $P = 1.000$), and nonsteroidal anti-inflammatory drugs (NSAIDs) ($\chi^2 = 3.770$, $P = 0.107$).

TABLE 2 Eating habits and lifestyle habits in the M_IBS and IBS groups.

	M_IBS	IBS	*p*-value
Number, n	16	13	
Smoking, *n* (%)	2(12.5)	2(15.4)	1.000
Alcohol, *n* (%)	2(12.5)	3(23.1)	0.632
Tea, *n* (%)	3(18.8)	3(23.1)	1.000
Coffee, *n* (%)	3(18.8)	1(7.7)	0.606
Breakfast per week			0.606
<3	3	1	
≧3	13	12	
Refined grain (Median [IQR], 50 g per day)	5 (1.75)	4(2)	0.401
Coarse grain (Median [IQR], 50 g per day)	1 (0)	1(1)	0.356
Take away food per week			1.000
<3	13	11	
≧3	3	2	
Beans per week			1.000
<3	12	10	
≧3	4	3	
Yogurt per week			0.192
<3	16	11	
≧3	0	2	
Meat			1.000
Occasionally	1	0	
Regularly	15	13	
Vegetable			1.000
Occasionally	1	0	
Regularly	15	13	
Fruit			1.000
Occasionally	1	0	
Regularly	15	13	
Fermented food per week			0.632
<3	14	10	
≧3	2	3	
BM per day			0.238
≧1	10	11	
2-3	6	2	
BMQ			0.119
Loose	8	3	
Normal	5	9	
Solid	3	1	
Exercise per week			0.588
<3	9	6	
≧3	7	7	
Stay up late per week			0.667
<3	13	9	
≧3	3	4	
Great pressure, *n* (%)	8(50)	6(46.2)	0.837
Happy mood per week			
<3	4	2	0.663
≧3	12	11	

IQR, interquartile range; BM, bowel movements; BMQ, bowel movement quality (loose: tend toward diarrhea; solid: tend toward constipation).

TABLE 3 Comorbidities and medications in the M_IBS and IBS groups.

	M_IBS	IBS	*p*-value
Comorbidities, *n* (%)			
Hypertension	1(6.3)	1(7.7)	1.000
Hyperlipidemia	3(18.8)	0(0)	0.232
Diabetes	1(6.3)	0(0)	1.000
Allergies	5(31.3)	3(23.1)	0.697
Asthma	1(6.3)	0(0)	1.000
Gastric ulcer	1(6.3)	1(7.7)	1.000
Medications, *n* (%)			
Antihypertensives	1(6.3)	1(7.7)	1.000
Statins	1(6.3)	0(0)	1.000
Antidiabetic Drug	1(6.3)	0(0)	1.000
NSAIDs	4(25)	0(0)	0.107

NSAIDs, nonsteroidal anti-inflammatory drugs.

TABLE 4 Clinical features of migraine in the M_IBS group.

Clinical features of migraine	M_IBS
AF (Median[IQR], times per month)	3.67(7.58)
DD, years	15.81 ± 11.11
VAS	7.88 ± 1.19
MIDAS, Median (IQR)	27(63.5)
MIDAS days	11(22.75)
MIDAS severity	7.88 ± 1.19

AF, attack frequency; DD, disease duration; VAS, visual analog scale; MIDAS, migraine disability assessment; IQR, interquartile range.

The clinical features of migraine in the M_IBS group are shown in Table 4. The median AF was 3.67 times per month, and the interquartile range (IQR) was 7.58. The average DD was 15.81 ± 11.11 years, and the average VAS score was 7.88 ± 1.19. The median MIDAS was 27, and the IQR was 63.5. The median number of MIDAS days was 11 days, and the IQR was 22.75. The average MIDAS severity was 7.88 ± 1.19.

Alpha and beta diversity between the M_IBS and IBS groups

Alpha diversity indices, including Chao1, observed species, phylogenetic diversity whole tree, and Shannon and Simpson indices, were analyzed to quantify species abundance and diversity based on OTU levels. There was no significant difference between the M_IBS and IBS groups in α-diversity indices (chao1: $P = 0.487$; observes_species: $P = 0.661$; PD_whole_tree: $P = 0.358$; Shannon: $P = 0.546$; Simpson: $P = 0.408$), indicating that the richness and diversity of the gut microbiota in migraine patients with IBS patients were not

different from that of patients with IBS. However, significant differences were found in β-diversity based on Bray–Curtis principal coordinate analysis (PCoA; $P = 0.041$; Figure 2A) and partial least squares discrimination analysis (PLS-DA; $P < 0.001$; Figure 2B) between the M_IBS and IBS groups, which meant that the gut microbial structure in the M_IBS group was significantly different from that in the IBS group.

Taxa alteration between the M_IBS and IBS groups

The relative abundance of the gut microbiota in the M_IBS and IBS groups at the phylum and genus levels is shown in Figure 3. Eleven phyla and 46 genera were evaluated in all subjects. We used the Wilcoxon rank-sum test to perform differential abundance analyses of differentially abundant phyla and genera between the M_IBS and IBS groups at a false discovery rate of 5%. At the phylum level, we identified a higher relative abundance of the phylum Bacteroidota ($P = 0.056$) and a lower relative abundance of the phyla Firmicutes ($P = 0.083$) and Actinobacteriota ($P = 0.072$) in the M_IBS group than in the IBS group, but the differences were not statistically significant (Figure 3A). The phylum Cyanobacteria was only found in the IBS group but not in the M_IBS group ($P < 0.001$, Figures 3A,C). At the genus level, the relative abundance of the genus Parabacteroides was higher in the M_IBS group, and the relative abundance of the genera Paraprevotella, Lachnospiraceae_UCG-010, Lactococcus, Collinsella, and Comamonas was higher in the IBS group ($P < 0.05$, Figures 3B,D,E). Differences in the taxa at the genus level are detailed in Figure 3. To identify important taxonomic differences between the M_IBS and IBS groups, we conducted linear discriminant analysis (LDA) effect size (LEfSe) analysis, and a logarithmic LDA score cutoff of 3.0 was used. We found significant abundance differences in the gut microbiota between the M_IBS and IBS groups. The relative abundance of the genus Parabacteroides was higher in the M_IBS group, while the relative abundance of the genus Paraprevotella was higher in the IBS group (LDA score (log10) > 3, $P < 0.05$, Figures 4A,B). These results indicated that migraine patients with IBS had a differential abundance of certain genera compared to that of patients with IBS.

Random forest predictive models

To evaluate the disease status of migraine patients with IBS based on an ensemble of decision trees, we used RF to build a predictive model based on gut microbiota and clinical data profiles using the significantly different taxa at each level and OTUs from the Wilcoxon rank-sum test as the input. In these models, four phyla and the Firmicutes/Bacteroidetes ratio (F/B ratio), three classes, four orders, four families, six genera, three species, 51 OTUs, and clinical data, including HAMA and HAMD scores, predicted migraine patients with IBS using the RF model (Figure 4C). The importance of correlated phylum-level abundance taxa, F/B ratio, and clinical data is shown in Figure 4D. According to this model, the phylum Bacteroidota shows the most important role in migraine patients with IBS.

Correlation between gut microbiota and clinical characteristics of migraine

We performed a correlation analysis between gut microbiota (significantly different taxa at the genus level, at a prevalence ≥10%) and migraine clinical data, including attack frequency (AF), disease duration (DD), pain severity (VAS), migraine disability (MIDAS), PSQI, and HAMA and HAMD scores but no statistical correlation was found ($P > 0.05$, Figure 5). The genus Parabacteroides has a possible positive correlation trend toward significance with PSQI ($r = 0.487$, $P = 0.056$), and the genus Paraprevotella has a possible negative correlation trend toward significance with DD ($r = -0.458$, $P = 0.075$) (Figure 5).

Discussion

Migraine is frequently reported in patients with IBS, which leads to a worse prognosis for these patients; however, biomarkers for migraine in patients with IBS have not yet been discovered. In this study, we found altered gut microbiota for the first time in migraine patients with IBS in the Chinese Han population, and no differentially expressed bacterial taxa were related to the clinical characteristics of migraine. The strength of our study lies in a detailed comparison of eating habits, lifestyle habits, comorbidities, and medications, which may largely mitigate the influence of confounding factors on the results.

In our study, no significant difference was found in α-diversity indices of gut microbiota in migraine patients with IBS compared with patients with IBS, but β-diversity indices of migraine patients with IBS differed significantly from those of patients with IBS qualitatively. A metagenomic shotgun-sequencing study on gut microbiota in elderly women with migraine showed that α-diversity was evidently decreased in the migraine group at both the genus and species levels, whereas the species richness was not significantly different in the migraine and control groups at either level (31). The species richness analysis results in the previous study were consistent with our results, but the results of the α-diversity indices were not consistent with our results. We speculate that there may be several reasons for the different α-diversity results. First, the study populations are different. The subjects of our

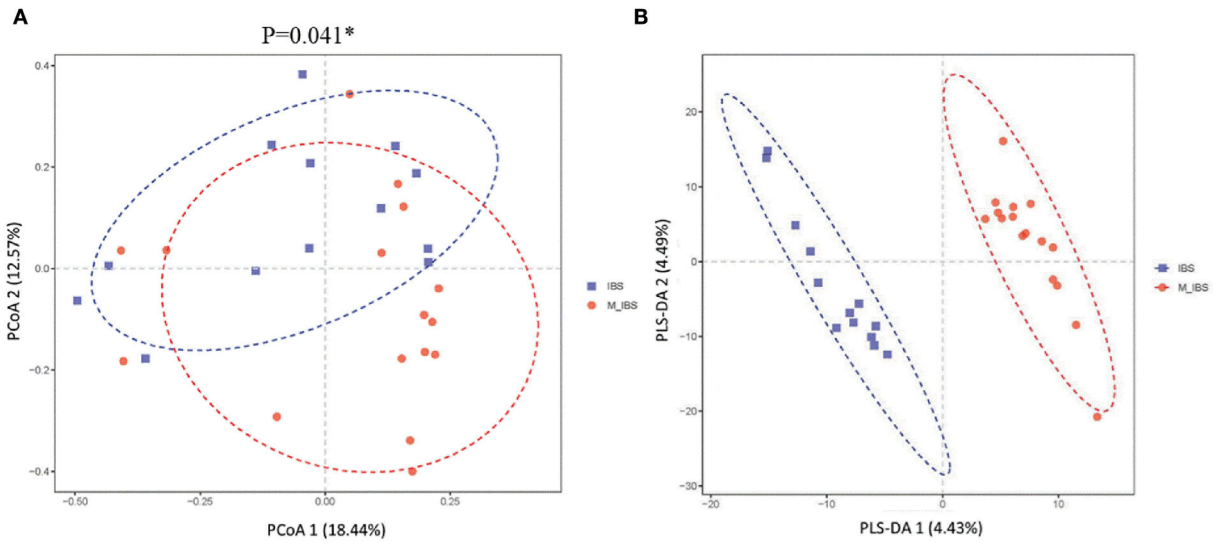

FIGURE 2
β-diversity indices of gut microbiota in the M_IBS and IBS groups. Differences in beta diversity indices between the M_IBS and IBS groups were measured using PCoA based on Bray–Curtis **(A)** and PLS-DA **(B)**, and significant *P*-values are indicated. The axes represent the two dimensions explaining the greatest proportion of variance in the communities. Each symbol represents a sample, and the points of different colors or shapes in the figure represent different groups. The scales on the horizontal and vertical axes are relative distances, while PCoA 1, PCoA 2, PLS-DA 1, and PLS-DA 2 represent the suspected influencing factors for the deviation of the microbial composition of the two groups of samples. M_IBS, migraine with irritable bowel syndrome; IBS: irritable bowel syndrome; OUT, operational taxonomic unit; PCoA, principal coordinates analysis; PLS-DA, partial least squares discrimination analysis.

study were migraine patients with IBS, and the control group consisted of patients with IBS, while in the previous study, the subjects were elderly female migraine patients, and the control group consisted of healthy individuals. Second, stool detection methods were different. The method in our study was 16S rRNA gene sequencing, whereas the method in the previous study was metagenomic shotgun sequencing. Third, diversity analysis is based on different data. The diversity analysis in our study was based on OTUs, while the diversity analysis in the previous study was based on genus and species levels. In short, diversity analyses suggest that the structure of the gut microbiota in migraine patients with IBS is different from that of patients with IBS.

Our results showed that at the phylum level, we found a higher abundance of the gram-negative phylum Bacteroidota and a lower abundance of the gram-positive phyla Firmicutes and Actinobacteriota in migraine patients with IBS, but the differences were not statistically significant. RF predictive models also underlined the importance of the phylum Bacteroidota in migraine patients with IBS. Some studies showed similar results to ours, and decreased Firmicutes and increased Bacteroidetes in the gut microbiota were found in some central nervous system diseases, including patients with Alzheimer's disease (32), Parkinson's disease (33), multiple sclerosis (34), major depressive disorder, and autism spectrum disorder (35). However, some differences were observed between our study and previous studies. Individuals with obesity have a greater F/B ratio, more Firmicutes, and fewer Bacteroidetes (36). Additionally, patients with IBS show increased Firmicutes and decreased Bacteroidetes abundance (37). A study on the gut microbiota of patients with migraine found that elderly female patients with migraine showed significantly higher levels of Firmicutes relative to the controls (38). We speculate that changes at the phylum level may be associated with migraine in IBS. Some species within Firmicutes can produce the metabolite butyrate, a short-chain fatty acid, which predominantly plays an immunoregulatory role. All species within Bacteroidetes are gram-negative and contain the toxin lipopolysaccharide (LPS) in their outer membrane, which is known for its proinflammatory properties. The imbalance of Firmicutes and Bacteroidetes may induce an immune inflammatory response, which may be related to the pathogenesis of migraine in IBS. The phylum Cyanobacteria was only found in patients with IBS but not in migraine patients with IBS; therefore, the depletion of Cyanobacteria may be related to the occurrence of migraine in patients with IBS. However, due to its low abundance, it has not been studied extensively to date.

At the genus level, the relative abundance of Parabacteroides was higher and the abundance of Paraprevotella, Lachnospiraceae_UCG-010, Lactococcus, Collinsella, and Comamonas was lower in migraine patients with IBS. LEfSe analysis found similar results, with more Parabacteroides and less Paraprevotella in the gut microbiota of migraine

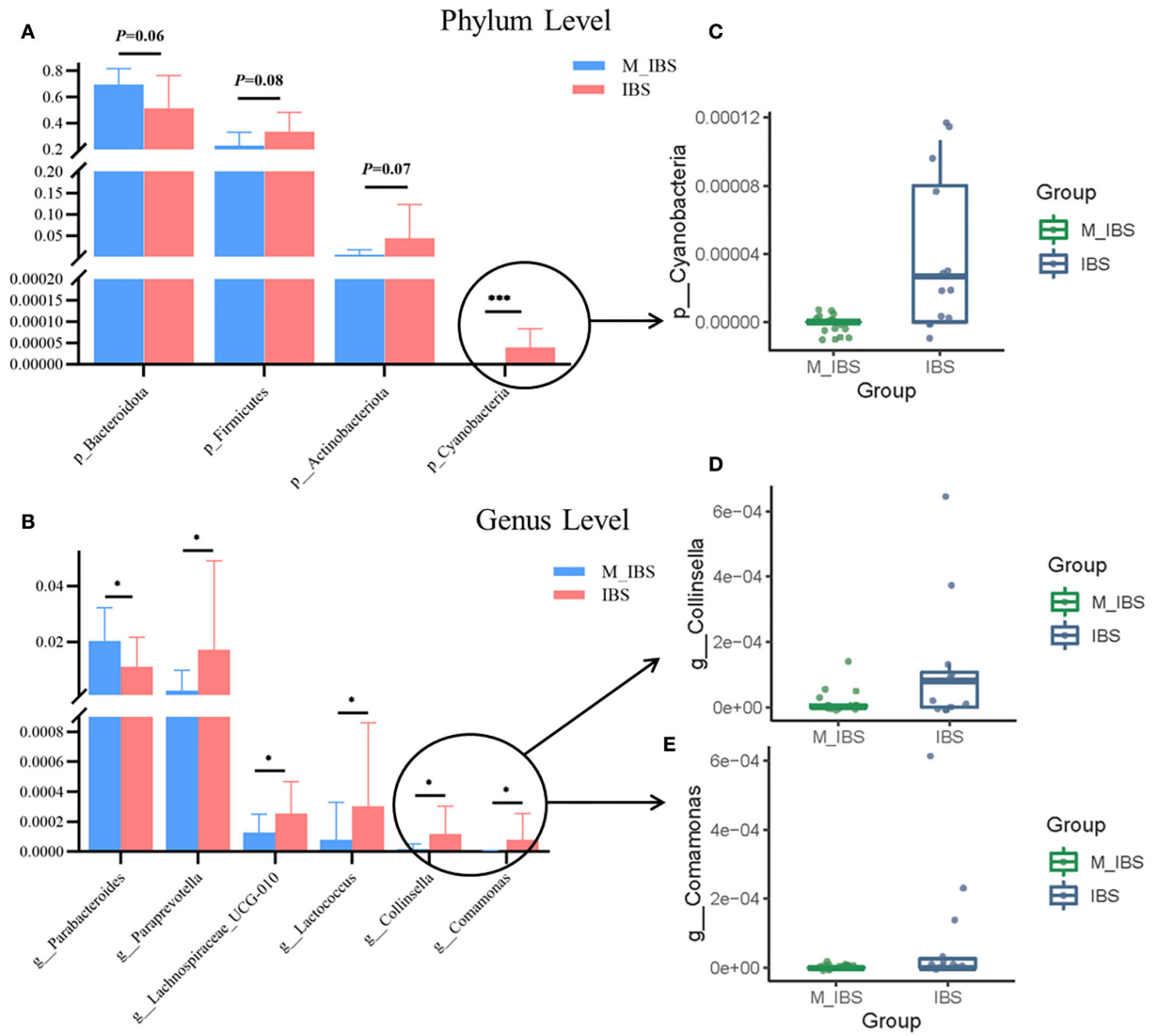

FIGURE 3
Relative abundances of gut microbiota in the M_IBS and IBS groups. **(A,B)** Bar plots comparing abundances of differentially abundant phyla and genera between the M_IBS and IBS groups, and only $p < 0.05$ or trending results are shown. **(C–E)** Box plots comparing differential phyla and genera with lower relative abundance values in bar plots between the M_IBS and IBS groups. These "signature" taxa were selected using Wilcoxon rank-sum tests and a false discovery rate of 5%. Error bars represent standard deviations, and phylum-level and genus-level taxa are plotted. *$P < 0.05$; ***$P < 0.001$. M_IBS, migraine with irritable bowel syndrome; IBS, irritable bowel syndrome.

patients with IBS. However, a metagenomic study on gut microbiota in elderly women with migraine showed that some detrimental species, especially Clostridium spp., were significantly enriched in migraineurs, and the controls held more beneficial microorganisms, such as Bifidobacterium adolescentis, Faecalibacterium prausnitzii, and Bacteroides intestinalis, and some "unfriendly" species, such as Odoribacter splanchnicus and Prevotella copri (31). Different results may be due to different research subjects and methods.

Parabacteroides is a group of gram-negative anaerobic bacteria in the phylum Bacteroidota that commonly colonize the gastrointestinal tract of humans. Parabacteroides exert proinflammatory effects through LPS and its metabolic end-product succinic acid (38). Paraprevotella in the phylum Bacteroidota contributes to the production of propionate by Phascolarctobacterium and then exerts an anti-inflammatory effect (39). There is limited information on the physiological role of Lachnospiraceae UCG-010 in the family Lachnospiraceae, phylum Firmicutes. Lachnospiraceae has previously been shown to be negatively correlated with new-onset, treatment-naive Crohn's disease in biopsy samples from the ileum and rectum (40). Lachnospiraceae

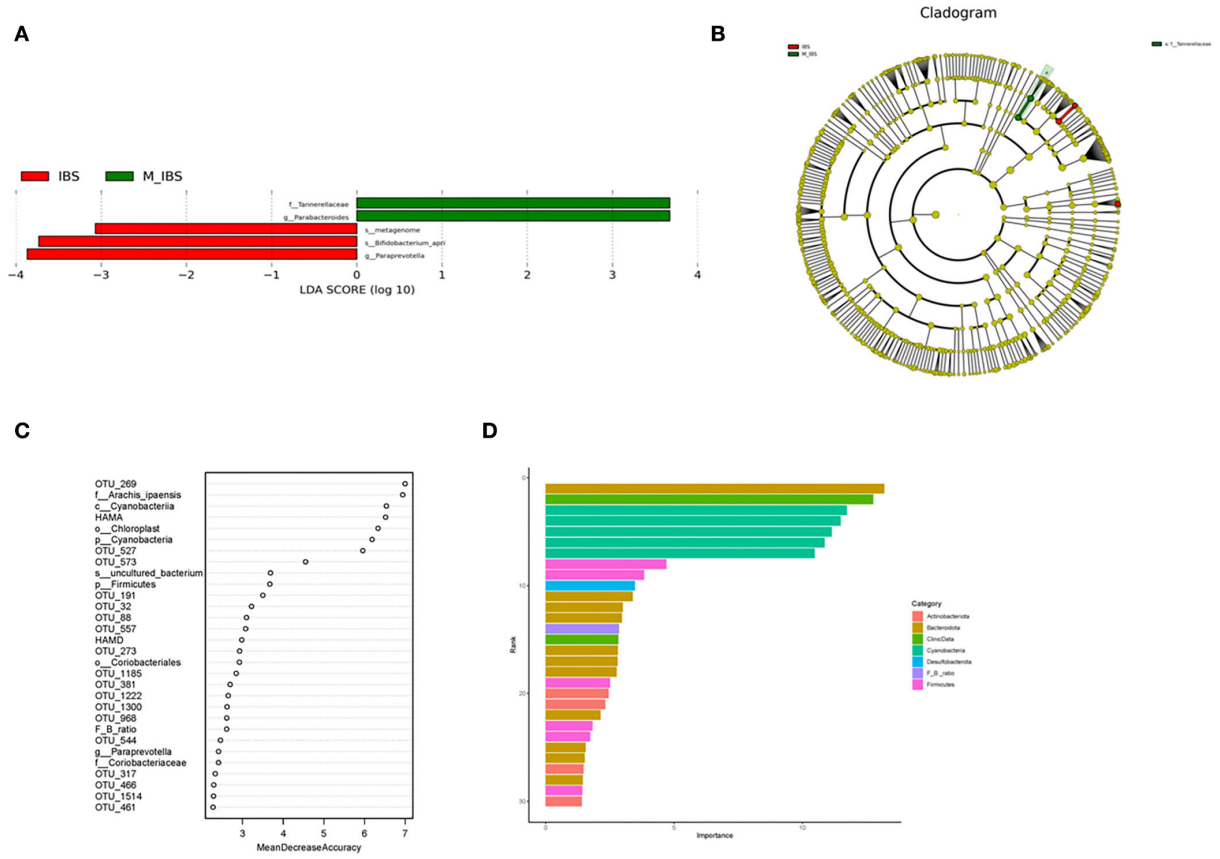

FIGURE 4
Taxonomic differences in gut microbiota in the M_IBS and IBS groups. **(A)** Linear discriminant analysis (LDA) effect size (LEfSe) analysis revealed significant taxonomic differences in gut microbiota between the M_IBS group (positive score) and the IBS group (negative score). The LDA scores (log10) >3 and $P < 0.05$ are listed. **(B)** Cladogram using the LEfSe method indicating the phylogenetic distribution of gut microbiota in the M_IBS and IBS groups. **(C)** The predictive model based on differentially abundant taxa and clinical data using an RF model. The relative importance of each index in the predictive model was determined using the mean decreasing accuracy and the Gini coefficient. **(D)** Variable importance of correlated phylum-level abundance taxa, F/B ratio, and clinical data. M_IBS, migraine with irritable bowel syndrome; IBS, irritable bowel syndrome; p, phylum; c, class; o, order; f, family; g, genus; s, species; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; RF, random forest; F/B ratio, Firmicutes/Bacteroidetes ratio.

UCG-010 increased significantly after grape powder intake for 4 weeks (41). Therefore, Lachnospiraceae UCG-010 may be a beneficial genus. Lactococcus is a genus of gram-positive facultative anaerobic bacteria in the phylum Firmicutes and is generally considered nonpathogenic toward humans in which some species produce antimicrobial compounds, such as bacteriocins, nisin, lactococcin, and recombinant proteins. Additionally, Lactococcus plays an important role in maintaining human intestinal health (42). A study found that the level of Lactococcus in the gut microbiota of nonobese patients with polycystic ovary syndrome (PCOS) was significantly lower than that of healthy controls and found that the gut microbiota changes in patients with PCOS were associated with sex hormone levels (43). Our study found that the relative abundance of Lactococcus in the gut microbiota of migraine patients with IBS was reduced, suggesting that Lactococcus may be involved in the pathophysiological process of migraine patients with IBS through changes in sex hormone levels. Comamonas in the phylum Proteobacteria is one of the few genera that can synthesize vitamin B12, which is important for normal physiological processes in humans (44). We speculate that Comamonas may be involved in the pathological process of migraine in patients with IBS through the reduction of vitamin B12 synthesis. The genus Collinsella in the phylum Actinobacteriota has been linked to proinflammatory dysbiosis in patients with type 2 diabetes (45), which is not consistent with our results. This may be due to the lower abundance of Collinsella, which is not sufficient to reverse the inflammatory effect of Parabacteroides and Paraprevotella. The changes in gut microbiota in this study suggest that migraine patients with IBS had an unhealthier gut microenvironment than patients with IBS, possibly related to inflammation, sex hormone changes, and vitamin B12 reduction.

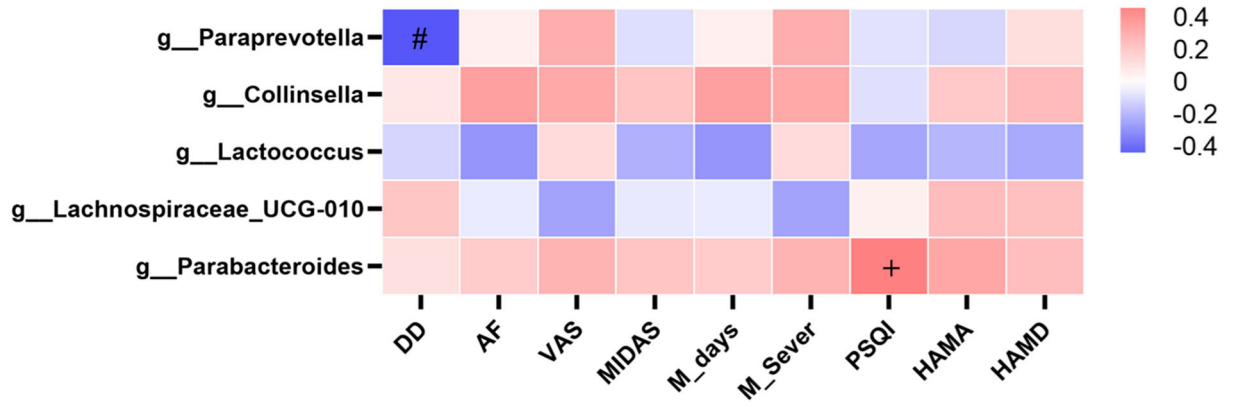

FIGURE 5
Heatmaps showing correlations between gut microbiota and clinical characteristics of migraine. Heatmap based on the abundance (sequence counts) of gut microbiota (prevalence≥10% in migraine patients with IBS) shows the correlations between significantly different taxa at the genus level and migraine clinical characteristics, including AF, DD, VAS, MIDAS, M_days, M_Sever, PSQI, HAMA, and HAMD. The intensity of the color represents the r value (correlation coefficient; negative score: blue; positive score: red). IBS, irritable bowel syndrome; AF, attack frequency; DD, disease duration; VAS, visual analog scale; MIDAS, the migraine disability assessment; M_days, MIDAS days; M_Sever, MIDAS severity; PSQI, Pittsburgh Sleep Quality Index; HAMA, Hamilton Anxiety Scale; HAMD, Hamilton Depression Scale. Spearman test, $^{+}P = 0.056$ in positive correlation, $^{\#}P = 0.075$ in negative correlation.

In our study, we found no correlation between the genus in the gut microbiota and clinical characteristics of migraine, including attack frequency, disease duration, pain severity, migraine disability, sleep, anxiety, and depression. The genus Parabacteroides has a possible positive correlation trend toward significance with PSQI scores, so there may be a positive correlation between genus Parabacteroides and PSQI scores in a large sample, which means that increased Parabacteroides may be associated with poorer sleep quality. Because Parabacteroides is a proinflammatory genus (38), poor sleep quality may be associated with inflammation in the gut microbiota. The genus Paraprevotella has a possible negative correlation trend toward significance with disease duration, so there may be a negative correlation between genus Paraprevotella and disease duration in a large sample, which means that the longer the duration of migraine, the lower the abundance of Paraprevotella, and the weaker the anti-inflammatory effect of Paraprevotella (39). We speculate that prolonged migraine duration may be related to a reduction in the anti-inflammatory genus.

In this study, we explored the composition of gut microbiota in migraine patients with IBS in a Chinese Han population and found altered gut microbiota in migraine patients with IBS. However, we cannot determine whether this alteration was the result of disease progression or the cause of disease, and animal experiments are needed to verify this problem. This study may provide a new direction for the treatment of migraine patients with IBS, and further clinical research and animal experiments on probiotics or fecal bacteria transplantation will be of great help to the treatment of this disease.

The limitations should be considered. First, the sample size was limited, and studies involving a larger sample size from different populations are needed to confirm our results. Second, cohort studies will be more convincing in terms of disease progression. Third, to obtain more in-depth results, shotgun metagenome analysis can provide more detailed information in functional analysis and deeper analysis at the species level and is needed in future studies on gut microbiota in migraine patients with IBS.

Conclusion

We find evidence for gut microbiota dysbiosis in a Chinese Han cohort of migraine patients with IBS for the first time. A well-matched control population in terms of eating habits, lifestyle habits, comorbidities, and medications is beneficial for the identification of disease-related microbiota. No correlation was found between gut microbiota and clinical characteristics of migraine. We could not clarify the detailed roles of gut microbiota in the pathogenesis of migraine in IBS from this cross-sectional study. Further studies are needed to verify whether gut microbiota can be used as a potential biomarker for migraine in patients with IBS so that novel therapeutic options aimed at regulating gut microbiota can be considered in a timely manner to improve the prognosis of migraine in IBS.

Data availability statement

The datasets presented in this study can be found in online repositories (46). The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Chinese PLA General Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL and WT contributed to the statistical analysis and writing of the manuscript. SY conducted the research design. ZD modified the manuscript, and all other authors contributed to collect the clinical data. All authors contributed to the article and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2022.899056/full#supplementary-material

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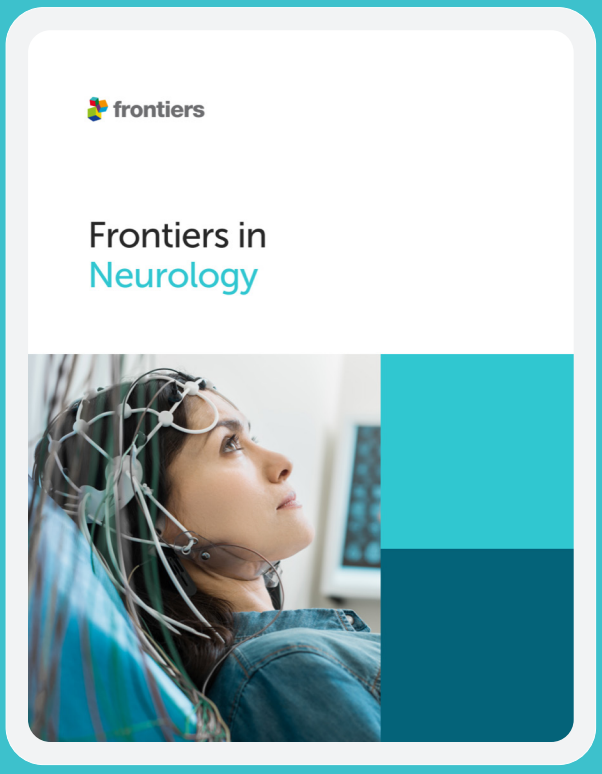

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