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RESEARCH

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Effect of fasting-induced headache on calcitonin gene related peptide (CGRP) and other clinical biomarkers on the first day of Ramadan: Sub-analysis from a randomized open label clinical trial

Abdulrahman Alwhaibi[1,2*†], Fawaz Alasmari[2,3†], Faris Almutairi[2,3], Mohammed A. Assiri[2,3], Feras S. Aldawsari[1,2], Saud T. Aloyayd[1,2], Abdullah A. Alhejji[1,2], Jawaher A. Alotaibi[2,3], Abdulrazaq Albilali[2,4], Omar A. Almohammed[1,2] and Sary Alsanea[2,3*]

Abstract

Background Fasting-induced headaches (FIHs) have been shown to occur on the first day of Ramadan and clearly decline thereafter. Despite the wealth of knowledge about different types of headaches (e.g., migraine-, cluster-, and tension-type headaches), research on the mechanism underlying FIHs, as well as their treatment, remains scarce. Our study aimed to investigate any association between FIHs during the first day of Ramadan and potential headache-related biomarkers, including fasting blood glucose (FBG), C-reactive protein (CRP), magnesium, vitamin B9, vitamin B12, homocysteine, and calcitonin gene related peptide (CGRP), and to assess whether a prophylactic use of paracetamol may influence these biomarkers.

Methods As part of a randomized, open-label clinical trial that evaluated the effect of paracetamol as a prophylactic therapy for FIH, blood samples from stratified subjects in the prophylaxis and control groups were withdrawn while fasting after the 1st dose of paracetamol (in the prophylaxis group) and prior to reporting headache occurrence.

Results Plasma and serum were separated for 61 subjects; 31 and 30 subjects from the prophylaxis and control groups, respectively. Overall, no significant differences were found in the levels of FBG, CRP, magnesium, vitamin B9, and vitamin B12 in headache-suffering subjects compared to those without headache despite the use of paracetamol for prophylaxis. Homocysteine, however, was significantly reduced in all subjects who experienced FIH compared to those without headache (median 6.9 [1.6] vs. 7.7 [2.7] umol/L; $p = 0.041$). On the contrary, when the CGRP was measured using immunoassay, it was found to be significantly elevated in all headache-suffering subjects compared to those without headache (median 126.1 [17.7] vs. 105.8 [19.6] pg/mL; $p \leq 0.0001$). This difference was maintained upon comparing the headache to non-headache subjects in both the prophylaxis (median 121.5 [15.4] vs.

†Abdulrahman Alwhaibi and Fawaz Alasmari contributed equally to this work.

*Correspondence:
Abdulrahman Alwhaibi
aalwhaibi@ksu.edu.sa
Sary Alsanea
salsanea@ksu.edu.sa
Full list of author information is available at the end of the article

105.8 [9.4] pg/mL; $p < 0.01$) and control groups (median 128.5 [28.3] vs. 105.8 [23.8] pg/mL; $p < 0.01$). Additionally, an elevated CGRP level was found to increase the odds of having a FIH [OR = 1.32; 95%CI 1.06–1.22].

Conclusions Our findings revealed the role of CGRP in FIHs for the first time and suggest further investigation in signaling pathways downstream CGRP receptors. Furthermore, the modulation CGRP or CGRP receptors could have a clinical application in the prevention of FIHs.

Trial registration This study was registered with the Saudi Food and Drug Authority in the Saudi Clinical Trials Registry (SCTR; No. 22122102).

Keywords Fasting, Headache, Ramadan, CGRP, Homocysteine

Key finding

- The level of CGRP in plasma is elevated during FIHs.
- An elevated level of CGRP could potentially provoke FIHs.

Introduction

Fasting during Ramadan is one of the fundamental rituals of Islam practiced by all healthy adult Muslims and requires refraining from eating, drinking (including water), smoking, and sextual relationship for 29 to 30 days from dawn (fajr) to dusk (maghrib) [1]. It is considered intermittent, short-term fasting given the alternate fasting time and length, which ranges between 13–18 h per day [2, 3]. According to the International Classification of Headache Disorders, 3rd edition (ICHD-3, 2018), a "headache attributed to fasting" is described as a non-pulsating headache that occurs during and caused by fasting ≥ 8 h and relieved after eating [4]. Since daily fasting during Ramadan lasts more than eight hours, headaches triggered by fasting during Ramadan (referred to as fasting-induced headaches [FIHs] thereafter) belong to the "headache attributed to fasting" category, a subtype of secondary headache disorder and is classified as a "headache attributed to disorders of homeostasis" [4].

Recently, we reported the occurrence of FIH during day 1 of Ramadan to reach 33% in an open-label, randomized controlled trial (RCT) that evaluated paracetamol as a preventative therapy for FIH [5]. Body compositions do change during fasting due to caloric deprivation and disturbance to lifestyle taking place during Ramadan [6, 7]. These changes bring about alternations in the homeostatic and biochemical profiles in both healthy and non-healthy subjects [2, 8]. Despite these alterations, the association between biomarkers and/or homeostatic changes attributed to fasting and the incidence of FIHs remains under-investigated.

Biomarkers are powerful tools that can be objectively measured and evaluated to screen for and monitor diseases [9]. They can be classified as biomarkers of disease, which are usually used to diagnose and measure the progress of the disease, or biomarkers of exposure, which often present before the disease develops [10]. Several studies have assessed the impact of Ramadan fasting on certain hematological and biochemical markers. Khaled et al. reported reduction in blood glucose, hemoglobin A1C, and high density lipoprotein in patients with type 2 diabetes mellitus while total cholesterol, low density lipoprotein cholesterol, and triglycerides were significantly elevated during fasting compared to pre- or post-Ramadan periods measures [11]. Al Hourani et al. reported significantly reduced platelet count and triglycerides levels among other hematological and biochemical markers when assessed while fasting during Ramadan versus pre-Ramadan levels [12]. A study conducted by Mughan et al. revealed significantly reduced C-reactive protein (CRP) reading beside other hematological biomarkers during fasting compared to the pre-Ramadan non-fasting values [13]. Furthermore, Gnanou et al. observed significantly reduced plasma adiponectin and insulin along with increased insulin sensitivity during Ramadan fasting compared to pre-Ramadan levels [14]. Additionally, Darazbi and Hejazi reported a significant increase in urea, uric acid, and osmolarity in the fasting group compared to non-fasting group in Ramadan [15]. Conversely, Khorsid et al. reported no significant changes in their levels [16]. Interestingly, Madkour et al. recently investigated the impact of Ramadan intermittent fasting (RIF) on inflammatory biomarkers. They observed a decline in systemic inflammation during RIF reflected by a reduction of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) and an elevation of interleukin-10 (IL-10) levels compared to pre-RIF levels [17].

In addition to the research on disturbances of biomarkers during Ramadan fasting, other studies have highlighted their clinical utility in different types of headaches, particularly migraine headaches. For example, Jang et al. and Cernuda-Morollon et al. showed calcitonin gene-related peptide (CGRP) to be significantly elevated in migraineurs compared to healthy individuals [18, 19], which drastically decreased after treating the migraine [20]. Later, CGRP was linked to the pain and pathophysiology of migraine given its vasodilatory activity [21]. Martami et al. demonstrated increasing TNF-α

levels among chronic migraineurs (cM) compared to healthy controls [22]. Moreover, Saricam reported an elevation of CRP beside other inflammatory modulators in migraineurs compared to healthy individuals [23]. Taken together, these disturbances suggest that inflammatory biomarkers play a role in the initiation and sustenance of headaches, including migraines through activation of pain pathways, triggering neurogenic inflammation and altering the predetermined proportion of other pro- and anti-inflammatory neurotransmitters [24]. Furthermore, Vitamin D and magnesium levels were reported to be significantly lower among migraineurs compared to healthy controls [25, 26]. Homocysteine and CRP were also found to increase while serum folate and vitamin B12 were observed to decrease in pediatric migraineurs compared to healthy controls [27].

Clearly, most of the literature focuses on the biomarkers associated with migraine headaches, while the research on what provokes FIHs has never been investigated. Indeed, whether the use of these biomarkers (particularly FBG, magnesium, vitamin B9, vitamin B12, CRP, homocysteine, and CGRP) is clinically useful with FIHs remains unclear. Therefore, we aimed in this study to assess changes in these biomarkers that could play a mechanistic role in the pathophysiology of FIHs and speculate whether therapeutic strategies based on their levels could be adopted to prevent FIH.

Materials and method

Study design and subjects

This study is based on serum and plasma samples obtained from 61 subjects; 30 and 31 who were randomly selected from the control and paracetamol groups, respectively. Those subjects represent 35.2% (61/173) of participants included in our recently published open-label parallel-group randomized controlled trial (RCT) that was conducted to investigate the prophylactic effect of paracetamol on FIHs during the first week of Ramadan. Further details about the design of RCT and occurrence of FIHs as well as baseline factors associated with its incidence are published previously [5].

All males and females who aged > 18 years and < 70 years and participated in the RCT were eligible for blood withdrawal. However, females who experienced their menses during the first day of Ramadan, all subjects who used pain reliving medications chronically including paracetamol, NSAID, or any other medication with neurological effects, and all those who had hypersensitivity to paracetamol were excluded from the study. Participants with at least one of the following: acute or chronic heart disease, kidney disease, liver disease, or active cancer were excluded from the study; those may not be able to fast and many of them are ritually excused from fasting for these medical reasons. A statement requesting participants to provide optional blood sample on the first day of Ramadan was included in the consent form for the RCT. In other words, subjects who agreed to provide a blood sample, had already been determined prior to study initiation, i.e. before the occurrence of headache, to avoid any selection bias.

Given the high cost of analyses, only 35 subjects from the control and paracetamol groups were randomly invited from the list of participants, in each group, who agreed and consented for blood analysis (50 and 52 participants, respectively) which was already stratified based on multiple criteria in the main RCT. These 70 subjects were contacted at the night before the 1st day of Ramadan and reminded about the blood withdrawal on the 1st day of Ramadan. Upon their attendance, 2 nurses were awaiting in a pre-set station to withdraw blood sample at the same time where FBG is checked. We have made all the efforts to warrant all 70 subjects to set for blood withdrawal, however 9 didn't show up. However, dropping out from the study and inability to provide blood samples for any reason was expected. A flow chart demonstrating the study design and subjects' selection is shown in Fig. 1.

This study abides by the Declaration of Helsinki and was approved by the King Saud University Institutional Review Board (approval no. E-22–7145) and registered as a clinical trial with the Saudi Food and Drug Authority in the Saudi Clinical Trial Registry (SCTR; No. 22122102).

Sample collection

On the first day of Ramadan, venous blood samples (6–10 ml) were drawn from the median cubital vein of subjects in 5 mL plain yellow-top and purple-top blood tubes. Serum samples were extracted from the yellow-top tubes to measure homocysteine. Plasma samples were extracted from the purple-top tubes for CRP, vitamin B9, vitamin B12, magnesium, and CGRP analysis. Plasma samples were aliquoted into two separate Eppendorf tubes; one for CGRP and the other for the rest of tests. Based on the literature evaluation, these biomarkers were considered the most potentially reliable ones for assessing FIHs and were measured using the corresponding kit's protocol. Serum and plasma samples were used immediately for analysis except those determined for CGRP, which were stored at − 80°C to be used later. After performing the laboratory assessments, samples were discarded according to the laboratory's biohazards materials protocols. A schematic diagram depicting the study's flow, study groups and tested biomarkers is shown in Fig. 2.

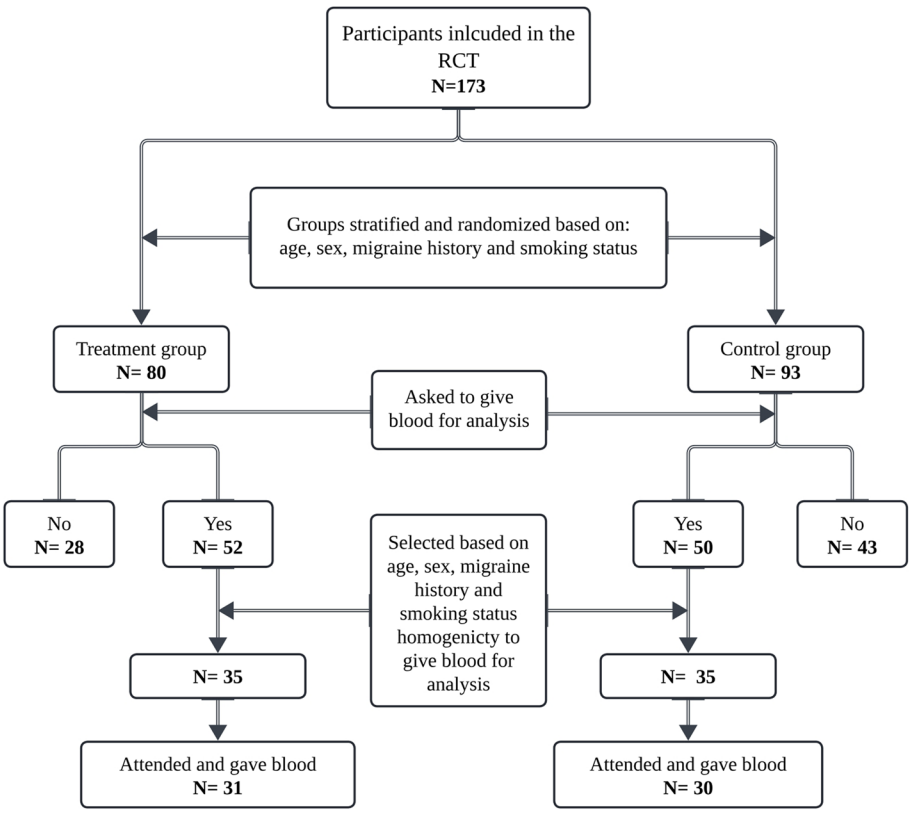

Fig. 1 Flowchart demonstrates the recruitment process of subjects for biomarkers analysis

Blood glucose test

Fasting blood glucose (FBG) levels were measured using the Accu-Chek Instant Blood Glucometer (Roche Diagnostics, Mannheim, Germany). This process was conducted at the same time as the blood samples were drawn for the other biomarker assays.

Enzyme Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay was performed to measure Human CGRP levels in the collected plasma samples using a CGRP enzyme-linked immunosorbent assay kit (ABclonal, Wuhan, China) according to the manufacturer's instructions. In brief, lyophilized CGRP was reconstituted in a 1 mL standard diluent to make a 2000 pg/mL CGRP concentration. After waiting 15 min with gentle agitation, different serial concentrations of CGRP were then prepared (1000, 500, 250, 125, 62.5, 31.25, and 0 pg/mL). In a 96-well plate, a 350 uL washing buffer was added three times in each well for aspiration for 40 s. The prepared serial concentrations (1000, 500, 250, 125, 62.5, 31.25, and 0 pg/mL) or the plasma samples were then added into each well (100 uL). The plate was then covered with an appropriate adhesive strip and kept at 37C^{o} for two hours. Working biotin conjugate antibody (ABclonal, Wuhan, China), streptavidin-HPR, TMB substrate, and stop solution were used during sample preparation according to the manufacturer's instructions. Microplate readings for the data at wavelengths of 450 nm and 630 nm were obtained, and the absorbance readings at 630 nm were subtracted from those at 450 nm. The concentrations of CGRP in plasma were determined using the established standard curve.

BN ProSpec System

Homocysteine-containing samples were combined with polystyrene particles coated with monoclonal antibodies specific to human homocysteine causing particles to be aggregated (Siemens BN ProSpec, Germany). A light beam passing through the samples was scattered by these aggregates. The relevant homocysteine concentration in the sample is directly correlated with the intensity of the dispersed light. The concentrations of homocysteine in the serum samples of subjects were determined using the established standard curve.

Particle Enhanced Immunoturbidimetric Assay

To evaluate the concentrations of CRP in subjects with/without FIH, we employed particle enhanced

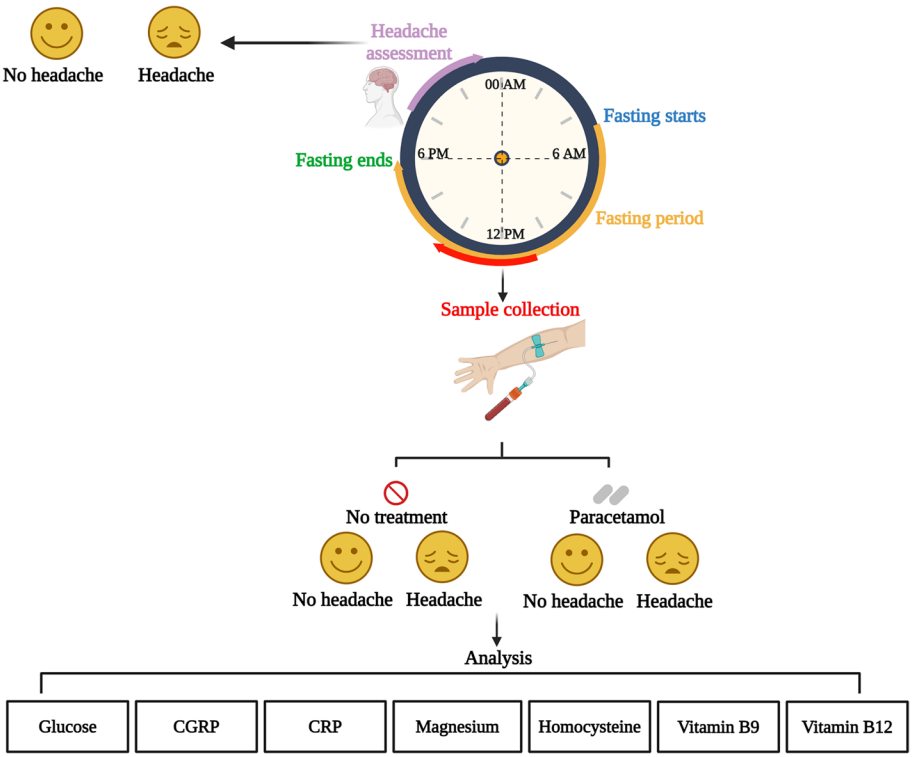

Fig. 2 Schematic diagram demonstrates the design of study and biomarkers association with fasting-induced headache. The subjects were divided into two groups: no treatment or extended-release paracetamol before fasting. Both groups underwent fasting from sunrise until sunset. The orange arrow represents the fasting period (4:30AM – 6PM). The red arrow represents the sample collection period (11AM – 2PM). The pink arrow represents the online headache assessment (8PM – midnight). The figure was created with BioRender®

immunoturbidimetric assay as performed in a previous study [28]. Turbidimetric measurement is used to determine the antigen–antibody agglutinate produced when anti-CRP antibodies and latex microparticles react with the CRP in the sample. Absorbance at 570 nm was used to measure light absorbance. A calibration curve was used to calculate the CRP concentration in the subjects' plasma.

Atomic Absorption Spectrometry

Atomic absorption spectrometry was used to determine the concentrations of magnesium in the plasma of subjects, as performed previously [29]. By applying specific electromagnetic radiation from a light source, atomic absorption spectrometry (Roche, Basel, Switzerland) was used to detect magnesium in the samples. Lanthanum chloride solution was used in sample preparations, and standards solutions containing magnesium were prepared to establish the calibration curve. The absorbance of magnesium was measured at 285.2 nm.

Electrochemiluminescence

For vitamin B9 and vitamin B12 determination, electrochemiluminescence (ECL) immunoassay technology was performed, as previously published [30]. For a full reaction and uniform distribution, the samples and ECL reagents for vitamin B9 or B12, including antibodies, were properly mixed. Mixing was accomplished by pipetting depending on the requirements of the assay. To enhance the reaction, the sample and the additional chemicals were kept at room temperature with shaking during the incubation period. Tripropylamine and a magnet were used to wash away unspecified conjugates. Calibration standards and controls were used to confirm the ECL assay's accuracy and precision. When a potential was applied to the electrode, the binding complex went into an exciting stage, leading to the initiation of signal generation. Vitamin B12 and vitamin B9 concentrations were then measured in the plasma of the subjects.

Statistical analysis

Data distribution for normality was inspected using the Shapiro–Wilk test and Kolmogorov–Smirnov test, and the group comparison was conducted accordingly via non-parametric statistics (Mann Whitney or Kruskal Wallis tests) or parametric statistics (unpaired t-test or one-way ANOVA) for continuous variables. A chi-square and Fisher's exact test were employed to investigate any

association between categorial variables and the subjects' group. Univariate logistic regression was used to examine predictors for FIHs on the first day of Ramadan, and the results were reported as an odds ratio (OR) with a 95% confidence interval (95%CI). SPSS software version 28 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 6.01 were used for statistical analysis. All *p*-values were two-sided and considered significant if < 0.05.

Results

Description of subjects' baseline characteristics

Of the sixty-one subjects included in our study, 38 were males who were near evenly distributed between the prophylaxis and control groups. The proportions of smokers and coffee drinkers were numerically higher in the control group, while the proportion of migraineurs and those complaining of other non-specified self-reported headaches were higher in the prophylaxis group, yet not significant. Median age and body mass index (BMI) were 34.7 years and 24.8 kg/m^2, respectively, with no significant difference between the prophylaxis and control groups. The average systolic and diastolic blood pressure was 120.7 and 75.4 mmHg, respectively, with no significant difference between the two groups. None of the subjects was diagnosed with hypertension, and the majority (73.8%) had no comorbidities, with a similar distribution between the groups. Overall, no significant differences in baseline characteristics were found between the groups. Further details about their baseline characteristics are provided in Table 1.

Subjects were further divided based on headache occurrence in two groups—the headache and non-headache groups. Interestingly, the non-headache group contained the majority of the smokers, while the proportions of coffee drinkers was numerically but not significantly higher in the headache group. The proportion of subjects who self-reported history of migraine was limited (8.2%; 5/61) while those who reported other non-specified headaches was high (55.7%; 34/61); but those were evenly distributed between the groups. Further details are provided in Table 1.

Subjects were also divided based on intervention and headache occurrence into four groups—control without headache ($n=20$), control with headache ($n=10$), prophylaxis without headache ($n=20$), and prophylaxis with headache ($n=11$) groups—as shown in Table 2**.** Half

Table 1 Baseline characteristics of study subjects overall, based on randomized allocation, or occurrence of headache on day 1 ($n=61$)

Characteristic	Overall ($n=61$)	Control vs. Prophylaxis		*p*-value*	Non-headache vs. Headache		*p*-value*
		Control ($n=30$)	Prophylaxis ($n=31$)		Non-headache Group ($n=40$)	Headache Group ($n=21$)	
Age, years	34.7 [15.7]	34.5 [15.3]	34.7 [15.7]	0.761	31.3 [15.6]	35.5 [9.0]	0.202
Sex				0.600			0.371
Male	38.0 (62.3)	20.0 (66.7)	18.0 (58.1)		26.0 (65.0)	12.0 (57.1)	
Female	28.0 (37.7)	10.0 (33.3)	13.0 (41.9)		14.0 (35.0)	9.0 (42.9)	
Body Mass Index, kg/m^2	24.8 [5.8]	24.8 [4.4]	24.8 [8.8]	0.783	24.3 [5.8]	26.3 [5.4]	0.179
Blood pressure, mmHg							
Systolic	120.7 ± 9.3	121.8 ± 8.8	119.7 ± 9.9	0.401	120.6 ± 9.5	121 ± 9.2	0.861
Diastolic	75.4 ± 8.1	75.2 ± 8.9	75.5 ± 7.4	0.893	74.7 ± 8.7	76.6 ± 6.7	0.528
Smoker	8.0 (13.1)	5.0 (16.7)	3.0 (9.7)	0.473	6.0 (15.0)	2.0 (9.5)	0.433
Coffee drinker	58.0 (95.1)	29.0 (96.7)	29.0 (93.5)	> 0.999	37.0 (92.0)	21.0 (100.0)	0.275
Self-reported history of headache							
Migraine	5.0 (8.2)	2.0 (6.7)	3.0 (9.7)	> 0.999	3.0 (7.5)	2.0 (9.5)	0.567
Other non-specified headaches	34.0 (55.7)	15.0 (50.0)	19.0 (61.3)	0.444	23.0 (57.5)	11.0 (52.4)	0.789
Comorbidities							
Diabetes Mellitus	1.0 (1.6)	0.0 (0.0)	1.0 (3.2)	> 0.999	0.0 (0.0)	1.0 (4.8)	0.344
Dyslipidemia	3.0 (4.9)	1.0 (3.3)	2.0 (6.5)	> 0.999	2.0 (5.0)	1.0 (4.8)	0.730
Other comorbid conditions	12.0 (19.7)	8.0 (26.7)	4.0 (12.9)	0.211	8.0 (20.0)	4.0 (19.0)	0.606

For continuous variables, results are presented as mean ± *SD* for normally distributed data and median [*IQR*] for non-normally distributed data

For categorial variables, results are presented as number of patients with the proportion (%) of patients having the outcome

* *p*-values are from unpaired t-test for normally distributed or Mann–Whitney test for non-normally distributed continuous data, and chi-square or fisher-exact tests for categorical data

Table 2 Baseline characteristics of study subjects according to intervention and headache occurrence

Characteristic	Control-NH (*n* = 20)	Control-H (*n* = 10)	Prophylaxis -NH (*n* = 20)	Prophylaxis -H (*n* = 11)	*p*-value*
Age, years	32.8 [15.6]	36.9 [11.1]	30.4 [16.2]	35.2 [17.3]	0.629
Sex					0.64
Male	13.0 (65.0)	7.0 (70.0)	13.0 (65.0)	5.0 (45.5)	
Female	7.0 (35.0)	3.0 (30.0)	7.0 (35.0)	6.0 (54.5)	
Body Mass Index, kg/m^2	24.2 [4.3]	27.6 [4.9]	24.4 [10.5]	25.7 [5.9]	0.387
Blood pressure, mmHg					
Systolic	121.1 ± 8.7	123.1 ± 9.3	120.2 ± 10.5	119.0 ± 9.1	0.778
Diastolic	73.4 ± 9.7	78.9 ± 5.8	76.1 ± 7.7	74.5 ± 7.1	0.348
Smoker	4.0 (20.0)	1.0 (10.0)	2.0 (10.0)	1.0 (9.0)	0.743
Coffee drinker	19.0 (95.0)	10.0 (100.0)	18.0 (90.0)	11.0 (100.0)	0.534
Self-reported history of headache					
Migraine	1.0 (5.0)	1.0 (10.0)	2.0 (10.0)	1.0 (9.1)	0.938
Other non-specified headaches	11.0 (55.0)	4.0 (40)	12.0 (60.0)	7.0 (63.6)	0.698
Comorbidities					
Diabetes Mellitus	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	1.0 (9.1)	0.202
Dyslipidemia	1.0 (5.0)	0.0 (0.0)	1.0 (5.0)	1.0 (9.1)	0.819
Other comorbid conditions	6.0 (30.0)	2.0 (20.0)	2.0 (10.0)	2.0 (18.0)	0.466

For continuous variables, results are presented as mean ± *SD* for normally distributed data and median [IQR] for non-normally distributed data

For categorial variables, results are presented as number of patients with the proportion (%) of patients having the outcome

NH Non-Headache, *H* Headache

* *p*-values are from one-way ANOVA for normally distributed or Kruskal–Wallis test for non-normally distributed continuous data, and from chi-square test for categorical data

of the smokers were in the non-headache control group, yet no significant difference was found when compared with the other groups. Despite the small variation in proportions of subjects with self-reported migraine or other non-specified headaches among the four groups, the difference was not statistically significant. Overall, baseline variables were numerically similar with no significant difference between the four groups. Further details are provided in Table 2.

Occurrence of FIH is not attributed to changes in FBG level

FBG levels were between 81 and 142 mg/dL, with no significant difference between the headache and non-headache groups or between the prophylaxis and control groups with/out headache (Fig. 3). This finding indicates that changes observed in biomarkers, particularly in the headache sufferers, are not attributed to disturbances in FBG or assigned treatment.

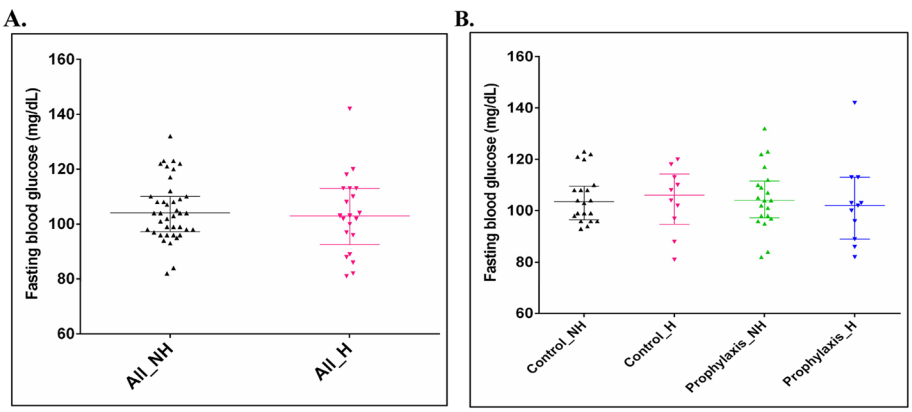

Fig. 3 Fasting blood glucose level assessment in groups according to headache occurrence in all cohort (A) and the randomized allocation (B)

CGRP plasma levels are elevated with FIH and not influenced by paracetamol

The analysis revealed significantly higher levels of plasma CGRP among subjects with a headache versus those without a headache (median 126.1 [17.7] versus 105.8 [19.6] pg/mL; $p < 0.0001$) (Fig. 4A). Moreover, the headache sub-groups with or without paracetamol had comparable plasma CGRP levels, and both were significantly higher than the respective non-headache sub-groups (median for treatment; 121.5 [15.4] vs. 105.8 [9.4] pg/mL; $p < 0.01$, and control 128.5 [28.3] vs. 105.8 [23.8] pg/mL; $p < 0.01$), as presented in Fig. 4B. This finding reiterates the notion that changes in CGRP are not influenced by the used treatment.

Elevated CGRP plasma levels increase the odds of developing FIH

Of all baseline characteristics and measured biomarkers, an elevated level of CGRP was found to increase the odds of having a FIH [OR = 1.32, 95% CI 1.06–1.22], as shown in Table 3. The levels of CGRP were also assessed based on the start time of the headache and the time of blood sample collection. Interestingly, CGRP levels in subjects who had a headache that started during the sampling time tended to be higher compared to those suffering from a headache after blood collection but not significantly so (median 128.1 [13.5] vs. 121.5 [18.5] pg/mL] (Fig. 5).

Homocysteine serum levels are reduced with FIH with no impact on the odds of FIH

The statistical analysis revealed a significant reduction in the serum homocysteine concentrations in subjects with a headache compared to those without a headache (median 6.9 [1.6] vs. 7.7 [2.6] umol/L; $p = 0.041$) (Fig. 6A). However, this significance was not observed when both the intervention and the occurrence of headache were considered in the analysis (Fig. 6B). Interestingly, unlike CGRP, when logistic regression was used to test for homocysteine's influence on FIHs, no significant effect was found [OR = 0.72; 95%CI 0.51–1.01], as shown in Table 3.

Changes in plasma levels of CRP have no influence on FIH occurrence

The statistical analysis did not reveal any significant differences in plasma CRP levels between the headache and non-headache groups (median 0.9 [1.4] vs. 0.8 [1.7] mg/dL, $p = 0.792$) (Fig. 7A). Moreover, the analysis did not show any significant change in the plasma levels of CRP when intervention and occurrence of headache were considered in comparison (Fig. 7B). Additionally, the odds of having a FIH seems not to be influenced by changes in CRP levels [OR = 0.97; 95%CI 0.80–1.18], as shown in Table 3.

Changes in plasma levels of magnesium have no influence on FIH occurrence

No significant changes were determined when magnesium plasma levels were compared between the headache and non-headache groups regardless of the intervention used (mean 0.8 ± 0.1 nmol/L vs. 0.8 ± 0.1 nmol/L, $p = 0.795$) (Figs. 8A) or when both intervention and headache occurrence were used to compare the groups (Figs. 8B). Furthermore, changes in the plasma levels of magnesium seems not to impact the odds of having a FIH [OR = 0.31; 95%CI 0.01–1939.2], as shown in Table 3.

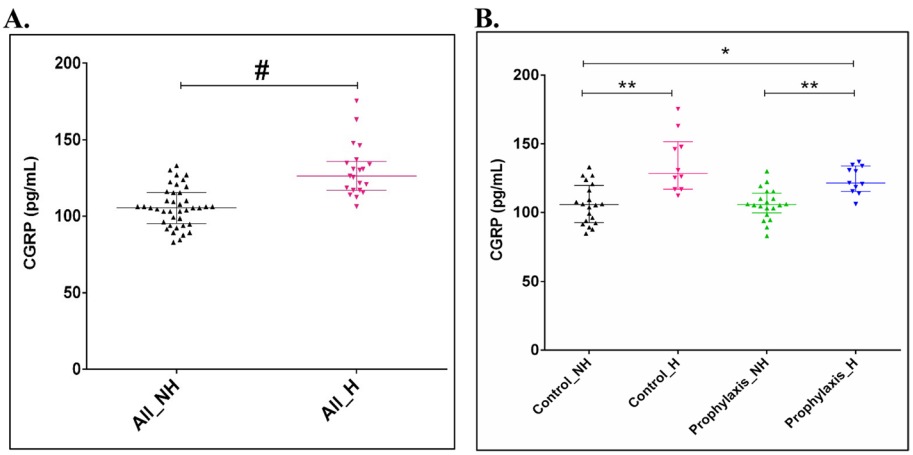

Fig. 4 Calcitonin gene related peptide (CGRP) level assessment according to headache occurrence in all cohort (A) and the randomized allocation (B). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; # $p \leq 0.0001$

Table 3 Factors associated with headache episode while fasting on the first day of Ramadan

Variables	Fasting Headache on Day 1		OR (95%CI)[a]
	No ($n=40$)	Yes ($n=21$)	
Age, years	31.3 [15.6]	35.5 [9.0]	1.04 (0.98 – 1.11)
Sex			
Male	26.0 (65.0)	12.0 (57.1)	Ref
Female	14.0 (35.0)	9.0 (42.9)	1.39 (0.47 – 4.11)
Body Mass Index, kg/m^2	24.3 [5.8]	26.3 [5.4]	1.02 (0.93 – 1.13)
Blood pressure, mmHg			
Systolic	120.6 ± 9.5	121.0 ± 9.2	1.00 (0.95 – 1.06)
Diastolic	74.7 ± 8.7	76.6 ± 6.7	1.27 (0.96 – 1.10)
Smoker			
No	34.0 (85.0)	19.0 (90.5)	Ref
Yes	6.0 (15.0)	2.0 (9.5)	0.59 (0.11 – 3.25)
Coffee drinker			
No	3.0 (7.5)	0.0 (0.0)	NA
Yes	37.0 (92.5)	21.0 (100.0)	NA
Migraine			
No	37.0 (92.5)	19.0 (90.5)	Ref
Yes	3.0 (7.5)	2.0 (9.5)	1.29 (0.20 – 8.45)
Other types of headache			
No	17.0 (42.5)	10.0 (47.6)	Ref
Yes	23.0 (57.5)	11.0 (52.4)	0.81 (0.28 – 2.35)
Comorbidities			
Diabetes Mellitus			
No	40.0 (100.0)	20.0 (95.2)	NA
Yes	0.0 (0.0)	1.0 (4.8)	NA
Dyslipidemia			
No	38.0 (95.0)	20.0 (95.2)	Ref
Yes	2.0 (5.0)	1.0 (4.8)	0.95 (0.08 – 11.13)
Other comorbid conditions			
No	32.0 (80.0)	17.0 (81.0)	Ref
Yes	8.0 (20.0)	4.0 (19.0)	0.94 (0.25 – 3.58)
Fasting blood glucose, mg/dL	104.0 ± 11.0	103.3 ± 14.4	0.98 (0.95 – 1.03)
CGRP, pg/mL	105.8 [19.6]	126.1 [17.7]	**1.32 (1.06 – 1.22)**
Homocysteine, umol/L	7.7 [2.7]	6.9 [1.6]	0.72 (0.51 – 1.01)
C-reactive protein, mg/dL	0.8 [1.7]	0.9 [1.4]	0.97 (0.80 – 1.18)
Magnesium, mmol/L	0.8 ± 0.1	0.8 ± 0.1	0.31 (0.01 – 1939.2)
Vitamin B9, nmol/L	27.2 ± 8.7	28.2 ± 9.5	1.01 (0.95 – 1.08)
Vitamin B12, pmol/L	210.5 [138.0]	220.5 [111.8]	1.00 (0.99 – 1.01)

For continuous variables, results are presented as mean ± *SD* for normally distributed data and median [IQR] for non-normally distributed data. For categorial variables, results are presented as number of patients with the proportion (%) of patients having the outcome

OR Odds ratio, *95%CI* 95% confidence interval, *CGRP* Calcitonin gene related peptide

[a] OR with 95%CI are from binary univariate logistic regression analysis

Changes in plasma levels of vitamin B9 and vitamin B12 have no influence on FIH occurrence

Vitamin B9 and vitamin B12 levels were numerically similar with no significant differences found when all headache sufferers were compared to the non-headache group (mean 28.2 ± 9.5 vs. 27.2 ± 8.7 nmol/L, $p=0.656$; median 220.5 [111.8] vs. 210.5 [138.0] pmol/L, $p=0.962$, respectively) (Fig. 9A and 10A). The same was observed when both the intervention and headache incidence were used to compare the groups of subjects (Figs. 9B and 10B). Thus, changes in the plasma levels of vitamin B9 and vitamin B12 seem not to influence the odds of having FIH

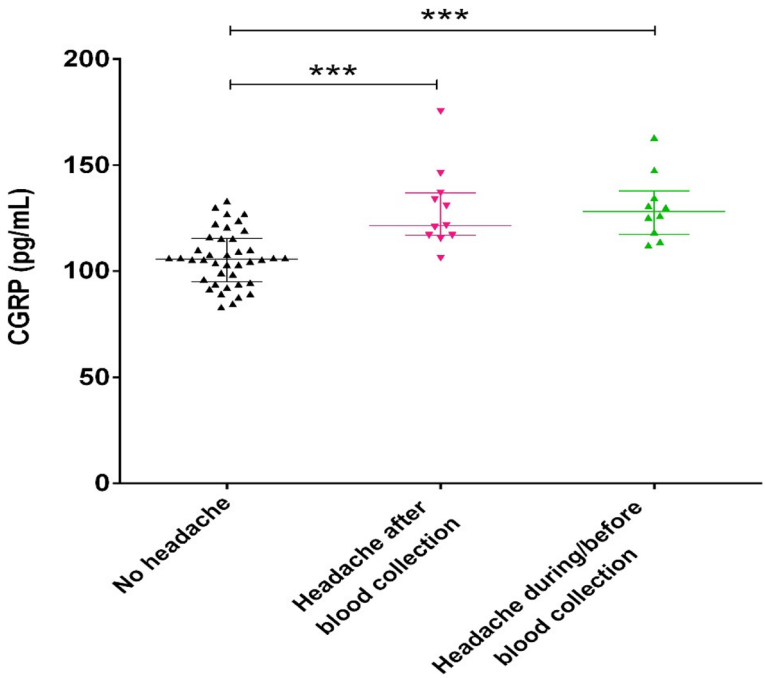

Fig. 5 Calcitonin gene related peptide (CGRP) level assessment according to headache occurrence and time of blood sample collection. *** $p \leq 0.001$

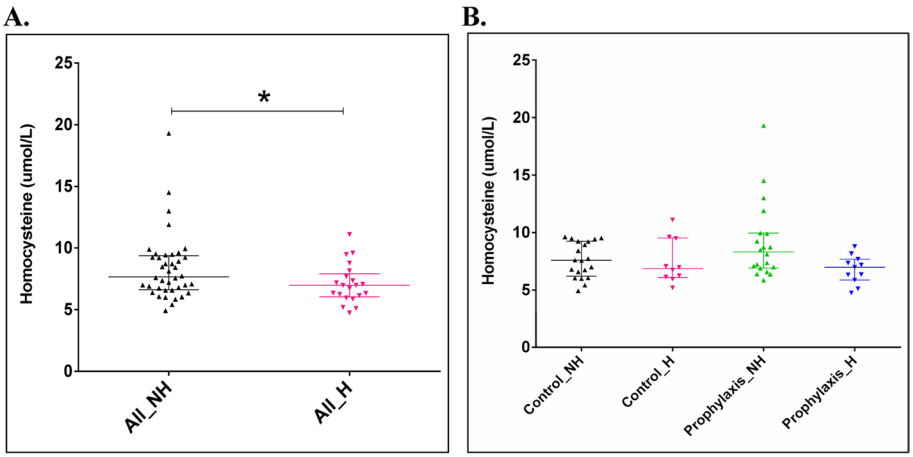

Fig. 6 Homocysteine level assessment according to headache occurrence in all cohort (A) and the randomized allocation (B). * $p = 0.041$

[OR = 1.01; 95%CI 0.95–1.08; OR = 1.00, 95%CI 0.99–1.01, respectively], as shown in Table 3.

Discussion

Our findings demonstrate that FIHs during the first day of Ramadan are significantly correlated with an elevation in plasma levels of CGRP and reduced serum levels of homocysteine, regardless of paracetamol usage. Additionally, our results show no significant difference in the FBG, plasma levels of CRP, magnesium, vitamin B9, or vitamin B12 between those with and without a FIH.

Studies published by researchers on CGRP, Drs. Lars Edvinsson and Peter Goadsby [31, 32], have guided and paved the way for several researchers to investigate the involvement of CGRP in different types of headaches. In their first reports, they demonstrated an elevation in CGRP plasma level during the headache phase of migraineurs compared to healthy controls [33], as well as in attacks of episodic cluster headache (eCH), which

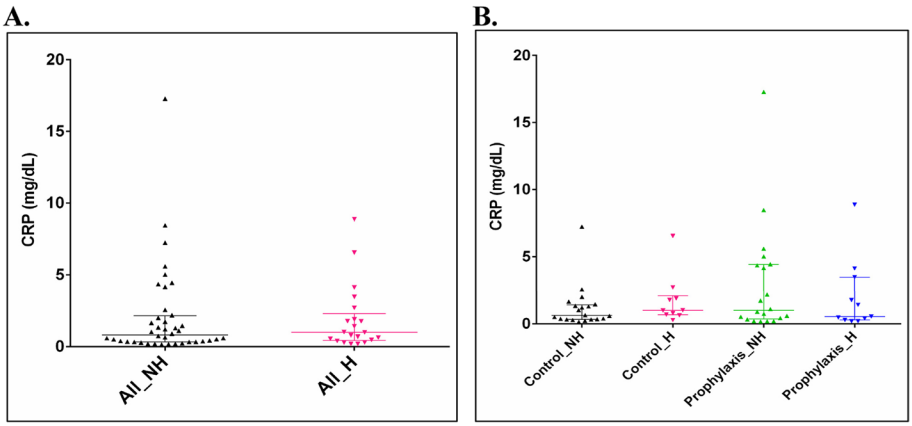

Fig. 7 C-reactive protein level assessment according to headache occurrence in all cohort (A) and the randomized allocation (B)

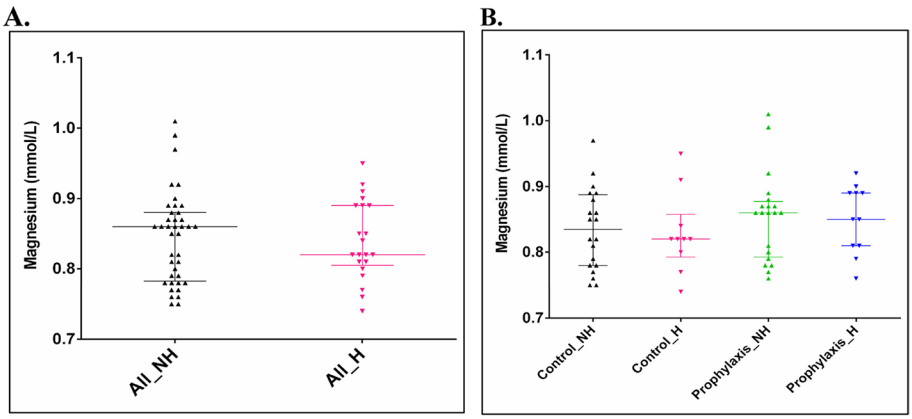

Fig. 8 Magnesium level assessment according to headache occurrence in all cohort (A) and the randomized allocation (B)

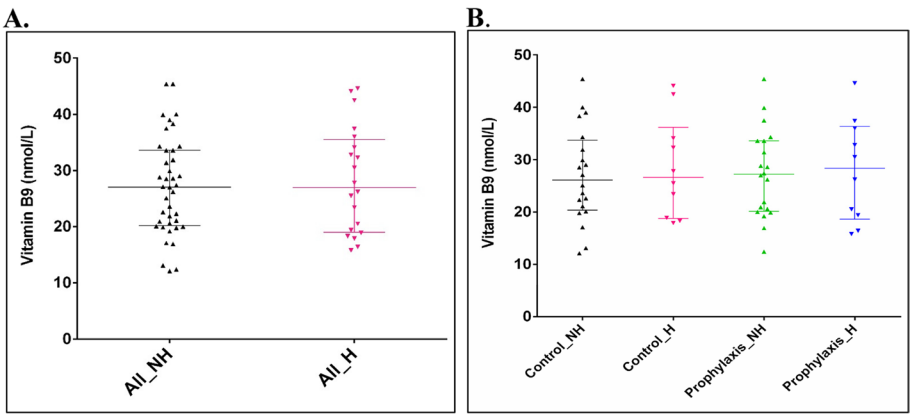

Fig. 9 Vitamin B9 level assessment according to headache occurrence in all cohort (A) and the randomized allocation (B)

was normalized with oxygen or sumatriptan and, consequently, relieved the headache [34, 35]. A subsequent study by Nicolodi and Bianco showed elevated levels of CGRP in saliva collected from migraineurs and eCH sufferers during the attack compared to resting intervals. The same elevation was also observed when CGRP levels

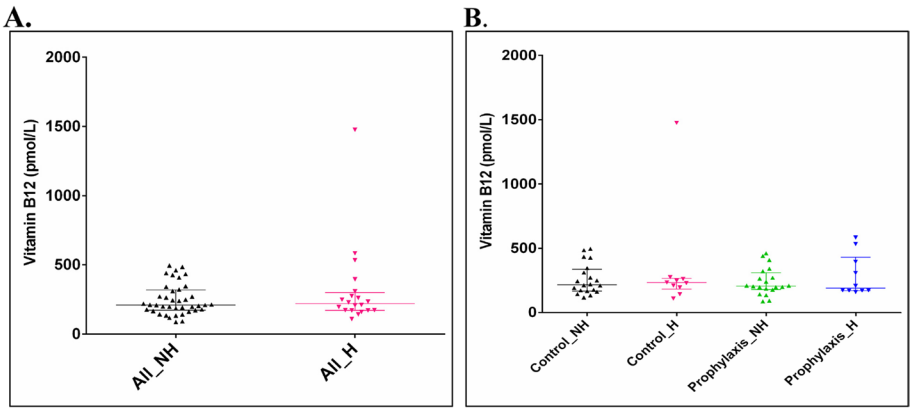

Fig. 10 Vitamin B-12 level assessment according to headache occurrence in all cohort (A) and the randomized allocation (B)

in eCH sufferers (but not migraineurs) were compared to levels in healthy participants [36]. Another study by Kamm et al. revealed higher levels of CGRP in the tear fluid of those suffering from active eCH or a chronic cluster headache (cCH) who were not administered abortive therapies in the last 48 h prior to its incidence compared to healthy individuals [37]. Further, Fanciullacci et al. confirmed an increase in the extracerebral plasma concentrations of CGRP (in subjects who were in the active period of cluster headache) during nitroglycerin-induced spontaneous cluster headache attack [38, 39], while the plasma level of CGRP declined later, regardless of whether it was spontaneous or due to the pharmacological effect of sumatriptan [39]. Intriguingly, administering nitroglycerin to those in the remission period did not induce the attack. Beside the role of CGRP in migraine and CH, a study conducted by Eggertsen et al. in 2024 revealed higher serum levels of CGRP among patients experiencing persistent post-concussion symptoms, including post-traumatic headache, compared to healthy individuals at a median time of 4 months after the trauma. Later and during the follow-up period of a median time of 11.4 months after the trauma, it declined when compared to its baseline level. Interestingly, when gender was considered in stratification, they found healthy males were having significantly higher median CGRP concentration compared to healthy females (201.8 pg/mL vs. 32.1 pg/mL, $p=0.0025$) [40].

Despite the existing evidence linking headaches to increased CGRP levels, few recently published studies, particularly on CH, negate that idea. Although Kamm et al. showed an elevated CGRP level in tear fluid from untreated patients diagnosed with eCH or cCH, this finding was not reflected in the plasma samples [37]. Another study by Peterson et al. showed a reduction in CGRP plasma levels among patients with CH compared to healthy controls, while no difference was found between those with eCH and cCH. [41]. Despite this finding, patients with eCH in active state (bout) had higher levels of CGRP compared to those with eCH in attack-free remission state, which make them respond to CGRP monoclonal antibodies. Pellesi et al. investigated whether cluster headaches are provoked by the activation of mast cells or elevated CGRP, which was induced by the administration of pituitary adenylate cyclase-activating polypeptide-38 (PACAP38) and vasoactive intestinal polypeptide (VIP) intravenously. Their results showed no difference in CGRP plasma levels among those who developed attacks versus those without a headache [42]. This was further supported by the findings reported by Guo et al., who found no difference in CGRP plasma level between migraineurs who have developed a migraine headache post-PACAP38 administration compared to those who did not [43]. Interestingly, Pellesi et al. also found no difference in the plasma levels of CGRP among patients with eCH in the active phase, patients with eCH in the remission phase, and patients with cCH, both at baseline (prior to PACAP38 or VIP administration) and during the provoked attacks of CH. Although the effect of fasting on CGRP is barely and unclearly discussed [41], a few studies have disputed the link between elevated CGRP and headache occurrence. This discrepancy could be attributed to the differences in the tested biological fluids and its sensitivity to minor changes in CGRP concentration (blood vs. saliva vs. tears), study methodologies (as some were comparing control healthy sample to during-attack samples or post-attack samples), states of headache episodes (eCH vs. cCH), radioimmunoassay used for samples analysis, and the possibility of the potential depletion of CGRP from the trigeminal nerve as the source of its synthesis and release [44, 45].

Our logistic regression analysis indicates that high CGRP levels would increase the odds of having a FIH. This finding is supported by the blood sampling, starting time of the headache, and detected levels of CGRP. In other words, subjects who reported experiencing a headache before and during blood sampling in our sample had numerically higher CGRP levels than those reporting a headache after blood collection. This notion is augmented by the studies published by Asghar et al. [46], Vollesen et al. [47], and Soner et al. [48] in which they confirmed the critical role of CGRP in initiating a migraine by administrating CGRP intravenously. This finding suggested the efficacy of anti-CGRP therapies, as abortive and preventative approaches, and supported the use of CGRP measurement to predict the response to CGRP-targeted therapies in both types of headache [46–48]. Indeed, following these studies, several anti-CGRP therapies were tested and approved for migraines [49–57] and CH [58]. Applying this concept to our findings, anti-CGRP could potentially be useful as a preventative therapy for FIH, yet administration of IV CGRP and its impact on the onset, severity, and frequency of FIH, as well as investigating signaling pathways downstream CGRP in FIH, is warranted in preclinical and clinical studies to proceed with such an approach.

In contrast to CGRP findings, our analysis showed significantly reduced homocysteine in the headache compared to non-headache group. However, that did not influence the odds of a FIH. This finding is contrasted by the Nelson et al. results who reported that children diagnosed with a headache, including migraine, had significantly increased homocysteine compared to the non-headache group [27]. This conflict could potentially be attributed to the higher proportion of males and almost close proportions of both genders in the non-headache and headache groups, respectively, since Nelson et al. reported that males with and without headache had higher serum homocysteine levels compared to females. Interestingly, when all our cohort regardless of headache occurrence was divided based on gender, it appeared that males had significantly higher homocysteine compared to females (median 8.44 [2.49] vs. 6.57 [1.02]; $p < 0.0001$), reiterating results observed by Nelson et al. Further classification of non-headache group only based on gender revealed the same findings where males did have a significantly higher level of homocysteine compared to females (median 8.97 [2.18] vs. 6.68 [0.93]; $p = 0.0004$), while significance was lost when the same comparison was conducted on the headache group only (median 7.13 [2.68] vs. 6.35 [1.78]; $p = 0.107$). In spite of this, gender distribution between headache and non-headache groups in our study was statistically balanced, and the male proportions in both headache and non-headache groups were numerically higher than females. Nevertheless, the effect of gender needs more investigation as the impact of small samples size in our study cannot be neglected.

CRP is an inflammatory marker that has an established role in several inflammation pathologies, including chronic neuroinflammatory diseases [59, 60]. Additionally, Ramadan fasting was found to reduce its level compared to pre-Ramadan period [13]. Despite the involvement of CRP in headaches, including migraine headache [27, 61], we found no difference in its levels between the headache and non-headache groups, as well as no association between its level and FIHs. Our finding is supported by the study published by Gudmundsson et al. in which no significant difference in serum level of CRP was noted between migraineurs and non-migraineurs [62]. This is also supported by Park et al. study, which found no significant difference in CRP levels among episodic migraineurs (eM) or cM upon comparing them to healthy groups [63, 64].

Unlike studies showing precipitated severe headache and migraine attacks secondary to reduction in vitamin B12 and vitamin B9 levels [27, 65, 66] and the use of vitamin B12 and B9 as an effective adjuvant therapy to cure or prevent episodic migraine [67], we found no difference in their levels between the headache and non-headache groups, which suggests the absence of their role in FIHs. Similarly, the levels of magnesium in our subjects were numerically comparable between the two groups, while it was reported to be significantly reduced with migraine [26, 68, 69]. Again, this finding could indicate the important impact of magnesium in migraine headaches, but not in FIH.

Our results showed no significant difference in FBG between headache and non-headache fasting subjects. Interestingly, although blood glucose level was reported to reduce with fasting Ramadan compared to pre-Ramadan readings [11, 14], few reports have negated this correlation [13, 15]. Cumulatively, these findings indicate that fasting might not significantly influence blood glucose, thus FBG has no potential role in headache occurrence.

Study limitation

Several limitations exist in our study; thus, our findings should be interpreted with caution. First, we did not determine the baseline level of CGRP prior to fasting in both the headache and non-headache groups. Fortunately, Eggertsen et al. investigated serum CGRP in healthy non-fasting individuals compared to those with persistent post-concussion symptoms [40]. Upon comparing their findings on healthy non-fasting individuals who had no headache to our control fasting non-headache subjects, the data suggest that fasting itself

could potentially elevate CGRP level (median 105.75 vs. 76.3 pg/mL). Nevertheless, understanding the impact of fasting on CGRP, beside the changes of CGRP level during FIHs, through a well-designed study is imperative given the evolving role of CGRP in different types of headaches. Second, the absence of successive samples throughout the month of Ramadan hindered our ability to correlate the reduced headache occurrence noted on day 7 compared to day 1 of the 1st week of Ramadan that we reported previously [5]. Third, our sample size is considered too small to produce robust statistics. However, it is almost comparable to other studies investigating the role of CGRP in headaches [33–37, 39, 46, 48]. Yet, a larger sample size is required to generate more conclusive results. Fourth, there is a predominance of male gender in our sample, which might have introduced some bias in CGRP changes, hence susceptibility to headache. Although Eggeretsen et al. have observed the impact of gender on CGRP level in healthy non-fasting individuals [40], considering gender in our analysis yielded no significant effect on CGRP among the control subjects without headache (median 106.14 [10.0] vs. 96.13 [30.76] pg/mL; $p=0.772$, in males vs. females, respectively) nor in the control groups with headache (median 130.75 [25.38] vs. 125.36 [31.53] pg/mL; $p=0.783$). Fifth, the blood samples in our study were collected from the median cubital vein (peripheral circulation) and not from the external jugular vein (cranial circulation). Therefore, interpretation of our findings could be arguable, as CGRP levels detected previously by Goadsby and Edvinsson in peripheral blood samples of migraineurs were significantly lower than external jugular vein samples, but they were comparable to levels in healthy individuals [33]. Nevertheless, recent evidence suggests otherwise [70]. Sixth, the half-life of CGRP in plasma is 7 min, which increases the risk of its degradation during separation [71]. However, a rapid process of separation followed by freezing the plasma samples at -80 C^0 was performed to overcome this issue. Finally, limited funding is certainly a major hurdle that affected our research, particularly when it comes to samples analysis. Despite these limitations, our study is the only of its kind that uncovers the association between changes in CGRP plasma levels and occurrence of FIHs.

Conclusion

Overall, given the enhanced plasma levels of CGRP among the FIH-suffering group compared to the non-headache group, the increased odds of a FIH associated with higher levels of CGRP and the absence of association between headache occurrence and changes in other investigated biomarkers, our results support the notion that CGRP could provoke FIHs, implicating its potential role in the pathophysiology and as a preventative therapy of FIHs.

Abbreviations

All-H	All subjects with headache
All-NH	All subjects without headache
CGRP	Calcitonin gene related peptide
cCH	Chronic cluster headache
CI	Confidence interval
CRP	C-reactive protein
ECL	Electrochemiluminescence
eCH	Episodic cluster headache
FBG	Fasting blood glucose
FIH	Fasting-induced headache
OR	Odds ratio
RIF	Ramadan intermittent fasting
RCT	Randomized controlled trial

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Authors' contributions

AW and FA generated and formulated the idea led the research team, interpreted the data, and wrote the manuscript. SO, AH, and FD, and JO recruited the participants and collected the data. AW and FA designed the study. FM, MA, and JO perform the lab analysis. AB designed the study, interpreted the data, and revised the manuscript. AB, OM, and SS participated in interpreted the data and manuscript writing and revision. All the authors have reviewed and approved the final manuscript.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declarations

Competing interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author details

[1]Department of Clinical Pharmacy, College of Pharmacy, King Saud University, P. O. Box 2457, 11451 Riyadh, Saudi Arabia. [2]King Saud University Medical City, King Saud University, Riyadh, Saudi Arabia. [3]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, P. O. Box 2457, 11451 Riyadh, Saudi Arabia. [4]Department of Internal Medicine, College of Medicine, Neurology Unit, King Saud University, Riyadh, Saudi Arabia.

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