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Nesfatin-1 and Ghrelin/GOAT as Potential Biomarkers in Adolescent Headache with Temporomandibular Disorders: A Cross-Sectional Study

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ABSTRACT

Introduction. Nesfatin-1 and Ghrelin O-acetyltransferase (GOAT) are peptide hormones involved in stress response and pain modulation beyond their metabolic functions. This study investigated whether these biomarkers differ between adolescents with headaches and healthy controls and examined their relationship with temporomandibular disorders (TMD).

Material and methods. This cross-sectional study measured Nesfatin-1 and Ghrelin/GOAT levels in the serum and saliva of 44 adolescents with headaches (aged 10-17 years, diagnosed per ICHD-3 beta criteria) and 20 age-matched healthy controls. TMD was assessed using the Diagnostic Criteria for TMD (DC/TMD). Statistical comparisons were performed using non-parametric tests, with significance set at $p < 0.05$.

Results. Nesfatin-1 was elevated in all headache patients compared to controls in both blood (patient median range: 39.5-45.0 ng/mL vs. control median range: 8.1-9.0 ng/mL, $p < 0.001$) and saliva (patient medi-

an range: 6.6-8.2 ng/mL vs. control median range: 1.8-2.3 ng/mL, p < 0.001) regardless of headache type (migraine vs. tension-type) or TMD status. Ghrelin/GOAT showed opposite patterns in blood versus saliva: serum levels were higher in patients (median range: 5.9-6.6 ng/mL vs. 1.4 ng/mL in controls, p < 0.001), while salivary levels were lower (median range: 0.2 ng/mL vs. 1.4 ng/mL in controls, p < 0.001). Gender differences emerged in clinical manifestations: females had more muscle pain (74% vs 38%, p = 0.016), while males reported more psychological distress (90% vs 48%, p = 0.003).

Conclusions. This study reveals altered Nesfatin-1 and Ghrelin/GOAT levels in adolescents with headaches, independent of headache type or TMD status. These findings suggest potential utility as pain-related biomarkers, though validation studies with larger sample sizes and receiver operating characteristic analyses are needed before clinical implementation. Gender specific clinical patterns underscore the importance of sex-stratified approaches in adolescent pain management.

Introduction

Headache is among the most common complaints in pediatric neurology clinics, affecting 54-58% of children and adolescents globally [1-3]. These headaches frequently lead to school absences and impaired academic performance, creating a substantial burden on young people during critical developmental years [2,3]. Both migraine and tension-type headache (TTH) can significantly impact quality of life and functioning, making early recognition and treatment essential [4,5].

Temporomandibular disorders (TMD) encompass pain and dysfunction of the masticatory muscles, the temporomandibular joint (TMJ), and surrounding structures. They are the most common cause of chronic orofacial pain and the primary source of non-dental pain in the craniofacial region [6]. A notable clinical overlap exists between TMD and headache, particularly tension-type headache (TTH), though this relationship is often overlooked in clinical practice [7,8]. The International Classification of Headache Disorders, 3rd edition (beta version) (ICHD-3 beta), acknowledges this diagnostic ambiguity, noting that when the TMD diagnosis is uncertain, the headache may be classified as 'TTH or one of its subtypes' (presumably with pericranial muscle tenderness) [9].

Current headache diagnosis in adolescents relies primarily on clinical history and examination, with a limited objective markers available to aid diagnosis or predict outcomes. The availability of reliable biomarkers would help resolve diagnostic uncertainty and potentially guide treatment decisions. While some biomarkers have been identified in adult migraine patients from peripheral blood and cerebrospinal fluid [10,11], research in paediatric populations remains limited.

Nesfatin-1, discovered in 2006, is a peptide derived from the nucleobindin-2 (NUCB2) gene that was initially characterised as a satiety signal [12]. However, subsequent research revealed broader roles in stress response, anxiety, and pain processing [13]. Nesfatin-1 is expressed in brain regions involved in both stress and pain processing, including the hypothalamus, periaqueductal grey and rostral ventromedial medulla. These areas are key components of the descending pain-modulatory system, suggesting that Nesfatin-1 may link stress and pain perception.

Similarly, ghrelin was identified in 1999 as a growth hormone-releasing neuropeptide that influences appetite and energy metabolism [14]. Ghrelin undergoes post-translational modification by ghrelin O-acyltransferase (GOAT), which attaches a fatty acid chain that is essential for biological activity [15]. Beyond its metabolic functions, ghrelin influences pain processing through central nervous system actions. The ghrelin system has been implicated in anxiety disorders and stress responses [16], conditions that frequently co-occur with chronic pain syndromes.

Adolescence represents a critical developmental period characterised by dramatic hormonal changes, brain maturation, and psychosocial transitions. These factors may influence both the pain experience and the expression of biomarkers. Additionally, gender differences in pain conditions emerge during adolescence, with females showing higher rates, including TMD [17,18].

Despite the clinical overlap between headache and TMD and the theoretical rationale for neuroendocrine biomarkers, no previous study has examined Nesfatin-1 and Ghrelin/GOAT levels in adolescents with headaches. This study aimed to address this gap with the following objectives:

› Primary Objective:
  To compare serum and salivary levels of Nesfatin-1 and Ghrelin/GOAT between adolescents with headaches and age-matched healthy controls.
› Secondary Objectives:
  1. To examine whether biomarker levels differ between migraine and TTH
  2. To assess whether biomarker levels vary across TMD diagnostic categories (myalgia, disc displacement with reduction, and no TMD)
  3. To evaluate gender-specific differences in biomarker expression and clinical manifestations

Materials and methods

Study Design and Ethical Approval

This cross-sectional study was performed at the Department of Developmental Neurology, Poznan University of Medical Sciences, from November 2017 to December 2020. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Bioethical Committee of Poznan University of Medical Sciences (approval number 550/17) [19]. Written informed consent was obtained from all participants and their legal guardians following a comprehensive explanation of the study protocol. Participant anonymity was maintained throughout the study, and participation was voluntary with no financial incentives offered. The study procedures were designed to minimise any potential burden on the participants.

Participation Selection and Recruitment

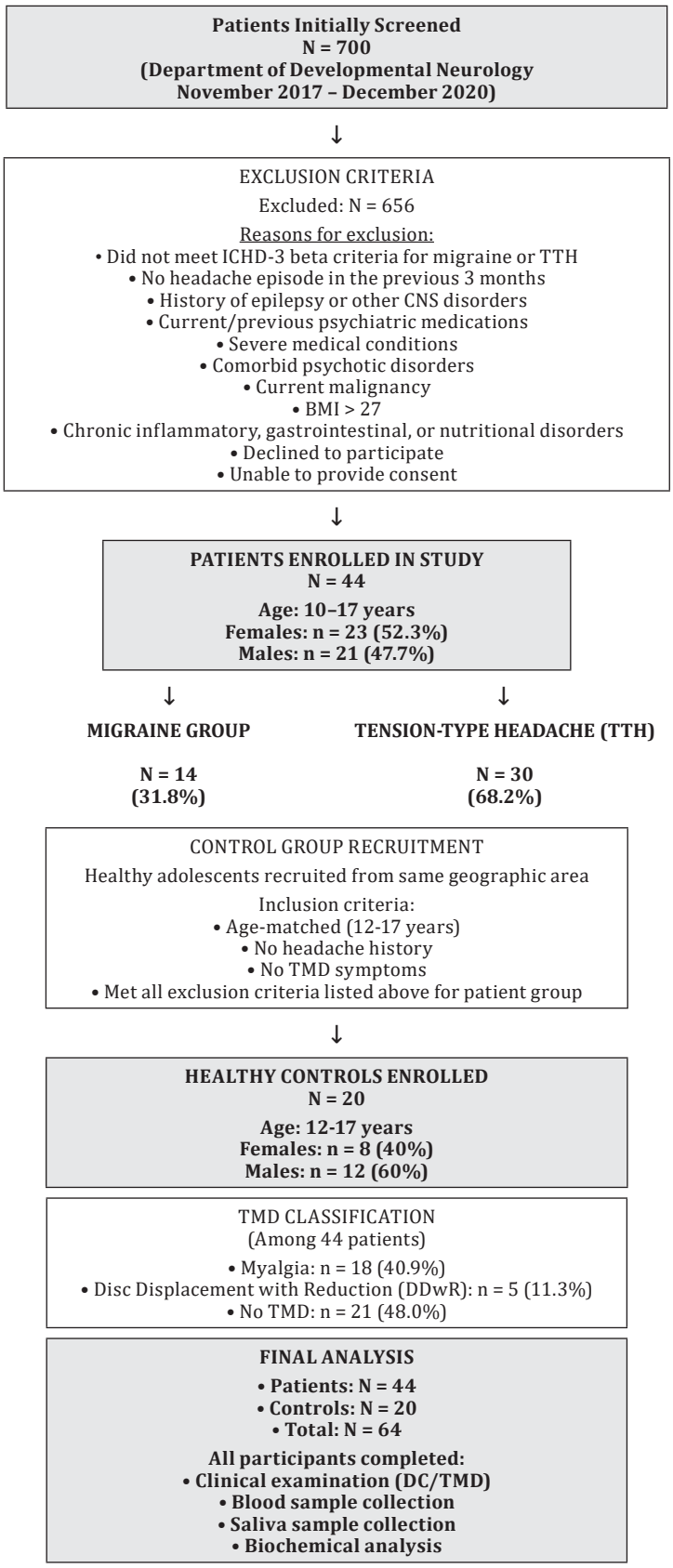
Of the 700 patients initially screened, 44 adolescents met the inclusion criteria and were enrolled in the study (see **Figure 1**). Inclusion criteria for patients: age 10-17 years; confirmed migraine or TTH according to the ICHD-3 beta criteria [9]; at least one headache episode in the previous 3 months; and ability to provide consent from themselves and their parents. Exclusion criteria for both groups: history of epilepsy or other central nervous system disorders; current or previous use of psychiatric medications; presence of severe medical conditions; comorbid psychotic disorders; current malignancy; body mass index (BMI) exceeding 27; and presence of chronic inflammatory, gastrointestinal, or nutritional disorders that could potentially affect Nesfatin-1 and Ghrelin levels. Additional exclusion criteria for the control group included any history of headaches or TMD symptoms. Subjects with a BMI greater than 27 were excluded from the study, as body mass significantly affects the secretion and circulating levels of both Nesfatin-1 and Ghrelin, which could confound the interpretation of the results [20,21].

The control group consisted of 20 age-matched healthy adolescents from the same geographic area, who did not have headache or TMD symptoms. Control patients met all exclusion criteria listed above. The patient age range (10-17 years) was defined according to ICHD-3 beta criteria. In comparison, the control group was recruited from ages 12 to 17 years to match the patient cohort's median age distribution. No significant age differences were observed between the final recruited patient group and the control group.

The smaller control group size (n = 20 vs. n = 44 patients) was determined based on power calculations. Using pilot data showing mean Nesfatin-1 levels of approximately 40 ± 16 ng/mL in patients versus 8 ± 2 ng/mL in controls, with $\alpha = 0.05$ and power = 0.80, a minimum of 15 controls were required to detect this significant effect (Cohen's $d \approx 2.5$). We recruited 20 controls to allow for potential sample quality issues. The unequal group sizes reflect pragmatic challenges in recruiting healthy adolescents for research procedures, including blood draws, which proved more difficult than enrolling patients already presenting for clinical care.

Headache diagnosis was established through a comprehensive assessment protocol that included investigator-designed parental questionnaires, a detailed medical history, and a clinical examination performed by a certified child neurologist (M.Z. and B.S.).

All diagnoses were made according to ICHD-3 beta criteria [9]. Patients were classified as having either migraine or tension-type headache.



BMI = Body Mass Index; CNS = Central Nervous System; DC/TMD = Diagnostic Criteria for Temporomandibular Disorders; ICHD-3 = International Classification of Headache Disorders, 3rd edition; TMD = Temporomandibular Disorders; TTH = Tension-Type Headache

Figure 1. STROBE flowchart showing participant recruitment, selection, and classification in the cross-sectional study of Nesfatin-1 and Ghrelin/GOAT as potential biomarkers in adolescent headache with temporomandibular disorders.

Temporomandibular Disorder Assessment

TMD examinations were performed by trained dentists (Y.B. and A.S.M.) who were blinded to participants' headache diagnoses and control-group status. The examination was conducted in accordance with the protocol outlined in the Diagnostic Criteria for TMD (DC/TMD) Axis II guidelines [22,23].

A comprehensive clinical assessment included medical and dental histories, with particular attention to parafunctional habits, such as bruxism. The intraoral examination assessed signs of bruxism, including dental wear facets, fractures, masseter hypertrophy, and soft tissue changes (linea alba and tongue scalloping). Bruxism was identified based on clinical signs during examination rather than polysomnography, and we did not differentiate between awake and sleep bruxism in this study.

Two major diagnostic categories were assessed: myalgia and disc displacement with reduction (DDwR). Myalgia was evaluated by palpation of the temporalis and masseter muscles and by pain during jaw opening, whereas DDwR was identified by joint sounds (clicking or popping). Following DC/TMD protocols, pain provocation during examination was confirmed as familiar pain matching the patient's primary complaint. Functional measurements included maximum mouth opening (measured from the maxillary to mandibular incisal edges at midline), with an opening of less than 40 mm considered restricted [24]. Mandibular deviation was defined as a displacement of 2 mm or more to either side during opening movement. The assessment also included a distress evaluation using the Patient Health Questionnaire-4 (PHQ-4), the PHQ-9, the Generalised Anxiety Disorder 7-item scale (GAD-7), and the Oral Behaviour Checklist (OBC) [23]. Parafunctional habits were systematically assessed using the OBC, which categorises oral behaviours into 'bite' parafunctions (clenching, grinding) and 'non-bite' parafunctions (nail biting, cheek chewing, lip biting). Physical symptoms were defined using the somatic symptom items from the PHQ-9 as part of the DC/TMD Axis II distress assessment [23].

Biochemical Sample Collection and Analysis

Fasting venous blood (5 mL) and unstimulated whole saliva (2 mL) samples were collected simultaneously between 08:00 and 09:00. Participants fasted overnight and refrained from eating, drinking (except water), and oral hygiene procedures for at least 1 hour before sample collection. Saliva was collected using the passive drooling method: participants were instructed to allow saliva to accumulate naturally in the floor of the mouth and drool into sterile collection tubes over approximately 5 minutes until 2 mL was obtained.

Saliva samples underwent double centrifugation (3046.4 g, 2 × 10 minutes), with supernatant collection between cycles, and were stored at -20°C in 0.5 mL aliquots. Blood samples were processed immediately and stored at -20°C until analysis, a temperature compatible with the ELISA kits used. Thyroid-stimulating hormone (TSH), fasting plasma glucose, and total cholesterol were analysed using a Cobas 8000 platform (Roche Diagnostics GmbH, Germany). Nesfatin-1 and Ghrelin/GOAT levels were duplicated using commercial *enzyme-linked immunosorbent* assay (ELISA) kits (Human NEFA/Nesfatin-1 and Human MBOAT4/GOAT).

Nesfatin-1 and Ghrelin/GOAT levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Human NEFA/Nesfatin-1, Cat# E8726h; Human MBOAT4/GOAT, Cat# E3525h; Wuhan EIAab Science Inc., China). Both kits had a detection range of 0.312-20 ng/mL. The Nesfatin-1 kit demonstrated a sensitivity of <0.12 ng/mL, with an intra-assay coefficient of variation (CV) of ≤6.2%, an inter-assay CV of ≤8.5%, and an average recovery of 94%. The Ghrelin/GOAT kit demonstrated a sensitivity of <0.1 ng/mL, with intra-assay CV ≤5.9%, inter-assay CV ≤10.1%, and an average recovery of 102%.

Statistical Analysis:

Descriptive statistics included means, standard deviations, and medians for continuous variables. The Shapiro-Wilk test was used to assess the normality of the distribution.

Due to non-normal distribution, between-group comparisons were conducted using non-parametric tests: the Mann-Whitney U test for two-group comparisons and the Kruskal-Wallis test for multiple-group analyses. Categorical variables were presented as counts (N) and percentages, and differences in proportions were

analysed using chi-square tests. Statistical significance was set at $p < 0.05$. All analyses were performed using Statistica v13.0 (Dell Inc., Round Rock, TX, USA).

Although the Shapiro-Wilk test indicated non--normal distribution for some variables, data are presented as both mean±standard deviation and median for several reasons: (1) to facilitate comparison with existing literature which predominantly reports means, (2) the large effect sizes observed reduce concern about violation of normality assumptions for descriptive purposes, and (3) means provide information about the magnitude of differences. However, all inferential statistical tests (Mann-Whitney U and Kruskal-Wallis) were nonparametric and did not require the assumption of normality.

Results

Study Population and Characteristics

The study cohort comprised 44 adolescents with headaches (23 females [52.3%] and 21 males [47.7%] aged 10-17 years) (Figure 1). All participants had experienced the last episodes of headaches within the previous three months, and this was the main reason for their admission to the Department. Based on ICHD-3 beta diagnostic criteria, 14 participants (31.8%) were diagnosed with migraine, while 30 (68.1%) met the requirements for TTH [9]. According to DC/TMD Axis I criteria [22], the distribution of TMD was as follows: 18 patients (40.9%) had myalgia, five patients (11.3%) had DDwR, and 21 patients (48.0%) had no TMD symptoms. The control group comprised 20 healthy adolescents (8 females [40%] and 12 males [60%], aged 12-17 years) without headache or TMD symptoms.

Table 1 presents the demographic and biochemical characteristics of patients with headaches compared to controls, stratified by sex. No statistically significant differences were observed between female patients and female controls, or between male patients and male controls, in anthropometric measurements (BMI, weight, height) or in basic biochemical parameters (TSH, total cholesterol, glucose). The mean BMI values were comparable between groups (20.78 kg/m² in female patients vs. 19.53 kg/m² in female controls; 19.05 kg/m² in male patients vs. 19.90 kg/m² in male controls).

Table 1. Characteristics of the investigated headache patients and controls.

Characteristic	Female (N = 23) mean ± SD (median)	Female control mean ± SD (median)	p-value	Male (N = 21) mean ± SD (median)	Male control mean ± SD (median)	p-value
BMI [kg/m^2]	21.05 ± 2.41 (20.78)	20.65 ± 4.10 (19.53)	0.603	20.23 ± 2.53 (19.05)	19.74 ± 2.63 (19.90)	0.708
Weight [kg]	51.91 ± 9.25 (52.00)	45.25 ± 16.74 (47.00)	0.416	48.67 ± 13.67 (50.00)	48.63 ± 17.42 (43.75)	0.721
Height [cm]	156.43 ± 10.41 (155.00)	144.88 ± 15.99 (153.50)	0.099	153.57 ± 14.04 (155.00)	154.08 ± 19.98 (149.50)	0.895
TSH level [µU/ml]	3.47 ± 1.73 (3.41)	3.08 ± 1.01 (2.60)	0.527	3.23 ± 1.38 (2.83)	3.37 ± 1.16 (3.34)	0.369
Total cholesterol [mg/dl]	161.70 ± 25.66 (158.00)	158.50 ± 29.49 (153.50)	0.635	162.81 ± 34.33 (167.00)	161.50 ± 24.25 (161.50)	0.808
Glucose level [mg/dl]	88.74 ± 6.05 (88.00)	90.63 ± 4.96 (88.50)	0.286	89.71 ± 7.46 (93.00)	93.92 ± 5.65 (94.50)	0.106
Nesfatin-1 (blood) [ng/mL]	39.12 ± 18.28 (39.48)	9.14 ± 0.63 (9.04)	**<0.001**	43.97 ± 14.47 (45.00)	7.52 ± 2.50 (8.14)	**<0.001**
Nesfatin-1 (saliva) [ng/mL]	10.57 ± 9.62 (6.61)	2.16 ± 0.68 (2.32)	**<0.005**	11.16 ± 9.19 (8.22)	1.85 ± 0.82 (1.76)	**<0.001**
Ghrelin/GOAT (blood) [ng/mL]	6.35 ± 2.75 (5.86)	1.47 ± 0.22 (1.43)	**<0.001**	6.21 ± 2.46 (6.64)	1.46 ± 0.45 (1.39)	**<0.001**
Ghrelin/GOAT (saliva) [ng/mL]	0.29 ± 0.24 (0.21)	1.65 ± 0.58 (1.41)	**<0.001**	0.24 ± 0.25 (0.18)	1.63 ± 0.78 (1.37)	**<0.001**

Data are presented as mean ± SD (median) for continuous variables. Between-group comparisons were performed using the Mann-Whitney U test. *p < 0.05 (statistically significant). BMI, Body Mass Index; GOAT, Ghrelin O-acetyltransferase; TSH, Thyroid-Stimulating Hormone.

Primary Outcome: Neuroendocrine Biomarker Levels

Blood Nesfatin-1 levels in blood were markedly elevated in both female (patients 39.12 ± 18.28 ng/mL (median 39.48) vs. controls 9.14 ± 0.63 ng/mL (median 9.04), p < 0.001, Cohen's d = 2.51) and male (patients 43.97 ± 14.47 ng/mL (median 45.00) vs. controls 7.52 ± 2.50 ng/mL, (median 8.14) p < 0.001, Cohen's d = 3.35) compared to their controls.

Similarly, salivary Nesfatin-1 levels were significantly higher in females (patients 10.57 ± 9.62 ng/mL (median 6.61) vs. controls 2.16 ± 0.68 ng/mL (median 2.32), p < 0.005, Cohen's d = 1.12) and males (patients 11.16 ± 9.19 ng/mL (median 8.22) vs. controls 1.85 ± 0.82 ng/mL (median 1.76), p < 0.001, Cohen's d = 1.30) (see **Table 1**).

A distinctive pattern was observed with Ghrelin/GOAT: blood levels were significantly elevated in both female (patients 6.35 ± 2.75 ng/mL (median 5.86) vs. controls 1.47 ± 0.22 ng/mL (median 1.43), p < 0.001, Cohen's d = 2.32) and male (patients 6.21 ± 2.46 ng/mL (median 6.64) vs. controls 1.46 ± 0.45 ng/mL, (median 1.39), p < 0.001, Cohen's d = 2.47). In contrast, salivary levels were markedly lower in both female (patients 0.29 ± 0.24 ng/mL (median 0.21) vs. controls 1.65 ± 0.58 ng/mL, (median 1.41), p < 0.001, Cohen's d=-3) and male (patients 0.24 ± 0.25 ng/mL (median 0.18) vs. controls 1.63 ± 0.78 ng/mL, (median 1.37), p < 0.001, Cohen's d = 2.50) compared to controls. The negative Cohen's d values for salivary ghrelin/GOAT indicate lower levels in patients than in controls (see **Table 1**).

Biomarker Levels Across Headache Types

No statistically significant differences were observed between the migraine patients (n = 14) and the TTH (n = 30) groups for any measured parameter (see **Table 2**).

Biomarker Levels Across TMD Categories

Comparison between TMD subgroups revealed significant differences in physical symptoms. Patients with myalgia showed a significantly higher prevalence of physical symptoms compared to the no-TMD group (89% vs 47%, p < 0.006). Similarly, the DDwR group demonstrated substantially more physical symptoms than the no-TMD group (100% vs. 47%, p < 0.035). The Kruskal-Wallis test revealed no significant differences in Nesfatin-1 levels (blood and saliva), Ghrelin/GOAT levels (blood and saliva), or TSH levels among the Myalgia, DDwR, and no-TMD groups (see **Table 3**).

Comparison of Neuroendocrine Markers Between All Patient Subgroups and Controls

Nesfatin-1 and Ghrelin/GOAT levels revealed consistent patterns across all headache subgroups compared to controls (see **Table 4**). Nesfatin-1 levels in blood were significantly elevated in migraine patients (42.94 ± 17.04 ng/ml), TTH patients (40.73 ± 16.58 ng/ml), those with TMD symptoms (41.74 ± 16.75 ng/ml), and those without TMD symptoms (41.10 ± 16.76 ng/ml) compared to controls (all p < 0.001). Similarly, salivary Nesfatin-1 levels were significantly higher in all patient subgroups (ranging from 10.13 ± 9.48 to

Table 2. Neuroendocrine Markers and Clinical Characteristics in Migraine versus Tension-Type Headache Patients.

Headache	Nesfatin-1 (ng/ml) Blood mean ± SD (median)	Nesfatin-1 (ng/ml) Saliva mean ± SD (median)	Ghrelin/ GOAT (ng/ml) Blood mean ± SD (median)	Ghrelin/ GOAT (ng/ml) Saliva mean ± SD (median)	TSH (uU/ml)	Myalgia TMD N(%)	DDwR TMD N(%)	Parafunctional bite or nonbite N(%)	Distress N(%)	Physical symptoms N(%)
Migraine (N = 14)	42.94 ± 17.04 (42.94)	10.98 ± 8.23 (8.62)	6.26 ± 2.65 (5.91)	0.25 ± 0.26 (0.16)	3.57 ± 1.95 (2,89)	8 (57%)	1 (7%)	14 (100%)	8 (57%)	23 (76%)
TTH (N = 30)	40.73 ± 16.58 (41.70)	10.79 ± 9.91 (4.52)	6.30 ± 2.60 (6.09)	0.27 ± 0.23 (0.21)	3.25 ± 1.36 (3.20)	10 (33%)	4 (13%)	18 (93%)	20 (66%)	8 (57%)
p-value	0.998	0.457	0.990	0.296	0.990	0.131	0.555	0.399	0.564	0.224

Data are presented as mean ± SD (median) for continuous variables and n (%) for categorical variables. Between-group comparisons were performed using the Mann-Whitney U test for continuous variables and the chi-square test for categorical variables. *p < 0.05 (statistically significant). DDwR, Disc displacement with reduction; GOAT, Ghrelin O-acetyltransferase; TMD, Temporomandibular Disorders; TSH, Thyroid-Stimulating Hormone; TTH, Tension-Type Headache.

Table 3. Comparison of Neuroendocrine Markers and Clinical Manifestations Across TMD Diagnostic Subgroups in Headache Patients.

TMD	Nesfatin-1 (ng/ml) Blood mean ± SD (median)	Nesfatin-1 (ng/ml) Saliva mean ± SD (median)	Ghrelin/ GOAT (ng/ml) Blood mean ± SD (median)	Ghrelin/ GOAT (ng/ml) Saliva mean ± SD (median)	Physical symptoms of TMD N(%) mean ± SD (median)	Distress N(%)	Parafun-ctional bite or nonbite N(%)	TSH (uU/ml) N(%)
Myalgia(M) N = 18	40.89 ± 17.23 (40.99)	11.59 ± 9.08 (10.30)	6.45 ± 2.85 (6.34)	0.18 ± 0.06 (0.16)	16 (89[a])	12 (67)	18 (100)	3.66 ± 1.88 (3.33)
Disc displacement with reduction (DDwR) N = 5	44.79 ± 16.32 (50.00)	11.22 ± 11.29 (8.22)	7.19 ± 3.58 (6.19)	0.25 ± 0.03 (0.25)	5 (100[a])	5 (100)	5 (100)	3.74 ± 1.34 (4.18)
No TMD N = 21	41.10 ± 16.76 (45.00)	10.13 ± 9.48 (4.48)	5.92 ± 2.13 (5.61)	0.33 ± 0.33 (0.21)	10 (47[b])	12 (57)	18 (85)	2.99 ± 1.27 (2.56)
p-value	0.628	0.823	0.764	0.067	***0.006***[a] 0.486 ***0.035***[a] ***vs No TMD***	0.522 0.149 0.077	0.119 0.393 0.392	0.289

Data are presented as mean ± SD (median) for continuous variables and n (%) for categorical variables. Multiple-group comparisons were performed using the Kruskal-Wallis test for continuous variables and the chi-square test for categorical variables. Post-hoc pairwise comparisons were conducted when the overall test was significant. *p < 0.05 (statistically significant). Superscripts indicate significant pairwise differences between groups (different letters denote p < 0.05). DDwR, Disc displacement with reduction; GOAT, Ghrelin O-acetyltransferase; TMD, Temporomandibular Disorders; TSH, Thyroid-Stimulating Hormone.

Table 4. Comparison of Nesfatin-1 and Ghrelin/GOAT Levels Between Headache Subgroups, TMD Categories, and Controls.

	Migraine (N = 14)	Control (N = 20)	p-value	TTH (N = 30)	Control (N = 20)	p-value	TMD (N = 23)	Control (N = 20)	p-value	No TMD (N = 21)	Control (N = 20)	p-value
Nesfatin-1 (ng/ml) **Blood** mean ± SD (median)	42.94 ± 17.04 (42.94)	8.16 ± 2.10 (8.64)	<0.001	40.73 ± 16.58 (41.70)	8.16 ± 2.10 (8.64)	***<0.001***	41.74 ± 16.75 (42.33)	8.16 ± 2.10 (8.64)	<0.001	41.10 ± 16.76 (45.00)	8.16 ± 2.10 (8.64)	***<0.001***
Nesfatin-1 (ng/ml) **Saliva** mean ± SD (median)	10.98 ± 8.23 (8.62)	1.97 ± 0.77 (2.01)	<0.001	10.79 ± 9.91 (4.52)	1.97 ± 0.77 (2.01)	***<0.001***	11.51 ± 9.32 (8.92)	1.97 ± 0.77 (2.01)	<0.001	10.13 ± 9.48 (4.48)	1.97 ± 0.77 (2.01)	***<0.001***
Ghrelin/ GOAT (ng/ml) **Blood** mean ± SD (median)	6.26 ± 2.65 (5.91)	1.46 ± 0.37 (1.41)	<0.001	6.30 ± 2.60 (6.09)	1.46 ± 0.37 (1.41)	***<0.001***	6.61 ± 2.95 (6.19)	1.46 ± 0.37 (1.41)	<0.001	5.92 ± 2.13 (5.61)	1.46 ± 0.37 (1.41)	***<0.001***
Ghrelin/ GOAT (ng/ml) **Saliva** mean ± SD (median)	0.25 ± 0.26 (0.16)	1.64 ± 0.69 (1.38)	<0.001	0.27 ± 0.23 (0.21)	1.64 ± 0.69 (1.38)	***<0,001***	0.19 ± 0.06 (0.19)	1.64 ± 0.69 (1.38)	<0.001	0.33 ± 0.33 (0.21)	1.64 ± 0.69 (1.38)	***<0.001***

Data are presented as mean±SD (median). Between-group comparisons were performed using the Mann-Whitney U test. *p < 0.05 (statistically significant). GOAT, Ghrelin O-acetyltransferase; TMD, Temporomandibular Disorders; TTH, Tension-Type Headache.

11.51 ± 9.32 ng/ml) compared to controls (1.97 ± 0.77 ng/ml) (all p < 0.001). Ghrelin/GOAT demonstrated a tissue-specific pattern: blood levels were significantly higher in all headache groups (ranging from 5.92 ± 2.13 to 6.61 ± 2.95 ng/ml) compared to controls (1.46 ± 0.37 ng/ml) (all p < 0.001), while salivary levels showed the opposite trend, being significantly lower in all patient groups (ranging from 0.19 ± 0.06 to 0.33 ± 0.33 ng/ml) compared to controls (1.64 ± 0.69 ng/ml) (all p < 0.001).

Gender Specific Clinical Manifestations

Gender-specific analysis revealed significant differences in TMD manifestations. Muscle pain upon palpation was significantly more prevalent in females than males (74% n = 17/23 vs 38% n = 8/21, p < 0.016). Conversely, males exhibited a higher prevalence of TMD-free cases than females (66%, n = 14/21 vs. 30%, n = 7/23, p < 0.016). Psychological distress was significantly more prevalent in males compared to females (90% n = 19/21 vs 48% n = 11/23, p < 0.003) (see **Table 5**).

Myalgia diagnosis, according to DC/TMD criteria, is also more frequent in females (52%/23 vs. 28%, n = 6/23), but there is no significant difference (p < 0.105). While both groups showed comparable rates of parafunctional habits, males demonstrated a higher frequency of both bite (52% vs. 21%, 43% vs. 23%, p < 0.550) and non-bite (90% vs. 21%, 82% vs. 23%, p < 0.447) parafunctions. Physical symptoms were similarly distributed between females and males (70% vs. 23% and 71% vs. 21%, respectively). No significant differences were observed in any of the other assessed parameters (see **Table 5**).

Discussion

The current study provides new perspectives into the relationship between neuroendocrine markers and headaches with TMD in adolescents. The most notable finding was the consistent and significant increase in Nesfatin-1 levels in both blood and saliva among all headache groups, regardless of headache type or TMD status. This pattern, together with the tissue-specific changes in Ghrelin/GOAT levels, suggests that these peptides may serve as biomarkers for headache disorders in adolescents.

Neuroendocrine Dysregulation in Adolescent Headache

The elevated Nesfatin-1 levels reported in our headache patients are consistent with recent evidence regarding this peptide's functions beyond energy regulation. Nesfatin-1 has been associated with multiple stress-related disorders, as evidenced by Hofmann et al., who identified positive associations between Nesfatin-1 levels and scores of anxiety, depression, and perceived stress in individuals of normal weight [25]. Xiao et al. showed a favourable correlation between plasma Nesfatin-1 levels and the degree of depression [26]. Considering the recognised correlation between psychological discomfort and pain

Table 5. Gender Distribution of TMD, Parafunctional Habits, and Associated Symptoms in Adolescents with Headache.

Signs and symptoms of TMD	Female (N = 23)	Male (N = 21)	p-value
Pain symptoms in the temple area	16 (70%)	18 (86%)	0.203
Muscle pain with palpation	17 (74%)	8 (38%)	***0.016***
Bite parafunctions	10 (43%)	11 (52%)	0.550
Non-bite parafunctions	19 (82%)	19 (90%)	0.447
Signs of bruxism	10 (43%)	10 (48%)	0.739
Physical symptoms N(%)	16 (70%)	15 (71%)	0.942
Distress (Depression&Anxiety) N(%)	11 (48%)	19 (90%)	***0.003***
Diagnosis of TMD – Myalgia	12 (52%)	6 (28%)	0.105
Diagnosis of TMD – DDwR	3 (13%)	2 (9%)	0.673
No TMD	7 (30%)	14 (66%)	***0.016***

Data are presented as n (%). Between-group comparisons were performed using the chi-square test. *p < 0.05 (statistically significant). DDwR, Disc displacement with reduction; TMD, Temporomandibular Disorders.

problems, our findings further substantiate that Nesfatin-1 may function as a neurobiological mediator connecting stress and pain perception in adolescents experiencing headaches.

The tissue-specific distribution of Ghrelin/GOAT, augmented in blood yet diminished in saliva, is particularly intriguing. The mechanisms underlying this differential expression across body fluids remain unclear and warrant further investigation. Potential explanations include differences in local production, degradation rates, or transport mechanisms between systemic circulation and salivary glands, though these hypotheses require direct experimental validation. Previous research has shown elevated plasma Ghrelin levels in children with anxiety disorders [16], but our study is the first to demonstrate this distinct pattern in adolescents with headaches. The mechanism underlying the inverse association between blood and salivary Ghrelin/GOAT levels warrants further examination and may yield insights into the pathophysiology of headache disorders.

Comparison with Established Headache Biomarkers

Our findings should be contextualised within the broader landscape of headache biomarker research. Calcitonin gene-related peptide (CGRP) is the most extensively studied biomarker for migraine in adults, and therapeutic agents targeting CGRP are now approved for migraine prevention [10, 11]. Pituitary adenylate cyclase-activating polypeptide (PACAP) also shows promise as a migraine marker [27]. However, these established biomarkers have limitations in pediatric populations, and their specificity for particular headache types may limit utility in mixed or uncertain diagnoses [3,10,11,27].

Compared to these established markers, Nesfatin-1 and Ghrelin/GOAT offer potential advantages: they can be measured in both blood and saliva (which is essential for pediatric populations, where minimally invasive sampling is preferred) [13,16,20], and they may reflect broader pain and stress mechanisms relevant to multiple headache types, rather than being specific to migraine alone [1-5]. However, without receiver operating characteristic (ROC) curve analysis, we cannot determine diagnostic accuracy parameters (sensitivity, specificity, optimal cutoffs) needed for clinical implementation.

Gender-Specific Clinical Manifestations

Our data revealed prominent gender-specific differences, with palpation-induced muscular discomfort occurring significantly more frequently in females (74%) than in males (38%; $p < 0.016$). In contrast, males demonstrated elevated levels of psychological distress (90% vs 48%, $p < 0.003$) and were more frequently asymptomatic for TMD (66% vs 30%, $p < 0.016$). These gender disparities align with previous research demonstrating higher TMD prevalence in females [28, 29], possibly due to hormonal influences, different pain thresholds, and gender-specific stress responses. The paradoxical observation of increased psychological distress in boys, despite lower incidences of TMD symptoms, indicates intricate gender-specific correlations between psychological factors and pain expression in adolescents.

Headache-TMD Comorbidity and Parafunctional Habits

A correlation between TMD and headache was apparent in our population, with 52% of patients with headache exhibiting TMD symptoms. This association corroborates the findings of Nilsen et al. regarding the strong correlation between TMD pain and headache in adolescents [30]. Recent Polish studies have also documented high prevalence and significant overlap between headaches and TMD, with headache being more frequent among those with painful TMD [7, 31]. Liljestrom et al. also reported connections between TMD and headaches in teenagers, urging the diagnosis of TMD when headaches are accompanied by ear pain, difficulty in mouth opening, and tiredness [32]. Our data support these suggestions, indicating that a thorough evaluation of TMD should be included in the standard assessment for adolescents presenting with headaches.

The lack of significant differences in Nesfatin-1 and Ghrelin/GOAT levels between patients with migraine and those with TTH is particularly noteworthy. This finding suggests that changes in these neuroendocrine markers may reflect overarching pain mechanisms rather than headache-specific pathways. Similarly, the lack of significant differences in these markers across TMD subgroups (myalgia, DDwR, no TMD) supports their potential as general biomarkers of pain and/or stress rather than as indicators of specific diseases.

Parafunctional habits were prevalent in our study group, with both bite and non-bite parafunctions observed in more than 80% of cases. This prevalence is more than the reported ranges in literature (5.9-49.6%) [33, 34] and highlights the possible contribution of these activities to the onset and persistence of TMD and headache symptoms. The elevated incidence of physical symptoms in individuals with myalgia (89%) and disc displacement with a decrease (100%) relative to those without TMD (47%) underscores the clinical importance of these conditions and their effect on adolescents' quality of life [34]. Recent evidence from Polish TMD populations further emphasises the complex relationships between sleep bruxism, reported pain, headache, and various health factors in patients with temporomandibular disorders [31].

Study Limitations

Several limitations of our study should be acknowledged. The limited sample size, particularly within specific subgroups, restricts the generalizability of our results. Subgroup analyses are statistically underpowered (e.g., DDwR, n = 5) and should be considered exploratory rather than confirmatory. The cross-sectional approach inhibits our ability to establish causality or ascertain whether the identified neuroendocrine abnormalities are primary factors or indirect effects of headache and TMD.

There is a slight difference in age ranges between the patient (10-17 years) and control (12-17 years) groups. However, it did not result in significant age differences in the final recruited cohorts, which warrants consideration of uniform age criteria in future studies.

Although TMD examinations were conducted according to standardised DC/TMD protocols, we did not formally assess inter-rater reliability between examiners, which would strengthen diagnostic accuracy in future studies.

We did not perform formal correlation analysis between blood and salivary biomarker levels. The opposing directional changes observed (Ghrelin/GOAT elevated in blood but decreased in saliva) indicate differential regulatory mechanisms in distinct biological compartments rather than simple linear relationships. Future mechanistic studies examining production, degradation, and transport in different tissues would better elucidate these tissue-specific patterns than correlation analysis.

We did not apply multiple-comparison corrections (e.g., Bonferroni or Benjamini-Hochberg) to our statistical analyses. Our primary comparisons (patients vs. controls for four biomarkers) were pre-specified, and the observed effect sizes were extensive (Cohen's d = 2.3-3.4), making false-positive findings highly unlikely. Subgroup analyses (headache type, TMD categories, gender) were clearly identified as exploratory. Applying strict multiple comparison corrections in this context would be overly conservative and risk false-negative conclusions in this hypothesis--generating study.

Moreover, although we controlled for several confounding factors (BMI via exclusion criteria; age, sex, and basic biochemical parameters via group balancing and stratification), our sample size (n = 44) precluded multivariable regression analyses that would have allowed simultaneous adjustment for multiple confounders. Future studies with larger cohorts are needed to perform multivariable modelling incorporating stress, parafunctional habits, TMD status, and headache type. Additionally, we did not assess pubertal stage (Tanner staging), which may influence neuroendocrine marker levels and represents a significant biological variable in adolescent research.

Clinical Implications and Future Directions

Future research should focus on longitudinal studies to investigate the temporal relationship between neuroendocrine indicators and headache/TMD symptoms, as well as the potential predictive value of these markers for treatment efficacy. Investigating the processes governing the tissue-specific patterns of Ghrelin/GOAT may yield significant insights into the pathophysiology of headache diseases. Ultimately, interventional studies focusing on these neuroendocrine pathways may provide novel therapeutic strategies for adolescents suffering from headaches and TMD.

Conclusion

This cross-sectional study demonstrates consistent elevations in Nesfatin-1 and contrasting patterns of Ghrelin/GOAT expression (elevated

in blood, reduced in saliva) in adolescents with headaches compared to healthy controls, independent of specific headache type or TMD status. These findings suggest that neuroendocrine dysregulation may represent a common pathway in adolescent headache rather than being specific to particular diagnostic subtypes.

In conclusion, our findings reveal substantial changes in Nesfatin-1 and Ghrelin/GOAT levels in adolescents experiencing headaches, irrespective of headache classification or TMD status. The persistent increase in these markers across various clinical manifestations indicates their potential utility as biomarkers in adolescent headache disorders. The gender-specific disparities in TMD symptoms and psychological suffering underscore the necessity of treating sex as a biological variable in pain studies. These findings enhance our comprehension of the intricate interactions among neuroendocrine variables, psychological distress, and pain perception in adolescents experiencing headaches and TMD.

Declarations

Authors' contributions

Conceptualisation: M.Z., B.S., B.D.B., A.S.: data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software; A.S., Y.B., M.Z.; supervision: M.Z., B.S., B.D.B.; validation, visualisation, writing: A.S., Y.B., O.Y., M.Z. All authors have read and approved the final version of the manuscript.

Conflicts of interest

Authors disclose any financial and personal relationships with others or organisations that could inappropriately influence the work.

Human ethics and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and received ethical approval from the Bioethical Committee of Poznan University of Medical Sciences (approval number 550/17). For child participants, written informed consent was obtained from their parents or legal guardians, and assent was obtained from the children themselves after a full explanation of the study procedures. Participant anonymity and confidentiality were maintained throughout the study. Participation was voluntary, with no financial or other obligations imposed on the participants. All procedures were designed to minimise any potential burden or risk to the participants.

Ethics approval declaration

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Bioethical Committee of Poznan University of Medical Sciences (approval number 550/17).

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Clinical trial number

Not applicable.

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