Serum levels of Autotaxin reveal its role as a novel biomarker of migraine

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Abstract

Background: Migraine is the most common neurological disorder and the second most disabling human condition. Autotaxin (ATX) is a plasma enzyme that leads to the formation of lysophosphatidic acid (LPA), which is involved in different functions involved in migraine, such as vascular tone control, inflammation, neuronal excitation, endothelial dysfunction, and neuropathic pain; among others. The vast majority of patients with migraine are females and, interestingly, ATX is physiologically higher in the serum of females compared to males.

Objective: As ATX may be a link between common mechanisms associated with migraine, we aim to determine the potential role of ATX in migraine by studying its concentrations in serum between patients with episodic (EM) and chronic migraine (CM) compared to healthy controls, as well as the correlation of ATX with clinical outcomes, and another biomarkers described in migraine.

Methods: In this cross-sectional study, healthy controls (n=62), EM (n=45), and CM (n=38) were studied. Clinical outcomes, such as migraine intensity as assessed on the Visual Analogue Scale (VAS), frequency of headaches (days/month), evolution time (months) and the duration of attacks (hours) were investigated together the serum biomarkers for inflammation (interleukin-6, [IL-6] and interleukin-10 [IL-10]), trigeminovascular system activation (calcitonin gene-related peptide, [CGRP]), endothelial dysfunction (pentraxin-3, [PTX-3], cellular fibrinogen [cFN], soluble tumor necrosis factor-like weak inducer of apoptosis [sTWEAK]), and ATX. Additionally, the serum lipidomic biomarkers profile was also analyzed.

Results: Serum ATX levels were found to be significantly elevated in both EM (310.7±79.7 ng/mL) and CM (336.7±66.9 ng/mL) compared to controls (212.3±53.2 ng/mL) ($p<0.001$). Elevated ATX levels were associated with migraine outcomes in CM, such as VAS (Spearman's coefficient=0.405, $p<0.05$), frequency (Spearman's coefficient=0.718, $p<0.001$), and evolution time (Spearman's coefficient=0.2257, $p<0.01$). ATX was correlated with CGRP (Pearson's coefficient=0.278, $p<0.001$), PTX3 (Pearson's coefficient=0.468, $p<0.001$), sTWEAK (Pearson's coefficient=0.242, $p<0.001$), cFN (Pearson's coefficient=0.252, $p<0.01$), and IL-6 serum levels (Pearson's coefficient=0.159, $p<0.001$). A drastic decrease in serum lysophosphatidylcholine (LPC) levels indicates high ATX activity in patients with migraine.

Conclusions: Serum levels of ATX were significantly increased in EM and CM. In addition, ATX correlates with clinical outcomes, as well as CGRP, endothelial dysfunction and inflammation biomarkers. Further studies are necessary to elucidate the potential role of ATX as a therapeutic target for migraine.

Keywords: Autotaxin, CGRP, endothelial dysfunction, inflammation, lysophospholipids, migraine, serum, vascular

Resume:

This study shows that patients with migraine have elevated serum levels of ATX compared to healthy controls, especially for patients with CM.

These findings could indicate the involvement of ATX in migraine attacks and its evolution.

Further studies are necessary to elucidate the potential role of ATX as a biomarker and therapeutic target for migraine.

1. Background

Migraine is a neurological disorder that affects 15% of the world's population, and it is considered the second most disabling human condition [1]. It is characterized by recurrent attacks of moderate to severe throbbing and pulsating pain on one side of the head. Migraine is diagnosed according to the International Headache Society (International Classification of Headache Disorders, ICHD-3) [2], which recognizes two types according to attack frequency: episodic migraine (EM) and chronic migraine (CM). Importantly, around 2% of patients with EM may eventually experience an increase in attack frequency, leading to the development of chronic migraine.

The pathophysiology of migraine involves both vascular and neural mechanisms. In this regard, two theories have been postulated about migraine's origin [3]. The neuronal theory, based on an over-excitation of trigeminal neurons that release factors that stimulate vasodilation and inflammation of the vascular endothelium; and the vascular theory, focused on the vascular tone changes of the endothelium that lead to the release of pro-excitatory factors for the neurons.

Migraine involves nociceptive inputs from the raphe and locus coeruleus nuclei [4], the cortical spreading depression phenomenon, and the trigeminovascular system activation [5]. The implication of inflammatory vasoactive peptides, such as calcitonin gene-related peptide (CGRP), is also well known [6], which promotes dilatation of the meningeal vessels and modulates endothelial function [7,8]. Particularly, CGRP acts as a very potent vasodilator through its interaction with specific receptors in smooth muscle and endothelium. Remarkably, the more effective treatments are focused on the blocking of CGRP or its receptors by monoclonal antibodies [9].

On the other hand, lysophosphatidic acid (LPA) is a bioactive lysophospholipid involved in physiological functions and pathological processes [10]. LPA, through specific membrane receptors, known as LPA receptors (LPARs), is involved in several mechanisms related to migraines, such as platelet aggregation, vascular tone control, inflammation, neuronal excitation, endothelial dysfunction, and neuropathic pain; among others [11–18]. In this regard, several studies have described that ATX-LPA axis promotes

neuropathic pain-like behavior [18–21]. Moreover, recent work demonstrated that ATX inhibition ameliorates neuropathic pain [22].

LPA is mainly produced locally by the secreted lysophospholipase D (LysoPLD), also known as autotaxin (ATX), which is encoded by the ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) gene [23]. ATX levels have been reported to be physiologically higher in females than in males [24], an interesting fact taking into account that migraine is more prevalent in females, with a female-male ratio of 3:1 [25]. Despite the apparent physiological relationship between both molecules, the implication of the ATX-LPA axis in migraine has not been addressed so far.

Considering all this evidence, this study aims to study the potential role of ATX in migraine for testing the hypothesis that ATX serum levels are elevated in patients episodic (EM) and chronic (CM) compared to healthy controls; as well as the association between ATX levels and clinical outcomes.

2. Methods

2.1 Parcipipants

All patients and control participants were prospectively recruited from the Headache Unit of Neurology Department at Hospital Clínico Universitario of Santiago de Compostela. Patients with migraine were classified according to the International Classification of Headache Disorders, 3rd edition criteria [2].

2.2 Study protocol

This cross-sectional study was conducted following the Declaration of Helsinki of the World Medical Association (2008) and approved by the Ethics Committee of the Servizo Galego de Saúde (2016/085). All patients and healthy controls were treated and supervised by expert neurologists according to the International Headache Society (International Classification of Headache Disorders, ICHD-3)[2] .Participants were recruited from 2017 to 2020. Patients with EM and CM, and healthy control participants, all over 18 years old who signed the informed consent, were included. Biomarkers were determined in peripheral venous blood. Patients with migraine were headache-free from 24-48 hours before the visit to 24 hours after the blood samples. If a migraine occurred within the first 24 hours, measurements were repeated in another headache-free period. Participants had not previously consumed anti-inflammatory or analgesic medication during this headache-free period.

Exclusion criteria were the following: 1) chronic inflammatory conditions; 2) severe systemic diseases; 3) pregnancy or lactation; 4) neuroendocrine tumors; 5) multisystemic trauma; 6) other neurological diseases; 7) vascular risk factors (arterial hypertension, diabetes mellitus, coronary disease, smoking, dyslipidemia and obesity with BMI $\geqq$ 35 kg/m^2; 8) excessive sports activity (Vigorous physical activity more than 5-6 days a week, 7-10 hours of vigorous exercise a week, or vigorous exercise for more than 2-3 hours daily) and 9) other forms of chronic headache.

2.3Clinical variables

All patients had a complete medical record including demographic data (age, sex), personal and family history and physical examination and neuroimaging. For patients with migraine, the type of migraine (episodic or chronic), time of evolution of the disease

(determined in years), the intensity of headaches (measured by the visual analog scale [VAS]), duration of attacks (quantified in hours) and frequency of headaches (number of days with pain per month), and neuroimaging were recorded. These clinical parameters were considered as an average of the patient's episodes.

2.4Laboratory tests

Markers of inflammation (interleukin-6, [IL-6], and interleukin-10 [IL-10]), trigeminovascular system activation (alpha-calcitonin gene-related peptide, [CGRP]), endothelial dysfunction (pentraxin-3, [PTX-3], [sTWEAK] and [cFN]), as well as ATX, were investigated. For serum level determination, 4.5 ml of blood from the antecubital vein was collected. Samples were obtained during a pain-free interval and after an overnight fast. All samples were kept in chemistry test tubes and centrifuged at 1700G for 15 minutes. The serum was immediately frozen and stored at -80ºC for further analysis.

Serum levels of IL-6 and IL-10 were measured by using an immunodiagnostic IMMULITE 1000 System (Siemens Healthcare Global, Los Angeles, CA, USA). Serum levels of PTX-3 (Assay Biotech, Sunnyvale, CA, USA; Ref: Lum-8346), CGRP (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA; Ref: FEK-015-02), cFN (Cusabio Life Science, Wuhan, China; Ref: CSB-E11850h), sTWEAK (Elabscience, Texas, USA)., ATX (R&D Systems, USA; Ref: DENP20), and LPA (Echelon Bioscience, Utah, USA, Ref: K-2800S) were determined with commercial ELISA kits following manufacturer instructions. ATX activity was determined by an enzymatic reaction kit (Echelon Bioscience, Utah, USA, Ref: K-4100). All the ELISA reactions and activity assay were measured with a Biotek HT plate reader. To determine the concentrations by ELISA, an extrapolation was performed with the internal standards of the kit. The intra-assay and inter-assay coefficients of variation (CV) for all biomarkers were <10%.

All biomarkers and cellular determinations were performed in a laboratory blinded to clinical data.

2.5Lipidomics

Lipids were extracted from serum samples and analyzed as described [26,27]. For phospholipids and neutral lipids, a total of 750 µL of a methanol-chloroform (1:2,

vol/vol) solution containing internal standards (16:0 D31_18:1 phosphocholine, 16:0 D31_18:1 phosphoethanolamine, 16:0 D31-18:1 phosphoserine, 17:0 lyso-phosphocholine, 17:1 lyso-phosphoethanolamine, 17:1 lyso-phosphoserine, 17:0 D5_17:0 diacylglycerol, 17:0/17:0/17:0 triacylglycerol and C17:0 choresteryl ester, 0.2 nmol each, from Avanti Polar Lipids) were added to 50 µL of serum. Samples were vortexed and sonicated until they appeared dispersed and extracted at 48°C overnight. The samples were then evaporated and transferred to 1.5 mL Eppendorf tubes after the addition of 0.5 mL of methanol. Samples were evaporated to dryness, and stored at -80°C until analysis. Before analysis, 150 µL of methanol was added to the samples, centrifuged at 13,000 g for 3 min, and 130 µL of the supernatants were transferred to ultra-performance liquid chromatography (UPLC) vials for injection and analysis. For sphingolipids, a total of 750 µL of a methanol-chloroform (2:1, vol/vol) solution containing internal standards (N-dodecanoylsphingosine, N-dodecanoylglucosylsphingosine, N-dodecanoylsphingosylphosphorylcholine, C17-dihydrosphingosine and C17-dihydrosphingosine-1-phosphate, 0.2 nmol each, from Avanti Polar Lipids) were added to 75 µL of serum. Samples were extracted at 48°C overnight and cooled. Then, 75 µL of 1 M KOH in methanol was added, and the mixture was incubated for 2 h at 37°C. Following the addition of 75 µL of 1 M acetic acid, samples were evaporated to dryness, and stored at -80°C until analysis. Before analysis, 150 µL of methanol was added to the samples, centrifuged at 13,000 g for 5 min and 130 µL of the supernatant were transferred to a new vial and injected.

Lipids were analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS). LC-HRMS analysis was performed using an Acquity ultra high-performance liquid chromatography (UHPLC) system (Waters, USA) connected to a Time of Flight (LCT Premier XE) Detector. Full scan spectra from 50 to 1800 Da were acquired, and individual spectra were summed to produce data points each of 0.2 sec. Mass accuracy at a resolving power of 10,000 and reproducibility was maintained by using an independent reference spray via the LockSpray interference. Lipid extracts were injected onto an Acquity UHPLC BEH C8 column (1.7 µm particle size, 100 mm x 2.1 mm, Waters, Ireland) at a flow rate of 0.3 mL/min and a column temperature of 30ºC. The mobile phases were methanol with 2 mM ammonium formate and 0.2% formic acid (A)/water with 2 mM ammonium formate and 0.2% formic acid (B). A linear gradient was programmed as follows: 0.0 min: 20% B; 3 min: 10% B; 6 min: 10% B; 15 min: 1% B;

18 min: 1% B; 20 min: 20% B; 22 min: 20% B. Positive identification of compounds was based on the accurate mass measurement with an error <5 ppm and its LC retention time, compared with that of a standard (92%). Quantification was carried out using the extracted ion chromatogram of each compound, using 50 mDa windows. The linear dynamic range was determined by injecting mixtures of internal and natural standards. Since standards for all identified lipids were not available, the amounts of lipids are given as pmol equivalents relative to each specific standard. For LCB-phosphate quantification, analysis of the extracts was performed by UPLC-MS/MS with a system consisting of an Acquity ultraperformance liquid chromatography (UPLC) system (Waters, USA) connected to a triple-quadrupole mass spectrometer (Xevo TQ-S, Waters, USA) and controlled with Waters/Micromass MassLynx software. Detection was performed with an electrospray interface operating in the positive ion mode, the capillary voltage was set to 3.1 kV, the source temperature was 150 °C and the desolvation temperature was 500°C, acquiring the following selected reaction monitoring transitions: C17 D-erythro-dihydrosphingosine- 1-phosphate, 368–252 Da, cone voltage 30V, collision energy 20 eV; and S1P, 380–264 Da, cone voltage 30V, collision energy 20 eV.

2.6Data and statistical analysis

Results were expressed as percentages for categorical variables, mean (SD) or median and interquartile range for continuous variables, depending on the normal or not normal distribution of data. Normality was determined by the Kolmogorov-Smirnov test. Two-tailed statistical tests were used to compare continuous data were independent t-test, Mann-Whitney U test, Kruskal-Wallis H to nonparametric data, or post-hoc ANOVA-Tukey test to normal distributed discrete/continuous variables for more than two groups. Categorical variables were reported as percentages and compared by X^2test. Pearson coefficient for continuous variables (duration of disease) and Pearson and Spearman (non-normally distributed) coefficient for categorical variables (intensity of pain and frequency of attacks) were applied. Logistic regression analysis, including all patients with migraine *vs.* controls, was used for identifying those biomarkers independently associated to migraine; effect size and directionality of the estimates from the logistic regression analyses are also reported as odds ratios with 95% confidence intervals. The sensitivity and specificity of the different biomarkers were represented graphically by receiver operating characteristic (ROC) curves. A value of $p < 0.05$ was considered significant. The cut-offs were determined by Youden's index. Statistical analysis was

performed using SPSS version 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 10.0 (GraphPad Software, Inc., San Diego, CA, USA). There were no missing data.

Since this is a secondary analysis from previous works [28,29], no formal sample size calculation was performed. The group's previous work studied different serum biomarkers in the blood of patients with migraine, both episodic and chronic. In this way, these samples, together a well-characterized clinical data-set, seemed like a good database to determine the relationship of new biomarkers with migraine. In addition, a post-hoc power analysis based on the results obtained from the present study and using our primary outcome (i.e., ATX concentrations) confirmed a 95% power to detect an 80.0 ng/mL difference in ATX levels between study groups (patients with migraine vs. controls), with an SD of 20.1. All statistical power analyses were done SPSS version 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 10.0 (GraphPad Software, Inc., San Diego, CA, USA).

2.7 Endpoints

The primary endpoint was the comparison of serum ATX levels between patients with migraine and healthy control participants, as well as between patients with EM and CM.

As secondary endpoints, we analyzed the association of ATX serum levels with the type of migraine (episodic or chronic), time of evolution of the disease (determined in years), intensity of headaches (measured by the visual analogic scale [VAS]), duration of attacks (quantified in hours) and frequency of headaches (number of days with pain per month).

Finally, we analyzed the correlation between ATX and serum levels of endothelial dysfunction and inflammation biomarkers, as well as lipidomics, to investigate the possible mechanism of action of ATX in migraine.

3. Results

A total of 62 controls and 81 cases were matched by age (Ctrl=41.2 ± 12.4; EM 42.3 ± 12.8; CM 45.1 ±11.1; n.s.), and by sex (Ctrl= 94% female, EM=93% female; CM=97% female; n.s.) (**Table 1**). Of patients with migraine, 45 were diagnosed as EM (attacks frequency 8.3 ± 5.5 days/month) and 38 as CM (attacks frequency 21.4 ± 4.7 days/month) according to the International Classification of Headache Disorders, 3rd edition criteria.

3.1. Autotaxin (ATX) levels were found elevated in both episodic and chronic migraine compared to control participants

ATX levels were significantly found elevated in both, EM (310.7 ± 79.7 ng/mL,) and CM (336.7 ± 66.9 ng/mL,) patients compared with controls (212.3 ± 53.19 ng/mL) ($p<0.001$); meanwhile the values were not significantly different between EM and CM ($p=0.187$) **(Fig. 1A)**. Our results were corroborated with a significant increase in plasmatic ATX activity **(Fig. 1B).** In addition, we analyzed the specificity and sensitivity of ATX levels for the diagnosis of the different types of migraine **(Fig. 1 C, D)**. In this regard, it was observed that ATX levels clearly distinguish from controls to EM **(Fig. 1C)** and CM **(Fig. 1D)**; specifically, ROC analysis showed an area under the curve (AUC) of 0.84 (95% CI: 0.76-0.91, $p<0.001$) and 0.98 (95% CI: 0.87-0.98, $p<0.001$), respectively for EM and CM. Furthermore, ATX levels ≥ 260 ng/mL identify patients with CM versus controls with a sensitivity of 85% and a specificity of 81%. Specifically, 89.5% of patients with CM had ATX levels ≥ 260 ng/mL, compared to 71.1% of patients with EM ($p<0.001$). Meanwhile, ATX levels ≥ 270 ng/mL identify patients with EM versus controls with a specificity of 85.2%. In a logistic regression model, using all patients with migraine *vs.* controls and ATX as a continuous non-categorical variable, ATX levels ≥ 260 ng/mL independently identify migraine (OR, 2.05; CI 95%: 1.02 – 3.04, $p<0.001$) **(Table 2).**

3.2 High levels of ATX were associated with clinical outcomes of chronic migraine

ATX levels were not correlated with an increase in the Visual Analogue Scale (VAS) scores when all patients were analyzed together; however, VAS correlated significantly with ATX levels in patients with CM (Spearman's coefficient=0.405,

p<0.05) **(Fig. 2A)**. Moreover, ATX levels were not associated with the frequency of migraine attacks in EM patients; nevertheless, ATX levels showed a significant positive correlation with the frequency of attacks in patients with CM (Spearman's coefficient=0.718, p<0.001) **(Fig. 2B)**. Additionally, there was a significant association between ATX levels and the chronicity of the disease, as a correlation was observed between ATX levels and the time of evolution in patients with CM (Spearman's coefficient=0.2257, p<0.01), but not in patients with EM **(Fig. 2C)**. On the contrary, no association was found between ATX levels and migraine duration **(Fig. 2D)**.

3.3 ATX and CGRP levels correlated but did not discriminate between episodic and chronic migraine

ATX and CGRP levels were determined in serum samples of healthy control, EM and patients with CM. No correlation was found between both biomarkers in patient with EM (Pearson's coefficient=0.04, p=0.2) and CM (Pearson's coefficient=$3 \cdot 10^{-6}$, p=0.99), since patients with patients showed elevated levels of both mentioned molecules **(Fig 3A)**. However, when the CGRP and ATX levels were analyzed together including healthy controls, a significant correlation was found (Pearson's coefficient=0.278, p<0.001) **(Fig. 3B)**.

3.4 ATX is associated with high levels of endothelial dysfunction biomarkers in migraine patients

Previously, different articles demonstrated the involvement of endothelial dysfunction in the pathophysiology of migraine [30,31]. In this regard, high levels of pentraxin-3 (PTX-3), a well-known biomarker for endothelial dysfunction, and ATX were found in both EM and CM compared with healthy controls **(Fig. 4A)**. In addition, a significant correlation between PTX-3 and ATX was found in both EM (Pearson's coefficient=0.323, p<0.001), but not inCM (Pearson's coefficient=0.038, p<0.001). Furthermore, this correlation was stronger when the migraine groups were analyzed together (Pearson's coefficient=0.468, p<0.001) **(Fig. 4B)**.

Moreover, high levels of soluble tumour necrosis factor-like weak inducer of apoptosis (sTWEAK), another endothelial dysfunction biomarker, were found in patients with CM, positively and significantly correlating with ATX levels (Pearson's coefficient=0.239, p=0.002). On the contrary, this association was not found for EM

(Pearson's coefficient=0.047, p=0.150) **(Fig. 4C)**. However, the overall (EM and CM together) correlation between ATX and sTWEAK was significant (Pearson's coefficient=0.242, p<0.001) **(Fig. 4D)**.

Finally, cellular fibrinogen (cFN) correlated significantly with ATX levels in CM (Pearson's coefficient=0.252, p<0.01); however, although the correlation in the EM group was stronger than in CM, it was not statistically significant (Pearson's coefficient 0.536, p=0.06) **(Fig. 4E)**. In addition, it was observed a significant overall correlation (EM and CM together) between ATX and cFN levels (Pearson's coefficient=0.425, p<0.001) **(Fig. 4F)**.

3.5 Association between the levels of ATX and inflammatory markers

Preliminarily, several studies have addressed the implication of the inflammatory response with an essential role in migraine pathogenesis [32–34]. As expected, a significant correlation was found between ATX and the pro-inflammatory IL-6 levels in both, patients with EM (Pearson's coefficient=0.145, p<0.001), and CM (Pearson's coefficient=0.100, p=0.056) **(Fig. 5A, B)**. However, ATX levels were not associated with the anti-inflammatory IL-10 levels when each study group was analyzed separately **(Fig. 5C)**, but when analyzing all the groups together, a correlation was found between the ATX and IL-10 levels (Pearson's coefficient=0.238, p<0.001) **(Fig. 5D)**.

3.6 Lipidomic showed a great reduction in lysophosphatidylcholine (LPC) levels that indicated high ATX activity in the serum of migraine patients

Considering that we found elevated levels of ATX in the serum of migraine patients, we analyzed the serum levels of a well-known ATX substrate, lysophosphatidylcholine (LPC), in order to determine ATX activity **(Table 3).** Importantly, a drastic reduction in LPC levels is observed in both EM and CM patients compared to healthy participants (**Fig 6A**). Furthermore, it occurs in all the different LPC species independently on their fatty acid chain and unsaturations (**Fig. 6B and C**); confirming the high activity of ATX in patients with migraine. Interestingly, the low levels of LPC are a highly sensitive and specific parameter to discriminate both patients with EM and CM(**Fig. 6B-C**). ROC analysis showed an AUC of 0.95 (95% CI: 0.90-1, p<0.001) with a Cut-Off of >79912

pmol/mL with a specificity of 90.5% for EM; and AUC of 0.87 (95% CI: 0.77-0.97, p<0.001), with a Cut-Off of > 88585 pmol/mL with a specificity of 85.7% for EM.

In addition, the low levels of LPC 20:3 are also highly sensitive and specific parameter to discriminate EM with an AUC of 0.82 (95% CI: 0.70-0.93, p<0.001) with a Cut-Off of >1742 pmol/mL with a specificity of 81%; but not for CM with an AUC of 0.63 (95% CI: 0.47-0.78, p=0.124) with a Cut-Off of >1880 pmol/mL with a specificity of 66.7%.

3.7Sphingolipidomic reveals a possible implication of LPPs in LPA degradation

Contradictorily, identical concentrations of LPA were observed in patients with migraine compared to healthy participants **(Fig. 7A).** In this regard, it is also well-known that LPA in plasma is rapidly degraded by different pathways, such as intracellular LPA acyltransferase (LPAAT), and endothelial membrane lipid phosphate phosphatase (LPP)[35,36]. Moreover, LPPs can also dephosphorylate important sphingolipids such as sphingosine 1-phosphate (S1P) or sphinganine 1-phosphate (Sph1-P) [37,38]. As observed, serum levels of S1P and Sph1P were found to be significantly reduced **(Fig. 7B, C).**

4 Discussion

To the best of our knowledge, this is the first study which demonstrates that serum levels of ATX are higher in both EM and CM compared to healthy control participants. This study supports serum ATX levels as a good diagnostic biomarker for migraine, and describes the correlation of ATX levels with the time of evolution of the disease, intensity of headaches, and frequency of headaches, but only in patients with CM.

When studying the clinical parameters of migraine with the levels of ATX we could see that there was a correlation between VAS, frequency of attacks and evolution time with ATX levels in CM patients. However, no correlation was observed when determined with both migraine groups together. Possibly, it is due to a limitation given the differences in clinical values between patients with CM and EM.

CGRP is a hallmark biomarker for migraine. In this regard, CGRP is considered an inflammatory vasoactive peptide released by trigeminal activation, that stimulates vasodilation of the meningeal vessels and modulates endothelial function [39]. Several studies have determined an elevation of CGRP serum levels during migraine attacks [40]. Moreover, our study demonstrated that CGRP levels ≥ 14.86 pg/mL identify patients with migraine versus controls with a sensitivity of 100% and a specificity of 97.87%. Based on this, the employment of blocking monoclonal antibodies against CGRP and its receptors is currently a common therapy in migraine [9].

In this regard, we have not observed a correlation between ATX and CGRP levels when EM and CM were study independently. However, we have determined a positive correlation between ATX and CGRP levels when all the groups, were analyzed together. Actually, CGRP was found elevated in blood samples from migraine animal models during trigeminal fibers stimulation [41]. Furthermore, it was previously described that both CGRP and ATX serum levels are higher in females than in males [24,42], consistent with a higher frequency of migraine in females. Particularly, CGRP acts as a very potent local vasodilator through its interaction with different receptors in smooth muscle and endothelium. Specifically, the interaction of CGRP with the endothelial cells induces the activation of endothelial nitric oxide synthase (eNOS) through the adenylyl cyclase (AC)/cAMPK/protein kinase A (PKA) pathway [40]. This pathway is also stimulated by the interaction of LPA with LPAR4 and 6 [23]. Moreover, LPA stimulates vasodilation by a mechanism mediated by endothelial LPAR1, phospholipase C and eNOS [43]. These

evidences point to a relationship between CGRP and ATX in migraine pathology, however, further studies should be carried out to elucidate the relevance of this correlation. Especially when, as mentioned, the most effective treatments today are based on blocking CGRP or its receptors through monoclonal antibodies [44]. Nowadays, there are different CGRP-specific monoclonal antibodies approved for their medical use, such as erenumab, fremanezumab, galcanezumab and eptinezumab; in addition to small molecules that block CGRP receptors, such as rimegepant, ubrogepant, atogepant and zavegepant. However, despite the high efficiency of these treatments, they only reduce around 50% of migraine attacks per month in 50% of patients, without significant improvements being observed in the rest of the patients [45,46]. Therefore, the search for new biomarkers, such as ATX is of special relevance in order to shed light on CGRP treatment variations.

Previously, our group and others have observed that endothelial dysfunction seems to be acutely pronounced in migraine [30,47–49]. Particularly, increased levels of endothelial dysfunction biomarkers, such as PTX-3, have been determined in chronic migraine [47]. Interestingly, our data shown that PTX-3 levels ≥ 510.4 pg/mL identify patients with migraine versus controls with a sensitivity of 100% and a specificity of 97.87%. As expected, we also found a significant correlation between PTX-3 and ATX in EM . Interestingly, these results are in line with those published by Gustin and co-workers, who demonstrated an upregulation of PTX-3 in human endothelial cells by LPA treatment [50].

sTWEAK is a potential biomarker of several diseases and endothelial dysfunction [51], including chronic migraine [47]. In concordance with our previous results, elevated levels of ATX and sTWEAK in serum were found in patients with CM, with a significant correlation, but not in patients with EM. However, ATX and sTWEAK levels were significantly consistent when all the groups were analyzed together.

In addition, we observed that cFN correlated significantly with ATX levels in both, CM and EM. Furthermore, it was observed a considered overall correlation between ATX and cFN. Interestingly, cFN has been described as a marker of inflammation, since it facilitates the secretion of cytokines by monocytes, in addition to stimulating platelet aggregation. Additionally, it was described that cFN decreases NO production by endothelial cells [52], and promotes endothelial permeability [53], thus contributing to endothelial dysfunction and blood-brain barrier disruption. Moreover, different studies

have observed the elevation of cFN levels in both serum [54] and cerebrospinal fluid [55] of patients with migraine.

As previously mentioned, the inflammatory response plays an essential role in migraine pathogenesis. Several works have described the inflammatory response as a key piece of evidence on the relationship between endothelial dysfunctions and migraine pathophysiology [32,33,56]. In this context, we found a strong connection between ATX and pro-inflammatory markers, such as IL-6, in both patients with EM and CM for both types of analysis; however, a significant correlation between ATX and IL-10 levels was only observed when analyzing the groups together with controls. In this regard, the release by the endothelium of vascular endothelial growth factor (VEGF), which was found elevated in migraine [57], by the endothelium stimulates the recruitment of cells from the immune system, leading to the release of various cytokines, such as IL-6 and IL-10 [56]. Furthermore, IL-6 upregulation under LPA stimulation was described in dermal cells [58].

Although LPC can be degraded by various enzymes, such as the intracellular enzymes lysophosphatidylcholine acyltransferase (LPCAT) and enzymes with lysophospholipase A1 activity, the main responsible for degrading LPC in serum is the enzyme ATX [59]. In this regard, it is well described that ATX has LysoPLD activity, and, as mentioned, mainly hydrolyzes LPC to produce LPA. Considering that we found an elevated concentration of ATX in serum, we analysed both, LPC and LPA serum levels, to determine the ATX activity. As expected, a drastic reduction in LPC levels is observed in both patients with EM and CM compared to healthy participants. Our data are in concordance with a recent study that showed low levels of LPC in serum, in a small cohort of patients with migraine, compared to healthy participants [60]. Contradictorily, no changes in LPA concentrations were observed in patients with migraine compared to healthy participants. In this regard, several studies showed that LPARs are rapidly endocytosed after interaction with LPA[61,62], which could explain our results. Furthermore, it is also well known that LPA in serum is rapidly degraded by different pathways, such as LPA acyltransferase (LPAAT), or lipid phosphate phosphatase (LPP) [35]. LPPs are a group of enzymes encoded by genes belonging to a phosphatase/phosphotransferase family. LPPs are localized on plasma membranes, highly expressed in endothelial cells and smooth muscle cells, and exert their catalytic activity on the outer leaflet. Interestingly, LPPs surface expression is dynamically upregulated in the context of vascular inflammation to regulate to modify the balance between phosphorylated and dephosphorylated lipids [37].

In addition, LPPs can also dephosphorylate important sphingolipids such as sphingosine 1-phosphate (S1P) or sphinganine 1-phosphate (Sph1-P) [37,38]. As we demonstrated the levels of S1P and Sph1P were found to be significantly reduced in migraine samples, which, together with the endocytosis of LPARs could explain the LPA concentrations observed.

We have to acknowledge some limitations concerning this investigation. Firstly, future prospective clinical studies with a formal sample size calculation are needed to confirm these results. In addition, although in apparent good general health, some of the patients and controls could have other undiagnosed conditions linked to increased systemic inflammation and endothelial dysfunction.

5 Conclusions

In conclusion, higher serum levels of ATX were found in both EM and CM compared to control participants. Notably, these higher ATX levels were also correlated with the time of evolution of the disease, intensity of headaches, and frequency of headaches, but only in patients with CM. In addition, ATX also correlated with serum biomarkers previously described in migraine when all the groups were analyzed together, such as CGRP, PTX-3, sTWEAK, cFN, and IL-6. Further studies are necessary to elucidate the potential role of ATX as a biomarker and therapeutic target for migraine.

6 Abbreviations

ATX:	Autotaxin
CGRP:	Calcitonin gene-related peptide
cFN:	Cellular fibrinogen
CM:	Chronic migraine
EM:	Episodic migraine
ENPP2:	Ectonucleotide pyrophosphatase/phosphodiesterase 2
IL-6:	Interleukin 6
IL-10:	Interleukin 10
LPA:	Lysophosphatidic acid
LPAAT:	LPA acyltransferase
LPAR:	Lysophosphatidic acid
LPC:	LysophosphatidylCholine
LPCAT:	Lysophosphatidylcholine acyltransferase
LPP:	Lipid phosphate phosphatase
LysoPLD:	Lysophospholipase D
PTX-3:	Pentraxin-3
S1P:	Sphingosine 1-phosphate
Sph-1P:	Sphinganine 1-phosphate
sTWEAK:	Soluble tumor necrosis factor-like weak inducer of apoptosis
VAS:	Visual Analogue Scale
VEGF:	Vascular Endothelial Growth Factor

7 Declarations

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article. Raw data supporting this study's findings are available from the corresponding author, upon reasonable request.

Author`s contribution

AO, JC, RL, and TS designed and conceptualized the research framework, interpreted the results of experiments, and drafted the manuscript; AO, MCM, MRA, and JC performed experiments; AO, MCM, MRA, MDM, DRS, MAN, RIR, JC, IL, JCB, RL, and TS analyzed data; AO and TS performed supervision and critical review; and TS got funding. All authors have contributed to editing the manuscript. All authors have revised and approved the final version of the manuscript.

Competing interest

The authors declare no conflict of interest.

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8 References

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9 Figures legends

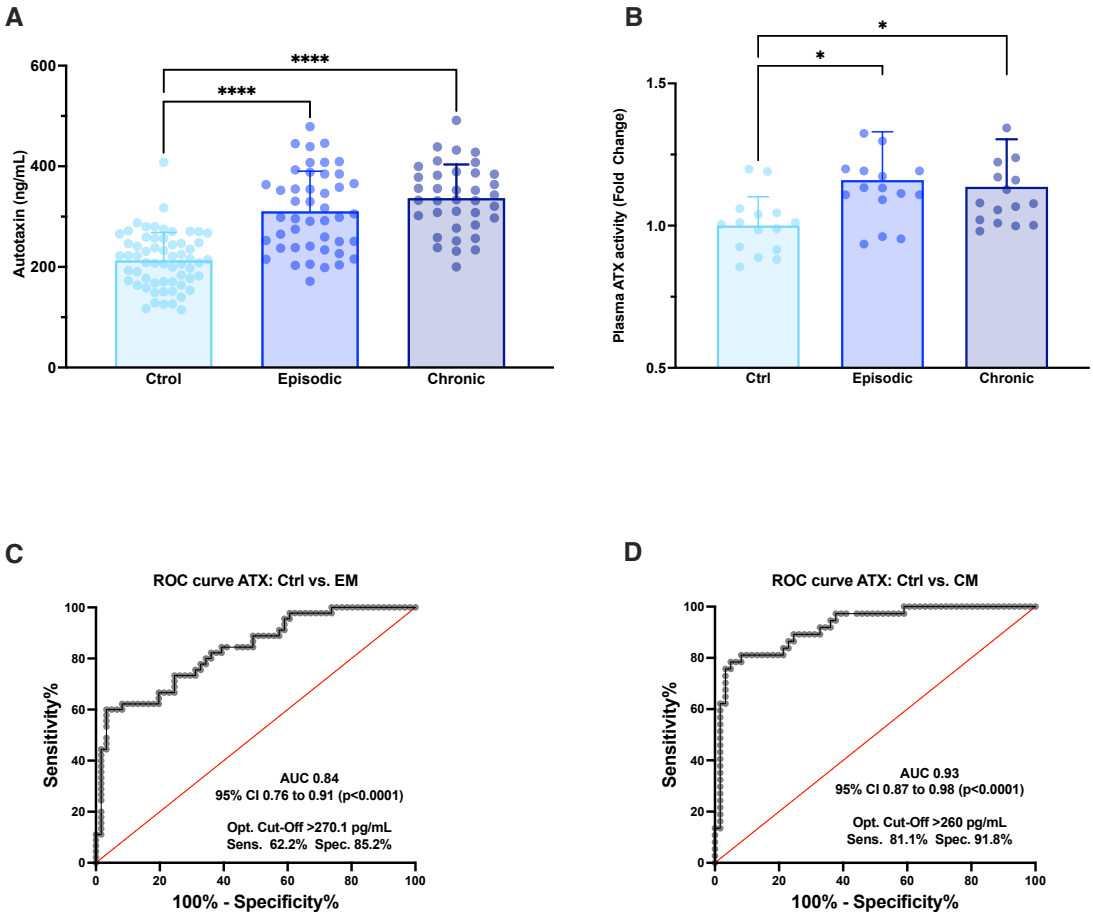

Fig. 1 Autotaxin (ATX) levels are increased in patients with migraine compared to controls. (A) Analysis of ATX levels in serum samples from healthy participants (Ctrl), patients with episodic migraine (EM), and patients with chronic migraine (CM), Results are expressed as the mean ± SD and analyzed by one-way ANOVA test (****p<0.001). (B) Serum ATX activity from healthy patients (Ctrl), patients with episodic migraine (EM), and patients with chronic migraine (CM), Results are expressed as the mean ± SD and analyzed by one-way ANOVA test (*p<0.05). (C) ROC curve analysis comparing ATX levels of controls with patients with EM. (D) ATX levels ROC curve analysis of controls versus patients with CM.

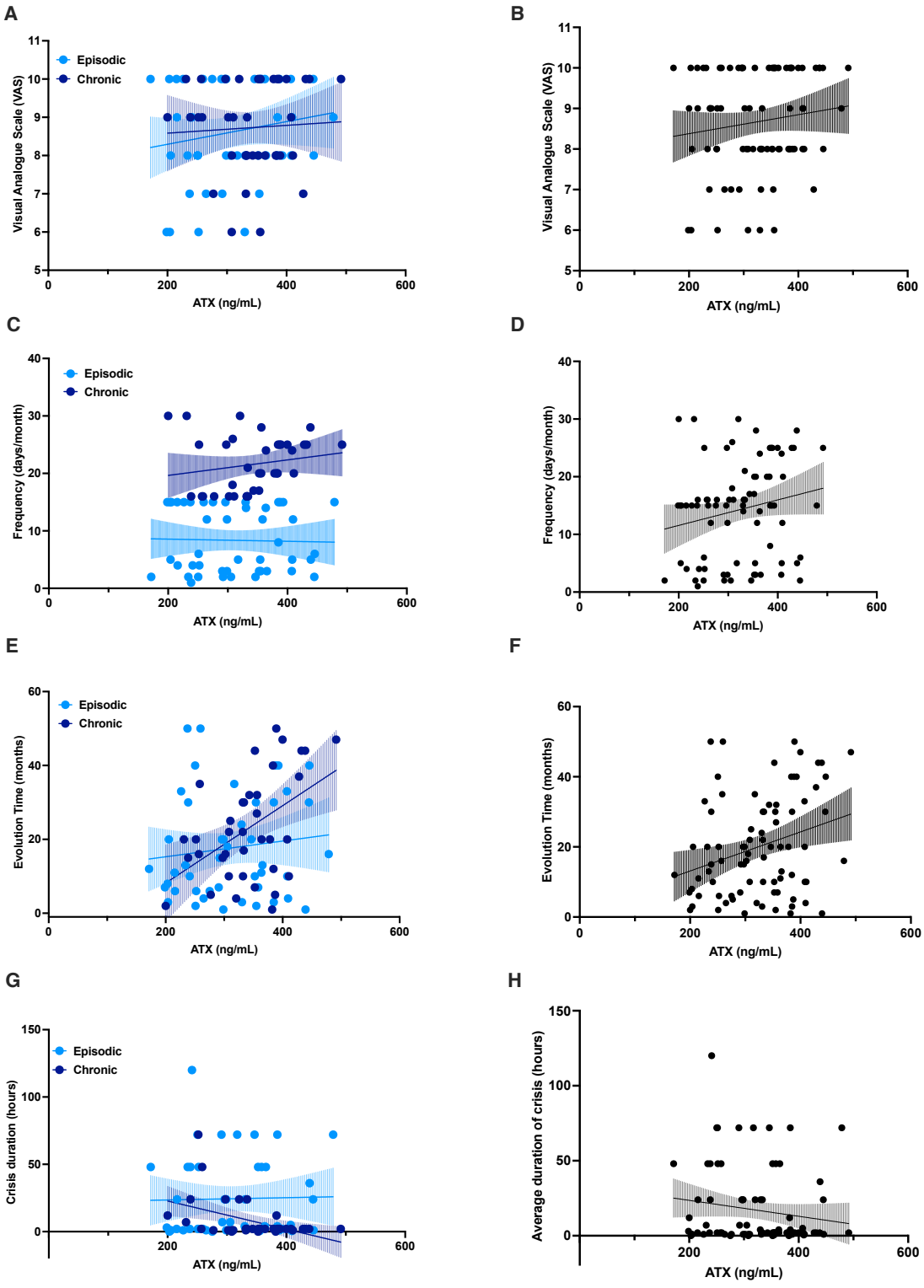

Fig. 2 Correlation between clinical outcomes and serum ATX levels in patients with migraine. (A) Representation of the Visual Analogue Scale (VAS) Score correlated with ATX values between patients with EM and CM; VAS

correlated significantly with ATX levels in patients with CM (Spearman's coefficient=0.405, p=0.012). (B) Correlation of VAS and ATX levels analyzing all the groups together, Pearson's coefficient=0.019, p<0.215. (C) Analysis of the frequency of days per month of migraine attacks against the ATX values of patients with EM and CM; ATX levels showed a significant correlation with the frequency of migraine attacks per month in patients with CM (Spearman's coefficient=0.718, p<0.001). (D) Correlation of frequency and ATX levels analyzing all the groups together, Pearson's coefficient=0.038, p<0.078. (E) Time of evolution of the patients and serum levels of ATX in patients with migraine; ATX levels also correlated with the time of evolution in patients with CM (Spearman's coefficient=0.226, p=0.003). (F) Correlation of time of evolution and ATX levels analyzing all the groups together, Pearson's coefficient=0.083, p=0.008 (G) Migraine duration in hours as a function of serum ATX levels. No correlation was found. (H) Correlation of frequency and ATX levels analyzing all the groups together, Pearson's coefficient=0.024, p=0.164

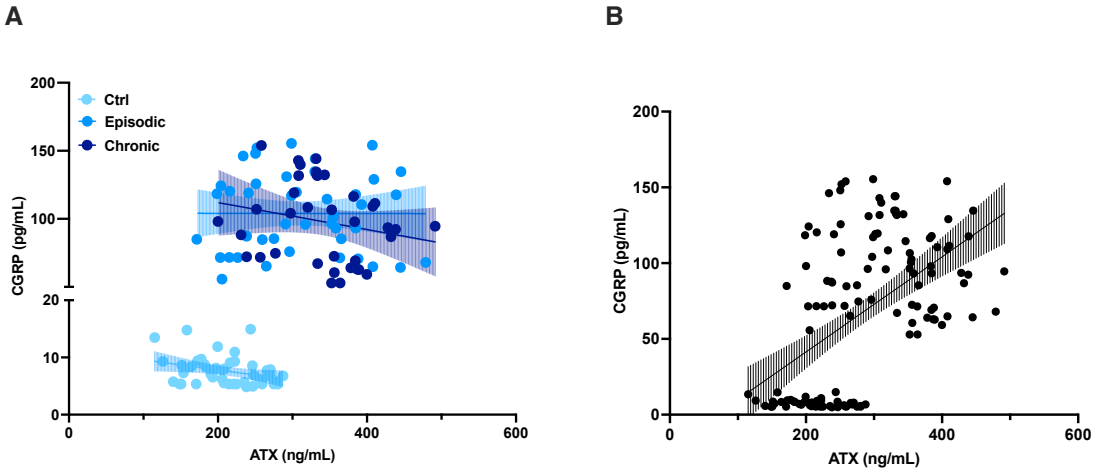

Fig. 3 ATX levels correlates with CGRP in serum samples. (A) Correlation analysis between the levels of CGRP and ATX in serum of the different study groups (Ctrl, EM and CM). Ctrl: Pearson's coefficient=0.106, p<0.05; EM: Pearson's coefficient=$3.5 \cdot 10^{-6}$, p=0.99; CM: Pearson's coefficient=0.046, p=0.2. (B) Correlation of CGRP and ATX levels analyzing all the groups together, Pearson's coefficient=0.278, p<0.001.

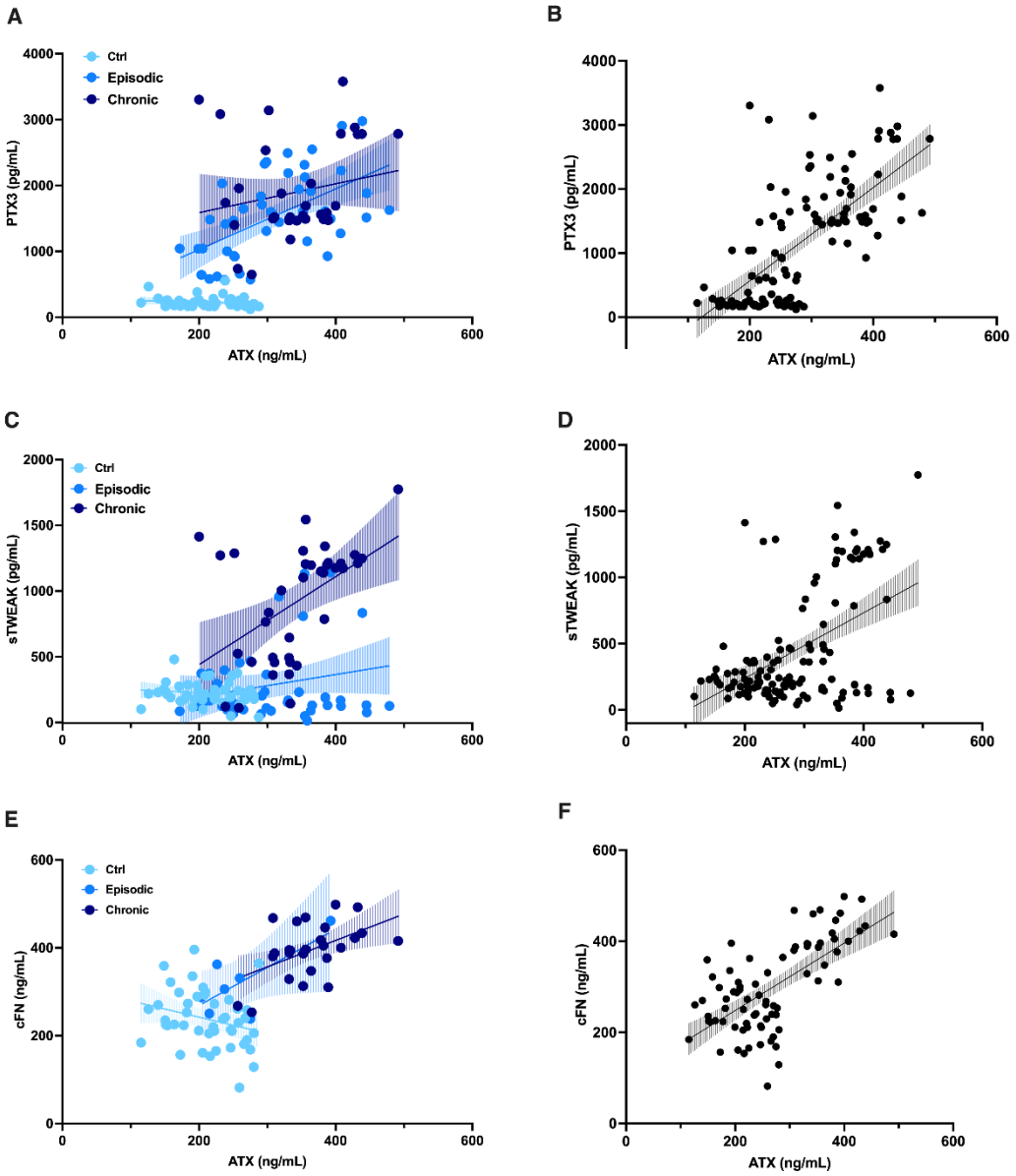

Fig. 4 Analysis of the association of ATX levels with endothelial dysfunction markers. (A) Correlation between PTX3 and ATX levels of the different study groups (Ctrl, EM and CM). Ctrl: Pearson's coefficient=0.023, p=0.317; EM: Pearson's coefficient=0.323, p<0.001; CM: Pearson's coefficient=0.038, p<0.001. (B) Correlation of PTX3 and ATX levels for all groups together; Pearson's coefficient=0.468, p<0.001. (C) Correlation between sTWEAK and ATX levels of the different study groups (Ctrl, EM and CM). Ctrl: Pearson's coefficient=0.016, p=0.394; EM: Pearson's coefficient=0.047, p=0.150; CM: Pearson's coefficient=0.239, p=0.002. (D) Correlation of sTWEAK and ATX

levels for all groups together; Pearson's coefficient=0.242, p<0.001. (E) Correlation between cFN and ATX levels of the different study groups (Ctrl, EM and CM). Ctrl: Pearson's coefficient=0.07, p=0.08; EM: Pearson's coefficient=0.536, p=0.06; CM: Pearson's coefficient=0.252, p=0.009. (F) Correlation of cFN and ATX levels for all groups together, Pearson's coefficient=0.425, p<0.001.

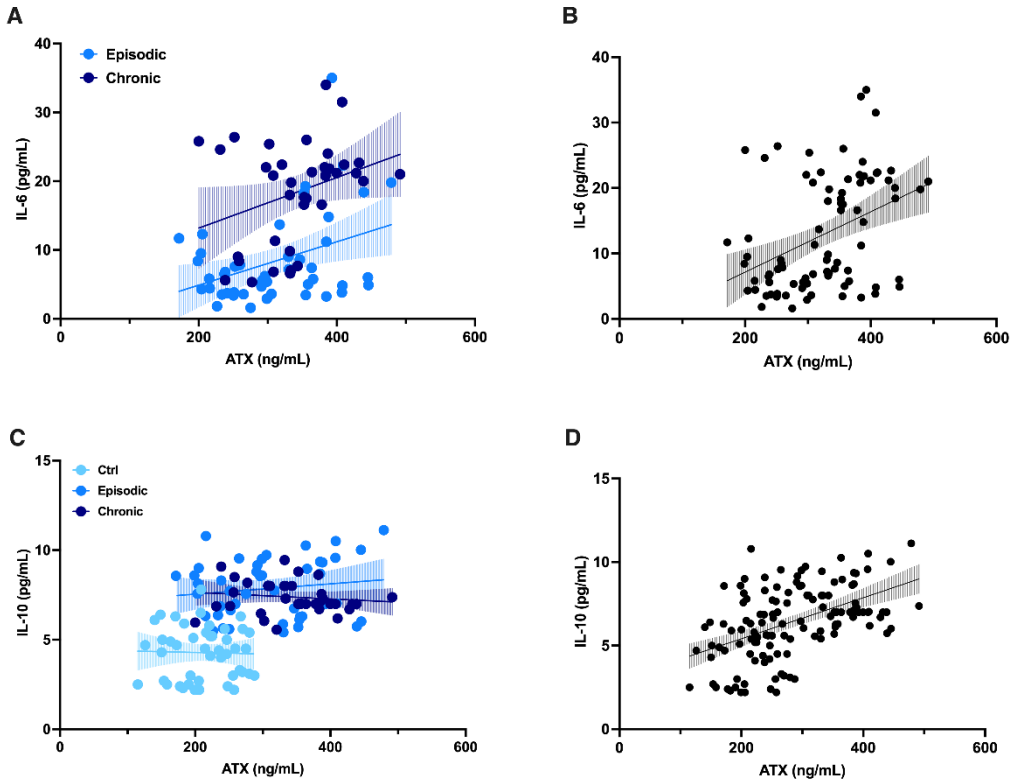

Fig. 5 Correlation of inflammatory molecules and ATX levels. (A) Correlation analysis between IL-6 and ATX levels of the different migraine groups (EM and CM). EM: Pearson's coefficient=0.145, p<0.05; CM: Pearson's coefficient=0.100, p=0.05. (B) Linear regression representation of IL-6 and ATX levels for all migraine groups, Pearson's coefficient=0.159, p<0.001. (C) Correlation analysis of IL-10 and ATX levels of the different study groups (Ctrl, EM and CM). Ctrl: Pearson's coefficient=0.0007, p=0.863; EM: Pearson's coefficient=0.019, p=0.357; CM: Pearson's coefficient=0.018, p=0.428. (D) Correlation analysis of IL-10 and ATX levels for all migraine groups together, Pearson's coefficient=0.238, p<0.001.

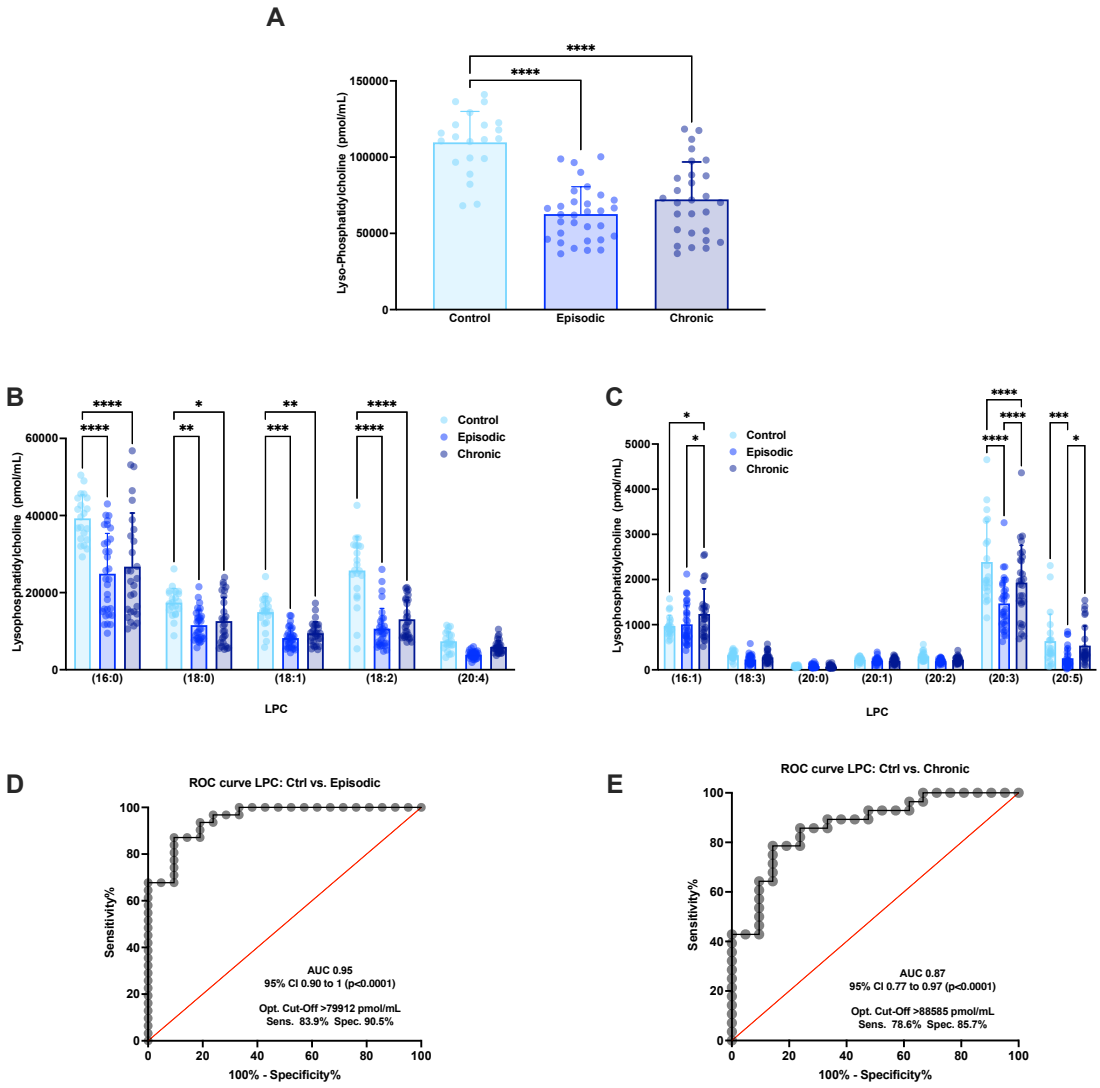

Fig. 6 Lipidomics showed a great reduction in lysophosphatidylcholine (LPC) levels that indicated high ATX activity in the serum of patients with migraine. (A) Representation in pmol/mL of the levels of total lysophosphatidylcholine, Results are expressed as the mean ±SD and analyzed by one-way ANOVA (****p<0.001) compared to the control group. (B-C) Representation in pmol/mL of the levels of lysophosphatidylcholine, Results are expressed as the mean ±SD and analyzed by one-way ANOVA (*p<0.05, **p < 0.01, p<0.001) compared to the control group. (D) ROC curve analysis comparing LPC levels of controls with patients with EM. (E) LPC levels ROC curve analysis of controls versus patients with CM.

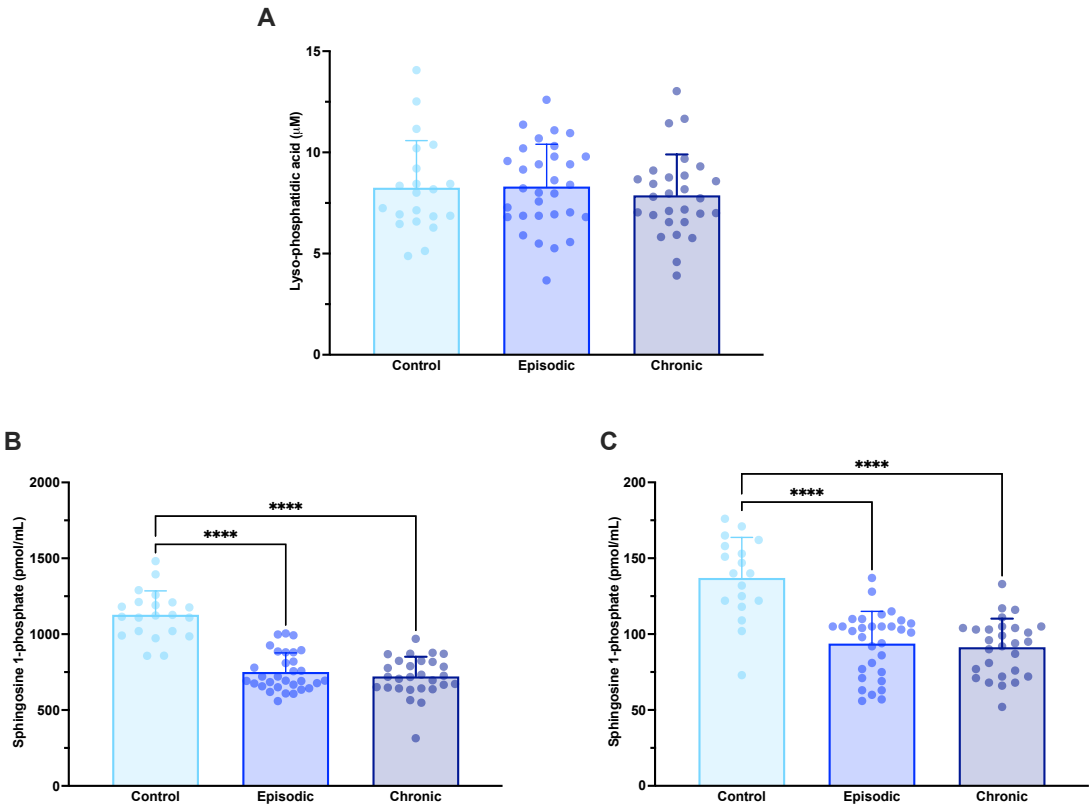

Fig. 7 Lipidomics reveals a possible implication of LPP in LPA degradation. (A) Representation in pmol/mL of the levels of total LPA, results are expressed as the mean ±SD and analyzed by one-way ANOVA compared to the control group. (B) Representation in pmol/mL of the levels of total S1P, results are expressed as the mean ±SD and analyzed by one-way ANOVA $p<0.001$) compared to the control group. (C) Representation in pmol/mL of the levels of total Sph-1P, results are expressed as the mean ±SD and analyzed by one-way ANOVA $p<0.001$) compared to the control group.

10 Tables

Table 1. Bivariate analysis by study groups.

Variable	Controls N = 62	EM N = 45	CM N = 38	p
Age, years	41.2 ± 12.5	42.4 ± 12.8	45.1 ± 11.1	
Ctrl vs EM				0.508
Ctrl vs CM				0.664
EM vs CM				0.676
Females, % (Freq.)	94 (58)	93 (42)	97 (37)	
Ctrl vs EM				>0.999
Ctrl vs CM				0.527
EM vs CM				0.424
Time since beginning (months)	-	17.6 ± 14.1	22.8 ± 14.1	
EM vs CM				0.193
Pain (days/month)	-	8.4 ± 5.6	21.4 ± 4.7	
EM vs CM				<0.001
VAS	-	8.6 ± 1.3	8.7 ± 1.2	
EM vs CM				>0.999
Evolution (months)	-	17.7 ± 14.1	22.8 ± 14.1	
EM vs CM				0.195
Autotaxin (ng/mL)	212.3 ± 53.2	310.7 ± 59.7	336.7 ± 60.6	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.182
CGRP (pg/mL)	7.6 ± 2.4	104 ± 28.3	98.33 ± 29.4	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.510
PTX3 (pg/mL)	224.9 ± 80.4	1537.9 ± 645.20	1940.4 ± 752.7	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				<0.001
sTWEAK (pg/mL)	221.9 ± 85.9	289.2 ± 309.9	919.3 ± 434.5	
Ctrl vs EM				=0.535
Ctrl vs CM				<0.001
EM vs CM				<0.001
cFN (ng/mL)	240.9 ± 64.8	319.7 ± 76.02	394.5 ± 62.8	
Ctrl vs EM				<0.05
Ctrl vs CM				<0.001
EM vs CM				<0.05
IL-6 (pg/mL)	-	8.4 ± 6.6	18.5 ± 7.4	
EM vs CM				<0.001
IL-10 (pg/mL)	4.3 ± 1.4	7.9 ± 1.6	7.4 ± 0.9	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.255

Table 2. Adjusted logistic regression model for all biomarkers. ATX was included as a continuous, non-categorical variable.

	Adjusted OR	CI 95%	p
Autotaxin	2.05	1.02 – 3.04	<0.001
CGRP	1.04	0.99 – 1.09	0.111
PTX	1.00	1.00 – 1.01	0.021
sTWEAK	1.04	0.98 – 1.11	0.172
cFn	1.81	1.05 – 3.12	0.031
IL6	6.61	0.77 – 56.71	0.085
IL10	0.87	0.59 – 1.28	0.472

Table 3. Sphingolipidomics data

Variable	Controls N = 21	EM N = 31	CM N = 28	p
LPC Total (pmol/mL)	109656 ± 20363	62680 ± 17939	72295 ± 24557	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.194
LPC 16:0 (pmol/mL)	39255 ± 6080	25264 ± 10428	26308 ± 13855	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.496
LPC 16:1 (pmol/mL)	971 ± 233	1006 ± 417	1237 ± 553	
Ctrl vs EM				0.939
Ctrl vs CM				<0.05
EM vs CM				<0.05
LPC 18:0 (pmol/mL)	17410 ± 3702	11222 ± 3506	12915 ± 13855	
Ctrl vs EM				<0.01
Ctrl vs CM				<0.05
EM vs CM				0.794
LPC 18:1 (pmol/mL)	14946 ± 4101	8075 ± 2505	9576 ± 2960	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.723
LPC 18:2 (pmol/mL)	25745 ± 8758	10570 ± 5316	13106 ± 4824	
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				0.293
LPC 18:3 (pmol/mL)	323 ± 87	229 ± 104	277 ± 35	
Ctrl vs EM				0.641
Ctrl vs CM				0.903
EM vs CM				0.872
LPC 20:0 (pmol/mL)	74 ± 20	79 ± 35	69 ± 34	
Ctrl vs EM				>0.999
Ctrl vs CM				>0.999
EM vs CM				0.994
LPC 20:1 (pmol/mL)	223 ± 52	201 ± 64	200 ± 67	
Ctrl vs EM				0.977
Ctrl vs CM				0.974
EM vs CM				>0.999
LPC 20:2 (pmol/mL)	297 ± 98	190 ± 46	222 ± 71	
Ctrl vs EM				0.569
				0.764

Ctrl vs CM				0.944
EM vs CM				
LPC 20:3 (pmol/mL)	2387 ± 895	1470 ± 574	1932 ± 827	<0.001
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				
LPC 20:4 (pmol/mL)	7385 ± 2422	3863 ± 901	5896 ± 1623	0.120
Ctrl vs EM				0.695
Ctrl vs CM				0.430
EM vs CM				
LPC 20:5 (pmol/mL)	636 ± 594	255 ± 225	535 ± 432	<0.001
Ctrl vs EM				0.614
Ctrl vs CM				<0.05
EM vs CM				
LPA Total (pmol/mL)	8.2 ± 2.3	8.3 ± 2.1	7.9 ± 2	0.120
Ctrl vs EM				0.695
Ctrl vs CM				0.430
EM vs CM				
S1P Total (pmol/mL)	1127 ± 157	754 ± 125	720 ± 130	0.679
Ctrl vs EM				<0.001
Ctrl vs CM				<0.001
EM vs CM				
Sph-1P Total (pmol/mL)	137 ± 27	93 ± 22	91 ± 19	<0.001
Ctrl vs EM				<0.001
Ctrl vs CM				0.908
EM vs CM				