JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 7, NO. 6, 2022

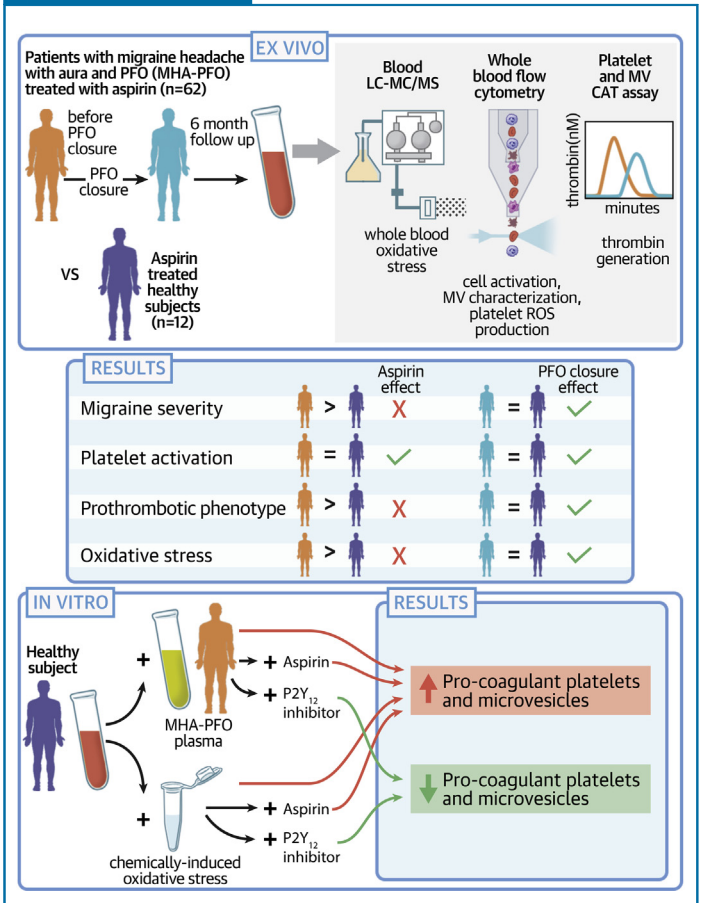
CLINICAL RESEARCH

Migraine in Patients Undergoing PFO Closure

Characterization of a Platelet-Associated Pathophysiological Mechanism: The LEARNER Study

Daniela Trabattoni, MD,[a] Marta Brambilla, PhD,[a] Paola Canzano, PhD,[a] Alessia Becchetti, MS,[a] Giovanni Teruzzi, MD,[a] Benedetta Porro, PhD,[a] Susanna Fiorelli, PhD,[a] Manuela Muratori, MD,[a] Calogero C. Tedesco, MS,[a] Fabrizio Veglia, PhD,[a] Piero Montorsi, MD,[a,b] Antonio L. Bartorelli, MD,[a,c] Elena Tremoli, PhD,[d] Marina Camera, PhD[a,e]

VISUAL ABSTRACT



Trabattoni D, et al. J Am Coll Cardiol Basic Trans Science. 2022;7(6):525-540.

HIGHLIGHTS

- **Patients experiencing MHA-PFO on aspirin are characterized by a marked thrombin generation capacity sustained by an elevated number of platelets and MVs expressing a functionally active tissue factor.**
- **MHA-PFO patients are also characterized by an altered oxidative stress status, ie, increased platelet ROS production and blood GSSG/GSH ratio.**
- **This prothrombotic condition fully reverts upon PFO closure and is associated with 100% migraine remission.**
- **MHA-PFO plasma and GSSG, added to blood of healthy subjects, mirrored the in vivo platelet activation and this effect is blunted by N-acetylcysteine, thus supporting the etiopathogenetic role of oxidative stress in this clinical setting.**
- **Aspirin had little effect on the platelet prothrombotic phenotype that was better controlled by $P2Y_{12}$ antagonist.**

ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2022.02.002

ABBREVIATIONS AND ACRONYMS

cTTE = contrast transthoracic echocardiography

GSH = reduced glutathione

GSSG = oxidized glutathione

HS = healthy subject(s)

MHA = migraine headache with aura

MV = microvesicles

NAC = N-acetylcysteine

PFO = patent foramen ovale

PS = phosphatidylserine

ROS = reactive oxygen species

TF = tissue factor

WB = whole blood

SUMMARY

The association between migraine and patent foramen ovale (PFO) has been documented. We aimed to investigate platelet activation, prothrombotic phenotype, and oxidative stress status of migraineurs with PFO on 100 mg/day aspirin, before and 6 months after PFO closure. Data show that, before PFO closure, expression of the classical platelet activation markers is comparable in patients and aspirin-treated healthy subjects. Conversely, MHA-PFO patients display an increased prothrombotic phenotype (higher tissue factor^{pos} platelets and microvesicles and thrombin-generation potential), sustained by an altered oxidative stress status. This phenotype, which is more controlled by $P2Y_{12}$-blockade than by aspirin, reverted after PFO closure together with a complete migraine remission. (pLatelEts And MigRaine iN patEnt foRamen Ovale [LEARNER]; NCT03521193) (J Am Coll Cardiol Basic Trans Science 2022;7:525–540) © 2022 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Migraine is a neurological disease associated with a multifactorial etiology and a prevalence of 12%-15% in the general population.[1] Several studies have described a strong relationship between migraine (especially with aura) and patent foramen ovale (PFO).[2] Patients experiencing migraine headache with aura (MHA) have a higher prevalence of PFO when compared with migraineurs without aura and to migraine-free subjects.[3]

Over the past 20 years, several observational studies highlighted the link between PFO and MHA, showing that in patients with high risk profile for paradoxical embolism, MHA can be significantly reduced after transcatheter PFO closure both in the short- and long-term follow-up.[4,5] Although migraine is not yet listed among the clinical indications of international guidelines (European Society of Cardiology; American Heart Association/American College of Cardiology) on PFO closure, a recently published pooled analysis of 2 prospective randomized clinical trials showed, for the first time, that the transcatheter closure significantly reduced the mean number of monthly migraine days and attacks. A greater number of subjects who experienced complete migraine cessation was also reported compared with patients treated with medical treatment alone. Finally, migraineurs with frequent aura are those who mostly benefit from PFO closure, suggesting that these may differ from other migraine subtypes.[6]

The pathophysiological mechanisms connecting PFO and MHA include shunting of humoral vasoactive factors that escape degradation in the pulmonary circulation or right-to-left shunt that permits paradoxical microemboli.[7,8] Among the vasoactive factors, serotonin was implicated in the pathophysiology of migraine for the first time by Sicuteri[9] in 1961 according to the elevated serotonin metabolites in urine during migraine attacks. The precise relationship between serotonin and migraine, however, remains unclear. In support of the presence of microemboli, there is evidence of a significantly increased platelet activation in migraine patients, first documented in 1971 by Hilton and Cumings[10] and then confirmed in several studies, suggesting alterations of both primary and secondary hemostasis.[11,12] Platelet activation, leading to the formation of platelet-leukocyte aggregates, can also promote the release of proinflammatory cytokines, further increasing sterile inflammation in the brain and facilitating pain signaling. The pathophysiological role of platelet activation in migraine is supported by the fact that, in cardiology practice, most of migraine PFO patients treated with aspirin, as primary preventive therapy or in lieu of PFO closure, experience a significant improvement in migraine with aura symptoms.[13]

From the [a]Centro Cardiologico Monzino IRCCS, Milan, Italy; [b]Dipartimento di Scienze Cliniche e di Comunità, Università degli Studi di Milano, Milan, Italy; [c]Dipartimento di Scienze Biomediche e Cliniche L. Sacco, Università degli Studi di Milano, Milan, Italy; [d]Maria Cecilia Hospital, Ravenna, Italy; and the [e]Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milan, Italy.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

Manuscript received November 9, 2021; revised manuscript received February 3, 2022, accepted February 3, 2022.

Finally, it must be considered that one of the major factors lowering the threshold of the first headache symptom is the impairment of oxidative metabolism,[14] which in turn may affect several cell functions, including platelet ones. However, no study has so far investigated these mechanisms in the context of PFO-associated migraine and the potential effects of PFO closure on them.

Thus, in the attempt to gain insights into the potential mechanisms linking MHA and PFO, we designed the LEARNER study (pLatelEts and migRaine iN patEnt foRamen ovale) to perform a comprehensive analysis of platelet activation, plasma and cell-associated thrombin generation capacity, together with assessment of inflammation mediator levels and oxidative stress status in patients with migraine on aspirin therapy before and 6 months after PFO closure. The primary endpoint of the study was to evaluate the rate of migraine regression, after PFO closure, in relation to these parameters. In addition, because we found a specific state of platelet activation and oxidative stress status, an additional aim of the study was to assess the in vitro capacity of oxidative stress to modulate the expression of platelet activation markers in the absence and presence of aspirin or a $P2Y_{12}$ antagonist.

METHODS

PATIENT SELECTION. We prospectively screened 93 consecutive patients addressed to PFO closure and experiencing migraine. Based on inclusion criteria—presence of PFO, migraine headache with aura (MHA), and aspirin treatment—78 patients were enrolled in the study (**Figure 1,** Supplemental Table 1). Data from 12 age-matched healthy subjects (HS) on daily-aspirin treatment, recruited at our Institute, with a negative cardiovascular history and free from migraine, were analyzed for comparison on platelet aggregation behavior and procoagulant potential (**Table 1, Figure 1**). All patients were informed and consented to the intervention. The study was approved by the local scientific review board and ethic committee (n. CCM769).

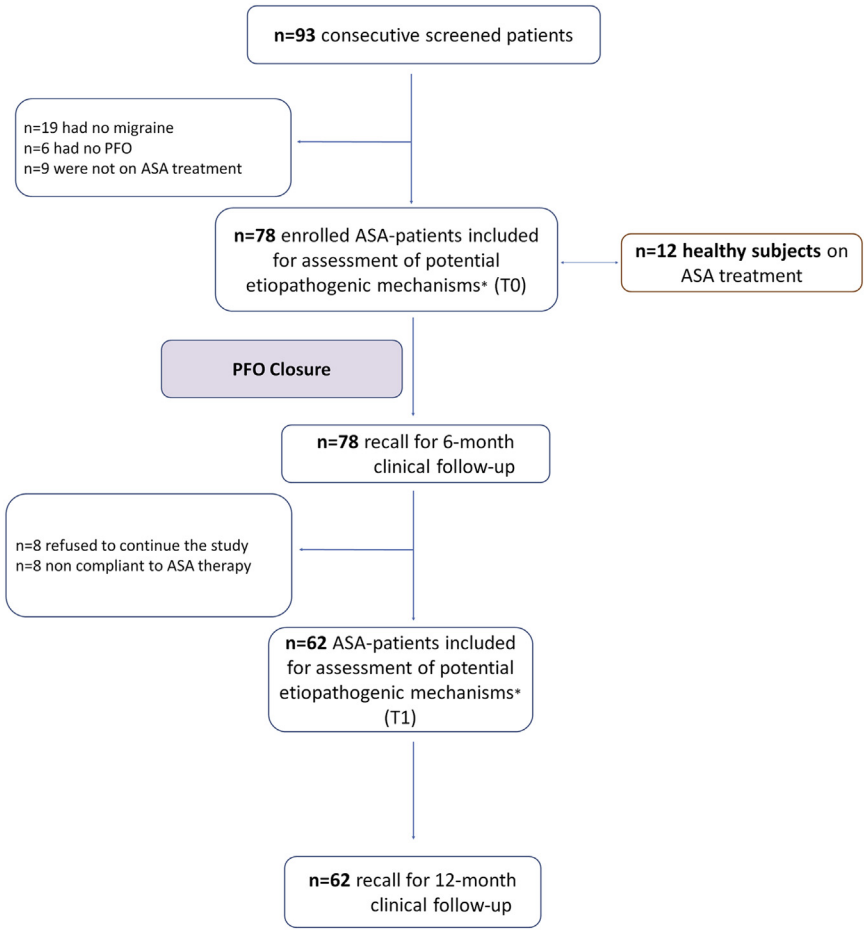
SAMPLE COLLECTION AND PREPARATION. The day before the procedure (T0) and at a 6 months follow-up visit (T1), in a headache-free time and under fasting conditions, whole blood (WB) was drawn from the antecubital vein with a 19-gauge needle without venous stasis into citrate (1/10 volume of 0.129M sodium citrate) or K_2 EDTA-containing tubes or in tubes without anticoagulant (Vacutainer, Becton Dickinson), discarding the first 4 mL. P-selectin^{pos}, activated-glycoprotein IIb/IIIa (aGPIIb/IIIa)^{pos}, tissue factor (TF)^{pos}, reactive oxygen species (ROS)^{pos} platelets, platelet-leukocyte aggregates (PLA), and microvesicles (MVs) were evaluated by whole blood flow cytometry (Supplemental Figures 1 and 2), thrombin generation (TG) by calibrated automated thrombogram assay, oxidative stress status by mass spectrometry, and serotonin and cytokines by enzyme-linked immunosorbent assay.

IN VITRO STUDIES. To evaluate the effect of MHA-PFO patients' plasma before PFO closure on platelet activation, blood from HS was centrifuged at 1,000 *g* for 10 minutes at RT, and plasma was removed and replaced in the centrifuged WB tube with MHA-PFO patients' plasma or with HS pool plasma as control. After 30 minutes of incubation, the blood thus reconstituted was analyzed by flow cytometry. Pretreatment of HS WB with N-acetylcysteine (NAC) (1 mmol/L; 30 minutes) before adding MHA-PFO patients' plasma collected at T0 was used to explore the possible involvement of WB oxidative stress in platelet activation.

The influence of oxidized glutathione (GSSG) (0.4-4 mmol/L) or serotonin (10 μmol/L) was assessed by incubation of WB for 30 minutes at RT. When the effect of antiplatelet drugs on platelet activation was tested, WB was incubated with aspirin (8 μmol/L, 120 minutes) or with $P2Y_{12}$ antagonist AR-C69931MX (1 μmol/L, 30 minutes) at RT before the addition of MHA-PFO patients' plasma or GSSG (4 mmol/L) or serotonin (10 μmol/L).

STATISTICAL ANALYSIS. A sample of 60 patients was estimated to provide 90% power to deem as significant ($P < 0.05$) a within-subject difference of at least 0.42 SDs (eg, Cohen's effect size) (eg, in the case of TF^{pos} platelets, this would correspond to a mean absolute variation of about 0.63%). Regarding comparisons with a sample of 12 HS, the minimum difference to be deemed as significant with 90% power was 1.0 SDs (corresponding to about 1.5% in the case of TF^{pos} platelets). Results are expressed as mean ± SD or median and first and third quartile as indicated. Categorical variables are presented as n (%) and were compared by chi-square, Fisher's exact test, or Exact McNemar's test, as appropriate. Within subject comparisons were made by Student's paired *t*-test or Wilcoxon signed rank test, as appropriate. Comparisons between 2 independent groups were made by unpaired Student's *t*-test or Wilcoxon rank sum test as appropriate. Spearman rank correlation coefficients were computed to assess associations between 2 variables. According to migraine resolution, responders were patients with 100% Anzola-scale reduction after 6 months and no responders were those with unchanged Anzola's score at follow-up;

FIGURE 1 The LEARNER Study Enrollment Flowchart



*Potential etiopathogenic mechanisms: evaluation of platelet activation and prothrombotic potential, oxidative stress status and biochemical analysis. ASA = acetylsalicylic acid; PFO = patent foramen ovale; T0 = day before the procedure; T1 = 6-month follow-up visit.

the remaining patients were considered to be partial responders. The ability of biomarkers to discriminate between complete or partial migraine remission was assessed using logistic regression to determine the area under the receiver-operating characteristics curve. Model results are presented using the OR with 95% CI. A P value <0.05 was considered to be statistically significant. Analyses were performed using Prism Graphpad version 9.0 (GraphPad Software) and SAS version 9.4 (SAS Institute).

For a complete Methods section, please see the Supplemental Appendix.

RESULTS

PATIENTS' CHARACTERISTICS AND MIGRAINE REGRESSION FOLLOWING PFO CLOSURE. We enrolled 78 consecutive patients who met the inclusion criteria (Figure 1). In total, 8 patients refused to continue the study and 8 were noncompliant to antiplatelet therapy during the follow-up. Final evaluation of the effect of PFO closure on MHA resolution was performed on 62 patients (79% women, mean age 41.8 ± 11.7 years). The leading indication for PFO closure was a positive brain magnetic resonance imaging, suggesting previous ischemic event not clinically detected, in 37 patients while an off-label indication was offered to 25 patients. Baseline demographics and PFO-related procedural characteristics are shown in Table 1.

Hematological parameters were within the normal range, and all were comparable before the procedure (T0) and at follow-up (T1) except for erythrocyte

TABLE 1 Demographic, Clinical, and PFO Characteristics of Enrolled Patients With Complete Follow-Up

	MHA-PFO patients (n = 62)	Healthy Subjects (n = 12)	*P* Value
Women	49 (79)	11 (91.6)	0.442
Age, y	41.8 ± 11.7	41.1 ± 11	0.997
Hypertension	5 (8)	0	0.584
Diabetes	1 (1.6)	0	1.000
Dyslipidemia	12 (19)	1 (8.3)	0.162
Smoking	11 (17.7)	3 (25)	0.687
Migraine with aura	62 (100)	0	≤0.001
Tunnel-like PFO	25 (40.3)	–	–
Atrial septal bulging	22 (35.5)	–	–
Atrial septal aneurysm[a]	15 (24.2)	–	–
cTTE mild RLS	1 (1.6)	–	–
cTTE moderate RLS	4 (6.4)	–	–
cTTE severe RLS	57 (91.9)	–	–

Values are n (%) or mean ± SD. Unpaired Student's *t*-test or Fisher exact test were used to compare groups. [a]Atrial septal aneurysm (>1 cm septal aneurysm excursion).

cTTE = contrast transthoracic echo; PFO = patent foramen ovale; RLS = right-to-left shunt.

count, hemoglobin levels, and hematocrit, which were significantly lower at T0 (Table 2).

All patients were on enteric-coated 100 mg/die aspirin. This treatment was effective in all enrolled subjects at T0 because it suppressed the production of serum and urinary thromboxane metabolites (Supplemental Figures 3A and 3B) as well as platelet aggregation in response to arachidonic acid (Supplemental Figure 3C), reflecting a complete COX-1 inhibition. At T1, serum TxB_2 levels were slightly above the cutoff value of 12 ng/mL[15] in a minority of patients (6.4%).

TABLE 2 Hematological and Inflammatory Parameters

	T0	T1	*P* Value
WBC count, 10^9/L	6.4 ± 1.6	6.3 ± 1.6	0.665
RBC count, 10^{12}/L	4.5 ± 0.6	4.7 ± 0.6	0.050
HGB, g/dL	13.1 ± 1.5	13.8 ± 1.4	0.010
HCT, %	38.3 ± 4.1	40.5 ± 3.4	0.002
PLT count, 10^9/L	231 ± 65	246 ± 70	0.256
PDW, fL	12.6 ± 2	12.3 ± 1.6	0.383
MPV, fL	10.6 ± 0.9	11.1 ± 3.5	0.277
IPF, %	4.7 ± 2.6	4.6 ± 3.5	0.817
IL-1β, ng/mL	1.23 ± 0.83	1.54 ± 1.77	0.487
IL-6, ng/mL	2.45 ± 2.18	2.58 ± 2	0.579
TNF-α, ng/mL	0.63 ± 0.37	0.76 ± 0.32	0.060
Serotonin, ng/mL	47.5 ± 44.4	46.4 ± 29.4	0.409

Values are mean ± SD.

HGB = hemoglobin; HCT = hematocrit; IL = interleukin; IPF = immature platelet fraction; MPV = mean platelet volume; PDW = platelet distribution width; PLT = platelet; RBC = red blood cell; T0 = day before the procedure; T1 = 6-month follow-up visit; TNF = tumor necrosis factor; WBC = white blood cell.

Plasma concentrations of interleukin-1β and -6 and TNF-α measured before PFO closure were within the reference range of HS and did not significantly change at follow-up. Notably, serotonin levels behaved similarly (Table 2).

Percutaneous PFO closure was successfully performed in all cases without major complications. Procedural characteristics and complications are described in Tables 3 and 4. A trivial and mild acute residual shunt was detected in 1 and 2 patients, respectively, whereas at 6-month follow-up, a significant residual shunt was observed in 1 case only (1.7%) at contrast transthoracic echo. Complete MHA resolution was observed in 43 (69.7%) patients, whereas only 2 (3.2%) patients were nonresponders. In the remaining 17 patients, a significant symptoms improvement was obtained with an overall migraine reduction of 84% ± 22% (% ratio between the Anzola's score at follow-up and at baseline) persisting at 6- and 12-month follow-up even after antiplatelet therapy discontinuation (Table 5).

PLATELET ACTIVATION MARKERS' EXPRESSION AND CELL-ASSOCIATED PROCOAGULANT POTENTIAL. A thorough evaluation of the activation status of circulating platelets in the enrolled patients was performed by assessing by flow cytometry analysis the expression of classical platelet activation markers, such as P-selectin and aGPIIb/IIIa, and the number of platelet-leukocyte aggregates before and after PFO closure. P-selectin (Figure 2A) as well as platelet-monocyte and platelet-granulocyte aggregate levels (Figures 2B and 2C) were comparable to those measured in HS on the same pharmacological treatment and remained unchanged after PFO closure. The percentage of circulating platelets with aGPIIb/IIIa was slightly, although significantly, greater in patients compared with HS (Figure 2D). This difference, however, was functionally irrelevant because it was not paralleled by an altered platelet aggregation in response to several agonists (Supplemental Table 2).

The procoagulant potential of circulating blood cells was next analyzed by evaluating the platelet-associated TF expression and annexinV binding to phosphatidylserine (PS) as well as the levels of TF^{pos}-leukocytes. Unlikely to what observed for the classical platelet activation markers, at T0 the number of platelets exposing TF and PS on their surface was twice than that measured in HS, and decreased following PFO closure (Figures 3A and 3B). Interestingly, the number of platelets with intracellularly stored TF was also significantly higher in patients at T0, reverting to HS values at T1 (Figure 3C). The

TABLE 3 Procedural Characteristics

Echocardiographic guidance	
Intracardiac echo	62 (100)
Procedural Time, min	13.5 ± 3.2
X-ray time, min	4.0 ± 3.53
DAP, cGy/cm^2	359 ± 417
Device size	
16/18 mm	26 (41.9)
23/25 mm	23 (37)
27/30 mm	11 (17.7)
33 mm	2 (3.2)
Acute residual shunt	3 (4.8)

Values are n (%) or mean ± SD.

decrease in TF and PS expression was paralleled by a statistically significant reduction in the platelet-associated thrombin formation. At T0, indeed, platelets had a greater endogenous thrombin potential (ETP), generated a greater amount of thrombin (peak) with a higher kinetic rate (velocity index) compared to T1 and HS (**Table 6, Figure 3D**). Furthermore, the time needed to produce thrombin, ie, the lag time that directly correlates with the intracellularly stored TF concentration ($r = -0.280$; $P = 0.046$), was significantly shorter at T0 than at T1. The TF dependence of thrombin generation in the calibrated automated thrombogram assay was further confirmed by the significantly prolonged lag time observed in the presence of a neutralizing anti-TF antibody (+4.5 ± 2.7 minutes; $P < 0.001$) (**Figure 3D**).

Unlike platelets, TF expression in monocytes, granulocytes, and platelet-leukocyte aggregates did not differ between MHA-PFO patients, measured at T0 and T1, and HS (data not shown).

Overall, these data show that in MHA-PFO patients on aspirin platelet aggregation as well as the expression of the classical platelet activation markers, measured before and after PFO closure, is comparable to that observed in HS. Conversely, the platelet procoagulant potential, which is significantly higher than in HS, is restored to physiological levels only upon PFO closure.

TABLE 4 Clinical Complications After PFO Procedure

Vascular complications	1 (1.6)
Pseudoaneurysm	0
Major hematoma (>5 cm)	1 (1.6)
Transient AF	5 (8)
Recurrent TIAs/stroke	0 (0)
Device malpositioning	0 (0)

Values are n (%). All AF occurred during/immediately after the procedure or ≤15 days afterwards.
AF = atrial fibrillation; TIA = transient ischemic attack.

It is worth mentioning on this regard that, as expected based on previous studies,[10,16] migraineurs not on aspirin show a platelet aggregation in response to low concentration of thrombin greater than that observed in patients at T0. They also show a trend toward a greater number of circulating TF^{pos} platelets compared with migraineurs on aspirin (Supplemental Figure 4). These data further suggest that the mechanisms leading to agonist-induced platelet aggregation are controlled by aspirin, which partly controls also platelet-associated TF expression.

ANALYSIS OF CIRCULATING MICROVESICLES. We also evaluated levels of circulating MVs, a well-known marker of cell activation, on a subgroup of patients (n = 26 before and n = 36 after PFO closure). Unpaired analysis showed that, before PFO closure, the concentration of total MVs was about twice the amount found in HS and significantly decreased upon PFO closure. Notably, the same significant decrease was confirmed by a paired analysis (**Figure 4A**, inset). Platelet-, monocyte-, and endothelium-derived MVs were all significantly higher before PFO closure reaching values similar to HS only after PFO correction (**Figure 4B**). Conversely, the concentration of erythrocyte-derived MVs was lower at T0 and increased significantly ($P = 0.003$) at T1 tending toward the value of HS (**Figure 4B**). This trend mirrored that observed in the number of circulating erythrocytes described in the previous text.

Finally, also the number of procoagulant TF^{pos}, PS^{pos}, and PS^{pos}/TF^{pos} MVs were all significantly higher at T0 compared with T1, becoming similar to HS value only after PFO closure (**Figures 4C to 4E**). This feature was paralleled by a thrombin generation time significantly faster at T0 than at T1 (lag time 30.5 ± 7.6 minutes and 36.5 ± 12.6 minutes at T0 and T1, respectively; $P = 0.041$; 35.7 ± 15.5 minutes in HS).

Overall, these data suggest that aspirin treatment of MHA-PFO patients is not able to fully control platelet activation in terms of MV release as well as MV-associated procoagulant potential. It is only after PFO closure that these indexes revert to physiological levels.

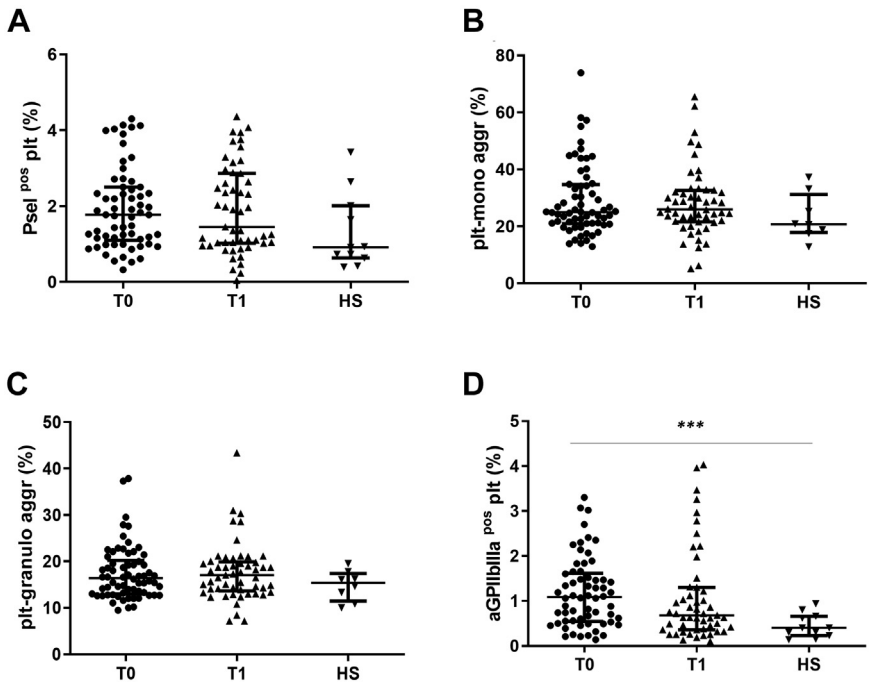
WHOLE BLOOD AND PLATELET-ASSOCIATED OXIDATIVE STRESS EVALUATION. Oxidative stress might play a role in the pathophysiology of migraine attack.[14] Indeed, analysis of the whole blood GSSG/reduced glutathione (GSH) ratio as well as of the number of reactive oxygen species (ROS)^{pos} platelets in a subgroup of patients showed values for both parameters significantly greater at T0 than at T1 when they became comparable to those found in HS (**Figures 5A and 5B**). Interestingly, the number of

TABLE 5 Anzola's and Global Migraine Severity Score (62 Points)

				P Value	
	Preprocedure	6 Months	12 Months	6 Months vs Preprocedure	12 Months vs Preprocedure
Anzola's score	7.2 ± 1.68	1.09 ± 1.47	1.14 ± 1.57	<0.001	<0.001
Migraine evaluation					
Intensity	2.4 ± 0.7	0.83 ± 0.7	0.79 ± 0.7	<0.001	<0.001
Mild	9 (14.5)	15 (24.2)	17 (27.4)	<0.001	<0.001
Severe	32 (51.6)	4 (6.4)	2 (3.2)		
Complete disability	21 (33.9)	0	0		
Duration, h	2.4 ± 0.8	0.93 ± 0.8	0.90 ± 0.9	<0.001	<0.001
<6	9 (14.5)	14 (22.6)	18 (29.0)	<0.001	<0.001
6-12	31 (50.0)	5 (8.0)	1 (1.6)		
>12	22 (35.5)	0	0		
Frequency, per mo	1.6 ± 0.8	0.45 ± 0.52	0.41 ± 0.48	<0.001	<0.001
1-6	18 (29.0)	18 (29.0)	19 (30.6)	<0.001	<0.001
7-14	28 (45.1)	1 (1.6)	0		
15-21	13 (21%)	0	0		
22-31	3 (4.8)	0	0		
Aura	62 (100.0)	4 (6.4)	2 (3.2)	<0.001	<0.001
Global score	6.49 ± 1.9	2.36 ± 2.0	2.13 ± 2.2	<0.001	<0.001

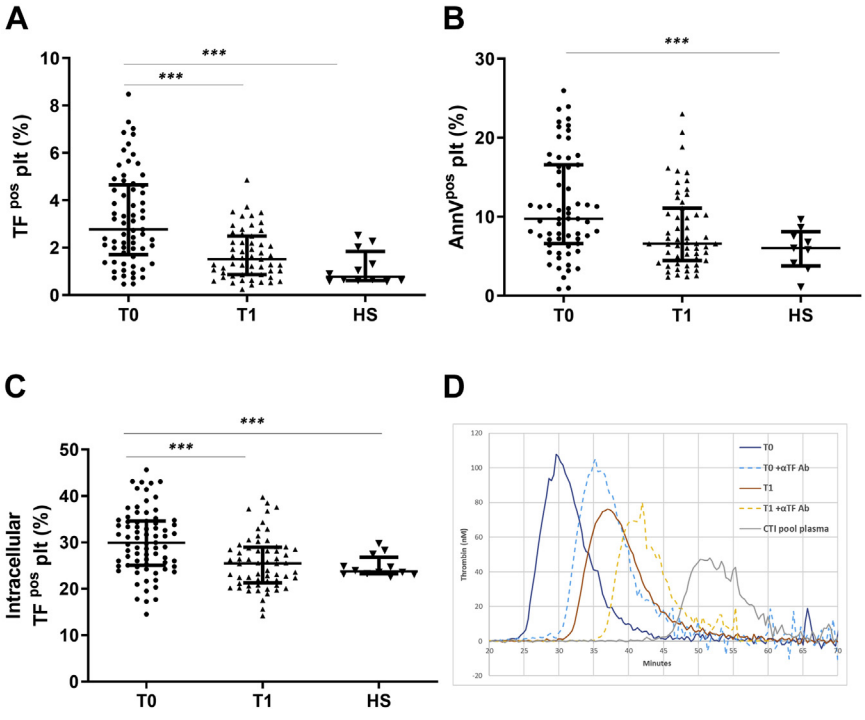
Values are mean ± SD or count (%).

FIGURE 2 Assessment of Platelet Activation



P-selectin expression **(A)**, platelet-monocyte **(B)**, and platelet-granulocyte **(C)** aggregate formation and aGPIIb/IIIa expression **(D)** were evaluated by flow cytometry in patients before (T0) and after (T1) PFO closure and in aspirin-treated healthy subjects (HS). The median value and 25th to 75th percentiles are reported. ***$P < 0.001$. PFO = patent foramen ovale; plt-granulo aggr - platelet-granulocyte aggregate; plt-mono aggr = platelet-monocyte aggregate; Pse = P=selectin expression.

FIGURE 3 Analysis of Platelet-Associated Procoagulant Potential



Surface expression of tissue factor (TF) **(A)**, phosphatidylserine **(B)**, and intracellular TF expression **(C)** was evaluated by whole blood flow cytometry in the enrolled subjects. Data are reported as median value and 25th to 75th percentiles. Platelet-associated thrombin generation was measured before (T0) and after (T1) patent foramen ovale closure by calibrated automated thrombogram assay **(D)**. The contribution of TF to thrombin generation was evaluated by preincubation of platelets with a neutralizing anti-TF antibody. Representative curves are shown. ***$P < 0.001$.

ROS^{pos} platelets correlated with that of TF^{pos} platelets (r = 0.533; $P = 0.009$) (Figure 5C) and their functional activity, ie, the amount of thrombin generated and the kinetic of thrombin formation, was positively associated with the GSSG/GSH ratio (lag time: r = −0.400; $P = 0.058$; ETP: r = 0.365; $P = 0.048$) (Figures 5D and 5E).

Thus, these data highlight a role for oxidative stress in sustaining the procoagulant phenotype of platelets in MHA-PFO patients.

By logistic regression analysis, GSH increase, platelet-associated thrombin generation lag time increase, and ETP reduction were the only biomarkers associated with 100% MHA remission with ORs of 2.1 (95% CI: 1.02-4.30), 3.02 (95% CI: 1.14-7.97), and 0.33 (95% CI: 0.13-0.81) for one SD increment, respectively, and with area under the receiver-operating characteristics curves of 0.68 (95% CI: 0.50-0.85), 0.74 (95% CI: 0.57-0.91) and 0.78 (95% CI: 0.62-0.94), respectively. Because this analysis suggests a link

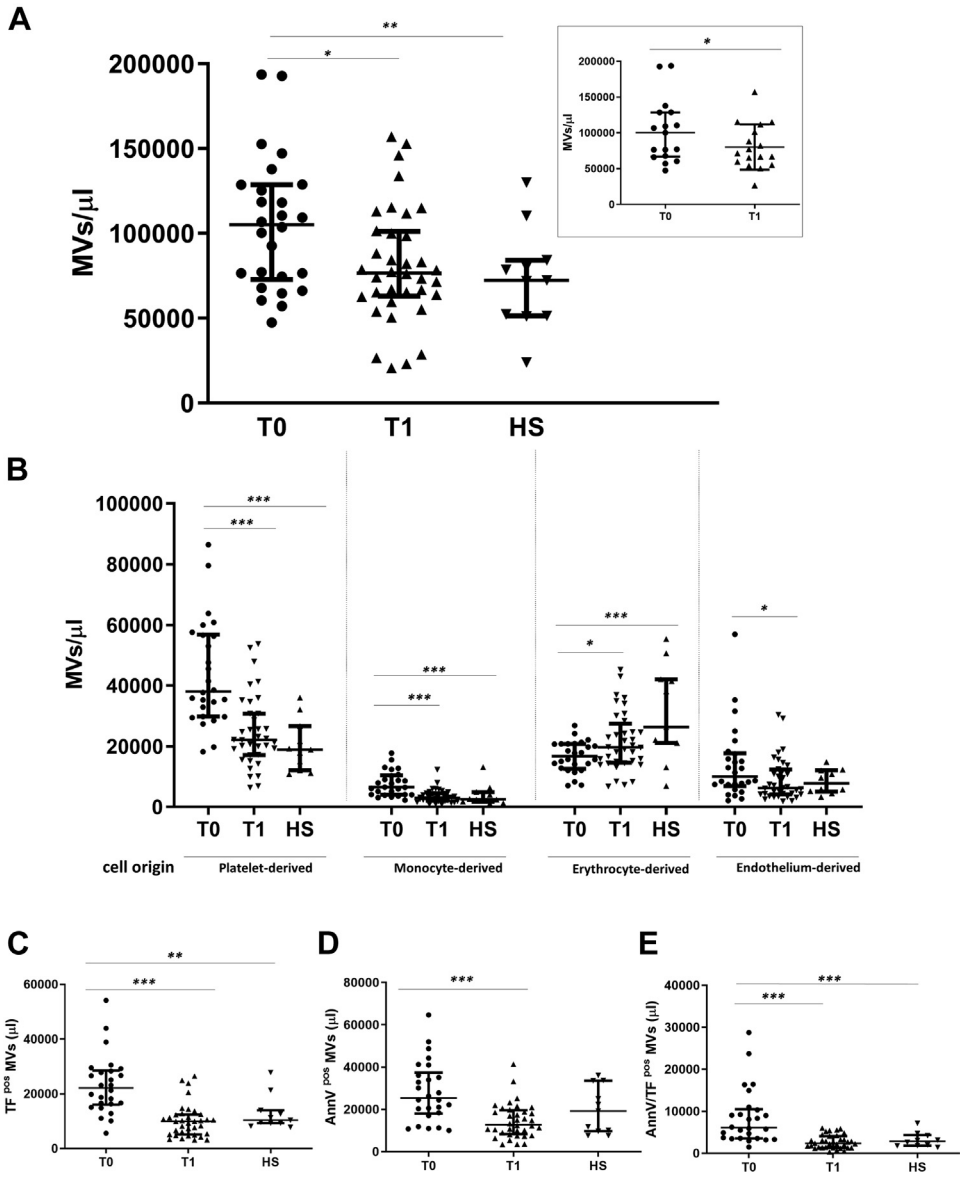
TABLE 6 Thrombin Generation Potential of Platelets

				P Value		
	T0	T1	HS	T0 vs T1	T0 vs HS	T1 vs HS
Lag time, min	26.9 ± 8.9	32.2 ± 12.4	30.6 ± 7.2	0.046	0.036	0.978
ETP, nmol/L × min	1,038 ± 352	797 ± 373	781 ± 319	0.005	0.039	0.619
Peak, nmol/L thrombin	115.2 ± 81.2	77.2 ± 59.2	63.4 ± 41.8	0.009	0.022	0.620
Time to peak, min	32.1 ± 9.5	37.8 ± 13	37.6 ± 10.2	0.039	0.030	0.871
Velocity index, nmol/L/min	29.1 ± 29	17.9 ± 19.9	12.3 ± 10.9	0.020	0.035	0.528

Values are mean ± SD.

ETP = endogenous thrombin potential; HS = healthy subjects; other abbreviations as in Table 2.

FIGURE 4 Quantification of Circulating MVs in Whole Blood

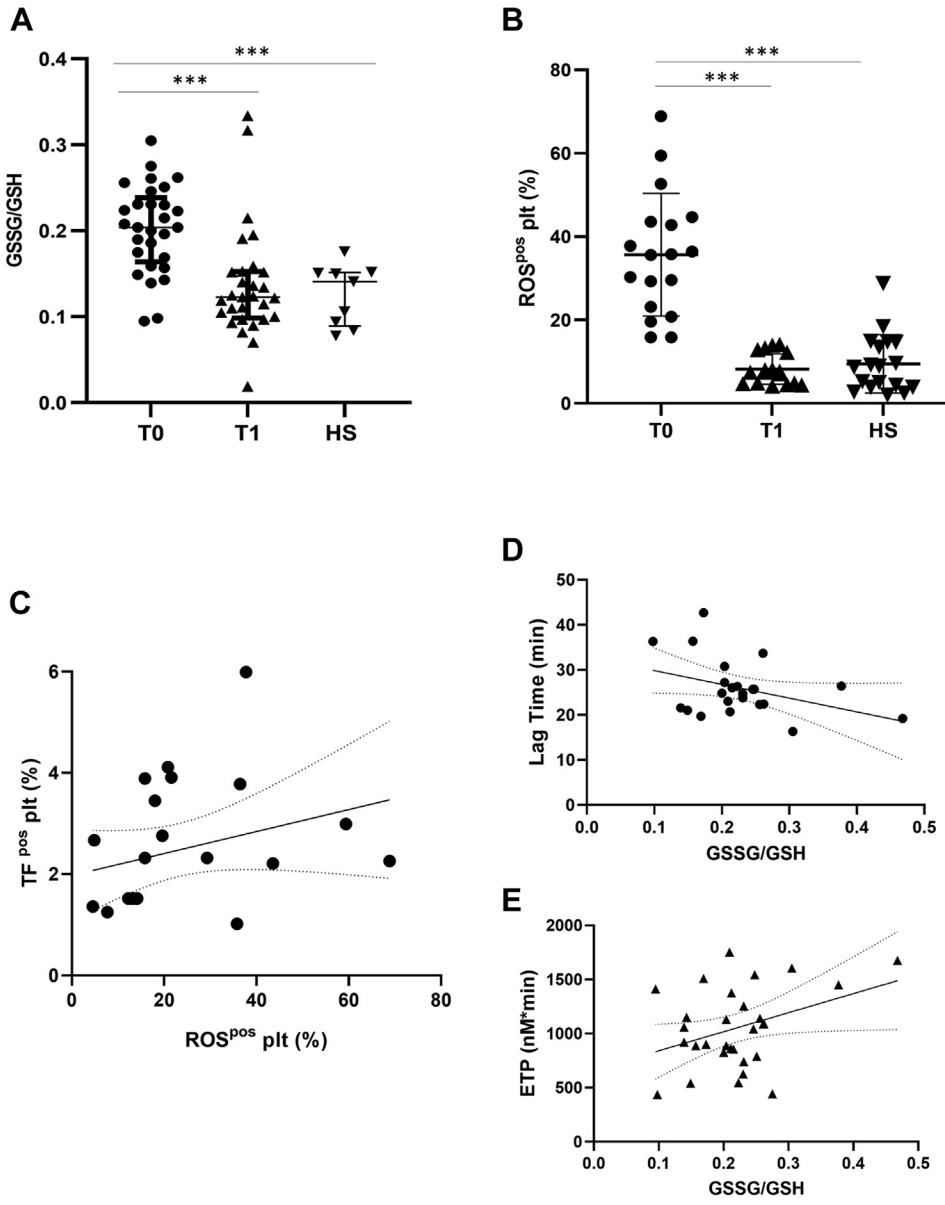


Microvesicles (MV) levels were evaluated by flow cytometry in a subgroup of patients before (T0) (n = 26) and after (T1) (n = 36) patent foramen ovale (PFO) closure and in healthy subjects (HS) (n = 11). Graphs report the median levels (25th to 75th percentiles) of total **(A)**, platelet-, monocyte-, erythrocyte-, and endothelium-derived MVs **(B)** as well as of procoagulant MVs **(C to E)**. **Inset in A** shows the results of total MVs analyzed in 17 paired patients. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

among MHA, platelet procoagulant phenotype, and oxidative stress, we explored this relationship by in vitro experiments.

IN VITRO STUDIES. We first tested whether plasma from MHA-PFO patients reproduced, on cells from HS, the peculiar platelet activation measured in patients before PFO closure. Blood from HS was plasma-depleted and reconstituted with plasma pools from MHA-PFO patients at T0 or from HS. Results showed that PFO plasma significantly increased the number of TF^{pos} and ROS^{pos} platelets and of TF^{pos} MVs (Figures 6A to 6C). By contrast, MHA-PFO plasma did not induce the expression of the other platelet activation markers (Figures 6D and 6E) nor the formation of platelet-leukocyte aggregates (Figures 6F and 6G), recapitulating the pattern observed in vivo. Interestingly, addition of NAC in the experimental setting blunted platelet- and MV-

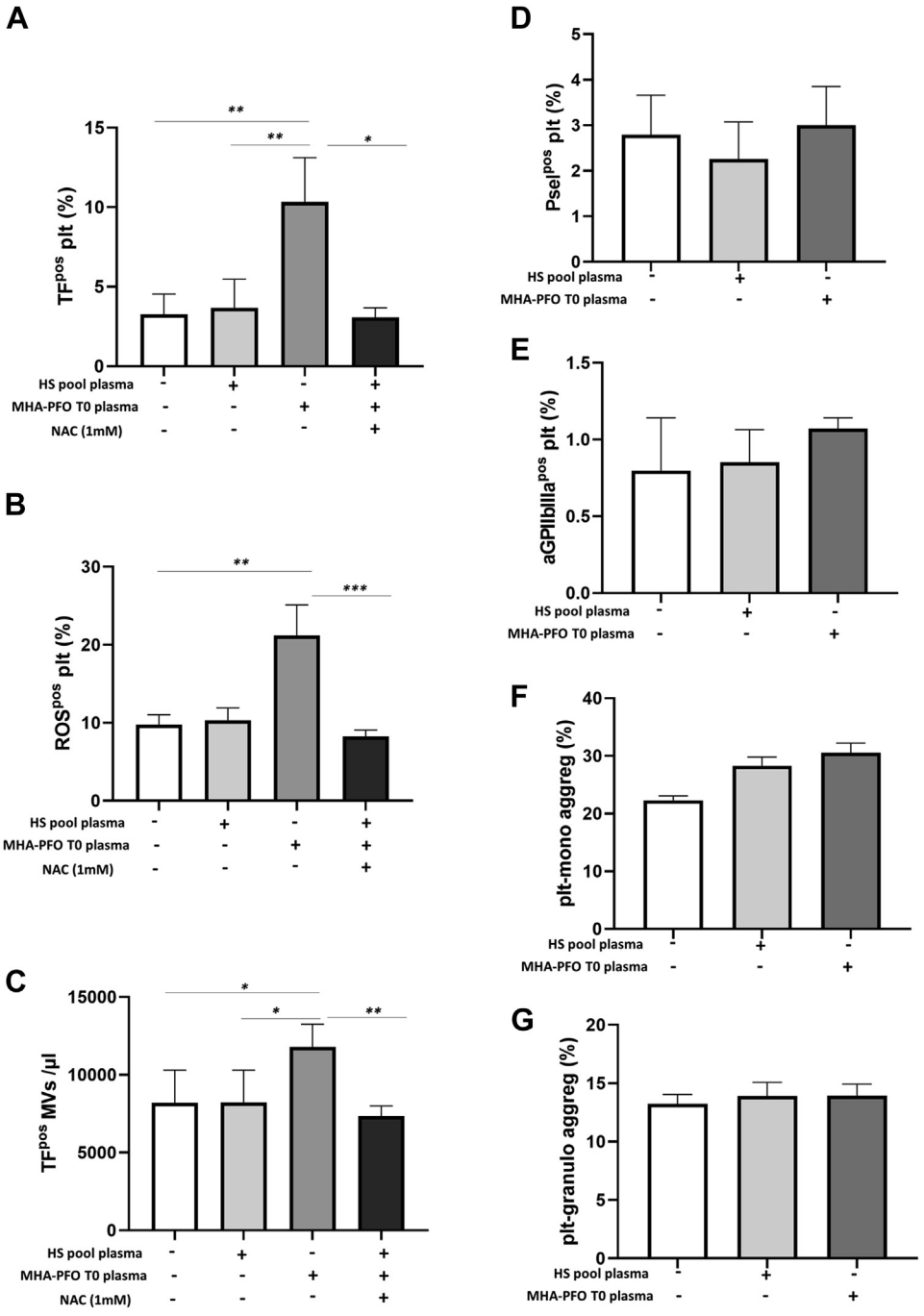
FIGURE 5 Evaluation of Oxidative Stress Status



The GSSG/GSH ratio **(A)** in MHA-PFO patients (n = 29) and in HS (n = 9) was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The platelet ROS production **(B)** was evaluated by whole blood flow cytometry on 18 MHA-PFO patients and 18 HS. Data are the median value and 25th to 75th percentiles. **(C to E)** The correlation between ROS^{pos} and TF^{pos} platelets and between GSSG/GSH ratio and the kinetic (lag time) and endogenous thrombin generation potential (ETP), respectively. ***$P < 0.001$. ETP = endogenous thrombin potential; GSH = reduced glutathione; GSSG = oxidized glutathione; HS = healthy subjects; MHA = migraine headache with aura; PFO = patent foramen ovale; ROS = reactive oxygen species; TF = tissue factor.

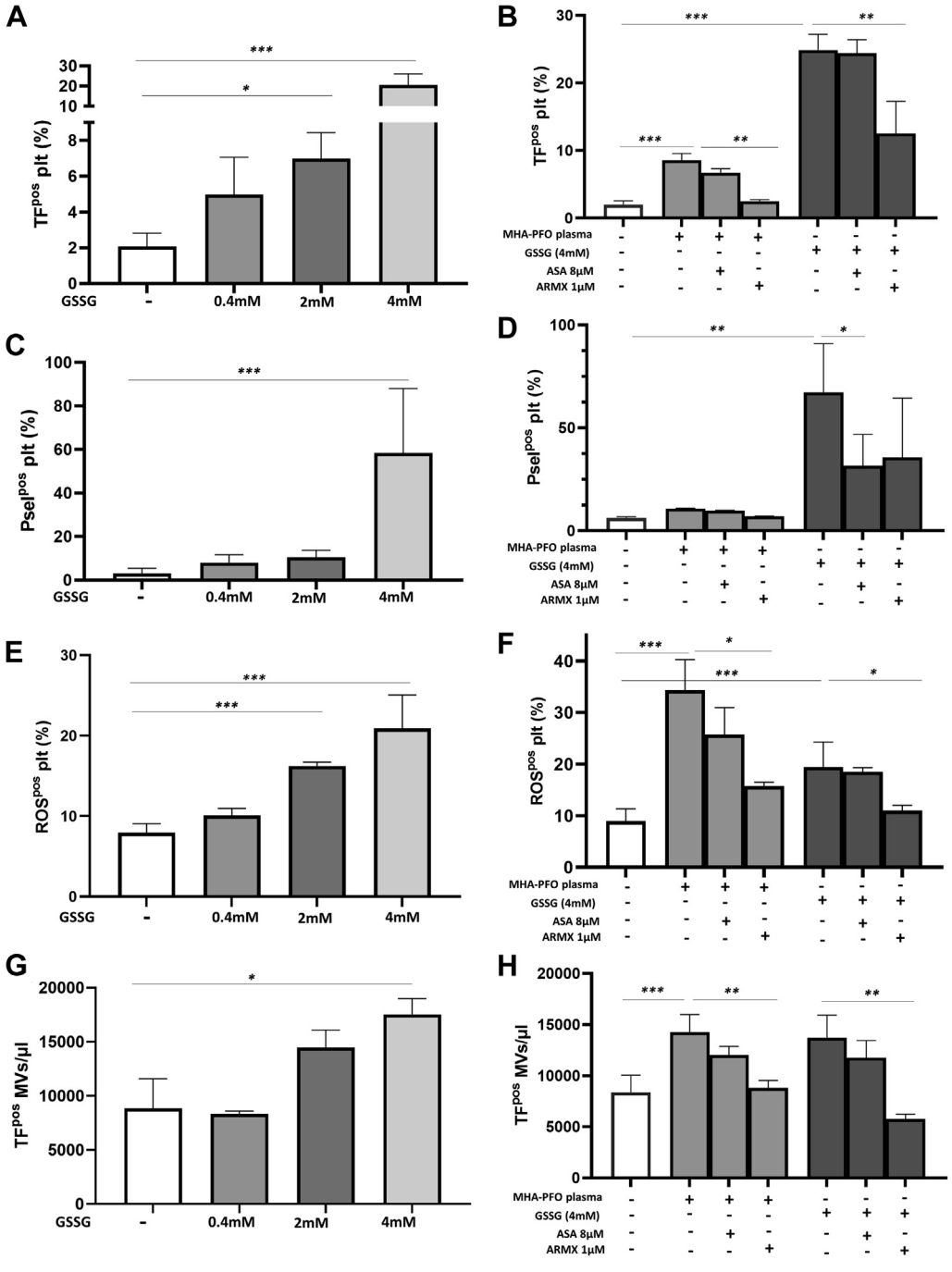
associated TF expression as well as platelet ROS production (Figures 6A and 6C), highlighting a plausible role of oxidative stress in this clinical condition. To further test this hypothesis, we verified whether GSSG-induced oxidative stress would activate platelets similarly. GSSG concentration dependently increased the percentage of TF^{pos} and ROS^{pos} platelets as well as the release of TF^{pos} MVs (Figures 7A, 7E, and 7G); P-selectin was up-regulated only at the highest concentration tested (Figure 7C) while aGPIIb/IIIa expression was never affected (data not shown).

FIGURE 6 **In Vitro Effect of Plasma From MHA-PFO Patients and NAC on HS Platelets**



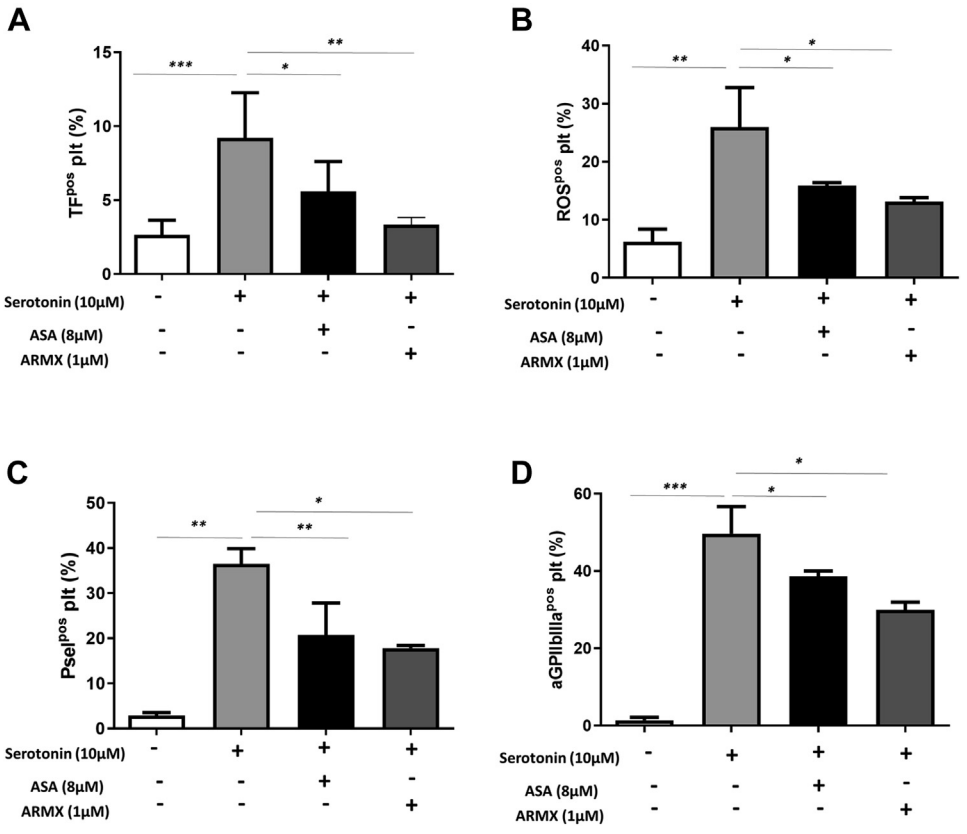
Plasma-depleted blood from HS (n = 5) was reconstituted with a plasma pool from HS or with a plasma pool from MHA-PFO patients in the presence or absence of N-acetylcysteine (NAC). Percentage of TF^{pos}-platelets **(A)**, platelet ROS formation **(B)**, TF^{pos}-MVs **(C)**, P-selectin^{pos} and aGPIIb/IIIa^{pos} platelets **(D and E)**, and platelet-monocyte and -granulocyte aggregates **(F and G)** was analyzed (n = 5). Data are mean ± SD. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Abbreviations as in Figures 3 and 4.

FIGURE 7 In Vitro Effect of GSSG on Platelet Activation and Modulation by Antiplatelet Drugs



GSSG concentration-dependent effect on the percentage of TF^{pos} **(A)**, P-$selectin^{pos}$ **(C)**, and ROS^{pos} platelets **(E)**, and TF^{pos} MV release **(G)**. The effect of aspirin and $P2Y_{12}$ antagonist AR-C69931MX (ARMX) on platelet activation markers induced by MHA-PFO plasma **(light gray bars)** or by GSSG (4mM) **(dark grey bars)** is reported in **B, D, F, and H.** Data are mean ± SD (n = 8). *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Abbreviations as in Figures 3 and 4.

FIGURE 8 **In Vitro Effect of Serotonin on Platelet Activation and Modulation by Antiplatelet Drugs**



Modulation by antiplatelet drugs of the serotonin-induced platelet TF exposure **(A)**, ROS production **(B)**, P-selectin **(C)**, and aGPIIb/IIIa expression **(D)**. Data are expressed as mean ± SD (n = 5). *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

Then we evaluated the effect of antiplatelet drugs on platelet activation preincubating WB with aspirin or a $P2Y_{12}$ antagonist before addition of MHA-PFO plasma or GSSG. In samples treated with MHA-PFO plasma aspirin showed a trend toward a small reduction in TF and ROS expression and MV release, whereas these parameters were significantly inhibited by the $P2Y_{12}$ antagonist. Interestingly, in the presence of a strong oxidative stress (GSSG 4 mmol/L) aspirin was able to inhibit P-selectin as much as the $P2Y_{12}$ antagonist, whereas it had no effect on TF and ROS expression which were down-regulated by the $P2Y_{12}$ antagonist (Figures 7B, 7D, 7F, and 7H).

Overall, these in vitro data, which closely mirror the ex vivo analyses of MHA-PFO patients, clearly point to oxidative stress as a mechanism of platelet activation differently modulated by antiplatelet drugs.

Because serotonin might also be implicated in migraine, we evaluated its in vitro effect on platelet activation in HS. Unlike MHA-PFO plasma, the serotonin-induced expression of TF and ROS was significantly inhibited by aspirin and $P2Y_{12}$ antagonist (Figures 8A and 8B). Moreover, serotonin also increased the number of P-selectin^{pos} and aGPIIb/IIIa^{pos} platelets, whose levels remained high despite a significant reduction by antiplatelet drugs (Figures 8C and 8D). Thus, these data, together with the finding that serotonin levels were within the reference range and did not change after PFO closure, downplay the role of serotonin while emphasizing the role of oxidative stress as etiopathogenic mechanism of the increased prothrombotic potential measured in these patients.

DISCUSSION

This study shows for the first time that blood of MHA patients with PFO on aspirin is characterized by a marked thrombin generation capacity, both in terms of the amount of thrombin produced as well as of its

rate of synthesis. This prothrombotic phenotype is caused by the presence in the circulation of an elevated number of platelets and their released MVs expressing a functionally active TF, able to trigger thrombin generation. PFO closure brings this phenotype back to the levels found in HS, together with resolution or reduction of migraine in 70% and 28% of patients, respectively.

MHA-PFO patients are also characterized by the presence of an altered oxidative stress status in terms of increased ROS production by platelets and increased GSSG/GSH ratio in whole blood, coupled with a significant reduction in the number of erythrocytes. As observed for the platelet activation phenotype, the antioxidant capacity and the erythrocyte count return to physiological levels after PFO closure supporting the etiopathogenic role of oxidative stress in this clinical setting.

Although it has been recently shown that PFO closure significantly improves MHA symptoms,[6] no pathophysiological mechanisms have so far explained how a PFO might trigger MHA.

A platelet activated phenotype in migraineurs has been first documented 40 years ago,[17] and confirmed in subsequent studies, in which platelet behavior was assessed in patients not taking any antiplatelet drug.[16,18-20] Later on, the role of platelet activation in the pathophysiological mechanisms linking PFO and MHA has been supported by the efficacy of aspirin therapy in improving symptoms in a percentage of patients.[13,21,22]

At present, the potential mediators of platelet activation in migraine have been only partially identified. Inflammatory cytokines may have a role, but they have not been found increased in our patients. Serotonin has also been closely linked to migraine, and its involvement has been supported by the efficacy of triptans, although only in one-third of treated patients.[23] Indeed, several studies exploring the circulating serotonin levels measured between headache attacks have provided opposite results, some reporting lower and other increased levels.[17,24] In our patient cohort, serotonin levels were within the physiological range and did not change with symptoms resolution after PFO closure. This finding, together with the fact that most of the patients reported no benefit from taking triptans weakens the serotonin etiopathogenic role. The possibility however that both serotonin and/or cytokine levels may increase during migraine attack cannot be excluded.

The finding that in the present study the platelet activated phenotype is documented despite aspirin treatment leads us to speculate that platelet activation might be triggered by the following: 1) stimuli that escape the aspirin effect (resulting in the up-regulation of all common platelet activation markers); or 2) stimuli that differently affect the expression of platelet activation markers (resulting in the up-regulation of some of them only). Alternatively, the following may also be hypothesized: 3) aspirin, depending on the stimulus, differently modulates the expression of platelet activation markers (some are down-regulated, but others are not).

The unique state of platelet activation observed in the migraineurs enrolled in our study seems to be supported by both the second and third hypotheses. Indeed, in these patients, who were all responsive to aspirin treatment based on the TxB_2 levels and on platelet aggregation tests, the classical markers of platelet activation, such as aGPIIb/IIIa, P-selectin, and platelet-leukocyte aggregates, were all expressed at levels comparable to those found in HS, except for TF^{pos} platelets and MVs and platelet ROS production. The stimulus that in vivo differently affects platelet activation and induces this prothrombotic phenotype is an altered oxidative stress status (GSSG/GSH). The etiopathogenic role of oxidative stress in MHA-PFO patients is strengthened by the following observations: 1) MHA-PFO patient plasma, when used to reconstitute a plasma-depleted blood from HS, reproduced the in vivo platelet phenotype, and this was blunted by NAC; 2) similar results were obtained when blood from HS was treated with GSSG; and 3) the GSSG/GSH ratio positively associates with the platelet-dependent thrombin generation. Aspirin has a negligible effect on these markers when induced by pathophysiological levels of oxidative stress. Interestingly, however, at higher levels, such as those reproduced in vitro with GSSG 4 mmol/L, aspirin is able to inhibit the P-selectin up-regulation, but has no effect on TF and ROS expression and on MV release. Furthermore, it is worth mentioning that also serotonin induces in vitro platelet TF and ROS expression. This effect, however, unlike that observed with oxidative stress, is significantly prevented by aspirin, thus weakening, in this cohort of patients, serotonin's contribution to the etiopathogenic mechanism linking platelet activation to migraine symptoms and to PFO.

It has been reported that patients experiencing MHA have vulnerability to oxidative stress.[25] Indeed, increased oxidative stress and/or decreased antioxidant capacity have been found in migraine patients,[25-28] thus supporting platelet activation.

In our patient cohort, the observed alteration in ROS production by platelets as well as in the GSH system might be related to the significantly lower

number of erythrocytes found before PFO closure, because they are the main source of this antioxidant system in the circulation.[29]

Whether the reduction in erythrocyte number, together with that of hemoglobin and erythrocyte-derived MVs, is a direct consequence of the mechanical stress related to the right-to-left shunt or is secondary to the oxidative stress status is currently unknown. Interestingly, a significantly higher erythrocyte oxidative stress status associated with left-to-right shunt congenital heart disease has been previously reported in children.[30] Furthermore, increased oxidative stress and reactive oxygen species accumulation in erythrocytes may induce hemolysis.[31] Free hemoglobin can also bind to GPIbα on platelets, leading to platelet activation and thrombus formation. Of note, the released heme up-regulates and binds to TF on macrophages as well, promoting TF-dependent coagulation activation.[32] Whether this mechanism may account also for the up-regulation of TF on platelet will be a matter of future research. Whatever the mechanisms, the number of erythrocytes and the alteration in GSH homeostasis reverted after PFO closure, reaching levels similar to those of HS. Of note, GSH increase and thrombin generation reduction were the only biomarkers associated with 100% migraine remission.

Thrombin, beside the crucial role in coagulation, has a more widespread role in inflammatory events mediated through proteinase-activated receptors (PARs).[33] PAR1 is expressed in endothelial cells, neutrophils, and platelets, and its activation leads to the release of pro-inflammatory mediators such as prostaglandins, interleukins, and nitric oxide, all involved in the thrombin-dependent edema.[33] Because anticoagulation has been reported to improve migraine, it is tempting to speculate that thrombin may have a role in the pathogenesis of migraine causing neurogenic inflammation that would favor and perpetuate a migraine attack.[34]

Finally, another interesting finding from the in vitro experiments is that although aspirin has no effect on oxidative stress-induced platelet-associated TF and ROS expression and on MV formation, these parameters are conversely efficiently inhibited by a $P2Y_{12}$ antagonist.

These data, in addition to further strengthening the hypothesis that a platelet-based mechanism might link MHA to PFO, would support, from a mechanistic point of view, findings of studies showing that $P2Y_{12}$ inhibition is effective in reducing MHA symptoms in patients with PFO.[35]

STUDY LIMITATIONS. Our findings should be interpreted in the context of their limitations. First, MHA is a multifactorial disease and we are well aware that other mechanisms may be involved. Nevertheless, the role of the oxidative stress appears consistent and further corroborated by the in vitro data. Second, the effect of oxidative stress and serotonin was tested on platelets from healthy subjects and not from patients because of the COVID-19 pandemic, which imposed restrictions on patient enrollment. Finally, thrombin generation capacity of platelets and MVs, as well as the MV characterization and platelet ROS production were included after the study had started to support the procoagulant phenotype evidenced by the first ad interim flow cytometry data analysis, and thus, were performed on a subset of patients. Finally, post hoc adjustment for type I error was not done; thus, results should be interpreted with caution.

CONCLUSIONS

This study suggests a pathophysiological mechanism linking PFO, or its associated right-to-left shunt, with MHA. Indeed, on the background of an altered oxidative stress status increased circulating levels of TF^{pos} platelets and MVs may play a primary role in triggering blood coagulation and thrombin generation, even in the absence of a lesion of the vessel wall and exposure of subendothelial cells expressing TF. This platelet activation, which can support an increased thrombotic risk that eventually leads to acute ischemic episodes, could play a primary role in producing the prodromal symptoms of migraine and it is switched off upon PFO closure.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

This work was supported by a grant from Italian Ministry of Health (Ricerca Corrente 2018-19). The authors have reported that they have no relationships relevant to the contents of this paper to disclose.

ADDRESS FOR CORRESPONDENCE: Dr Marina Camera, Department of Pharmaceutical Sciences, Università degli Studi di Milano, Via Balzaretti, 9, 20133 Milan, Italy AND Centro Cardiologico Monzino IRCCS, Via Parea 4, 20138 Milan, Italy. E-mail: marina.camera@unimi.it OR marina.camera@ccfm.it. Twitter: @marinacamera. OR Dr Daniela Trabattoni, Centro Cardiologico Monzino, IRCCS, Via Parea, 4, 20138 Milano, Italy. E-mail: daniela.trabattoni@ccfm.it. Twitter: @d_trabattoni.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Blood of MHA patients with PFO on aspirin is characterized by a unique state of platelet activation that sustains a marked thrombin generation capacity positively associated with an altered oxidative stress status. This condition fully reverts upon PFO closure and is associated with 100% migraine remission.

TRANSLATIONAL OUTLOOK: Due to the multifactorial etiology of migraine, the combined assessment of the systemic oxidative stress status (GSSG/GSH) together with the evaluation of platelet- and MV-associated TF expression may help the clinician to identify patients who would most benefit from PFO closure.

REFERENCES

1. Headache Classification Subcommittee of the International Headache Society. The international classification of headache disorders: II edition. *Cephalalgia*. 2004;24(Suppl 1):1–160.

2. Rigatelli G. Migraine and patent foramen ovale: connecting flight or one-way ticket? *Expert Rev Neurother*. 2008;8:1331–1337.

3. Schwedt TJ, Demaerschalk BM, Dodick DW. Patent foramen ovale and migraine: a quantitative systematic review. *Cephalalgia*. 2008;28:531–540.

4. Reisman M, Christofferson RD, Jesurum J, et al. Migraine headache relief after transcatheter closure of patent foramen ovale. *J Am Coll Cardiol*. 2005;45:493–495.

5. Trabattoni D, Fabbiocchi F, Montorsi P, et al. Sustained long-term benefit of patent foramen ovale closure on migraine. *Catheter Cardiovasc Interv*. 2011;77:570–574.

6. Mojadidi MK, Kumar P, Mahmoud AN, et al. Pooled analysis of PFO occluder device trials in patients with PFO and migraine. *J Am Coll Cardiol*. 2021;77:667–676.

7. Jesurum JT, Fuller CJ, Kim CJ, et al. Frequency of migraine headache relief following patent foramen ovale "closure" despite residual right-to-left shunt. *Am J Cardiol*. 2008;102:916–920.

8. Sandler M. Migraine: a pulmonary disease? *Lancet*. 1972;1:618–619.

9. Sicuteri F. Biochemical investigations in headache: increase in the hydroxyindoleacetic acid excretion during migraine attacks. *Int Arch Allergy Appl Immunol*. 1961;19:55–58.

10. Hilton BP, Cumings JN. An assessment of platelet aggregation induced by 5-hydroxytryptamine. *J Clin Pathol*. 1971;24:250–258.

11. Danese E, Montagnana M, Lippi G. Platelets and migraine. *Thromb Res*. 2014;134:17–22.

12. Tietjen GE, Al-Qasmi MM, Athanas K, Utley C, Herial NA. Altered hemostasis in migraineurs studied with a dynamic flow system. *Thromb Res*. 2007;119:217–222.

13. Biglione B, Gitin A, Gorelick PB, Hennekens C. Aspirin in the treatment and prevention of migraine headaches: possible additional clinical options for primary healthcare providers. *Am J Med*. 2020;133:412–416.

14. Borkum JM. Migraine triggers and oxidative stress: a narrative review and synthesis. *Headache*. 2016;56:12–35.

15. Frelinger AL, Li Y, Linden MD, et al. Aspirin 'resistance': role of pre-existent platelet reactivity and correlation between tests. *J Thromb Haemost*. 2008;6:2035–2044.

16. Lechner H, Ott E, Fazekas F, Pilger E. Evidence of enhanced platelet aggregation and platelet sensitivity in migraine patients. *Cephalalgia*. 1985;5(Suppl 2):89–91.

17. Hanington E, Jones RJ, Amess JA, Wachowicz B. Migraine: a platelet disorder. *Lancet*. 1981;2:720–723.

18. Hanington E, Jones RJ, Amess JA. Migraine and platelets. *Lancet*. 1982;1:1248.

19. Buttinelli C, Lazzaro MP, Lenzi GL, Paolucci S, Prencipe M. Correlation between migraine and circulating platelet aggregates. *Cephalalgia*. 1985;5(Suppl 2):87–88.

20. Kozubski W, Walkowiak B, Cierniewski CS, Prusinski A. Platelet fibrinogen receptors in migraine patients. *Headache*. 1987;27:431–434.

21. Buring JE, Peto R, Hennekens CH. Low-dose aspirin for migraine prophylaxis. *JAMA*. 1990;264:1711–1713.

22. Diener HC. Low-dose aspirin for migraine prophylaxis in women. *Cephalalgia*. 2001;21:167–168.

23. Zeller JA, Lindner V, Frahm K, Baron R, Deuschl G. Platelet activation and platelet-leucocyte interaction in patients with migraine. Subtype differences and influence of triptans. *Cephalalgia*. 2005;25:536–541.

24. Ribeiro CA, Cotrim MD, Morgadinho MT, Ramos MI, Santos ES, de Macedo Tdos R. Migraine, serum serotonin and platelet 5-HT2 receptors. *Cephalalgia*. 1990;10:213–219.

25. Tozzi-Ciancarelli MG, De Matteis G, Di Massimo C, Marini C, Ciancarelli I, Carolei A. Oxidative stress and platelet responsiveness in migraine. *Cephalalgia*. 1997;17:580–584.

26. Bolayir E, Celik K, Kugu N, Yilmaz A, Topaktas S, Bakir S. Intraerythrocyte antioxidant enzyme activities in migraine and tension-type headaches. *J Chin Med Assoc*. 2004;67:263–267.

27. Alp R, Selek S, Alp SI, Taskin A, Kocyigit A. Oxidative and antioxidative balance in patients of migraine. *Eur Rev Med Pharmacol Sci*. 2010;14:877–882.

28. Bernecker C, Ragginer C, Fauler G, et al. Oxidative stress is associated with migraine and migraine-related metabolic risk in females. *Eur J Neurol*. 2011;18:1233–1239.

29. Giustarini D, Milzani A, Dalle-Donne I, Rossi R. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells Mol Dis*. 2008;40:174–179.

30. Le GZ, Dong XY, Shen Y, Chen YQ, Lu JP. [Erythrocyte oxidative stress in children with left to right shunt congenital heart disease]. *Zhongguo Dang Dai Er Ke Za Zhi*. 2010;12:440–443.

31. Wang Q, Zennadi R. oxidative stress and thrombosis during aging: the roles of oxidative stress in RBCs in venous thrombosis. *Int J Mol Sci*. 2020;21(12):4259. https://doi.org/10.3390/ijms2112425

32. Bahl N, Winarsih I, Tucker-Kellogg L, Ding JL. Extracellular haemoglobin upregulates and binds to tissue factor on macrophages: implications for coagulation and oxidative stress. *Thromb Haemost*. 2014;111:67–78.

33. Cicala C, Cirino G. Linkage between inflammation and coagulation: an update on the molecular basis of the crosstalk. *Life Sci*. 1998;62:1817–1824.

34. Carramate JF, Fragoso YD, de Souza Carvalho D, Gabbai AA. The elusive role of thrombin in migraine. *Headache*. 2001;41:609–611.

35. Sommer RJ, Nazif T, Privitera L, Robbins BT. Retrospective review of thienopyridine therapy in migraineurs with patent foramen ovale. *Neurology*. 2018;91:1002–1009.

KEY WORDS migraine with aura, oxidative stress, patent foramen ovale, platelets, tissue factor

APPENDIX For an expanded Methods section as well as supplemental tables and figures, please see the online version of this paper.