Neurochemistry International 154 (2022) 105301

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

The effect of clopidogrel and aspirin on the severity of traumatic brain injury in a rat model

Firas Kobeissy [a,*,1], Khalil Mallah [a,b,1], Kazem Zibara [c,d], Fatima Dakroub [a,e], Zeinab Dalloul [a], Mohammad Nasser [a,e], Leila Nasrallah [a], Zahraa Mallah [a,e], Ghewa A. El-Achkar [a], Naify Ramadan [a], Wael Mohamed [f,g], Stefania Mondello [h], Hala Darwish [i,j], Eva Hamade [e,k,**], Aida Habib [l,***]

[a] Department of Biochemistry and Molecular Genetics, Faculty of Medicine, American University of Beirut, Beirut, Lebanon
[b] Department of Microbiology and Immunology, Medical University of South Carolina, 173 Ashley Avenue, BSB 204, MSC 504, Charleston, SC, 29425, USA
[c] ER045, Laboratory of Stem Cells, DSST, PRASE, Lebanese University, Beirut, Lebanon
[d] Department of Biology, Faculty of Sciences-I, Lebanese University, Beirut, Lebanon
[e] Molecular Biology and Cancer Immunology Laboratory, Faculty of Sciences-I, Lebanese University, Beirut, Lebanon
[f] Clinical Pharmacology Department, Menoufia Medical School, Menoufia University, AlMinufya, Egypt
[g] Basic Medical Science Department, Kulliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang, Malaysia
[h] Department of Neurosciences, University of Messina, Messina, Italy
[i] Nehme and Therese Tohme Multiple Sclerosis Center, Faculty of Medicine, American University of Beirut Medical Center, Lebanon
[j] Hariri School of Nursing, American University of Beirut, Lebanon
[k] Department of Biochemistry, Faculty of Sciences-I, Lebanese University, Beirut, Lebanon
[l] Department of Basic Medical Sciences, College of Medicine, QU Health, Qatar University, Doha, Qatar

ARTICLE INFO

Keywords:
Controlled cortical impact
Traumatic brain injury
Clopidogrel
Aspirin
Intracerebral haemorrhage
Neuroprotection

ABSTRACT

Traumatic Brain Injury (TBI) is one of the leading causes of death and disability worldwide. Aspirin (ASA) and clopidogrel (CLOP) are antiplatelet agents that inhibit platelet aggregation. They are implicated in worsening the intracerebral haemorrhage (ICH) risk post-TBI. However, antiplatelet drugs may also exert a neuroprotective effect post-injury. We determined the impact of ASA and CLOP treatment, alone or in combination, on ICH and brain damage in an experimental rat TBI model. We assessed changes in platelet aggregation and measured serum thromboxane by enzyme immune assay. We also explored a panel of brain damage and apoptosis biomarkers by immunoblotting. Rats were treated with ASA and/or CLOP for 48 h prior to TBI and sacrificed 48 h post-injury. In rats treated with antiplatelet agents prior to TBI, platelet aggregation was completely inhibited, and serum thromboxane was significantly decreased, compared to the TBI group without treatment. TBI increases UCHL-1 and GFAP, but decreases hexokinase expression compared to the non-injured controls. All groups treated with antiplatelet drugs prior to TBI had decreased UCH-L1 and GFAP serum levels compared to the TBI untreated group. Furthermore, the ASA and CLOP single treatments increased the hexokinase serum levels. We confirmed that αII-spectrin cleavage increased post-TBI, with the highest cleavage detected in CLOP-treated rats. Aspirin and/or CLOP treatment prior to TBI is a double-edged sword that exerts a dual effect post-injury. On one hand, ASA and CLOP single treatments increase the post-TBI ICH risk, with a further detrimental effect from the ASA + CLOP treatment. On the other hand, ASA and/or CLOP treatments are neuroprotective and result in a favourable profile of TBI injury markers. The ICH risk and the neuroprotection benefits from antiplatelet therapy should be weighed against each other to ameliorate the management of TBI patients.

* Corresponding author.
** Corresponding author.
*** Corresponding author. Molecular Biology and Cancer Immunology Laboratory, Faculty of Sciences-I, Lebanese University, Beirut, Lebanon.
E-mail addresses: firasko@gmail.com (F. Kobeissy), eva.hamade@ul.edu.lb (E. Hamade), aida.habib@qu.edu.qa (A. Habib).
[1] Equal contribution.

https://doi.org/10.1016/j.neuint.2022.105301
Received 10 October 2021; Received in revised form 7 January 2022; Accepted 31 January 2022
Available online 2 February 2022

1. Introduction

Traumatic brain injury (TBI) is a major cause of mortality and morbidity worldwide with an estimated 69 million individuals experiencing TBI each year (Dewan et al., 2018). Moreover, TBI crude mortality rates range from 3.3 to 24.4 per 100,000 population per year in Europe (Brazinova et al., 2021). In elderly patients, neurological injuries are associated with higher rates of hospitalizations, long-term disability, and mortality (Floyd et al., 2014; Roozenbeek et al., 2013). Moreover, elderly patients frequently have multiple comorbid conditions that require antiplatelet or anticoagulant treatment (King et al., 2010). In particular, aspirin and CLOP are two common adjuncts to the medical care of elderly patients with cardiovascular diseases. ASA irreversibly inhibits the production of thromboxane (TX) A_2 by platelets (Floyd et al., 2014). CLOP inhibits ADP-mediated platelet activation and aggregation (Sangkuhl et al., 2010).

To date, several studies have evaluated the effect of antiplatelet agents on TBI with inconsistent and even conflicting results. Some reported increased mortality rates in patients on antiplatelet therapy following TBI (Gaetani et al., 2012; Ivascu et al., 2008). Fabbri et al. demonstrated an increased risk of unfavorable outcomes in adult patients on CLOP, independent of ASA treatment (Fabbri et al., 2013). Clinical studies showed that patients on antiplatelet drugs before head trauma were at an increased risk to develop intracerebral haemorrhage (ICH) leading to higher neurosurgical intervention rates (Joseph et al., 2014; McCammack et al., 2015; Moore et al., 2012; Peck et al., 2014). Other studies showed that anticoagulant treatments had no impact on mortality (Ahmed et al., 2009), and revealed a protective role of ASA against ICH following TBI (Gangavati et al., 2009). Interestingly, a dual effect of antiplatelet or anticoagulant therapy on TBI was revealed in literature, suggesting neuroprotection despite an increased risk of haemorrhage. Albrecht et al. demonstrated that warfarin therapy resumption post-TBI reduced the stroke risk while increasing that of ICH (Albrecht et al., 2014). Exerting a neuroprotective effect, dalteparin significantly decreased the number of apoptotic neurons following experimental TBI in rats (Hasan et al., 2018). In mice, warfarin pre-treatment increased intracerebral blood volumes post-TBI without significantly worsening the functional outcome (Foerch et al., 2012).

We aimed to test the 'dual role' hypothesis concerning the antiplatelet therapy's effect on TBI in a rat model. For this purpose, we assessed the effect of single or combined treatment of ASA and/or CLOP on TBI-induced haemorrhage and neuronal injury. Platelet aggregation and TXB_2 were measured to assess platelet function. TXB_2 also represents a direct measure of the pharmacological effect of ASA and CLOP (Brun et al., 2016; Good et al., 2015; Kidson-Gerber et al., 2010). Blood-brain barrier (BBB) dysfunction is a secondary outcome of ICH characterized by the presence of blood components in the brain (Nadeau et al., 2019). To evaluate the BBB dysfunction post-TBI, we monitored the unspecific entrance of transferrin - a blood-borne factor-into the brain.

To evaluate neuronal and glial damage, we explored a panel of markers reflecting different pathobiological processes. We assessed the levels of ubiquitin C-terminal hydrolase-L1 (UCH-L1), glial fibrillary acidic protein (GFAP), hexokinase, and αII-spectrin cleavage products. UCH-L1 is a neuron-specific ubiquitin recycling enzyme (Tongaonkar et al., 2000), and GFAP is an intermediate filament protein expressed almost exclusively in astrocytes (Mondello et al., 2014). Substantially elevated levels of these markers after TBI have been associated with injury severity and outcome (Brophy et al., 2009; Kobeissy et al., 2008; Mondello et al., 2011, 2012; Mrozek et al., 2019; Wang et al., 2021) and were also found elevated after stroke (Ren et al., 2016). Serum GFAP is increased in the acute phase post-TBI and is correlated with worse clinical outcomes (Nylen et al., 2006; Shahim et al., 2021). Using a neuroproteomics platform, we previously demonstrated a reduction in hexokinase levels 48 h after TBI using an experimental rat controlled cortical impact (CCI) TBI model (Kobeissy et al., 2006). αII-spectrin is cleaved by several caspases during the apoptotic process, making its cleavage an indicator of ongoing apoptosis (Elmore, 2007).

2. Materials and methods

2.1 Animals: This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (Approval # 13-05-257). Adult male Sprague-Dawley rats (~70 days old, 250–300 g) were housed at the AUB Animal Care Facility under constant temperature (22 ± 2 °C) and humidity (60 ± 5%) control. Rats were fed ad libidum with Teklad traditional standard rodent diet (Envigo cat # 7012). They were kept in same-sex groups of three per cage (1354G Eurostandard Type IV cage with floor area of 1820 cm^2) to prevent crowding, while facilitating social interaction. To detect physiological or behavioral abnormalities, rats were monitored regularly. Those with the same intervention were housed in the same cage to prevent eliciting a stress response in non-treated animals. To decrease the stressful effects of surgery, rats were habituated earlier to gentle handling by the experimenter. Wood shavings were used as bedding material that was changed regularly. Cages, water bottles and bedding were sterilized by autoclaving.

2.2. Experimental design

Rats received a daily single dose of ASA (20 mg/kg *i.p.*), CLOP (10 mg/kg p.o.), or the combination of both for two days prior to TBI. They were sacrificed 48-h later as described previously (Abou-Abbass et al., 2016). Since it is not highly soluble in water, ASA (Acetylsalicylic acid, Sigma, USA) was reconstituted in ethanol, and then diluted with saline to a concentration of 10 mg/ml. Animals received 0.5 ml of the ASA preparation by intraperitoneal (*i.p.*) injection at a dose of 20 mg/kg. Clopidogrel tablets (Sanofi Aventis, France) were crushed, dissolved in saline, and 1ml/rat was administered by oral gavage at a dose of 10 mg/kg (Giachini et al., 2014). Drug concentrations were chosen based on previous studies in rats (Ma et al., 2016; Schoemaker et al., 1998; Umemura et al., 1995). Rats were sedated with 5% isoflurane and sacrificed by decapitation for brain protein extraction. For platelet aggregation experiments, fresh rat blood was collected via cardiac puncture directly after euthanization. Control groups (no TBI) consisted of four groups (n = 5 in each group): untreated (saline) or treated rats (ASA, CLOP, or both). For TBI groups (n=5 per group), control sham rats corresponded to untreated rats subjected to surgery without TBI. The experimental TBI groups included a group that received saline and three other groups that received ASA, CLOP, or the combination of both (ASA + CLOP).

2.3. Traumatic Brain Injury model

Controlled cortical impact (CCI) was performed using the Leica Angle Two system (Leica Biosystems Inc., Wetzlar, Germany) to model TBI in rats as described previously (Abou-Abbass et al., 2016). Surgical procedures were performed under anesthesia induced by an intraperitoneal (*i.p.*) injection of a xylazine (100 mg/kg) and ketamine (10 mg/kg). Proper anesthesia was verified by the response lack after toe pinching. All surgical tools were decontaminated by a dry sterilizer (Germinator 500). The stereotaxic frame was cleaned by ethanol, and the head of the rat was secured with ear bars to stabilize the surgical intervention. The eyes were lubricated using a sterile ointment and the head was shaved before making a midline scalp incision. The Angle Two™ software was used to determine the target's coordinates on the exposed skull according to Bregma and Lambda (−1.8 mm Anteroposterior (AP) and 3.6 mm Right (R) to Bregma ipsilateral). Craniectomy (7 mm diameter) was performed midway between bregma and lambda on the right ipsilateral cortex. TBI was induced by a 2 mm diameter impactor with a velocity of 4 m/s and a depth of 1 mm. The dwell time was 0.8 s, after which the surgical site was sutured. Sham rats

were exposed to an identical surgical procedure without the impact injury. We monitored postoperative behavior and body weight during the light phase of the cycle.

2.4. Platelet aggregation

Washed platelets were prepared from fresh blood as described previously (Gallet et al., 1999; Hirz et al., 2012). Briefly, blood samples (9 ml) were collected on acid citrate dextrose, and platelets were washed and resuspended in Hanks buffer, pH 7.4 containing 1 mg/ml of bovine serum albumin (BSA). Aggregation of washed platelets was determined using light transmittance aggregometry (Chrono-Log Corp., Havertown, PA). An increase in light transmission corresponds to an increase in platelet aggregation. Total platelets of 0.4×10^9 platelet/ml were incubated with an optimal concentration of arachidonic acid (AA) (as determined in Supplementary Fig. 1). For the CLOP experiments, platelets were triggered with 15 μM adenosine diphosphate (ADP), a $P2Y_{12}$ receptor agonist, as previously described (Hirz et al., 2012).

2.5. Enzyme immunoassay

During the sacrifice, rat blood was immediately collected and centrifuged and sera were stored at −80 °C. TXB_2 - the stable metabolite of TXA_2-was measured by enzyme immunoassay (EIA) as previously described (Pradelles et al., 1985) according to the manufacturer's instructions (Cayman Chemicals). Each serum sample was measured twice at two different diltutions. Results are expressed as means of TXB_2 measurements obtained from each rat.

2.6. Western blot

Brains were dissected in phosphate-buffered saline (PBS) on ice. The area surrounding the lesion epicenter on the ipsilateral cortex was rapidly collected, frozen in liquid nitrogen, and stored at −80 °C. Cortical tissue was crushed by mortar and pestle, then homogenized for 1 h in 1X diluted RIPA buffer containing a mix of protease inhibitors. The lysate was centrifuged and the supernatant was collected for protein quantification. Protein extracts from tissue or serum were resolved on a 12% SDS PAGE and transferred onto a nitrocellulose membrane using the Trans-Blot® SD Semi-Dry® (BioRad) transfer system. Membranes were incubated with antibodies against (UCH-L1) (cat # MCA-Bh7, EnCor Biotechnologies, FL), GFAP (cat # 556320, BD Biosciences), αII-spectrin/fodrin (cat # BML-FG6090, Enzo Life Sciences Lausen, Switzerland), Hexokinase (cat #AB3543, Millipore) or Transferrin (cat #ab82411, Abcam). Positive protein bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson Immuno Research, PA, USA) and ECL (Roche). GAPDH detection was used as an internal quantitative control for brain tissue proteins and its expression levels were used to normalize those of each investigated protein in the densitometry analysis. Protein loading was controlled by Coomassie blue staining of a gel run in parallel with same loading conditions. Positive signals were detected using the Chemidoc imaging system (BioRad). Regarding the serum Western blot data, equal loading was confirmed by staining with rabbit polyclonal anti-beta-actin (Abcam, 1801, 1/10000). The expression levels were used to normalize for each investigated protein in the densitometry analysis. We also ran separate gels with the same loading conditions and confirmed equal loading by Coomassie blue staining.

2.7. Immunofluorescence

For the immunofluorescence experiments, rats were intracardially perfused with 4% paraformaldehyde (PFA) and brains were extracted and stored at 4 °C in PFA solution. 24 h later, the brains were placed in 30% sucrose in PFA maintained at 4 °C. The brrains were sliced by a microtome into 40 μm-thick sections. The latter were stained and washed with PBS-tween (PBST) and then blocked with 5% fetal bovine serum (FBS) for 1 h at room temperature. Sections were then incubated with rabbit anti-GFAP antibody (1:1000) (Cat. # ab7260, Abcam antibodies) overnight at 4 °C. The following day, sections were washed with PBST, then incubated for 1 h with an Alexa-fluor 532 fluorescent anti-rabbit secondary antibody (Cat. # A11009, Thermofisher). After washing with PBS, sections were mounted on Superfrost™ slides. Images were obtained using confocal microscopy.

2.8. Statistical analysis

TXB_2 measurements were expressed as mean ± S.E.M. (n = 3). Densitometric analyses were performed using Scion Image (Scion Corporation, MD) and data was expressed as mean ± SEM (n = 5). The ratio of the different analyzed bands to GAPDH or beta actin was determined. Data was represented as percentage change compared to the sham group (no treatment and no TBI). Densitometric analysis of transferrin was performed using Image J (NIH) and its data was represented as fold change compared to the sham group (no treatment and no TBI). Statistical analysis was performed using One Way Anova followed by the Dunnet test using Sigma Plot (Systat Software Inc., San Jose, CA). Probability values were 2 sided, with statistical significance set at p-value<0.05.

3. Results

3.1. Aspirin, clopidogrel, or a combination of both inhibit platelet aggregation

To normalize the aggregation experiments, we first assessed the ASA treatment effect on platelet aggregation in animals without TBI. Blood platelets from animals treated with ASA (20 mg/kg) injected intravenously (*i.v.*) or *i.p.* were prepared and platelet aggregation was performed in the presence of arachidonic acid (AA) (50 μM). Platelets from these animals (from both *i.v.* or *i.p.* administration) showed complete inhibition of aggregation (0%) compared to control untreated rats (only saline + AA), which exhibited 70% aggregation as depicted in Fig. 1A. Similarly, we tested the effect of clopidogrel (CLOP) treatment on platelet aggregation in animals without TBI. Blood collected from animals treated with CLOP (10 mg/kg) was prepared in the presence or absence of adenosine diphosphate (ADP, 15 μM). The latter is an agonist of platelet activation that was shown to bind P2Y12 receptors. Platelets from rats treated with clopidogrel and activated with ADP showed complete inhibition of aggregation (0%) in comparison to control untreated rats (saline + ADP) (50%) (Fig. 1B). Moreover, platelet aggregation was not observed in platelets purified from control rats, neither treated with CLOP, nor activated by ADP (baseline). We finally evaluated the effect of co-administration of ASA + CLOP on platelet aggregation in animals without TBI. Washed platelets were obtained from treated rats and incubated with 50 μM AA and 15 μM ADP. Platelet aggregation was absent in the ASA + CLOP rats group (0%) and significantly higher (~80%) in untreated rats (saline) triggered by both ADP and AA (Fig. 1C).

3.2. Aspirin, clopidogrel, or the combination of both inhibit platelet aggregation and decrease serum TXB_2 levels post-TBI

Fig. 2A presents the experimental timeline used to carry out the in vivo experiments. To evaluate aspirin/clopidogrel effects on platelet aggregation following TBI, platelets were isolated from control rats (CTRL, without TBI) and four TBI groups: saline, ASA, CLOP, and combined treatment with aspirin and CLOP. Platelet aggregation was inhibited (0%) in rats treated with ASA alone or with ASA + CLOP prior to TBI (Fig. 2B). Clopidogrel treatment alone also resulted in the inhibition of platelet aggregation (0%) (data not shown). The TBI- rats (saline) showed around 30% platelet aggregation. We then measured

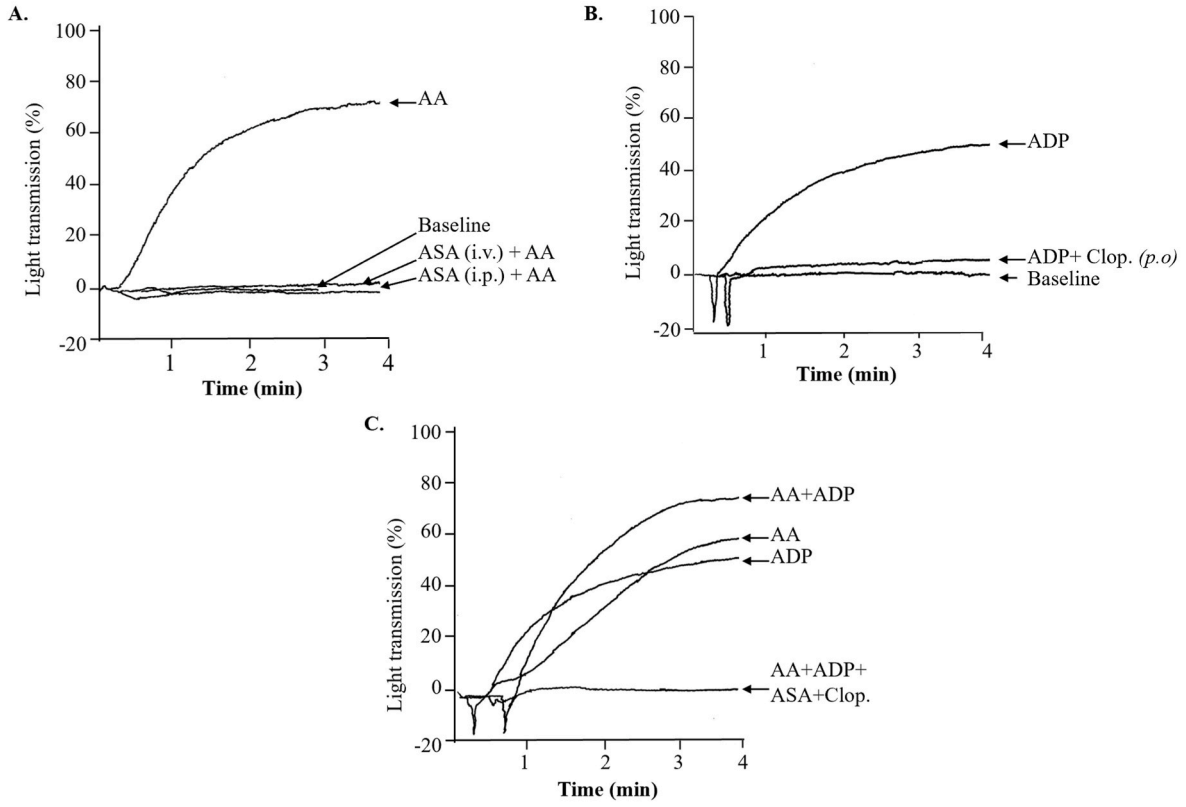

Fig. 1. Effect of aspirin, clopidogrel, or the combination of both on washed platelet aggregation curves.
Aggregation curves represented as percent of light transmission with the progression of time (in min) for washed platelets (0.4 × 109 platelets/m). **A**) Effect of ASA administered (*i.v.*) or (*i.p.*) on arachidonic acid-dependent aggregation of washed platelets. Curves represent saline-treated (*i.p.*) positive control rats (with AA), ASA-treated rats (*i.p.*) for 48 h (with AA), ASA-treated rats (*i.v.*) for 48 h (with AA), and saline-treated (*i.p.*) negative control rats (without AA)(Baseline). **B**) Effect of clopidogrel (CLOP) on ADP-dependent platelet aggregation. Curves represent washed platelets of the following conditions: saline-treated (*i.p.*) positive control rats (with ADP), rats treated with CLOP (*p.o.*) for 48 h (with ADP), and saline-treated (*i.p.*) negative control rats (without ADP) (baseline). **C**) ASA + CLOP treatment. Curves represent washed platelets of the following conditions: saline-treated (*i.p.*) positive control rats (with ADP), saline-treated (*i.p.*) positive control rats (with AA), saline-treated (*i.p.*) positive control rats (with AA and ADP), and rats treated with ASA (*i.p.*) and CLOP (*p.o.*) for 48 h (with AA and ADP).

serum TXB_2 levels in sera collected from rats in the different treatment groups. Fig. 2C shows that rats treated with ASA, CLOP, or both had significantly decreased levels of TXB_2 post-TBI, compared to the control untreated group (saline with TBI). The latter had the highest TXB2 concentration among all TBI groups (serum TXB2 > 200 ng/ml). Fig. 2D shows higher transferrin expression levels in the brain tissue of rats treated with aspirin or CLOP, compared to TBI rats treated with saline. Surprisingly, transferrin levels in the ASA + CLOP treatment group were similar to those of the non-injured group.

3.3. Aspirin alone and when combined with clopidogrel result in a favourable effect on post-TBI αII-spectrin cleavage

We analyzed proteins by immunoblotting to assess the impact of ASA and/or CLOP on the expression levels of serum TBI biomarkers (Fig. 3). The control untreated rat group (saline with TBI) had a significant increase in the serum levels of UCH-L1 (Fig. 3A), and a significant decrease in Hexokinase levels in comparison to control rats without TBI (Fig. 3B). GFAP levels were significantly elevated in the TBI untreated group compared to the sham control (Fig. 3C). In the ASA group, the UCHL-1 and GFAP expression levels post-TBI were similar to those of non-injured animals. Similarly, CLOP administration restored UCHL-1 and hexokinase levels to those observed in rats without TBI. Although CLOP did not restore GFAP to pre-injury levels, it still resulted in lower levels compared to the untreated TBI rats. Similarly, both UCHL-1 and GFAP levels in the ASA + CLOP treatment group were lower than those in the TBI untreated group. We also assessed the expression of GFAP in astrocytes at the perilesional area by immunofluorescence. Fig. 3D shows that the astrocytes in all treatment groups with TBI form more extensive networks compared to the TBI untreated group.

3.4. The single aspirin and ASA + CLOP treatment have a favourable effect on post-TBI αII-spectrin cleavage

To determine the ASA/CLOP implication in post-injury apoptosis, we performed Western blot analysis on brain protein extracts. αII-spectrin/Fodrin is a cytoskeleton protein that is essential in maintaining the structural stability of cellular membranes. During apoptosis, this protein is cleaved by caspases into smaller fragments. A 145 kDa αII-spectrin/Fodrin cleavage product —an indicator of apoptosis — was detected in all rats with TBI. The highest expression was detected in the TBI CLOP group (Fig. 4). A second cleavage product of 150 kDa was detected in all groups. ASA alone, as well as the ASA + CLOP treatment, resulted in lower cleavage of αII-spectrin/Fodrin compared to untreated rats.

4. Discussion

Worldwide, TBI represents a major public health issue that is increasingly recognized as a global health priority (Collaborators, 2019). No pharmacological treatment has demonstrated clinical effectiveness following TBI, and medications are usually directed at treating post-TBI cognitive-behavioral symptoms (Plantier et al., 2016). Thus, it is relevant to identify risk factors for a poor TBI prognosis. Previous studies showed that mortality rates increased in TBI patients maintained

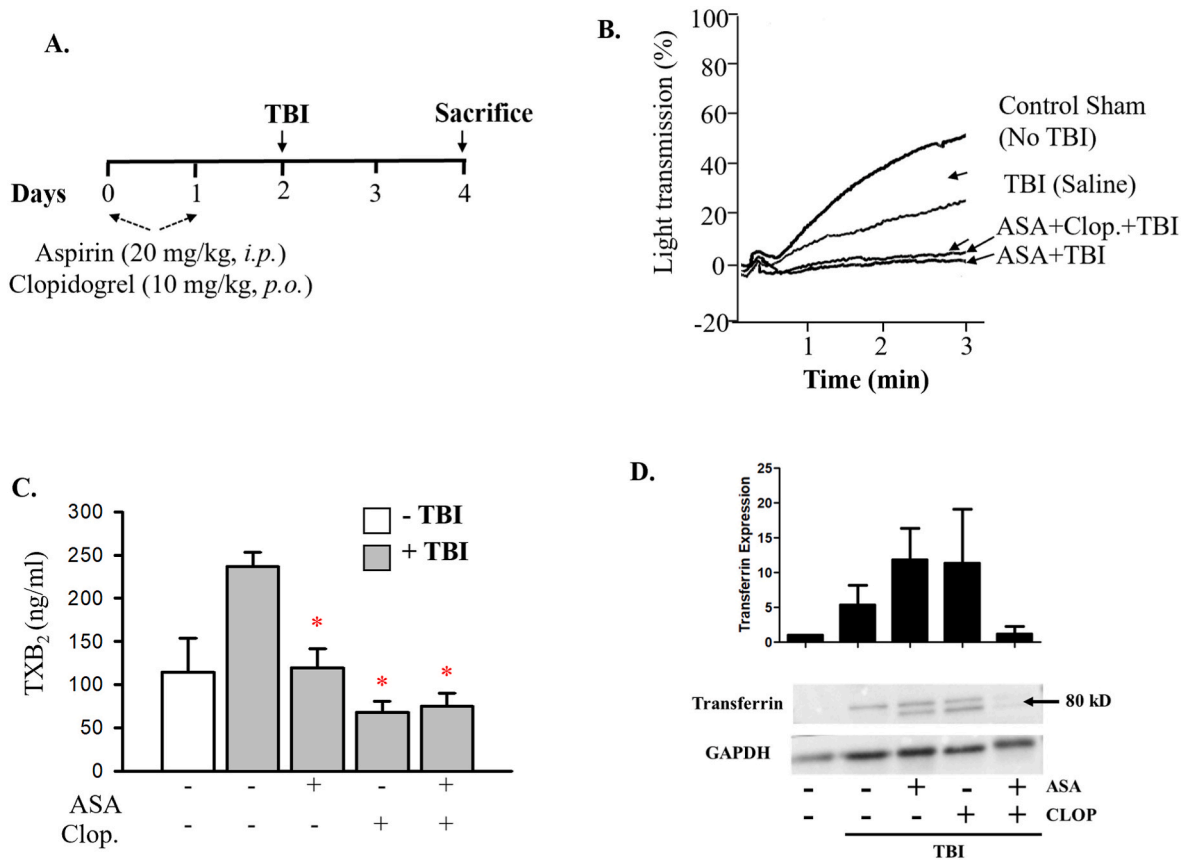

Fig. 2. Effect of aspirin, clopidogrel, or the combination of both on platelet aggregation, following TBI.
A) Animal experimental workflow used in the study. **B**) Platelet aggregation represented as percent of light transmitted with the progression of time (in min) for washed platelets collected from the following animal conditions: saline-treated (*i.p.*) negative control rats without TBI (with AA and ADP), saline-treated (*i.p.*) TBI positive control rats (with AA and ADP), ASA treated (*i.p.*) TBI rats for 48h (with AA and ADP), AS + ACLOP treated (*i.p./p.o.*) TBI rats for 48h (with AA and ADP). **C**) Quantification analysis of TXB_2 from collected serum. Values represent the average TXB_2 concentration detected in the EIA experiments (n = 3) and are reported as mean ± S.E.M. * = $p < 0.05$. **D)** Western blot analysis for transferrin expression levels with its corresponding quantification. GAPDH was used to ensure equal loading of proteins. Values represent the average fold change expression, normalized to GAPDH (which was used as a loading control), and relative to control. Results are representatives of three independent experiments (n = 3) performed for each treatment condition and reported as mean ± S.E.M.

on antiplatelet medications (Gangavati et al., 2009; Mondello et al., 2014; Tongaonkar et al., 2000), while others contradicted these findings (Ahmed et al., 2009; Fakhry et al., 2021). We aimed to determine the effect of ASA and/or CLOP administration on TBI severity in a rat model of TBI. We also aimed to elucidate the underlying molecular mechanistic pathways. For this purpose, we assessed changes in the serum protein expression levels of major TBI markers as well as platelet aggregation as illustrated in Fig. S2.

ASA or CLOP administration alone or in combination prior to TBI resulted in complete inhibition of aggregation when used with ADP or AA as an aggregation agonist. We further supported our observation by assessing serum TXB_2, a platelet activation marker. TXB_2 levels were reduced in the aspirin, CLOP, or combined ASA + CLOP treated rats with TBI. This disturbance in normal platelet activity induced by antiplatelet therapy may worsen the TBI-induced haemorrhage through a mechanism in which thromboxane is involved. Moreover, evidence of a worse BBB disruption was provided by the increased detection of transferrin in the brain tissue of rats treated with either ASA or CLOP. Moreover, the morphological changes observed in astrocytes after antiplatelet therapy are interesting. Astrocytes are key players in the maintenance of the BBB phenotype and function (Lecuyer et al., 2016). They induce brain endothelial cells through several astrocytic factors and pro-inflammatory mediators (Sobue et al., 1999; Zhang et al., 2000) which explains the more extensive astrocytes networks observed as a response to BBB damage (Fig. 3D). The above results confirm the first assumption of the dual role hypothesis indicating that antiplatelet therapy has a negative ICH outcome in TBI. They are in line with other studies on patient cohorts that demonstrate an increased risk to develop ICH in patients on antithrombotic therapy prior to a head injury (Joseph et al., 2014; McCammack et al., 2015; Moore et al., 2012; Peck et al., 2014). It is interesting to conduct clinical investigations of platelet aggregation alteration in TBI patients treated with anti-platelets drugs. Future studies should also measure and compare platelet activation markers levels in sera from TBI patients on different types and doses of antiplatelet treatments.

This work offers promising explanations on the underlying mechanisms following TBI and may offer better insights into the actual role of ASA and CLOP in this specific context. ASA or clopidogrel administered alone restored UCH-L1 and Hexokinase serum expression levels post TBI to those similar to non-injured levels. The combination of both drugs resulted in lower UCHL-1 expression compared to the untreated TBI group. These results demonstrate the neuroprotective role of antiplatelet therapy in the context of TBI which represents the second assumption of the dual role hypothesis. In all treatment conditions prior to TBI, GFAP levels were lower than those of the untreated TBI group, with aspirin even restoring GFAP to non-injured levels. These findings indicate that the aspirin and CLOP use in single or combination may be protective to glial cells as well. We showed that aspirin and CLOP were more protective when administered individually rather than together. We confirmed that αII-spectrin cleavage — an early marker of apoptosis — was increased after TBI in all groups compared to the non-injured control. It is unclear how aspirin or CLOP exert an effect on αII-spectrin

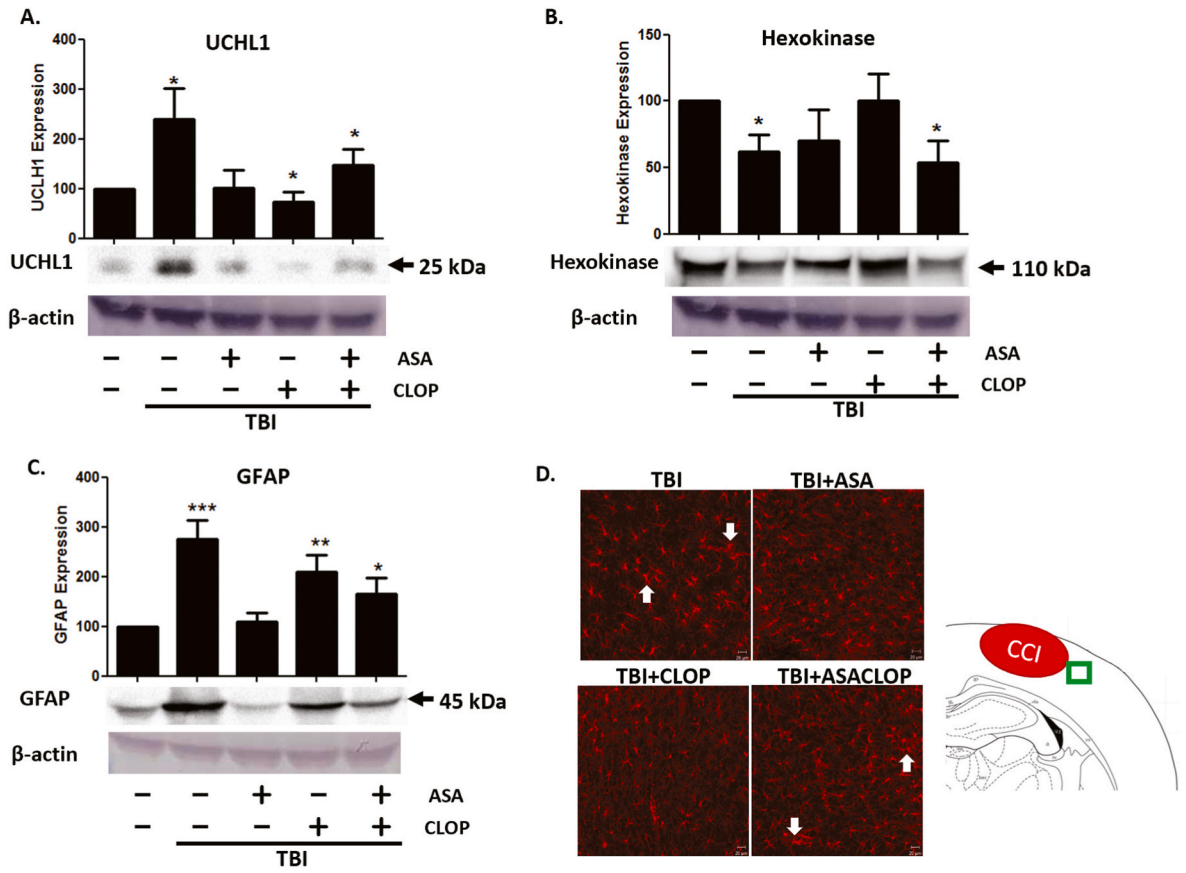

Fig. 3. Aspirin and clopidogrel modulate UCHL1, GFAP and Hexokinase serum protein levels after TBI.
A) Western blot analysis for UCHL1 expression levels with its corresponding quantification. **B**) Analysis for Hexokinase; **C**) GFAP expression. Immunoblottings are representative of five independent experiments performed for each treatment condition and with similar results. The same membrane was blotted for UCHL1 and hexokinase after stripping. The beta actin bands in panels a and b are identical for illustration purposes. The GFAP membrane was blotted separately. Quantification of Western blots is reported as mean ± S.E.M. *, **, *** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$; respectively. Values represent the average fold change expression, normalized to beta actin, and relative to control. **D**) Representative immunofluorescence images of GFAP for the different conditions. The green square on the atlas image represents the area imaged to obtain the represented immunofluorescence images. The white arrows refer to the astrocytes networks forming following TBI. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cleavage. Investigating the involved pathways can explain why clopidogrel treatment was the most potent in inducing αII-spectrin cleavage. In addition, it is interesting to investigate the impact of each drug on other apoptosis markers.

The dual role hypothesis is not an uncommon concept in its essence. Beynon et al. questioned the antiplatelet therapy position as a double-edged sword in head injury. They even suggested that the antiplatelet therapy's neuroprotective effects may be outweighed by the increased bleeding tendency in acute post-TBI haemorrhage (Beynon and Sakowitz, 2013). But it is worth considering the compensation of the ICH risk through platelet transfusion in TBI patients on antiplatelet therapy. Based on a systematic review pertaining to the matter, Thorn et al. concluded the need for randomized controlled trials investigating the effect of platelet transfusion in these patients (Thorn et al., 2019). Intensive-care unit (ICU) patients with TBI are at high risk of developing deep vein thrombosis (DVT) (Praeger et al., 2012) with venous thromboembolism (VTE) occurring in one out of every five patients with TBI treated in the ICU (Skrifvars et al., 2017). Anti-coagulant pharmacological prophylaxis may be effective against these post-TBI complications. Moreover, the benefits of antithrombotic agents in stroke or myocardial infarction prevention should be considered in relation to the risk of haemorrhage in those at increased risk for TBI.

This is the first study to examine the dual impact on altered bleeding and neuronal damage after TBI, with the administration of ASA/CLOP alone or together in a rat model. A strong point of our study is that we did not limit our assessment to a single post-TBI outcome. Alternately, we analyzed biomarkers of different systemic processes like apoptosis, inflammation, and bleeding. Assessing total protein offers distinct advantages over housekeeping genes as it is unbiased with respect to changes in the expression of a single (or multiple) housekeeping protein such as GAPDH (Bass et al., 2017). Moreover, our injury and animal models were suitable to address the study's goals. The histopathological and functional changes induced by the CCI in rats are consistent with what occurs in clinical TBI cases (Osier and Dixon, 2016), making it easier to translate our results clinically. The CCI model provided us with quantitative control over important key TBI parameters, which guaranteed that each rat was subject to the same TBI scale. The inclusion of sham controls allowed establishing control over confounding variables such as the effect of anesthesia or craniectomy on biochemical markers.

This study includes some limitations with the lack of functional outcomes and behavioral tests being the major one. The absence of brain imaging/histological outcomes of the TBI epicenter represents a limitation in this study for clinical translation. A histopathological analysis of the injury area in the brain in the context of each treatment will be considered for future studies. The TBI induced in all of our rats was moderate. It is interesting as well to determine the effect of antiplatelet therapy on severe TBI by modifying the impact's velocity and depth in our CCI model. Moreover, the analysis can be expanded to include a wide variety of apoptosis and inflammation markers. The dose diversity can be increased to allow the comparison of treatments with the same

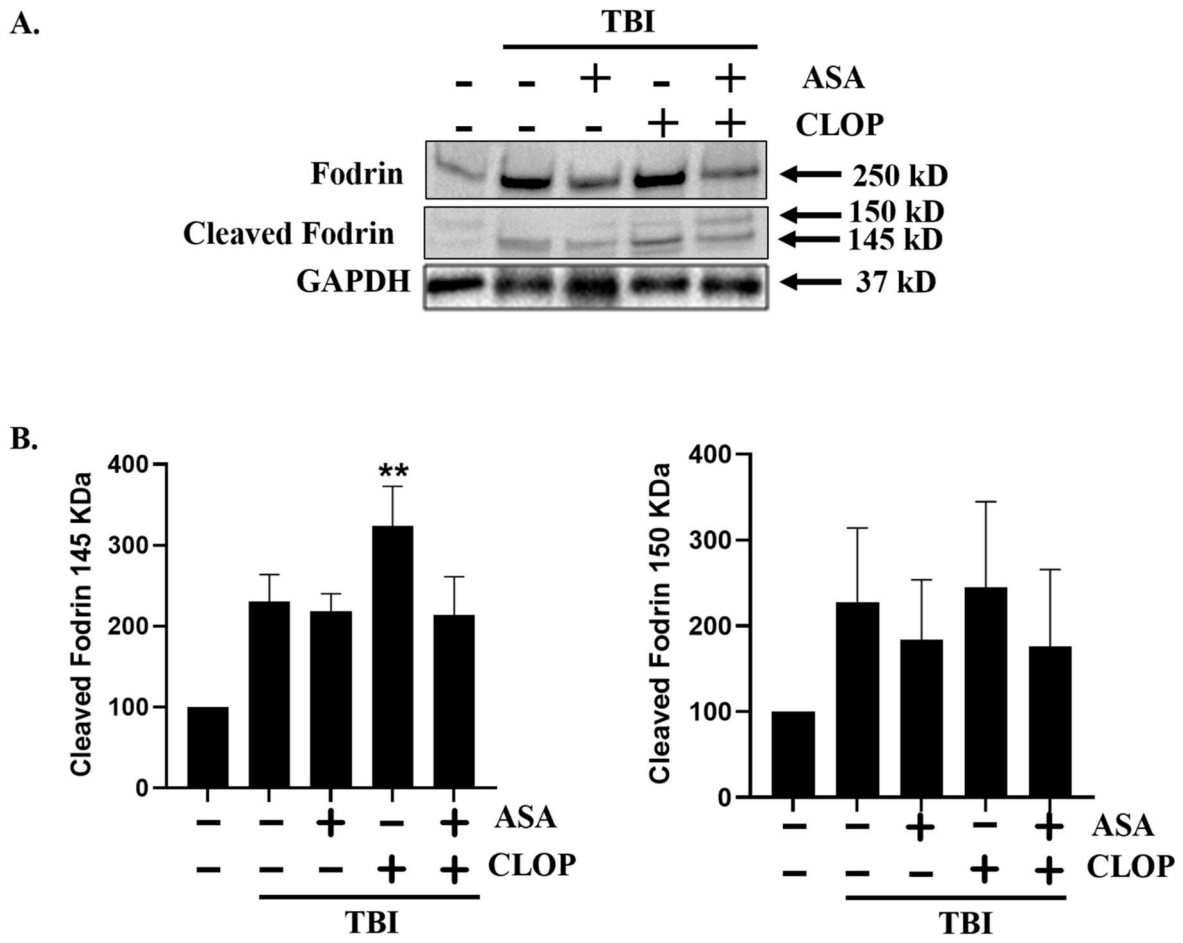

Fig. 4. Aspirin and clopidogrel modulate αII-spectrin/Fodrin protein levels after TBI.
A) Western blot for αII-spectrin/Fodrin. Positions of αII-spectrin/Fodrin and its cleavage products are indicated. GAPDH was used to ensure equal loading of proteins. **B**) Quantification analysis of the 145 and 150 kDa cleaved αII-spectrin/FodrinWestern blots. Values represent the average fold change expression, normalized to GAPDH (which was used as a loading control), and relative to control, and for 5 rats. Data are reported as mean ± S.E.M. ** indicate $p < 0.01$.

drug. Ultimately, it is interesting to assess the impact of anticoagulant drugs or their combination with antiplatelet agents on TBI.

In conclusion, we showed that combined treatment of ASA and CLOP was further detrimental in terms of ICH in an experimental TBI rat model. A higher risk was revealed when both drugs were simultaneously administered. Our results offer new insights into the management of TBI patients. Understanding the relationship between ASA/CLOP and their potential additive or synergistic role in exacerbating the bleeding event would have an impact on the prescription of these drugs. This includes the antiplatelet drug type, its dose, and the administration with other antiplatelets. In light of our results, there should be more caution when prescribing antiplatelet therapy in combination. Especially that the neuroprotective role of antiplatelets in the TBI-context is more evident with the single rather than combination treatment. Thus, the management of TBI patients should be adapted to the type and potency of the anti-platelet regime received by the patient prior to the insult.

Ethics approval and consent to participate

This study involves animals and was approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (approval # 13-05-257). All experiments were conducted in compliance with current Good Clinical Practice standards and in accordance with relevant guidelines and regulations and the principles set forth under the Declaration of Helsinki (1989). There is no human participation and thus consent to participate is not applicable.

Consent for publication

All authors read and approved the manuscript.

Availability of data and material

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

Funding

This work was supported by grants from the Lebanese National Council for Scientific Research (PI:FK), and by the American University of Beirut-Medical Center Practice Plan (MPP).

Author contributions

FK, AH, HD and EH conceptualize the study. EH, LN, ZD, FD, MN, GEA, NR, ZM and KM performed experiments. FK, KM, SM, WM, and AH prepared Figures. AH, EH, and FK designed the study. AH, EH, FD, WM, KZ, KM, and FK analyzed data and wrote the manuscript. All authors reviewed the manuscript.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgment

Authors would like to acknowledge the Lebanese National Council for Scientific Research and the American University of Beirut; Medical Practice Plan (MPP) for providing funding. Fig. S2 was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2022.105301.

References

Abou-Abbass, H., Bahmad, H., Abou-El-Hassan, H., Zhu, R., Zhou, S., Dong, X., Hamade, E., Mallah, K., Zebian, A., Ramadan, N., Mondello, S., Fares, J., Comair, Y., Atweh, S., Darwish, H., Zibara, K., Mechref, Y., Kobeissy, F., 2016. Deciphering glycomics and neuroproteomic alterations in experimental traumatic brain injury: comparative analysis of aspirin and clopidogrel treatment. Electrophoresis 37, 1562–1576.

Ahmed, N., Bialowas, C., Kuo, Y.H., Zawodniak, L., 2009. Impact of preinjury anticoagulation in patients with traumatic brain injury. South. Med. J. 102, 476–480.

Albrecht, J.S., Liu, X., Baumgarten, M., Langenberg, P., Rattinger, G.B., Smith, G.S., Gambert, S.R., Gottlieb, S.S., Zuckerman, I.H., 2014. Benefits and risks of anticoagulation resumption following traumatic brain injury. JAMA Intern. Med. 174, 1244–1251.

Bass, J.J., Wilkinson, D.J., Rankin, D., Phillips, B.E., Szewczyk, N.J., Smith, K., Atherton, P.J., 2017. An overview of technical considerations for Western blotting applications to physiological research. Scand. J. Med. Sci. Sports 27, 4–25.

Beynon, C., Sakowitz, O.W., 2013. Antiplatelet therapy: a double-edged sword in head injury? Crit. Care 17, 135.

Brazinova, A., Rehorcikova, V., Taylor, M.S., Buckova, V., Majdan, M., Psota, M., Peeters, W., Feigin, V., Theadom, A., Holkovic, L., Synnot, A., 2021. Epidemiology of traumatic brain injury in Europe: a living systematic review. J. Neurotrauma 38, 1411–1440.

Brophy, G., Mondello, S., Robicsek, S., Gabrielli, A., Papa, L., Tepas, J., Buki, A., Wang, K., Hayes, R., 2009. Biokinetic analysis of ubiquitin C-terminal hydrolase (Uch-L1) in severe traumatic brain injury patient biofluids. J. Neurotrauma 26. A14-A14.

Brun, C., Daali, Y., Combescure, C., Zufferey, A., Michelson, A.D., Fontana, P., Reny, J.L., Frelinger 3rd, A.L., 2016. Aspirin response: differences in serum thromboxane B2 levels between clinical studies. Platelets 27, 196–202.

Collaborators, G.B.D.N., 2019. Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 18, 459–480.

Dewan, M.C., Rattani, A., Gupta, S., Baticulon, R.E., Hung, Y.C., Punchak, M., Agrawal, A., Adeleye, A.O., Shrime, M.G., Rubiano, A.M., Rosenfeld, J.V., Park, K.B., 2018. Estimating the global incidence of traumatic brain injury. J. Neurosurg. 1–18.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Fabbri, A., Servadei, F., Marchesini, G., Bronzoni, C., Montesi, D., Arietta, L., Societa Italiana di Medicina d'Emergenza Urgenza Study, G., 2013. Antiplatelet therapy and the outcome of subjects with intracranial injury: the Italian SIMEU study. Crit. Care 17, R53.

Fakhry, S.M., Morse, J.L., Garland, J.M., Wilson, N.Y., Shen, Y., Wyse, R.J., Watts, D.D., 2021. Antiplatelet and anticoagulant agents have minimal impact on traumatic brain injury incidence, surgery, and mortality in geriatric ground level falls: a multi-institutional analysis of 33,710 patients. J Trauma Acute Care Surg 90, 215–223.

Floyd, C.N., Goodman, T., Becker, S., Chen, N., Mustafa, A., Schofield, E., Campbell, J., Ward, M., Sharma, P., Ferro, A., 2014. Increased platelet expression of glycoprotein IIIa following aspirin treatment in aspirin-resistant but not aspirin-sensitive subjects. Br. J. Clin. Pharmacol. 78, 320–328.

Foerch, C., You, Z., Wang, H., Lo, E.H., Whalen, M.J., 2012. Traumatic brain injury during warfarin anticoagulation: an experimental study in mice. J. Neurotrauma 29, 1150–1155.

Gaetani, P., Revay, M., Sciacca, S., Pessina, F., Aimar, E., Levi, D., Morenghi, E., 2012. Traumatic brain injury in the elderly: considerations in a series of 103 patients older than 70. J. Neurosurg. Sci. 56, 231–237.

Gallet, C., Rosa, J.P., Habib, A., Lebret, M., Levy-Toledano, S., Maclouf, J., 1999. Tyrosine phosphorylation of cortactin associated with Syk accompanies thromboxane analogue-induced platelet shape change. J. Biol. Chem. 274, 23610–23616.

Gangavati, A.S., Kiely, D.K., Kulchycki, L.K., Wolfe, R.E., Mottley, J.L., Kelly, S.P., Nathanson, L.A., Abrams, A.P., Lipsitz, L.A., 2009. Prevalence and characteristics of traumatic intracranial hemorrhage in elderly fallers presenting to the emergency department without focal findings. J. Am. Geriatr. Soc. 57, 1470–1474.

Giachini, F.R., Leite, R., Osmond, D.A., Lima, V.V., Inscho, E.W., Webb, R.C., Tostes, R. C., 2014. Anti-platelet therapy with clopidogrel prevents endothelial dysfunction and vascular remodeling in aortas from hypertensive rats. PLoS One 9, e91890.

Good, R.I., McGarrity, A., Sheehan, R., James, T.E., Miller, H., Stephens, J., Watkins, S., McConnachie, A., Goodall, A.H., Oldroyd, K.G., 2015. Variation in thromboxane B2 concentrations in serum and plasma in patients taking regular aspirin before and after clopidogrel therapy. Platelets 26, 17–24.

Hasan, Ç., Zeynep, N.O., Cem, O., E Nursen, K., Serdar, Y.M.Ç., 2018. Neuroprotective effects of dalteparin on experimental traumatic brain injury in rats. J. Anesth. Inten. Care Med.

Hirz, T., Khalaf, A., El-Hachem, N., Mrad, M.F., Abdallah, H., Creminon, C., Gree, R., Merhi, R.A., Habib, A., Hachem, A., Hamade, E., 2012. New analogues of 13-hydroxyocatdecadienoic acid and 12-hydroxyeicosatetraenoic acid block human blood platelet aggregation and cyclooxygenase-1 activity. Chem. Cent. J. 6, 152.

Ivascu, F.A., Howells, G.A., Junn, F.S., Bair, H.A., Bendick, P.J., Janczyk, R.J., 2008. Predictors of mortality in trauma patients with intracranial hemorrhage on preinjury aspirin or clopidogrel. J. Trauma 65, 785–788.

Joseph, B., Sadoun, M., Aziz, H., Tang, A., Wynne, J.L., Pandit, V., Kulvatunyou, N., O'Keeffe, T., Friese, R.S., Rhee, P., 2014. Repeat head computed tomography in anticoagulated traumatic brain injury patients: still warranted. Am. Surg. 80, 43–47.

Kidson-Gerber, G., Weaver, J., Gemmell, R., Prasan, A.M., Chong, B.H., 2010. Serum thromboxane B2 compared to five other platelet function tests for the evaluation of aspirin effect in stable cardiovascular disease. Heart Lung Circ. 19, 234–242.

King, M.D., Laird, M.D., Ramesh, S.S., Youssef, P., Shakir, B., Vender, J.R., Alleyne, C.H., Dhandapani, K.M., 2010. Elucidating novel mechanisms of brain injury following subarachnoid hemorrhage: an emerging role for neuroproteomics. Neurosurg. Focus 28, E10.

Kobeissy, F.H., Ottens, A.K., Zhang, Z., Liu, M.C., Denslow, N.D., Dave, J.R., Tortella, F. C., Hayes, R.L., Wang, K.K., 2006. Novel differential neuroproteomics analysis of traumatic brain injury in rats. Mol. Cell. Proteomics 5, 1887–1898.

Kobeissy, F.H., Sadasivan, S., Oli, M.W., Robinson, G., Larner, S.F., Zhang, Z., Hayes, R. L., Wang, K.K., 2008. Neuroproteomics and systems biology-based discovery of protein biomarkers for traumatic brain injury and clinical validation. Proteonomics Clin. Appl. 2, 1467–1483.

Lecuyer, M.A., Kebir, H., Prat, A., 2016. Glial influences on BBB functions and molecular players in immune cell trafficking. Biochim. Biophys. Acta 1862, 472–482.

Ma, N., Liu, X.W., Yang, Y.J., Shen, D.S., Zhao, X.L., Mohamed, I., Kong, X.J., Li, J.Y., 2016. Evaluation on antithrombotic effect of aspirin eugenol ester from the view of platelet aggregation, hemorheology, TXB2/6-keto-PGF1alpha and blood biochemistry in rat model. BMC Vet. Res. 12, 108.

McCammack, K.C., Sadler, C., Guo, Y., Ramaswamy, R.S., Farid, N., 2015. Routine repeat head CT may not be indicated in patients on anticoagulant/antiplatelet therapy following mild traumatic brain injury. West. J. Emerg. Med. 16, 43–49.

Mondello, S., Linnet, A., Buki, A., Robicsek, S., Gabrielli, A., Tepas, J., Papa, L., Brophy, G.M., Tortella, F., Hayes, R.L., Wang, K.K., 2012. Clinical utility of serum levels of ubiquitin C-terminal hydrolase as a biomarker for severe traumatic brain injury. Neurosurgery 70, 666–675.

Mondello, S., Papa, L., Buki, A., Bullock, M.R., Czeiter, E., Tortella, F.C., Wang, K.K., Hayes, R.L., 2011. Neuronal and glial markers are differently associated with computed tomography findings and outcome in patients with severe traumatic brain injury: a case control study. Crit. Care 15, R156.

Mondello, S., Schmid, K., Berger, R.P., Kobeissy, F., Italiano, D., Jeromin, A., Hayes, R.L., Tortella, F.C., Buki, A., 2014. The challenge of mild traumatic brain injury: role of biochemical markers in diagnosis of brain damage. Med. Res. Rev. 34, 503–531.

Moore, M.M., Pasquale, M.D., Badellino, M., 2012. Impact of age and anticoagulation: need for neurosurgical intervention in trauma patients with mild traumatic brain injury. J Trauma Acute Care Surg 73, 126–130.

Mrozek, S., Delamarre, L., Capilla, F., Al-Saati, T., Fourcade, O., Constantin, J.M., Geeraerts, T., 2019. Cerebral expression of glial fibrillary acidic protein, ubiquitin carboxy-terminal hydrolase-L1, and matrix metalloproteinase 9 after traumatic brain injury and secondary brain insults in rats. Biomark. Insights 14, 1177271919851515.

Nadeau, C.A., Dietrich, K., Wilkinson, C.M., Crawford, A.M., George, G.N., Nichol, H.K., Colbourne, F., 2019. Prolonged blood-brain barrier injury occurs after experimental intracerebral hemorrhage and is not acutely associated with additional bleeding. Transl Stroke Res 10, 287–297.

Nylen, K., Ost, M., Csajbok, L.Z., Nilsson, I., Blennow, K., Nellgard, B., Rosengren, L., 2006. Increased serum-GFAP in patients with severe traumatic brain injury is related to outcome. J. Neurol. Sci. 240, 85–91.

Osier, N., Dixon, C.E., 2016. The controlled cortical impact model of experimental brain trauma: overview, research applications, and protocol. Methods Mol. Biol. 1462, 177–192.

Peck, K.A., Calvo, R.Y., Schechter, M.S., Sise, C.B., Kahl, J.E., Shackford, M.C., Shackford, S.R., Sise, M.J., Blaskiewicz, D.J., 2014. The impact of preinjury anticoagulants and prescription antiplatelet agents on outcomes in older patients with traumatic brain injury. J Trauma Acute Care Surg 76, 431–436.

Plantier, D., Luaute, J., group, S., 2016. Drugs for behavior disorders after traumatic brain injury: systematic review and expert consensus leading to French recommendations for good practice. Ann. Phys. Rehabil. Med. 59, 42–57.

Pradelles, P., Grassi, J., Maclouf, J., 1985. Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: an alternative to radioimmunoassay. Anal. Chem. 57, 1170–1173.

Praeger, A.J., Westbrook, A.J., Nichol, A.D., Wijemunige, R., Davies, A.R., Lyon, S.M., Wills, J.L., Bailey, M., Rosenfeld, J.V., Cooper, D.J., 2012. Deep vein thrombosis and pulmonary embolus in patients with traumatic brain injury: a prospective observational study. Crit. Care Resusc. 14, 10–13.

Ren, C., Kobeissy, F., Alawieh, A., Li, N., Li, N., Zibara, K., Zoltewicz, S., Guingab-Cagmat, J., Larner, S.F., Ding, Y., Hayes, R.L., Ji, X., Mondello, S., 2016. Assessment of serum UCH-L1 and GFAP in acute stroke patients. Sci. Rep. 6, 24588.

Roozenbeek, B., Maas, A.I., Menon, D.K., 2013. Changing patterns in the epidemiology of traumatic brain injury. Nat. Rev. Neurol. 9, 231–236.

Sangkuhl, K., Klein, T.E., Altman, R.B., 2010. Clopidogrel pathway. Pharmacogenetics Genom. 20, 463–465.
Schoemaker, R.G., Saxena, P.R., Kalkman, E.A., 1998. Low-dose aspirin improves in vivo hemodynamics in conscious, chronically infarcted rats. Cardiovasc. Res. 37, 108–114.
Shahim, P., Politis, A., Van der Merwe, A., Moore, B., Ekanayake, V., Lippa, S., Chou, Y., Pham, D., Butman, J., Diaz-Arrastia, R., Zetterberg, H., Blennow, K., Gill, J., Brody, D., Chan, L., 2021. Time course and diagnostic utility of Nfl, Tau, GFAP, and UCH-L1 in Subacute and chronic TBI. Neurology 96, 593.
Skrifvars, M.B., Bailey, M., Presneill, J., French, C., Nichol, A., Little, L., Duranteau, J., Huet, O., Haddad, S., Arabi, Y., McArthur, C., Cooper, D.J., Bellomo, R., investigators, E.-T., the, A.C.T.G., 2017. Venous thromboembolic events in critically ill traumatic brain injury patients. Intensive Care Med. 43, 419–428.
Sobue, K., Yamamoto, N., Yoneda, K., Hodgson, M.E., Yamashiro, K., Tsuruoka, N., Tsuda, T., Katsuya, H., Miura, Y., Asai, K., Kato, T., 1999. Induction of blood-brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. Neurosci. Res. 35, 155–164.
Thorn, S., Guting, H., Mathes, T., Schafer, N., Maegele, M., 2019. The effect of platelet transfusion in patients with traumatic brain injury and concomitant antiplatelet use: a systematic review and meta-analysis. Transfusion 59, 3536–3544.
Tongaonkar, P., Chen, L., Lambertson, D., Ko, B., Madura, K., 2000. Evidence for an interaction between ubiquitin-conjugating enzymes and the 26S proteasome. Mol. Cell Biol. 20, 4691–4698.
Umemura, K., Ishihara, H., Nakashima, M., 1995. Anti-platelet effects of clopidogrel in rat middle cerebral artery thrombosis model. Thromb. Res. 80, 209–216.
Wang, K.K.W., Kobeissy, F.H., Shakkour, Z., Tyndall, J.A., 2021. Thorough overview of ubiquitin C-terminal hydrolase-L1 and glial fibrillary acidic protein as tandem biomarkers recently cleared by US Food and Drug Administration for the evaluation of intracranial injuries among patients with traumatic brain injury. Acute Med Surg 8, e622.
Zhang, W., Smith, C., Howlett, C., Stanimirovic, D., 2000. Inflammatory activation of human brain endothelial cells by hypoxic astrocytes in vitro is mediated by IL-1beta. J. Cerebr. Blood Flow Metabol. 20, 967–978.