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Deciphering glycomics and neuroproteomic alterations in experimental traumatic brain injury: Comparative analysis of aspirin and clopidogrel treatment

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Abstract

As populations age, the number of patients sustaining traumatic brain injury (TBI) and concomitantly receiving preinjury antiplatelet therapy such as aspirin (ASA) and clopidogrel (CLOP) is rising. These drugs have been linked with unfavorable clinical outcomes following TBI, where the exact mechanism(s) involved are still unknown. In this novel work, we aimed to identify and compare the altered proteome profile imposed by ASA and CLOP when administered alone or

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in combination, prior to experimental TBI. Furthermore, we assessed differential glycosylation PTM patterns following experimental controlled cortical impact model of TBI, ASA, CLOP, and ASA + CLOP. Ipsilateral cortical brain tissues were harvested 48 h postinjury and were analyzed using an advanced neuroproteomics LC-MS/MS platform to assess proteomic and glycoproteins alterations. Of interest, differential proteins pertaining to each group (22 in TBI, 41 in TBI + ASA, 44 in TBI + CLOP, and 34 in TBI + ASA + CLOP) were revealed. Advanced bioinformatics/ systems biology and clustering analyses were performed to evaluate biological networks and protein interaction maps illustrating molecular pathways involved in the experimental conditions. Results have indicated that proteins involved in neuroprotective cellular pathways were upregulated in the ASA and CLOP groups when given separately. However, ASA + CLOP administration revealed enrichment in biological pathways relevant to inflammation and proinjury mechanisms. Moreover, results showed differential upregulation of glycoproteins levels in the sialylated N-glycans PTMs that can be implicated in pathological changes. Omics data obtained have provided molecular insights of the underlying mechanisms that can be translated into clinical bedside settings.

Keywords

Aspirin; Clopidogrel; Neuroproteomics; PTMs; TBI

1 Introduction

Geriatric traumatic brain injury (TBI) has been and will continue to be a burden to any society [1]. It is widely known that the elderly population is susceptible to an increased risk of falling and sustaining a head trauma. Such kind of injuries, affecting the central nervous system (CNS), is associated with substantial patient mortality and everlasting disability [2]. Moreover, about 40% of the aged population suffers from several comorbidities including, but not limited to, cardiovascular diseases that may require an overwhelming prescription of different medications together with an antiplatelet or anticoagulant therapy. Nearly one fifth of the geriatric population managed for head trauma have been found to be on warfarin, hence complicating the condition and yielding mixed outcomes following TBI [1].

Two of the most widely used antiplatelet therapies include aspirin (ASA) and clopidogrel (CLOP). ASA irreversibly inhibits the platelets from producing thromboxane A₂ through acetylation of the cyclo-oxygenase-1 enzyme at Ser-529 residue [3] while CLOP acts by binding specifically and irreversibly to the platelet P2RY₁₂ purinergic receptor, inhibiting ADP-mediated platelet activation and aggregation [4]. Numerous studies assessing the impact of antiplatelet agents on TBI have been proposed. Ahmad et al. has shown that anticoagulation treatments do not have any impact on mortality irrespective of the anticoagulant type [5], whereas Gaetani et al. [6] and Ivascu et al. [7] have reported a significant increase in mortality rates following TBI in patients on antiplatelet therapy. Moreover, Fabbri et al. have reported that the use of antiplatelet drugs increased the risk of mortality by two folds. This risk was particularly high in subjects on CLOP treatment, independent of the association with ASA [8]. Similarly, antiplatelet drugs have been shown to substantially attenuate hemostatic functions through their association with increased

intracranial hemorrhage rates and/or progression; situations that result in an increased need for neurosurgical intervention [9–12]. On the contrary, a protective role of ASA following TBI has been proposed by Gangavati et al. [13] and Thaler et al. [14]. Finally, it is noteworthy to be stated that TBI-induced coagulopathy is shown to be associated with platelet dysfunction independent of antiplatelet medication. This has been shown by Davis et al. and Ploplis et al.'s work where they reported that a significant decrease in the platelet thrombotic activity is noticed in responses to ADP and arachidonic acid in TBI rat research models when compared to controls [15, 16].

A number of neuroproteomic studies have been conducted to study the mechanism(s) of injury post-TBI. The proteomic alterations in mitochondrial proteins have been evaluated by one study by Opii et al. where the role of oxidative stress following TBI has been highlighted upon [17]. Other studies have focused on biomarker discovery with little emphasis on mechanistic role elucidation [18, 19]. However, not many studies have assessed the role of post-translational modifications (PTMs) in the process of TBI especially glycosylation and glycoprotein investigation, which have been known to be highly implicated in neural functions [20]. On the other hand, studies evaluating PTMs induced by antiplatelet therapy in the realm of TBI are still rare. Floyd et al. [3] and Finamore et al. [21] conducted several studies concerning the alteration that ASA and CLOP induce on PTMs. Floyd et al. has demonstrated that glycoprotein IIIa (A isoform) expression has been increased in lysed platelets [3]. Besides, a significant increase in the acetylation rate and a significant decrease in glycation have been reported by Finamore et al. [21]. Dual antiplatelet therapy has been found to alter the expression of several proteins including but not limited to actin-binding protein isotopes 2 and 5, lactate dehydrogenase, and serotransferrin isotype 4 [22].

In our study, we compared the neuroproteomic profiles of four conditions (ASA versus CTRL; CLOP versus CTRL; ASACLOP versus CTRL and TBI versus CTRL (SHAM)) and evaluated changes on the glycomic-PTM level. An advanced systems biology platform was used to study biological processes, molecular functions, and unique protein interaction networks associated with these experimental conditions in an attempt to understand the underlying pathways associated with ASA and CLOP administration following TBI. To the best of our knowledge, this study is among the first ones to assess the effect of ASA and/or CLOP administration with TBI. Data from this work can be translated in clinical conditions highlighting the detrimental effect of these drugs following clinical head injuries.

2 Materials and methods

2.1 Animal housing conditions

Experiments were performed on adult male Sprague–Dawley rats (250–300 g) in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals) and approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (AUB). Rats were maintained and housed under pathogen-free conditions with constant temperature and humidity control at the AUB Animal Care Facility (ACF). Surgical procedures were performed under deep

anesthesia. Postoperative behavioral and body weight monitoring was conducted during the light phase of the cycle by a researcher blind to the treatment conditions.

2.2 In vivo model of experimental TBI

A controlled cortical impact (CCI) device was used to model TBI in rats as described previously [23, 24]. Briefly, adult male (250–300 g) Sprague–Dawley rats were anaesthetized with a mix of xylazine (100 mg/kg) and ketamine (10 mg/kg). Animals were supported in a stereotactic frame in a prone position and secured by ear and incisor bars. A midline scalp incision was made and the skin was retracted from the skull surface. Thereafter, a midline cranial incision was made, the soft tissues exposed, and a unilateral (ipsilateral to the site of impact) craniotomy (7 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda. Using the software associated with the device, a target site between bregma and lambda was set. Impact was induced by a 2 mm diameter impactor with a velocity of 4 m/s and a depth of 1 mm to produce moderate TBI. The impact duration was 0.8 s, after which the surgical site was sutured. Sham-injured control animals were exposed to an identical surgical procedure but did not receive an impact injury.

2.3 Drugs administration

Acetyl salicylic acid (ASA, Sigma, USA) was reconstituted in ethanol and then diluted with saline to the desired concentration, before being injected intraperitoneally (i.p.) at a dose of 30 mg/kg [25–27]. Clopidogrel (CLOP, Sigma, USA) was purchased as tablets (75 mg), crushed, dissolved in saline and given orally at a dose of 10 mg/kg [28, 29].

2.4 Experimental design

A total of 12 rats were divided into four groups of three rats each. Each rat was given daily treatments of: saline/sham (i.p.), ASA (30 mg/kg, i.p.), CLOP (10 mg/kg, Orally), or both ASA (30 mg/kg, i.p.), and CLOP (10 mg/kg, Orally). Two days subsequent to ASA and/or CLOP administration (either alone or together), rats were subjected to experimental TBI (CCI). Ipsilateral cortical brain tissue samples were harvested 48 h postinjury and kept at –80°C for further analysis.

2.5 Rat brain tissue harvesting

Following sedation with 5% isoflurane, all experimental groups were sacrificed by decapitation at 48 h post-CCI. Brains were isolated and dissected in PBS on ice; the area surrounding the lesion epicenter on the ipsilateral cortex was rapidly collected and stored at –80°C for further neuroproteomics analysis.

2.6 Chemicals

DTT, iodoacetamide (IAA), ammonium bicarbonate (ABC), sodium deoxycholate (SDC), DMSO, sodium hydroxide beads (20–40 mesh), iodomethane, and MS-grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade water was acquired from Mallinckrodt Chemicals (Phillipsburg, NJ). HPLC grade acetonitrile was acquired from J. T. Baker (Phillipsburg, NJ). Trypsin/Lys-C mix, MS grade was obtained from Promega

(Madison, WI) and PNGase F (Glycerol-free, 500 000 units/mL) from New England Biolab (Ipswich, MA).

2.7 Extraction and tryptic digestion of proteins

Brain tissue samples (30–100 mg) were washed with 200 μ L of 50 mM ABC buffer to remove blood. An extraction buffer (5% SDC) was then added to the sample at a 1:10 w/v ratio. Next, the samples were homogenized using a Bead-bug microtube homogenizer (Benchmark Scientific, Edison, NJ). Briefly, three to five triple high impact zirconium beads ($\varnothing$: 3.0 mm) were mixed with each brain tissue sample and extraction buffer in a 2 mL microtube. Homogenization was performed six times at 40 000 rounds per minute for 3 min with a 30 s rest in between. The homogenate was centrifuged at 21 000 g for 10 min. The supernatant was collected and denatured at 80°C for 30 min. SDC concentration was diluted to 0.5% with 50 M ABC buffer.

The extracted protein concentration was determined by BCA protein assay (Thermo Pierce). A 25- μ g aliquot of proteins was reduced by adding DTT to a final concentration of 5 mM and incubated for 45 min at 60°C. The mixture was alkylated by adding IAA to a final concentration of 20 mM and incubated in the dark for 45 min. The reaction was quenched with another addition of 5 mM DTT. Proteolytic digestion was performed by addition of Trypsin/Lys-C mixture (Promega, Madison, WI), 1:25 enzyme to protein ratio and incubated at 37.5°C for 18 h. The digestion was quenched, and the SDC was precipitated by adding 1% v/v neat formic acid. The mixture was centrifuged at 21 000 g for 10 min. The supernatant was collected, vacuum dried and kept at –20°C. The samples were resuspended in solvent A (2% ACN and 0.1% formic acid in HPLC water) immediately before LC-MS injection.

For glycomic profiling, a 200 μ g aliquot of proteins was prepared. Five hundred units of PNGase F were added to each of the samples, followed by incubation at 37°C water bath for 18 h. After PNGase F digestion, detergents were removed by acidic precipitation using the protocol described above. After that, glycans were further purified by protein precipitation and dialysis. Ninety percent ice-cold ethanol aqueous solution was used to precipitate proteins and a home-made dialysis device fitted with 500–1000 Da MWCO dialysis membrane was used to dialyze glycan samples. Purified N-glycans were then reduced and permethylated following our routine protocol [30, 31]. Briefly, dried reduced glycans were mixed with 0.3 μ L of water, 30 μ L of DMSO and 20 μ L iodomethane, and the mixture was incubated in a spin column packed with sodium hydroxide beads for 40 min. Thereafter, another 20 μ L iodomethane was added after 20 min of incubation. After derivatization, dried samples were resuspended in 20 μ L of 20% CAN and 0.1% formic acid in HPLC water before LC-MS/MS analysis.

2.8 Untargeted LC-MS/MS analysis

LC-MS/MS was performed using Dionex 3000 Ultimate nano-LC system (Dionex, Sunnyvale, CA) interfaced to LTQ Orbitrap Velos (Thermo Scientific, San Jose, CA) equipped with nano-ESI source. The separation was attained using Acclaim PepMap RSLC columns (75 μ m i.d. $\times$ 15 cm, 2 μ m particle sizes, 100 Å pore sizes, Dionex, Sunnyvale, CA)

with a flow rate of 350 nL/min. For the analysis of protein digest mixture, 1 µg of protein digest was injected. During the separation, the column compartment was maintained at 29.5°C. The LC elution gradient of solvent B used in both LC-MS/MS analysis was: 5% over 10 min, 5–20% over 55 min, 20–30% over 25 min, 30–50% over 20 min, 50–80% over 1 min, 80% over 4 min, 80–5% over 1 min and 5% over 4 min. Solvent B consisted of 100% ACN with 0.1% formic acid, while solvent A was composed of 2% ACN with 0.1% formic acid added to HPLC grade water.

The LTQ Orbitrap Velos mass spectrometer was operated in positive ion-mode with the ESI voltage set to 1600 V. Data-dependent acquisition mode was employed to achieve two scan events. The first scan event was a full MS scan of 400–2000 m/z at a mass resolution of 60 000. The second scan event was CID MS/MS of precursor ions selected from the first scan event with an isolation width of 3.0 m/z , at normalized collision energy of 35%, and a Q activation value of 0.250. The CID MS/MS scans were performed on the ten most intense ions observed in the MS scan event. The dynamic exclusion was set to have a repeat count of 2, repeat duration of 30 s, exclusion list size of 200 and exclusion duration of 90 s. A 60-min LC method was used for the separation of permethylated glycan on the C18 column. The multigradient utilized for elution was as follows: 20% mobile phase B from minute 0–10, then the percentage of B was brought up to 38 in 1 min, after that B was elevated to 60% in 30 min, then a 10-min 90% mobile phase B push was employed. Finally, the percentage of B was brought back to 20. The same parameters were set for nano-ESI, as described above. Full MS was operated at 15 000 resolution and a scan range was set to 700–2000 m/z . MS2 were conducted in data-dependent acquisition mode. Top 4 intensity precursor ions from full scan were subjected to both CID and HCD. Those MS2 spectrums assist further confirmation of glycan structures.

2.9 LC-MS/MS data analysis

For the analysis of protein digest, LC-MS/MS raw data were transformed to mascot generic format file (*.mgf) by Proteome Discover version 1.2 software (Thermo Scientific, San Jose, CA) were searched using UniProt database (2015_06, Rattus, 7937 entries) in MASCOT version 2.4 (Matrix Science Inc., Boston, MA). IAA modification of cysteine was set as a fixed modification while oxidation of methionine was set as a variable modification. The MS, MS/MS, and m/z tolerance, were set to 6 ppm and 0.8 Da, representatively. Maximum peptide missed cleavage value was set to 2. MASCOT search result was validated and quantified by spectral counting using Scaffold (version Scaffold_3.6.3, Proteome Software Inc., Portland, OR). Peptide identifications were accepted at greater than 95.0% probability as specified by the Peptide Prophet algorithm [32]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [33]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The normalized spectral count was used to quantify the relative abundance of each identified protein among different samples. An independent *t*-test was performed to evaluate the statistical significance. Proteins showed a significant difference ($p < 0.05$) in spectral counts, and quantitation results were further investigated by a systems biology analysis.

The glycomics data were processed by MultiGlycan [34, 35]. The mass tolerance was set to 5 ppm; isotope envelope tolerance was set to 15 ppm, and a minimum number of peaks in a cluster were set to 3. Peaks corresponding to glycans in different adduct forms and charge states were accumulated to represent the intensity of each glycan.

2.10 Multiple reaction monitoring (MRM) transitions design for targeted quantitation of proteins

The proteins, which were determined by untargeted analysis to be significantly altered as a result of TBI, were further evaluated using the following procedure. Targeted quantitative proteomics using LC-MRM experiment was applied to a set of proteins, including transthyretin, ceruloplasmin, myelin proteolipid protein, serine protease inhibitor A3K, haptoglobin, hemopexin, protein S100-B, protein RUFY3, contactin-1, dynactin subunit 2, actin-related protein 3, E3 ubiquitin-protein ligase UBR4, and mitochondrial import inner membrane translocase subunit TIM44. Peptides and transitions were selected using Pinpoint (version 1.4.0, Thermo Scientific). For each targeted protein, one or two associated peptide were selected using the following rules [36]: (1) identified in the untargeted analysis with a Scaffold probability greater than 99%, (2) completely tryptically digested peptide, (3) contains seven to 25 amino acid residues, (4) excluding the first 25 amino acids at the N-terminus of proteins, (5) excluding peptides with M, RP, KP, and glycosylation site (NXS/T), (6) excluding peptides with ragged ends on both C- and N- terminus (tryptic peptides cleaved between R/K, K/R, R/R, and K/K), and (7) peptides that contains cysteines were fixed modified by carbamidomethylation. Next, three transitions for each selected peptide were determined using the following rules: (1) precursor ions with charge states of +2 or +3, (2) y series of fragment ions greater than y3 with a charge state of 1, (3) the three most intense y ions in the MS/MS spectra from untargeted analysis, and (4) m/z values of precursor and transition ions are between 300 and 1200. A complete list of the MRM transitions used here is provided in Supporting Information Table 1.

2.11 LC-MRM-MS analysis

The same LC condition for peptide separation described previously in the untargeted analysis was used in the LC-MRM-MS analysis. A 3- μ g aliquot of peptide mixture derived from previous tryptic digestion was injected. The TSQ Vantage mass spectrometer (Thermo Scientific) was operated in in positive mode with an ESI voltage of 1.8 kV. Data-independent acquisition mode was used for MRM experiment. Selected precursors and transition ions list was imported into the acquisition setting with a cycle time of 6.0 s. The chromatogram filter peak width was set to 10.0s; the Q1 FWHM was set to 0.7 Da, and the collision gas pressure was 1.5 mTorr in Q2. The collision energy for each precursor was predicted by Pinpoint using the following equation: $CE(+2) = 0.034 * m/z + 3.314$ (eV), $CE(+3) = 0.044 * m/z + 3.314$ (eV).

2.12 LC-MRM-MS data analysis

The LC-MRM-MS data was analyzed using Pinpoint (version 1.4.0, Thermo Scientific). Peptide peak areas were calculated by the peak area summation of its corresponding transitions. Protein quantitative value was determined by summing the peak areas of its

corresponding peptides. An independent *t*-test was performed to evaluate the statistical significance of the values determined for each experimental group versus control.

2.13 Bioinformatics, subnetwork enrichment pathway analyses, and statistical testing

The Elsevier's Pathway Studio version 9.0 (Ariadne Genomics/Elsevier) was used to deduce relationships among differentially expressed protein candidates using the Ariadne ResNet database [37,38]. "Subnetwork Enrichment Analysis" (SNEA) algorithm was selected to extract statistically significant altered biological and functional pathways pertaining to each identified set of protein hits among each experimental group. Fisher's statistical test was utilized by SNEA to determine if there are nonrandom associations between two categorical variables organized by specific relationship. SNEA operates in the following manner. First, a central "seed" from all relevant entities in the database is created, and retrieving associated entities are retrieved based on their relationship with the seed (i.e. binding partners, expression targets, protein modification targets, and regulation). The subnetwork distribution is compared to the background distribution using one-sided Mann-Whitney *U* test, and a *p*-value is calculated indicating the statistical significance between two distributions. In our analysis, "GenBank ID and gene symbols from each set were imported to the software to form an experimental dataset. For the reconstruction of networks of pathways, both biological processes and molecular function were evaluated for each single protein hit and its associated targets (networks and pathways) [39, 40]. Integrated Venn diagram analysis was performed using "the InteractiVenn" that is a web-based tool for the analysis of complex datasets. Categorization of proteins by molecular function and biological processes was performed using Protein Analysis through Evolutionary Relationship (PANTHER) system.

3 Results

3.1 Differential protein expression among the different groups

In our study, a neuroproteomics platform analysis was used to identify differences in the protein expression pattern in each of the four conditions: ASA versus Ctrl, CLOP versus Ctrl, ASACLOP versus Ctrl, and TBI versus Ctrl. A global protein expression profile for each of the four groups was performed in comparison to the control (Fig. 1). First, in the ASA versus Ctrl group, 41 differentially expressed proteins (21 upregulated and 20 downregulated proteins) were revealed by protein expression analysis as shown in Fig. 1A and Supporting Information Table 2. Second, for the CLOP versus Ctrl group, 44 altered proteins (25 upregulated and 19 downregulated) were shown by protein expression analysis (Fig. 1B and Supporting Information Table 3). Third, in the ASACLOP versus Ctrl group, 34 proteins were found to be altered (16 upregulated and 18 downregulated) (Fig. 1C and Supporting Information Table 4). Finally, protein expression analysis of the TBI versus Ctrl group was shown to have 22 altered proteins (five upregulated and 17 downregulated) (Fig. 1D and Supporting Information Table 5). In this study, it was hypothesized that specific functional neuroproteins can undergo differential splicing and PTMs such as glycosylation, which is known to impact protein turnover and localization, protein interactions, signaling pathways, and cell division. Thus, an MS/MS analysis was used for peptide sequencing and protein identification. In Supporting Information Fig. 1 the MS/MS analyses for eight of the differentially expressed proteins was demonstrated in our experimental conditions and the

amino acid sequence for each of these proteins was shown along with the mass difference of the reference peptide of each of these proteins.

3.2 Unique and common upregulated and downregulated proteins among the different groups

In order to further scrutinize the differences that each drug has at the cellular level in TBI, unique and common upregulated and/or downregulated proteins were looked for among the four different groups. Through neuroproteomics platform analysis, several unique upregulated proteins were revealed in each group (16 proteins in ASA versus Ctrl, 19 in CLOP versus Ctrl, 12 in ASACLOP versus Ctrl, and 16 in TBI versus Ctrl) (Fig. 2A), and uniquely downregulated proteins were also seen in each group (14 proteins in ASA versus Ctrl, 10 in CLOP versus Ctrl, 13 in ASACLOP versus Ctrl, and 5 in TBI versus Ctrl) (Fig. 2B). Interestingly, some of the differentially expressed proteins were also found to be commonly expressed among the different experimental groups (Fig. 2). Indeed, three proteins were identified to be upregulated in both “ASA versus Ctrl” and “CLOP versus Ctrl” groups, two proteins between “ASA versus Ctrl” and “ASACLOP versus Ctrl,” one protein between “CLOP versus Ctrl” and “TBI versus Ctrl,” and two between “CLOP versus Ctrl” and “ASACLOP versus Ctrl” (Fig. 2A and Table 1A). Besides, four proteins were identified to be commonly downregulated in “ASA versus Ctrl” and “CLOP versus Ctrl” groups, three proteins between “CLOP versus Ctrl” and “ASACLOP versus Ctrl,” and two between “ASA versus Ctrl,” “CLOP versus Ctrl” and “ASACLOP versus Ctrl” (Fig. 2B and Table 1B). Interestingly, some proteins were found to be upregulated in one group and downregulated in another. For example, importin subunit β -1 was upregulated in the TBI group but downregulated in the ASACLOP group. In addition, actin-related protein 3 was upregulated in the TBI group, it was however downregulated in the CLOP group. Collectively, our data suggest that many of the differentially expressed proteins in the ASA and CLOP groups were found to be neuroprotective against cellular injury following experimental brain trauma as compared to the TBI cohort as shown in Table 2. However, in the ASACLOP group, which combines the two drugs ASA and CLOP, prosurvival functions were exhibited by some of the altered proteins while in other cells cell death mechanisms were reflected. Such mechanisms are similar to what is observed in TBI. Hence, analysis of the molecular pathways related to those proteins was performed.

3.3 Confirmation and validation of differentially altered proteins among the different groups via LC-MRM-MS

In order to confirm our results and to strengthen our data confidence, LC-MRM-MS was performed on seven randomly selected proteins, namely actin-related protein 3, mitochondrial import inner membrane translocase subunit TIM44, serine protein inhibitor A3K, ceruloplasmin, transthyretin, hemopexin, and haptoglobin. MRM technique is considered among the best confirmatory platforms for MSMS results comparable to other immune biochemical assays such as Western blotting and enzyme essays. This is due to the MRM technique’s high sensitivity, specificity, accuracy, assay speed, and sample throughput for quantification of peptides [41] in complex biological samples [42]. MRM was used by Chambers et al. for quantifications of 97 proteins in dried blood samples, and it was reported that this assay method was efficient in achieving targeted protein quantification and is

suitable for biomedical research application [43]. Likewise, in Silajdzic et al. MRM to antibody-based techniques was compared for detection of inflammatory biomarkers in Huntington's disease and it was reported that their findings were correlated with utilized clinical measures [44]. In our confirmatory analysis, it was revealed that actin-related protein 3 and mitochondrial import inner membrane translocase subunit TIM44 were found to be decreased in the TBI group when compared to the CTRL group (Supporting Information Fig. 2A and B). Serine protein inhibitor A3K, ceruloplasmin, and transthyretin were found to be increased in the ASA group compared to the CTRL group (Supporting Information Fig. 2C, D, and E). Hemopexin and haptoglobin were found to be significantly elevated in the CLOP group compared to the CTRL group (Supporting Information Fig. 2F and G). Remarkably, MRM data show strong agreement with the normalized spectral count quantitation (Supporting Information Fig. 2).

3.4 Enrichment pathways analyses among the different groups

To further understand the effect of antiplatelet drugs (ASA, CLOP, or ASACLOP) at the cellular level, analysis of the specific enriched pathways was performed. Our results revealed that several altered proteins were associated with different biological pathways among the groups, some of which were neuroprotective while others reflected injury-inducing mechanisms (Supporting Information Fig. 3). These combined pathways were subcategorized using a Venn diagram to indicate the number of "Shared" versus "Unique" pathways involved (Supporting Information Fig. 4). Of those pathways, many were unique to each of the four studied groups (58 in ASA versus Ctrl, 45 in CLOP versus Ctrl, 66 in ASACLOP versus Ctrl, and 58 in TBI versus Ctrl); yet some pathways were also found to be shared and common among the different groups as shown in Supporting Information Fig. 4, Supporting Information Tables 6 and 7. Interestingly, pathways influenced by treatment with ASA + CLOP treatment encompassed functions related to nerve injury and cell death, but when ASA was administered alone, results showed activation of pathways that promote DNA protection and cell survival against apoptosis was shown.

3.5 Glycosylation modifications and their relation to neuronal injury

To quantify the glycomic changes of each group, LC-ESI-MS experiments were performed on a total of 58 detected N-glycans. Differential quantitative glycosylation PTM patterns were observed among the groups, as shown in Supporting Information Fig. 5. Of the detectable species, six high mannose glycoforms were detected (Supporting Information Fig. 5A). There was a lesser number of high mannose N-glycans in the treated groups compared to the control except for GlcNAc2Man9. This is particularly significant for GlcNAc2Man5 species. Terminal mono-, di-, tri-, and tetrasialylation of N-glycans (Supporting Information Fig. 5B, GlcNAc4Man3Gal2Sia2, GlcNAc4Man3Gal2Fuc1Sia2, and GlcNAc4Man4Gal2Fuc1Sia1 among the high abundant structures) were found to be increased in the treated groups, in comparison to controls. This was also significant in the CLOP group in the high abundant structures based on the calculated independent *t*-test with *p*-value less than 0.005 (Supporting Information Table 8). However, in the medium and low abundant structures (Supporting Information Fig. 5C and D), it was significant to the ASACLOP and TBI groups. It is worth noting that N-glycan composition GlcNAc3Man4Gal1Fuc1Sia1 was an exception, as it was shown to be almost the same for

CLOP and control groups. Similarly, GlcNAc4Man3Gal2Sia1 and GlcNAc5Man3Gal3Sia1 were found to be increased in the control group compared to the other treated groups.

Overall, the N-glycan compositions elevated in the experimental groups compared to controls were of the complex type, possessing either a terminal galactose or GlcNAc. The composition GlcNAc4Man3Gal2 terminating in galactose is increased in all experimental groups. Similarly, GlcNAc6Man3Fuc1, GlcNAc6Man3Gal1Fuc1, GlcNAc4Man3Gal1Fuc1, GlcNAc4Man3Gal2Fuc1, and GlcNAc5Man3Gal2Fuc1 are shown to follow the same trend. Examples of extracted ion chromatograms of two detected glycans and their annotated MS/MS tandem are shown in Supporting Information Figs. 6 and 7.

4 Discussion

TBI represents a major public health issue worldwide in terms of mortality and morbidity. Several factors contribute to the increased mortality in TBI concerning the management or exposure to drug therapy. Among these factors is the administration of antiplatelet drugs. It has been reported that elevated mortality rates in TBI patients are observed in those maintained on antiplatelet medications, and this leads to an alteration in the clinical outcomes [5, 6, 14]. However, the pathological mechanism(s) involved remain obscure. In this study, a rat experimental TBI model coupled with ASA and/or CLOP administration has been used. In order to elucidate molecular mechanistic pathways, cortical brain tissues from experimental cohorts have been assessed for changes in their protein expression levels by applying an advanced neuroproteomic/systems biology platform. In addition, glycosylation PTMs have also been studied, due to their pivotal contribution in controlling neural functions and their putative role in mediating pathogenesis [45, 46]. A principal component analysis (PCA) has also been performed on the spectral count data to investigate internal variation among the different treated groups (Fig. 3A). PCA plots have revealed that the different experimental and control groups parted from each other. Indeed, the data points of the different-treated groups have seemed to be far-apart from the control group suggesting that these treatments have an effect different from controls. Similarly, PCA analysis performed on glycoproteins has shown that the samples parted from each other (Fig. 3B).

In the ASA-treated group, four out of the 21 upregulated proteins have been found to be unique to ASA: T-kininogen 2, ceruloplasmin, transthyretin, and transaldolase. Studies done in vitro to determine the role of the activation of the kallikrein-kinin system have demonstrated an increased buildup of kininogen and vasoactive kinins, that are responsible for the development of brain edema and vascular injury following spinal cord injury [47, 48]. Interestingly, inconsistent data on the selective blockade of β_2 receptor, receptor for bradykinin, and other kinins, using Hoe140 have been reported, which was not the case for β_1 receptor [49, 50]. Likewise, C1-inhibitor, a serine protease inhibitor, is reported as one of the potential therapeutic approaches due to its role in inhibiting the early pathways of the kallikrein-kinin cascade [51]. Interventions targeting these mechanisms show a promising neuroprotective role in improving the outcome and recovery post-TBI. On the other hand, the exceptional upregulation of ceruloplasmin protein indicates a neuroprotective role in brain injury. Ceruloplasmin is an effective antioxidant in the CNS that protects neural cells from oxidative stress in vitro [52, 53]. This antioxidant function of ceruloplasmin is mostly

vital during CNS injury with elevated reactive oxygen species (ROS) [54]. In addition, transthyretin protein has been implicated with neuroprotective functions in mouse models of brain injury and ischemic stroke [55]. Transthyretin acts a carrier for transportation of both thyroid hormones and retinol. Data suggests that CSF transthyretin induces a protective mechanism against neuronal cell death and inflammation [55]. As for the transaldolase, a key enzyme in the pentose phosphate pathway, it has been shown to be responsible for NADPH generation for maintenance of reduced glutathione [56]. Our results show high expression of this protein in the ASA group. This validates what has been previously mentioned about a protective role of ASA in brain injury, as it is well known that transaldolase protects from cellular damage that is induced by different ROS [56].

Likewise, in the group of rats supplied with oral CLOP, several proteins have shown to be uniquely and highly expressed such as haptoglobin, hemopexin, and protein S100-B. Studies on these proteins validate their neuroprotective role at cellular levels following brain injury [57–60]. The endogenous hemoglobin-binding protein in blood plasma, haptoglobin, has been found to be upregulated after intracranial hemorrhages where it aids in reducing potential secondary injury [57]. Besides, studies conducted on mouse models in vitro have revealed that hemopexin has neuroprotective roles following stroke and intracerebral hemorrhage by protecting neurons against heme or ROS toxicity [58, 59]. Additionally, analysis of the significance of S100B in acute brain injury uncovers the beneficial effect of this protein in decreasing neuronal injury and contributing to repair after TBI as well as being a candidate biomarker of TBI occurrence [60]. On the other hand, fascin protein, an actin-binding protein implicated in different disease pathologies [61–63], has been found to be downregulated in the CLOP group. It has been shown that this protein is weakly expressed following brain ischemia, thus proposing a potential role in ischemic tolerance [62]. Using an in vitro model, Meller et al., showed that loss of fascin promotes actin filament reorganization that could be involved in the acquisition of rapid ischemic tolerance [63]. In accordance, downregulation of fascin in the CLOP group endorses a certain possible role of CLOP in TBI through triggering alteration in the protein expression profile, and this favors a neuroprotective role. Recent studies have reported the use of microRNA (miR)-133a in the inhibition of fascin [64, 65].

Further analysis has revealed low expression of actin-related protein 3 in the CLOP group that was, however, upregulated in the TBI group. A high percentage of the proteins that are expressed after brain injury are actin-related proteins. The Rho family takes part in the regulation of the actin cytoskeleton in both neuronal and nonneuronal cells [66]. It has been shown that inhibition of Rho family members, such as Rho kinase, improves axonal regeneration of the injured neurons [67]. Inhibition of the Rho kinase, which is involved in actin regulation, has been found to enhance CNS axonal regeneration postinjury [68]. Thus, low expression of actin-related protein 3 in the CLOP group, in contrast to its high levels in TBI group, may be attributed to the inhibition of the Rho pathway and thus define a protective role in CLOP.

Upon combination of ASA and CLOP together, neuroproteomics analysis has identified a subgroup of proteins, some of which were neuroprotective while others demonstrated cell death functions. For example, Contactin-1 has been shown to interact specifically with the

disulfated chondroitin sulfate E to induce neurite outgrowth [69]. Chondroitin sulfate has been known to play a critical role in CNS development and regeneration [70]. Our results have displayed upregulation of this protein in the ASACLOP group, which explains a prosurvival function of contactin-1 in this setting. Additionally, importin subunit β -1 protein, was highly expressed in the TBI group that did not receive any antiplatelet drug but showed low expression in the ASACLOP group. Importins have been reported to play pivotal roles in nuclear transport processes [71]. Otis et al. proved that following injury, mRNA's of importin β have been found to be elevated in the distal segments of the injured axons, thus indicating importin involvement in delayed regenerative outgrowth in vitro [72]. This is in accordance with what was previously mentioned about a possible neuroprotective function of both ASA and CLOP in TBI, where low expression of importin subunit β -1 has been established in the ASACLOP group compared to the TBI group.

Nonetheless, not only does ASA have been demonstrated to act on peripheral tissues but it has also been shown to act on the CNS by crossing the blood–brain barrier (BBB). Besides ASA, the minimal transfer of CLOP across an intact BBB, may explain, in part, the observed results independent of brain injury [73]. In fact, ASA has been reported to cause a remarkable decrease in periventricular glial cytoplasmic sulfur-rich Gomori-positive material [74]. Similarly, it has also been reported to upregulate ciliary neurotrophic factor (CNTF) in astrocytes through inducing the activation of cAMP-response element-binding protein [75]. The same study has also reported that ASA-induced the astroglial CNTF mice, but not CNTF null mice in increasing the expression of the oligodendrocytes myelin-associated proteins that protected them from the effects of TNF- α [75]. Another study has shown that acetylsalicylic acid inhibited prostaglandin E2 synthesis in primary rat microglia and this pinpoints to the protective role of ASA [76].

Analysis of protein networks using enriched pathways has revealed several altered proteins associated with different biological pathways. Upon treatment with ASA alone, enriched pathways have included cell survival, DNA fragmentation, and DNA protection (Supporting Information Fig. 3A). These three pathways help in promoting cell survival against apoptosis. Similarly, TBI resulted in the activation of pathways involving lipid degradation and protein targeting, in addition to the activation of inflammatory cytokines and phagocytosis (Supporting Information Fig. 3D). However, our results have also shown enrichment of pathways related to cell blebbing, mitochondrial damage, nerve injury, and nuclear fragmentation in the group of rats receiving ASA and CLOP together (Supporting Information Fig. 3C). This indicates that simultaneous use of ASA with CLOP may have mediated proinjury neural pathways. This is in agreement with what has been mentioned in clinical studies that ASA + CLOP administration corroborates a worse prognosis among trauma patients who are on combined antiplatelet therapy [77–79]. For instance, retrospective cohorts by Moore et al. and Ivascu et al. assessing trauma in elderly patients have shown that combination antiplatelet therapy (ASA + CLOP) has been associated with increased mortality rate and a higher need for neurosurgical intervention [78, 79].

On the glycoprotein level, it has been reported that the high mannose form of intercellular adhesion molecule-1 (ICAM-1) is less abundant whereas the sialylated counterpart is elevated in the cerebrospinal fluid of TBI patients [80]. Also, mannose-binding lectin

(MBL), a component of innate immunity, has been documented to be upregulated in TBI patients and has been proved to be an independent predictor of mortality and unfavorable outcomes [81,82]. These studies have also shown that MBL is associated with the severity and outcome postinjury and its modulation through therapeutic interventions confers long-lasting protection [83, 84]. Kizuka et al. has reported that the loss of branched O-mannose glycan on protein tyrosine phosphatase-beta (RPTP β) promotes the recovery process after myelin injury in the brain [85]. In this study, orthogonal glycomic analysis has confirmed an approximately equal decrease in the levels of high mannose N-glycans in the experimental groups compared to the control (Supporting Information Fig. 5A). This indicates that the reduction of mannose-rich glycans has shown to facilitate recovery and neuronal survival postinjury. This mechanism might have served to counteract the induced injury by hiding mannose-rich epitopes that, when present, trigger leukocyte rolling and extravasation, eventually leading to inflammation. Nevertheless, these adhesion molecules that aid in leukocyte homing to sites of injury, are subsequently subjected to the posttranslational addition of N-glycans. The extent of modification and how glycosylation contributes to cellular rolling and adhesion remains largely understudied.

Sialic acids were reported to function as an antioxidant by scavenging free radicals due to their strong negative charge [86, 87]. They were also reported to reduce organic peroxides and derivatives of arachidonic acid in addition to decreasing the cytotoxic effects of ROS [88, 89]. Recent evidence showed that the hypersialylated erythropoietin was able to reduce TNF- α -induced ROS and activation of JNK and MSK1, upstream kinase of NFkB [90]. Several sialic acid-containing glycosphingolipid species, such as monosialoganglioside GM1, were reported to prevent neuronal death whereas asialo GM1 (lacking sialic acid) was ineffective [91]. These findings highlight the importance of sialic acid in promoting neural survival. Serum sialic acid was also proposed to be used as a biomarker to evaluate the severity of brain injury [92]. Our results showed a significant increase in the mono-, di-, tri-, and tetrasialylation of N-glycans especially in the ASA + CLOP group suggesting its role in counteracting the trauma-induced damaging pathways.

Neuroproteomic analysis is rapidly emerging as an important tool for deciphering the complex mechanisms of human diseases. Of particular interest, the integration of proteomics-systems biology into the field of neurological diseases and brain trauma would specifically enable researchers to identify novel diagnostic markers indicative of disease progression and severity. Our study has applied an extra dimension of neuroproteomics in identifying global glycan moiety changes post-ASA/CLOP-TBI experimental condition. This work represents the first of its kind to evaluate the differential protein expression, cellular pathways, and PTMs following experimental TBI in animals maintained on combined or individual antiplatelet therapy. Interestingly, we have found that ASA or CLOP administered alone presents a neuroprotective role evident through the differentially expressed protein profile and the associated protein interaction networks. However, protein enrichment and pathway analysis for cortical samples in rats administered both ASA and CLOP have reflected a mixed neuroprotective functions accompanied by several biological pathways related to neural cell injury and neuronal damage, which may partly explain clinical data after such treatment. Finally, our differential glycosylation PTMs profile observed among the different groups may have contributed to the unfavorable clinical

outcomes post-TBI in patients on combined ASA and CLOP antiplatelet therapy. This work offers promising explanations on the underlying mechanisms following TBI and may offer better insights to the actual role of ASA and/or CLOP in this context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ASA	aspirin
BBB	blood–brain barrier
CCI	controlled cortical impact
CLOP	clopidogrel
CNS	central nervous system
CNTF	ciliary neurotrophic factor
IAA	iodoacetamide
i.p.	intraperitoneally
MRM	multiple reaction monitoring
PCA	principal component analysis
PTMs	post-translational modifications
ROS	reactive oxygen species
SDC	sodium deoxycholate
SNEA	subnetwork enrichment analysis
TBI	traumatic brain injury

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Figure 1. Heat map representation of the neuroproteomics analysis of the differentially expressed proteins among the ASA, CLOP, ASACLOP, and TBI groups, in comparison to control. Hierarchical clustering analysis of protein expression profiles. (A) Hierarchical clustering analysis of the differentially expressed proteins between ASA and the Ctrl groups. (B) Hierarchical clustering analysis of the differentially expressed proteins between CLOP and the Ctrl groups. (C) Hierarchical clustering analysis of the differentially expressed proteins between ASACLOP and the Ctrl groups. (D) Hierarchical clustering analysis of the differentially expressed proteins between TBI and the Ctrl groups. Each column represents one sample, and each horizontal line refers to a protein. Color legend is on the bottom-right of the figure. Red indicates proteins with a greater expression relative to the geometrical means; green indicates proteins with a lower expression relative to the geometrical means.

Figure 2. Venn diagram analysis depicting shared and unique protein hits among the experimental groups. Venn diagrams showing the number of common and unique proteins detected in the four different groups of study: ASA versus Ctrl, CLOP versus Ctrl, ASACLOP versus CTRL, and TBI versus Ctrl. (A) The common and unique upregulated proteins. Statistically significant unique proteins were identified including 16 proteins (ASA versus CTRL); 19 proteins (CLOP versus CTRL); 12 proteins (ASACLOP versus CTRL); and 16 proteins (TBI versus CTRL). (B) The common and unique downregulated proteins. Statistically significant unique proteins were identified including: 14 proteins (ASA versus CTRL); 10 proteins (CLOP versus CTRL); 13 proteins (ASACLOP versus CTRL); and five proteins (TBI versus CTRL). This work was done using “the InteractiVenn” software.

Figure 3. Principal component analysis (PCA) corresponding to glycomics changes and protein data among the experimental groups. (A) PCA corresponding to glycomics changes. Data points of Ctrl (green rectangle), ASA (red diamond), CLOP (purple triangle), TBI (blue circle), and combination of ASA and CLOP (white square) are shown. The number following the hyphen indicates the replicate number. (B) PCA corresponding to protein data. Data points

of Ctrl (blue triangle), ASA (blue circle), CLOP (red square), TBI (green diamond), and combination of ASA and CLOP (white triangle) are shown.

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Table 1

Common upregulated and downregulated differentially altered proteins between the different groups. (A) In the upregulated group, three proteins were identified to be upregulated in “ASA versus Ctrl” and “CLOP versus Ctrl,” two proteins between “ASA versus Ctrl” and “ASACLOP versus Ctrl,” one protein between “CLOP versus Ctrl” and “TBI versus Ctrl,” and two proteins between “CLOP versus Ctrl” and “ASACLOP versus Ctrl.” (B) In the downregulated group, four proteins were identified to be downregulated in “ASA versus Ctrl” and “CLOP versus Ctrl,” three proteins between “CLOP versus Ctrl” and “ASACLOP versus Ctrl,” and two proteins between “ASA versus Ctrl,” “CLOP versus Ctrl” and “ASACLOP versus Ctrl”

Upregulated/Downregulated protein	Accession number	p-value
<i>A. Upregulated proteins</i>		
“ASA versus Ctrl” and “CLOP versus Ctrl”		
• Serine protease inhibitor A3N	P09006	0.00026
• Alpha-1-inhibitor 3	P14046	0.00734
• Serotransferrin	P12346	0.00113
“ASA versus Ctrl” and “ASACLOP versus Ctrl”		
• F-actin-capping protein subunit alpha-1	B2GUZ5	0.00481
• Sodium channel protein type 2 subunit alpha	P04775	0.00615
“CLOP versus Ctrl” and “TBI versus Ctrl”		
• 26S proteasome non-ATPase regulatory subunit 11	O88761	2.59×10^{-8}
“CLOP versus Ctrl” and “ASACLOP versus Ctrl”		
• 60S ribosomal protein L12	P23358	0.00192
<i>B. Downregulated proteins</i>		
“ASA versus Ctrl” and “CLOP versus Ctrl”		
• E3 ubiquitin-protein ligase UBR4	Q2TL32	0.00745
• Rab GDP dissociation inhibitor alpha	P50398	0.00804
• Mitochondrial import inner membrane translocase subunit TIM44	O35094	0.0066
• Protein NDRG3	Q6AYR2	0.00815
“CLOP versus Ctrl” and “ASACLOP versus Ctrl”		
• Leucine-rich PPR motif-containing protein, mitochondrial	Q5SGE0	0.00173
• Ketimine reductase mu-crystallin	Q9QYU4	0.00834
• 26S protease regulatory subunit 4	P62193	0.00023
“ASA versus Ctrl” and “CLOP versus Ctrl” and “ASACLOP versus Ctrl”		
• Glutaredoxin-1	Q9ESH6	0.00222
• Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	P63088	1.58×10^{-5}

Table 2

List of some of the upregulated differentially expressed proteins in the ASA, CLOP, ASACLOP, and TBI groups that were found to be neuroprotective against cellular injury following experimental TBI

Protein	Experimental group	Neuroprotective role	References
Transthyretin	ASA	Transthyretin in the CSF regulates neuronal cell death, inflammation, and edema through protective mechanisms in mouse models of brain injury and ischemic stroke	[55]
Transaldolase	ASA	A major enzyme in the pentose phosphate pathway that is responsible for NADPH generation, maintenance of reduced glutathione, and guarding integrity of cells against reactive oxygen intermediates	[56]
Ceruloplasmin	ASA	Upregulation of ceruloplasmin has a neuroprotective role in brain injury as it protects neural cells from oxidative stress in vitro and acts as an effective antioxidant in the CNS	[52,53]
Myelin proteolipid protein	ASA	Myelin proteolipid aids paranodal axonal cytoskeletal organization of individual myelin internodes and offers axonal tropic support	[93]
Serine protease inhibitor A3K	ASA	Serine protease inhibitor A3K protects retinal neuronal cells and Muller cells from oxidative stress-induced cells death, through blocking of the calcium overload induced by the PLC pathway	[94]
Haptoglobin	CLOP	Haptoglobin is an endogenous Hb-binding protein present in the blood plasma that is upregulated in hematoma-affected brain following intracerebral hemorrhage to reduce potential secondary injury	[57]
Hemopexin	CLOP	Hemopexin guards neurons in vitro against heme or reactive oxygen species (ROS) toxicity in mouse models subjected to stroke and intracerebral hemorrhage	[58,59]
Protein S100-B	CLOP	S100-B has a key role in reducing neuronal injury and contributing to repair after TBI	[60]
Ras-related protein Rap-2b	CLOP	Ras family small GTPases as RAS and RAP-2 are activated after ischemic insults and serve as intrinsic switches to regulate survival and regeneration of neurons	[95]
Protein RUFY3	CLOP	RUFY3 is involved in single axon formation of growing neurons by inhibiting PI3K in minor neuronal processes and preventing the development of more axons	[96]
Contactin-1	ASACLOP	Contactin-1 has a critical role in CNS development and regeneration by interacting with the disulfated chondroitin sulfate E (CS-E) to induce neurite outgrowth	[69,70]
Dynactin subunit 2	ASACLOP	Dynactin subunit 2 triggers dynein-mediated retrograde axonal transport and augments microtubule stability via anti-catastrophe mechanism	[97]
Glutaredoxin-1	TBI	Glutaredoxin has a role in protecting neurons in the cerebellum from dopamine-induced apoptosis by activating the Ras/PI3K/Akt and JNK/AP-1 pathways, thus stimulating nuclear factor kappa B (NF-B)	[98]
Actin-related protein 3	TBI	Actin-related protein 2/3 complex is involved in formation of lamellipodia and filopodia as it is a major component of the actin-based protrusion in neuronal growth cones and it also sways neuritogenesis during neuronal differentiation.	[99]
E3 ubiquitin-protein ligase UBR4	TBI	It is incorporated in regulating mitochondrial morphology, activating mitochondrial self-repair mechanisms, and preventing oxidative damage to mtDNA	[100,101]
Mitochondrial import inner membrane translocase subunit TIM44	TBI	Tim44 associates the Tim23 complex and mortalin, which is a stress response protein involved in protecting cells and maintaining neuronal health against neurodegeneration through calcium channel regulation	[102,103]