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Peripheral microRNA alteration and pathway signaling after mild traumatic brain injury

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Abstract. Discovering novel diagnostic biomarkers and signatures for traumatic brain injury (TBI) represents a major challenge in the brain trauma research. Detailed analysis of post-concussive molecular pathways based on experimental data could provide a new insight into the pathophysiological sequelae and mapping of recovery mechanisms involved in TBI. MicroRNAs (miRNAs) detectable in peripheral body fluids after TBI are promising carriers of this missing knowledge. In order to define the signature of peripheral miRNAs signaling associated with mild TBI (mTBI), we performed a comprehensive meta-analysis of miRNA profiles in mTBI patients using multiple curated pathway databases. Using a bioinformatic pipeline with integrated data analysis we identified a set of genes that are connected to deregulated circulating miRNAs following the mTBI. Identified genes belong to specific pathways of MAPK, TGF-β, WNT, TLR2/4, PI3K/AKT, insulin, and growth factor signaling. Since the enriched pathways markedly overlap among the various biological fluids, signaling associated with mTBI that is concomitantly reflected in serum, plasma and saliva is robust and unique. Furthermore, we identified a network of 33 validated interacting proteins and their regulatory miRNAs that link the post-mTBI signaling in peripheral fluids with neurodegeneration-associated interaction pathways. Presented data provide a comprehensive insight into molecular events following mTBI, and the top predicted genes represent a group of novel candidate targets to be validated in connection with mTBI.

Key words: Mild traumatic brain injury — TBI — miRNA — Signaling pathways — Meta-analysis

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Introduction

Traumatic brain injury (TBI) is defined by a disruption of normal brain function as a reaction to external physical force (Menon et al. 2010). The estimated incidence of TBI *per* 100 000 people represents 948 cases in Asia, 1012 cases in Europe and the numbers are even higher (1299 cases) in North America (Dewan et al. 2019). Patients with mild TBI (mTBI) represent majority of the hospital-treated adult head injuries (70–90%); however, the overall numbers are likely underestimated since many mild head injuries remain unreported and thus undiagnosed (Cassidy et al. 2004; McCrea et al. 2017). Evaluation by the Glasgow coma scale (GCS) is a widely used diagnostic approach to determine the severity of TBI by observing patient's motor, eye, and verbal responses (Teasdale and Jennett 1976). The score of 13–15 refers to mTBI, 9–12 points to moderate TBI, and less than 8 to severe TBI (Iankova 2006). In addition to GCS, computed tomography (CT) and magnetic resonance imaging (MRI) are the most available and used neuroimaging techniques in the clinical management of brain injury (Shetty et al. 2016). However, the CT and MRI conclude very often normal findings reporting no abnormalities in mTBI patients (Iverson et al. 2000; Wintermark et al. 2015). Typically, most patients after mTBI or sport-related concussion fully recover after 7–10 days, although this period can be prolonged in children and adolescents (McCrory et al. 2005).

Recent research indicates that TBI may increase the risk for the development of neurodegenerative disorders evolved due to longitudinal sequelae of concussion or repetitive sub-concussive head impacts (Bailes et al. 2013; Manley et al. 2017; Alosco et al. 2018). For instance, single moderate, single severe TBI or even repeated mild head injuries are associated with the development of chronic traumatic encephalopathy (CTE) as it was reported in professional contact sport athletes (Omalu et al. 2005; McKee et al. 2009; Smith et al. 2013; Omalu and Hammers 2021). Moreover, the risk of Alzheimer's disease has been reported as 1.5 times higher following head trauma (Li et al. 2017). Similarly, the increased risk of Parkinson's disease has been described in military personnel suffering various severities of TBI (Gardner et al. 2018). Additional to the neurodegeneration, head trauma has a significant impact on mental health of TBI individuals as well. Patients with TBI frequently suffer from sleep disturbance (Gilbert et al. 2015) and psychiatric disorders including depression (Jorge et al. 1993), aggression (Gallant et al. 2018) or posttraumatic stress disorder (Bryant and Harvey 1998).

Although it is not possible to accurately predict the consequences of TBI, peripheral biomarkers could represent a valuable diagnostic and prognostic asset. In particular, the group of peripheral microRNAs (miRNAs) has been shown to be associated with mTBI (Redell et al. 2010; Hicks et al. 2020b) and various neurodegenerative disorders (Sheinerman et al. 2017; Siedlecki-Wullich et al. 2019; Uwatoko et al. 2019). The diagnostic potential of miRNAs originates from their general abundance, resistance against degradation, stability in different biofluids (Keller et al. 2017) and sensitive methods of detection. Furthermore, their regulatory role at post-transcriptional level enables identification of specific disease patterns and molecular signaling pathways associated with pathophysiological processes known to be involved in neurodegenerative diseases.

In the present study, we present a meta-analysis of circulating miRNA profiles in patients experiencing mTBI. Using an integrated bioinformatic pipeline we aimed to identify the molecular signature of candidate target genes, associated signaling pathways and involved protein interactions in the mTBI cascade. Special emphasis was placed on clarifying the link between post-concussive signaling and induction of neurodegeneration-associated interaction pathways. This approach helps to improve our understanding of the molecular events present in the brain and eventually mirrored in peripheral fluids during early post-traumatic period and to discover the novel candidate markers/targets associated with mTBI.

Methods

Search strategy, selection criteria and data extraction

A systematic search of relevant studies describing the altered miRNA expression levels in human peripheral biofluids of patients diagnosed with mTBI was performed in the PubMed database using the following query: ("traumatic brain injury"[Title/Abstract] OR "traumatic brain injuries"[Title/Abstract] OR "TBI"[Title/Abstract] OR "head trauma"[Title/Abstract] OR "head traumas"[Title/Abstract] OR "concussion"[Title/Abstract] OR "concussions"[Title/Abstract] OR "head impact"[Title/Abstract] OR "head impacts"[Title/Abstract]) AND ("microRNA"[Title/Abstract] OR "microRNAs"[Title/Abstract] OR "miRNA"[Title/Abstract] OR "miRNAs"[Title/Abstract] OR "mirna biomarker"[Title/Abstract] OR "mirna biomarkers"[Title/Abstract]). In total, 220 articles published before December 31, 2020, were identified.

The inclusion criteria for the articles were as follows: (1) original research, (2) mTBI patients clinically diagnosed according to GCS ≥ 13, (3) miRNA expression study in human peripheral biofluids, (4) acute post-TBI time <15 days. First, the titles and abstracts of searched articles were screened, followed by review of full texts to assess the data eligibility. Articles were excluded based on the following criteria: (1) not TBI or miRNA-related articles, (2) reviews,

meta-analyses, observational studies, or reports, (3) tissue or animal and cell experiments, (4) studies not published in English. Second, after full-text evaluation, articles were excluded if the severity of TBI was not determined with the GCS at all or did not meet a criterion of GCS score 13–15 that defines mTBI (Iankova 2006). Additionally, the articles with unclear regulation of miRNA (data constraints) or mixed mild to severe TBI cohorts, as well as those describing the longer post injury time (>15 days after the injury) or studies without a non-TBI cohort as a control group, were excluded (Fig. 1). The search strategy resulted in 8 articles included in the meta-analysis (Redell et al. 2010; Yang et al. 2016; Di Pietro et al. 2017; Qin et al. 2018; Sun et al. 2018; Yan et al. 2019; Hicks et al. 2020b; Tas et al. 2020).

From the final set of selected studies, the following data of interest were extracted: study details (authors, publication year), list of deregulated miRNAs, type of biofluid as a sample source, methodology used to detect miRNAs levels including the normalisation of the data, information about patients and control cohorts, GCS score of the patients, time of sample collection after the injury, *p*-value, and regulation status of miRNAs (upregulated or downregulated). Following the statistical method in the articles, a *p*-value <0.05 was considered significant while also taking to account the predictive models for miRNAs as mTBI diagnostic candidates. Curated source miRNA data including cohort details are summarized in Supplementary Material (Supplement 1).

miRNA:target identification

Prediction of gene targets for differentially expressed miRNAs was performed using the mirDIP: microRNA Data Integration Portal (http://ophid.utoronto.ca/mirDIP) version 4.1.11.1 (Database version 4.1.0.3.), which integrates 30 different algorithms (Tokar et al. 2018). Identification of miRNA putative targets involved all 30 databases at very high confidence level (39 miRNAs), except for the 4 miRNAs where a high score class filter was employed to query the predictions. The miR-12136 was the only miRNA that is not included in the mirDIP database and thus did not yield putative targets in any of the used resources. All raw data and search results are included in Supplement 2.

Comprehensive pathway enrichment

Putative target genes defined by mirDIP were used to identify significantly enriched pathways performing a pathway enrichment analysis *via* pathDIP: pathway Data Integration Portal http://ophid.utoronto.ca/pathDIP version 4.0.21.2 (Database version 4.0.7.0) (Rahmati et al. 2020). We have used all 22 pathway sources and only known literature curated (core) pathway memberships considering *q*-value <0.05 (false discovery rate: Benjamini-Hochberg method). Significantly enriched pathways were then processed to identify enriched terms in the pathway titles (word enrichment analysis; Supplement 3). To visualize

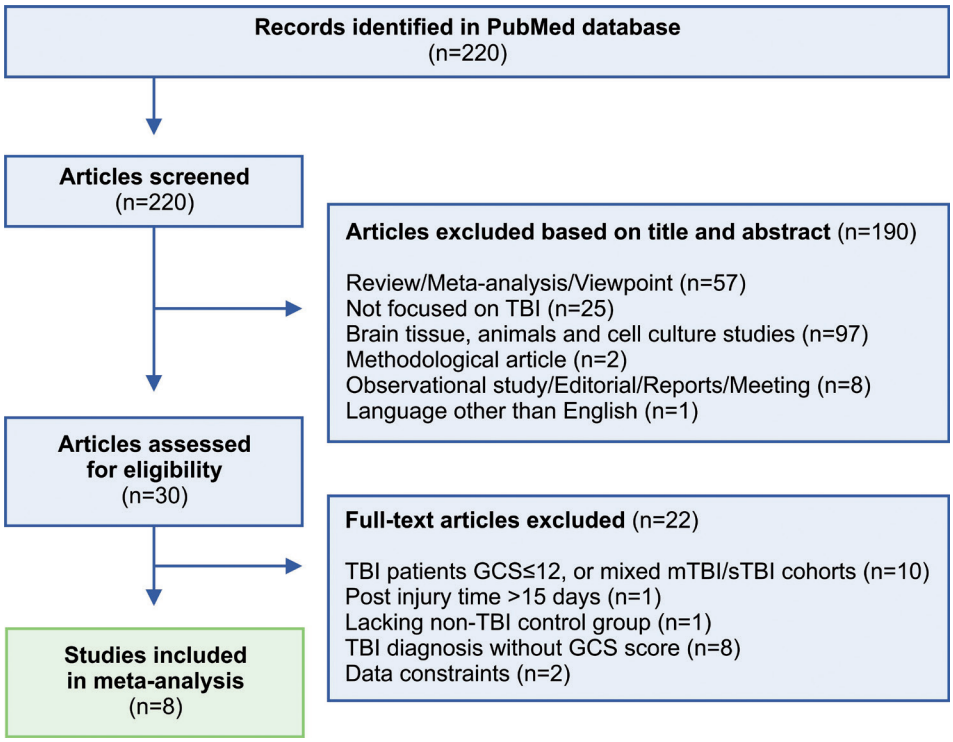

Figure 1. Flowchart of the meta-analysis selection criteria.

both common and unique terms we used NAViGaTOR ver. 3.0.11, highlighted terms of significantly enriched pathways (blue, purple and black refers to low-to-higher q-value) and produced the SVG file as output. The SVG file was post-processed in CorelDRAW X7 version 17.1.0.572 to its final form (Fig. 4B).

Protein-protein interactions

Identified gene targets unique for particular biofluid (Supplement 2) were used to query Integrated Interactions Database (IID) (http://ophid.utoronto.ca/iid) version 2018-11 (Kotlyar et al. 2019) to retrieve direct physical protein interactions among them. Using DisGeNet (Pinero et al. 2017), edges were annotated with relevance to neurodegenerative disease, nervous system disease, tauopathy and Alzheimer's disease (data integrated in IID). NAViGaTOR was then used to visualize and analyse the resulting network. To identify the most important proteins within the network, we computed centrality and node degree. Closeness centrality provides ranking for each node based on the average length of any breadth-first search path. The most central proteins (and those with degree >20) were selected. To reduce network complexity, other nodes and edges were removed, resulting in the final network in Figure 5 (Supplement 4).

Network analysis and visualization

NAViGaTOR version 3.0.11 (Brown et al. 2009) was used to visualize and annotate miRNA:gene network and physical protein interaction network. Final graphs were exported in SVG format, and completed in CorelDRAW X7 version 17.1.0.572 to produce the final images with legends.

Results

To identify signaling pathways associated with mTBI, we analysed the differentially expressed circulating miRNAs in mTBI with integrative bioinformatic tools. The analytic pipeline combined the comprehensive miRNA:gene target prediction (mirDIP), pathway enrichment analysis (pathDIP) and final integration with physical protein-protein interactions (IID) (Fig. 2A).

Following the selection criteria, the data for bioinformatic evaluation were extracted from 220 searched articles resulting in 8 studies focusing on altered miRNA levels in peripheral biofluids of subjects with mTBI. For a more detailed analysis, we separately examined the deregulated miRNAs based on their origin using three biofluid sources, i.e., serum, plasma, and saliva. In total, data for 12 different miRNAs were col-

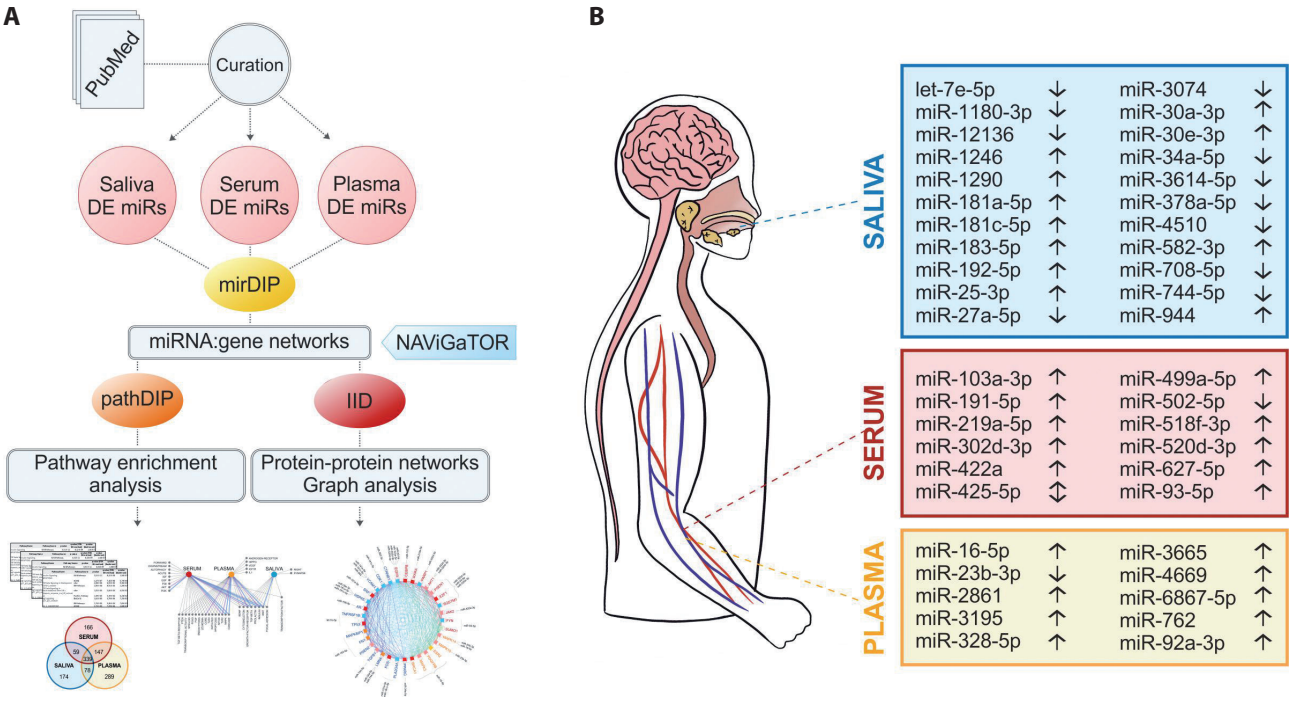

Figure 2. Design of the study. Pipeline of the bioinformatic analysis (**A**). Overview of the differentially expressed (DE) circulating miRNAs following mild TBI evaluated in the study (**B**). Arrows in the panel B represent direction of deregulation in particular biofluid, as reported in the original studies. mirDIP, microRNA Data Integration Portal; pathDIP, pathway Data Integration Portal; IID, Integrated Interactions Database.

Table 1. Key deregulated circulating miRNAs involved in the peripheral signaling after mild traumatic brain injury (mTBI)

miRNA	Sequence (5'-3')	Regulation	Biofluid	Number of targeted genes
hsa-miR-103a-3p	AGCAGCAUUGUACAGGGCUAUGA	↑	Serum	2087
hsa-miR-302d-3p	UAAGUGCUUCCAUGUUUGAGUGU	↑		1673
hsa-miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU	↑		1566
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG	↑		2540
hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG	↑	Plasma	2761
hsa-miR-23b-3p	AUCACAUUGCCAGGGAUUACCAC	↓		2284
hsa-miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU	↑		1238
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU	↓	Saliva	1387
hsa-miR-181a-5p	AACAUUCAACGCUGUCGGUGAGU	↑		1597
hsa-miR-181c-5p	AACAUUCAACCUGUCGGUGAGU	↑		1798
hsa-miR-25-3p	CAUUGCACUUGUCUCGGUCUGA	↑		1407
hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU	↓		1330
hsa-miR-944	AAAUUAUUGUACAUCGGAUGAG	↑		1178

lected in serum, 10 in plasma, and 22 in saliva (Fig. 2B). These deregulated miRNAs served as peripheral indicators of molecular regulatory mechanisms involved after the mTBI. We identified the putative target genes for deregulated miRNAs in all three biofluids using the mirDIP database portal, and visualized the miRNA:gene networks illustrating the top genes targeted by the most of the deregulated miRNAs in serum *vs*. saliva (Fig. 3A), serum *vs*. plasma (Fig. 3B) and saliva *vs*. plasma (Fig. 3C). Combined, we predicted 9,903 target genes for deregulated serum miRNAs, 6,544 target genes for plasma miRNAs, and 12,343 for salivary miRNAs.

Key involved miRNAs targeting the highest number of genes in particular biofluids are summarized in Table 1, suggesting their higher predictive value for peripheral signaling after mTBI. Gene targets for each biofluid were separately analysed using pathDIP portal to identify enriched pathways. Overall, we identified 771 significantly enriched pathways in serum, 757 in plasma, and 710 in saliva after mTBI. Our analysis revealed that majority of identified enriched pathways is shared among the biofluids and only a fraction of enriched pathways is unique to a particular biological fluid (Fig. 4A). Additionally, word enrichment analysis uncovered that most of the highly ranked pathway terms (blue and purple edge colour) are common among two or all three biofluids. The top scored pathways associate with MAPK, TGF-β, WNT, TLR2/4, PI3K/AKT, insulin, and growth factor signaling. Main mTBI-associated pathophysiological pathways are listed in Table 2. However, we also identified enriched pathways unique for each of the studied biofluids (Fig. 4B). A comprehensive list of identified enriched pathways is provided in Supplement 3.

To investigate the link between TBI and neurodegeneration, we mapped identified gene targets of deregulated miRNAs (1,123 targets in serum, 1,007 in plasma and 1,792 in saliva) to proteins. Using IID we retrieved 52,605 direct, human physical protein-protein interactions among unique targets across the biofluids. The resulting network was

Table 2. Representative pathophysiological pathways associated with mild traumatic brain injury (mTBI) identified by pathway enrichment analysis

Pathway name	Pathway source	*q*-value
PI3K/AKT/mTOR	ACSN2	6.08E−17
TGF-beta signaling	WikiPathways	2.35E−12
Starvation_autophagy	ACSN2	2.59E−11
Canonical WNT	ACSN2	3.10E−11
EGF/EGFR signaling	WikiPathways	4.46E−11
Insulin signaling	WikiPathways	6.34E−11
VEGFA-VEGFR2 signaling	WikiPathways	3.33E−09
BDNF signaling	WikiPathways	5.51E−09
MAPK signaling	KEGG	3.76E−08
FoxO signaling	KEGG	1.40E−07
Endocytosis	KEGG	5.15E−07
Regulation of actin cytoskeleton	KEGG	6.37E−07
Toll-like receptor signalling network	systems-biology.org	8.84E−07
Focal adhesion	WikiPathways	9.27E−07
Immunostimulatory core	ACSN2	3.42E−06
FGF signaling	Panther_Pathway	4.11E−06
Apoptosis	ACSN2	7.16E−06
IGF1R signaling cascade	REACTOME	2.03E−03
Growth factors signaling	ACSN2	6.95E−03
PTEN regulation	REACTOME	7.85E−03
Synaptic vesicle trafficking	Panther_Pathway	1.67E−02
IL-1 pathway	stke	2.26E−02

Literature curated (core) pathways considering *q*-value <0.05 using the false discovery rate (FDR): Benjamini-Hochberg (BH) method.

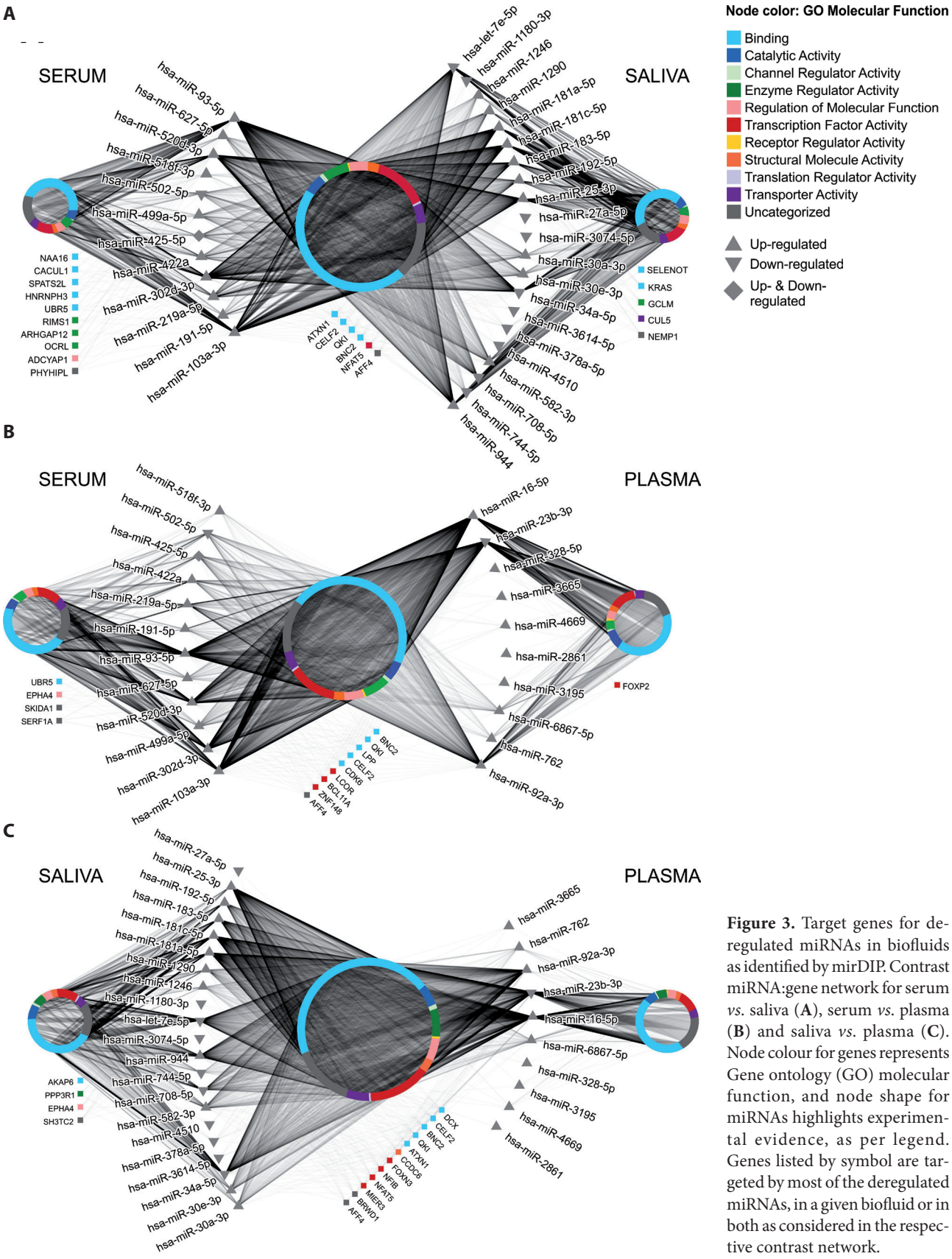

Figure 3. Target genes for deregulated miRNAs in biofluids as identified by mirDIP. Contrast miRNA:gene network for serum *vs.* saliva (**A**), serum *vs.* plasma (**B**) and saliva *vs.* plasma (**C**). Node colour for genes represents Gene ontology (GO) molecular function, and node shape for miRNAs highlights experimental evidence, as per legend. Genes listed by symbol are targeted by most of the deregulated miRNAs, in a given biofluid or in both as considered in the respective contrast network.

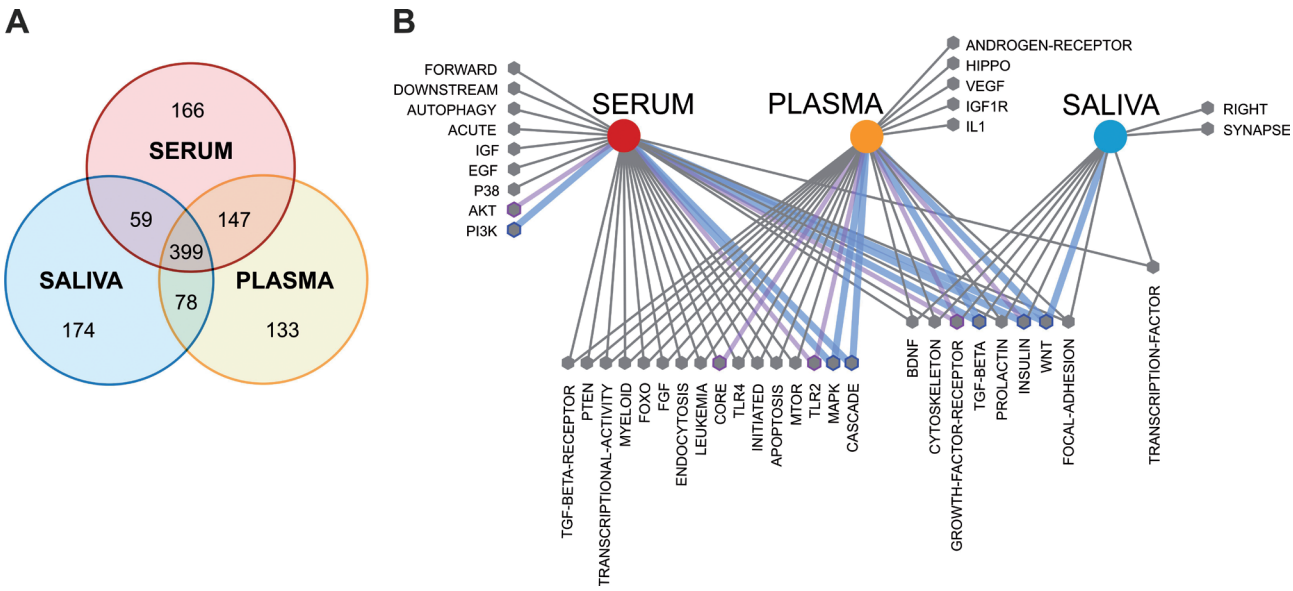

Figure 4. Pathway enrichment analysis using pathDIP. **A.** The number of identified enriched pathways and their overlap among the serum, plasma, and saliva. **B.** The most overrepresented enriched pathways show many shared terms. Edge colour and thickness represents significance of term enrichment (blue, purple, and black refers to low-to-higher *q*-value).

further filtered by selecting only the interactions related to neurodegeneration and focusing on central proteins within the network. Final network comprised 33 key hub proteins connected with interactions relevant in neurodegeneration, nervous system disease, tauopathy and Alzheimer's disease. Furthermore, we highlighted in the network the deregulated miRNAs that are linked to these key hub proteins associated with neurodegeneration (Fig. 5). Interestingly, among the interactions we identified several dominant connections that link deregulated miRNAs and identified proteins. Specifically, overrepresentation of the miR-93-5p, miR-302d-3p, miR-520d-3p in serum, miR-16-5p, miR-23b-3p in plasma and let-7e-5p, miR-34a-5p, miR-181a-5p, mir-181c-5p, miR-183-5p, miR-744-5p in saliva with neurodegeneration-associated proteins was observed. These deregulated miRNAs represent a group of candidate molecules linking the post-mTBI signaling reflected in peripheral fluids with neurodegeneration-associated interaction pathways. Final list of interacting proteins is attached in the Supplement 4.

Discussion

We performed a systematic review of deregulated miRNA profiles in peripheral fluids of individuals suffering mild traumatic brain injury aiming to identify molecular signaling associated with pathophysiology of mTBI. The meta-analysis specifically focused on studies that summarize the altered miRNA level in serum, plasma, and saliva of mTBI individuals. Following selection criteria, the search strategy identified 8 studies providing data on 44 deregulated miRNAs that were further analysed by bioinformatic pipeline. Integrated bioinformatic workflow enabled both increased coverage and depth of our analysis by simultaneous querying of multiple databases for more comprehensive prediction of putative gene targets and higher confidence of the outcomes when compared to recent analyses (Di Pietro et al. 2017, 2018; Atif and Hicks 2019).

miRNA:gene networks

Highlighted subgroups of identified genes visualized in the miRNA:gene networks (Fig. 3) represent a subset of genes targeted by the highest number of deregulated miRNAs across all biofluids after mTBI. Moreover, one of the target genes, *CACUL1*, was previously described as a target of miR-219a-5p that has been found deregulated after TBI. Experimental validation of *CACUL1* expression in the neuronal cell injury model revealed caspase-3-dependent induction of apoptosis regulated by Akt/Foxo3a and p53/Bcl-2 signaling pathways (Yan et al. 2019). Upregulation of another predicted target gene, *GCLM*, has been described in the TBI mice model as one of the downstream genes of Nrf2 signaling pathway, playing a neuroprotective role following TBI (Dong et al. 2018). Furthermore, decreased level of *CUL5* as measured in rat cerebral cortex and hippocampus 7 days post-TBI was linked to the inhibition of ubiquitin-proteasome system (Yao et al. 2006). The neu-

roprotective effect of *ADCYAP1*, also known as pituitary adenylate cyclase activating polypeptide (PACAP), has been investigated in the rat model of diffuse axonal injury. Intracerebroventricular administration of PACAP following TBI resulted in a reduction of beta-amyloid precursor protein-immunopositive axons in the corticospinal tract (Farkas et al. 2004; Tamas et al. 2006). Involvement of *EPHA4* has been observed in pro-inflammatory response to TBI *via* regulation of Akt, mTOR, and NF-κB signaling pathways (Kowalski et al. 2019).

Deregulation of miRNA levels in brain or peripheral fluids was extensively studied in various animal models of TBI. Despite a certain overlap of miRNA findings, the accumulated evidence indicates major difference between animal models and human TBI studies (Pinchi et al. 2020; Herrold et al. 2021). Nevertheless, animal models still carry the translation potential of research findings for diagnostic or therapeutic benefits in human medicine. In particular, upregulation of 2 miRNAs that are highly expressed in the brain, the miR-9-3p and miR-136-3p was reported in plasma of TBI rats and mTBI patients suggesting their biomarker potential (Das Gupta et al. 2021). Other promising miRNAs, such as miR-142-3p (Liu et al. 2014b), let-7i (Balakathiresan et al. 2012), miR-23b (Sun et al. 2018), miR-181a-5p and miR-191-5p (Weisz et al. 2020) were identified in both human and animals, indicating shared post-concussive signaling between TBI animal models and clinical studies.

Our findings suggest an association of identified genes with development and functioning of central nervous system indicating specific molecular signaling involved in mTBI. Employing the integrative approach, we were able to predict the gene candidates linked to the post-traumatic sequelae of TBI depicting the brain injury pathways together with particular healing processes. Our data are partially supported by experimental evidence; however, vast majority of drawn gene predictions is novel and remains to be explored by further functional and clinical studies. Moreover, we hypothesize that identified top ranked genes might represent a group of potential novel biomarkers to be further explored in connection with TBI conditions.

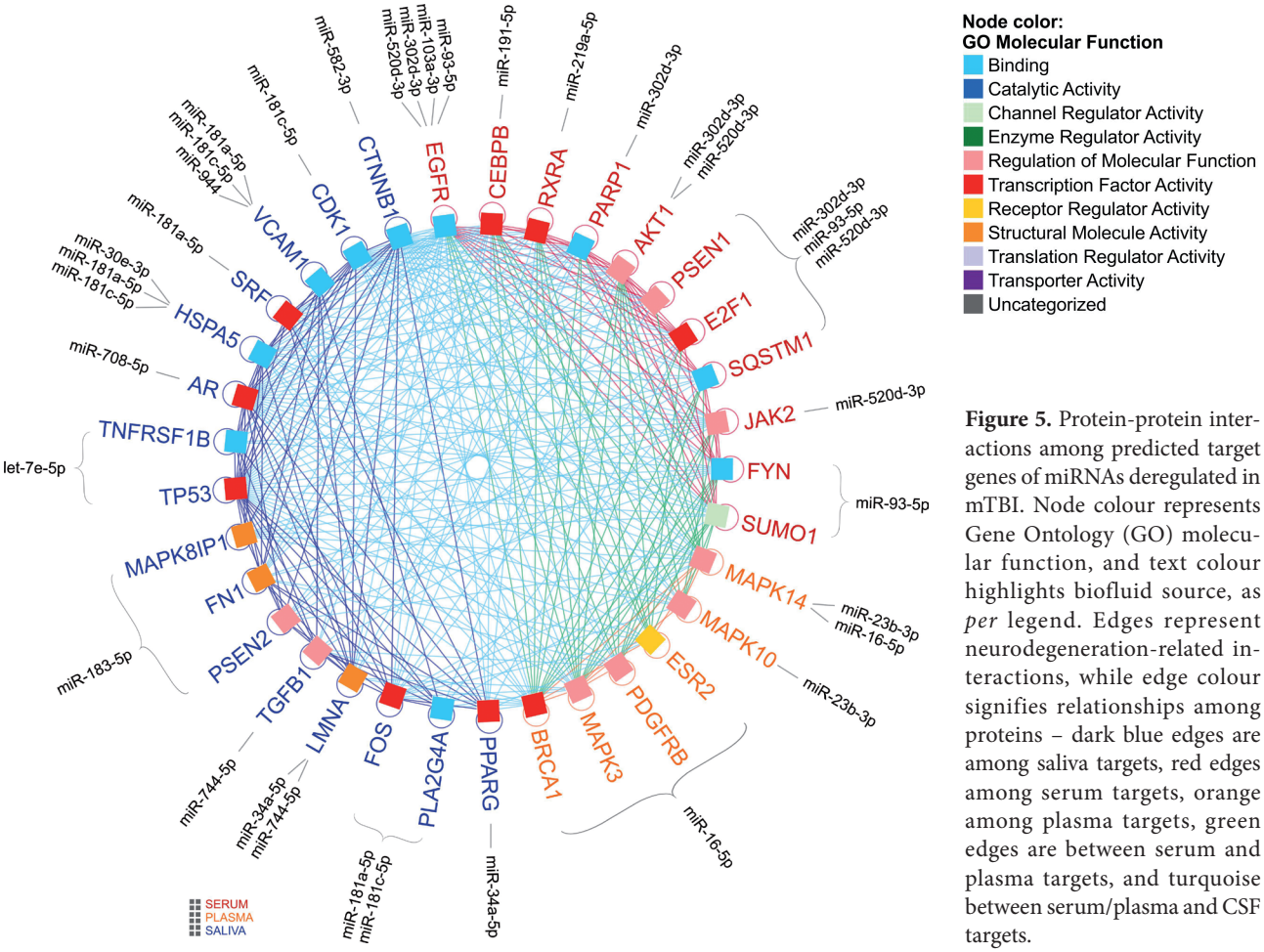

Figure 5. Protein-protein interactions among predicted target genes of miRNAs deregulated in mTBI. Node colour represents Gene Ontology (GO) molecular function, and text colour highlights biofluid source, as *per* legend. Edges represent neurodegeneration-related interactions, while edge colour signifies relationships among proteins – dark blue edges are among saliva targets, red edges among serum targets, orange among plasma targets, green edges are between serum and plasma targets, and turquoise between serum/plasma and CSF targets.

Signaling pathways altered after mTBI

Interestingly, the majority of the signaling pathways as derived from the deregulated peripheral miRNAs after mTBI are shared among the investigated biofluids of serum, plasma and saliva. According to these findings, dysregulated circulating miRNAs target the genes implicated in the same fundamental mechanisms simultaneously manifested in all assessed biofluids. These data support the idea that different biological fluids carry the molecular information on pathophysiological processes related to brain injury and might help to better elucidate the gene signaling or predict targets for therapeutical intervention of TBI.

Among the significantly enriched pathways the disturbance of hormone signaling represents the most prominent feature identified by bioinformatic analysis. Posttraumatic neuroendocrine dysfunction, e.g., hypopituitarism is a well-documented consequence of TBI (Benvenga et al. 2000; Kelly et al. 2000). Besides disturbances of other anterior pituitary hormones, higher prolactin level has been reported in soldiers who sustained blast-induced TBI (Baxter et al. 2013). Likewise, mild hyperprolactinemia was observed in mild to severe TBI patients (Aimaretti et al. 2004). A higher level of prolactin following TBI can be explained by transport repression of prolactin inhibitory factor into the pituitary gland (Scranton and Baskin 2015). The pituitary dysfunction has been associated with a decreased serum level of insulin like growth factor 1 (IGF-1), suspected to be also involved in the progression of cognitive dysfunction (Bondanelli et al. 2004; Berg et al. 2010). Inversely, administration of IGF-1 leads to stimulation of anti-apoptotic pathways mediated by PI3K/Akt and MAPK signals and prevents spatial memory deficits following mTBI in mice (Rubovitch et al. 2010, 2011). Stimulation of these cascades appears to be a promising strategy positively affecting the recovery mechanisms such as angiogenesis, anti-apoptotic survival, synaptic plasticity and neurogenesis (Mangiola et al. 2015).

Findings identified by our analysis are further supported by a recent study that revealed association of 5 deregulated miRNAs with insulin, IGF-1R and TGF-β signaling in military personnel with history of mTBI (Devoto et al. 2020).

Pleiotropic cytokine, transforming growth factor-beta (TGF-β), plays a role in neurogenesis, removal of amyloid-β protein and modulates the actions of various growth factors (Devoto et al. 2020). The general effect of TGF-β in TBI models remains questionable, but its neuroprotective function relates to the restriction of chemokines production after brain injury (Dobolyi et al. 2012). Potential pro-apoptotic function of TGF-β through activation of the caspase-3 in rats with induced mTBI was also reported (Patel et al. 2017).

The enrichment of TGF-β signaling, as reflected by all examined biofluids in our study, supports the idea that this pathway plays an important role in post-concussive signaling and might represent a viable candidate for management of post-traumatic sequelae in mTBI patients.

Another highly over-represented class of signaling pathways identified by our meta-analysis includes the group of various growth factors and receptors. One of the identified enriched signaling pathways, the vascular endothelial growth factor (VEGF), was previously reported in connection with angiogenesis, neurogenesis and neuroprotection in TBI models (Nag et al. 1997; Sun et al. 2003; Thau-Zuchman et al. 2010; Lu et al. 2019). A higher concentration of VEGF has been also reported in humans suffering traumatic brain injury with various severities (Mellergard et al. 2010; Helmy et al. 2011; Meabon et al. 2020). Recently, elevated plasma levels of VEGF, together with IL-6 and TNF-α were associated with CT lesions in patients following mTBI. It seems that a combination of these three molecules could represent a biomarker panel to distinguish mTBI cases with CT findings from mTBI without CT findings (Edwards et al. 2020b).

TBI was shown to evoke the sensitivity of cells to epidermal growth factor (EGF) cascade *via* increase of EGFR levels (Addington et al. 2015). Furthermore, neuroprotective effect of EGF in post-concussive period is mediated by stimulation of astroglial cells, which leads to improved cognitive function and reduced neuronal loss (Sun et al. 2010). Similarly to EGF, the pro-survival function of fibroblast growth factor (FGF) relates to its mitogen activity restricting the apoptosis and necrosis *via* inhibition of autophagy (Addington et al. 2015; Tang et al. 2017). A study on patients also confirmed the role of FGF in neurogenesis and protection of blood brain barrier (Chaban et al. 2020). Brain-derived neurotrophic factor (BDNF) plays a regulatory role in neuronal survival, neurogenesis, and synaptic plasticity (Martinowich and Lu 2008). This effect is mediated *via* binding to TrkB receptor and activation of three signaling pathways: PI3K, MAPK/ERK, and PLCγ (Chao 2003). The elevated plasma level of BDNF was previously observed in children with a concussion suggesting its potential use as a marker of mild head trauma. However, the BDNF levels did not differentiate the severe TBI with loss of consciousness from mTBI without loss of consciousness suggesting that BDNF increase indicates rather functional disturbance related to TBI as such and is not related to the severity of head trauma (Tylicka et al. 2020).

Growth factor signaling is associated with another important cascade of PI3K/Akt/mTOR. When activated, PI3K stimulates Akt serine/threonine kinase 1 (Akt) to trigger mTOR pathway that plays a crucial role in functional recovery process following a traumatic CNS injury (Don et al. 2012). Regulation of mTOR pathway *via* suppression of PTEN activates PI3K/Akt signaling leading to neuroprotection through regulation of apoptosis-related proteins (Ge et al. 2014; Han et al. 2014).

Neuroinflammation represents an important mechanism in pathophysiology of TBI leading to secondary brain damage in post-traumatic period (Singh et al. 2016; Sun et al. 2019). Our results from bioinformatic analysis agree with previously identified molecules of inflammatory pathways activated in response to TBI, e.g., IL-1, TLR2 and TLR4. Pro-inflammatory cytokine, interleukin 1 (IL-1), is an important mediator of neuroinflammatory response after TBI (Basu et al. 2004). Both IL-1α and IL-1β have been described previously as rapidly elevated after brain injury (Griffin et al. 1994; Fan et al. 1995). Furthermore, the genetic ablation of IL-1 receptor showed a more positive effect on cognitive function in TBI mice models than ablation of IL-1α and IL-1β alone (Newell et al. 2018). High level of inflammatory cytokines including IL-1β, IL-6 and CCL2 was acutely elevated in mTBI patients relative to controls. However, the elevated CCL2 level was also associated with greater severity of post-concussion symptoms negatively affecting the outcome of mTBI patients (Sun et al. 2019). These observations highlight the deleterious effect of pro-inflammatory signaling in post-injury period when persisting chronically.

Toll-like receptors (TLR) represent another group of highly enriched signaling pathways identified by our analysis. TLRs have been recognized as essential players of innate immune signaling and regulation of inflammatory responses (Kawai and Akira 2006). Even in the post-TBI conditions activation of TLR 2/4 involves signaling *via* MAPKs and subsequent stimulation of NF-κB leading to expression of inflammation responsive genes (Downes and Crack 2010; Ved et al. 2021). TLR2/4-mediated secondary brain injury was observed proposing their role in neuroinflammation and cell death (Krieg et al. 2017), while the TLR4/MyD88/NF-κB signaling pathway might be efficiently targeted by PACAP reducing the secondary inflammatory response and neuronal death after TBI (Mao et al. 2012).

Metabolic disturbance is another invariant feature of post-concussive condition in TBI patients.

Studies have shown occurrence of hyperglycolysis early after injury, followed by a hypometabolic phase lasting for days (Yoshino et al. 1991; Bergsneider et al. 2001). A high glucose level during the first hours after TBI can predict a poor neurologic outcome and mortality rate (Liu-DeRyke et al. 2009; Terzioglu et al. 2015). Additionally, the association between diabetes and increased risk of neurodegeneration developed due head trauma has been reported in veterans with type 2 diabetes and a history of TBI (Zimering et al. 2019). Possible molecular explanation suggests that elevated pro-inflammatory cytokines inhibit insulin receptor signaling by targeting the Akt that negatively affects the neuronal energy metabolism. Furthermore, insulin resistance following TBI deprives this neuroprotective pathway and thus renders the individuals vulnerable to neurodegeneration (Karelina and Weil 2016).

WNT signaling is involved in several critical processes in the brain, including cell proliferation, differentiation, inflammation, neurogenesis, synaptogenesis, axon guidance, neuron maintenance, and regeneration (Salinas 2012; Marchetti and Pluchino 2013). Various studies implicate the WNT pathway as a neuroprotective cascade that upon stimulation might represent a prospective treatment of post-TBI conditions (Zhao et al. 2016; Zhang et al. 2018). Intranasal administration of recombinant Wnt3a in TBI mice model during first 24 hours was shown to inhibit the autophagy and upregulate the expression of growth factors including GDNF and VEGF responsible for neurogenesis and angiogenesis, respectively. In our meta-analysis, WNT pathway has been the top ranked prediction in all examined fluids, suggesting a key role of WNT pathway in post-traumatic signaling and recovery.

Previous studies used various bioinformatic approaches to identify signaling pathways linked to deregulated miRNAs after TBI. However, the published pathways were derived from a single database, e.g., KEGG or Ingenuity Pathway analysis, respectively, leading to only partial results due to small overlap across pathway databases (Rahmati et al. 2017). Despite this methodological limitation the mTOR, TGF-beta, IGF1R, neurotrophin and inflammatory signaling represent major overlap with findings of our meta-analysis and support their involvement in post mTBI conditions by multiple evidence (Qin et al. 2018; Atif and Hicks 2019; Devoto et al. 2020; Hicks et al. 2020a; Di Pietro et al. 2021).

Link between mTBI and neurodegeneration

The history of mTBI, and especially the repetitive head traumas are associated with increased risk of development of neurodegenerative disorders (McKee and Robinson 2014; Manley et al. 2017; Alosco et al. 2018). To elucidate the relationship between prior TBI with subsequent dementia we tested the hypothesis how molecular signaling identified after mTBI relates to the protein interactions seen in neurodegeneration including Alzheimer's disease and tauopathies. Bioinformatic analysis revealed group of genes targeted by deregulated miRNAs after mTBI with a known interaction in neurodegenerative conditions. Within this subpopulation several central proteins implicated in the neurodegenerative disorders were identified. In particular, the Alzheimer's disease-associated proteins, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) showed major interactions within the network.

PSEN1 *via* increase of cytoplasmic CTNNB1, another identified key hub protein within the network, negatively regulates the WNT and Notch signaling (Murayama et al. 1998; Marambaud et al. 2002). Since these pathways play an important role in neuroregeneration and brain homeostasis, downregulation of this signaling by TBI might negatively affect the process of recovery and contribute to the longi-

tudinal sequelae of TBI, which might eventually progress towards neurodegeneration. Furthermore, targeting of gamma-secretase complex including presenilin to reduce amyloid beta production represents a perspective therapeutic strategy for treatment of Alzheimer's disease (De Strooper et al. 2012), and might be potentially employed also for the attenuation of neurotrauma following TBI (Mannix et al. 2011; Lin et al. 2017).

In addition, among the key hub proteins identified within the protein interaction network, there are several other candidates that have been previously reported in connection with TBI. Up-regulation of AKT1 (Edwards et al. 2020a), FOS (Wang et al. 2014), FYN (Liu et al. 2014a), MAPK3 (Ou et al. 2014), CEBPB (Sandhir and Berman 2010), PLA2G4A (Sarkar et al. 2020), RXRA (Zhao et al. 2019), E2F1 (Liu et al. 2013), HSPA5 (Truettner et al. 2007) and downregulation of AR (Golz et al. 2019), MAPK10 (Long et al. 2013) was observed in various animal TBI models or human TBI cases. While the increase of HSPA5 is thought to be neuroprotective in neurodegeneration (Casas 2017), the activation of E2F1 is associated with post-traumatic neuronal apoptosis (Liu et al. 2013). Activation of JAK2-STAT3 pathway together with upregulation of CEBPB couples the activation of astrocytes with growth factor and neuroinflammatory responses after TBI (Oliva et al. 2012). Along with these findings the TNFRSF1B and SRF-deficient mice exhibit poorer outcome following TBI (Yang et al. 2010; Forstner and Knoll 2020). On the other side experimental administration of EGF (Sun et al. 2010) and FN1 (Tate et al. 2007) in animal TBI models suggests their neuroprotective role. Furthermore, the upregulation of CDK1 by dexmedetomidine (Wu et al. 2018; Yang et al. 2021) and inhibition of PARP1 (Stoica et al. 2014) in TBI models also appears to be viable neuroprotective strategy.

Our study also identified a subgroup of proteins, e.g., BRCA1, LMNA and ESR2 that have not been associated with TBI so far, and thus might represent novel candidates for validation in TBI conditions.

Presence of validated targets within the protein interaction network supports the findings of integrated bioinformatic analysis with experimental evidence. Interestingly, the interaction network of neurodegeneration-associated proteins highlights the TGF-β, AKT1, MAPK, TP53, TNF and growth factor signaling that were also identified among the pathways enriched in mTBI. This overlap indicates overrepresentation of signaling related to these proteins and implicates their central role in molecular regulatory mechanisms shared between mTBI and neurodegeneration.

To our knowledge this is the first integrative bioinformatic analysis systematically combining comprehensive miRNA-target prediction, multiple pathway sources, and rich annotated physical protein interactions aiming to identify peripheral miRNA signaling associated with mild traumatic brain injury. Results of the integrative analysis reveal specific signaling pathways and highlight the key enriched molecular mechanisms reflected across the serum, plasma and saliva of individuals after mTBI. Findings of the meta-analysis suggest that peripheral miRNAs could represent relevant biomarkers with added value to the currently used diagnostic approaches and prognostics of recovery after TBI.

Strengths and limitations

Despite the supportive data for our findings in the scientific literature we assessed the risk of bias, among which the input miRNA data represent reasonable source of variability to be considered. In particular, various methodological approaches used for the measurement of miRNA, such as next-generation sequencing, real-time PCR or microarray techniques, provide a variable depth of information and sensitivity of detection for evaluated miRNAs. Furthermore, many studies lack the sample quality check or provide no statement about exclusion of low-quality samples prior analysis (e.g., hemolysis for plasma, contamination of saliva with cellular RNA). Third, use of different endogenous calibrators and normalization procedures for calculation of miRNA fold change has to be considered as it has a direct impact on accuracy of reported miRNA expression values. Fourth, variable statistical methods used in the research articles or lacking validation step of primary data obtained by whole transcriptome analysis might result in distinct pattern of false positives and negatives affecting the inclusion of miRNAs in the integrated analyses. We summarize these issues to be critically considered by the readers during interpretation of our findings.

On the other hand, the process of data curation accounted for multiple variable factors and data constraints providing more consistent data input when compared to previous meta-analyses. Evaluation of the data under the strict statistical and predictive parameters supports obtained findings at high confidence level. Moreover, we consider the use of integrative bioinformatic pipeline as a major advantage of the employed meta-analytic approach since it enables the identification of enriched signaling pathways and molecular mechanisms based on cumulative support from multiple database sources and thus to a substantial degree corrects for the variabilities of input experimental data.

Conclusions

Integrated bioinformatic analysis revealed enrichment of common signaling pathways involved in the post-concussive conditions as reflected by the profile of circulating miRNAs in various body fluids. Since the identified pathways sub-

stantially overlap among the biofluids we hypothesize that the specific signaling associated with mTBI is concomitantly reflected in various biological fluids. Furthermore, our data indicate that biofluid-specific miRNA profiles could serve for detailed characterization of molecular pathways involved in the pathophysiology and healing processes of mild traumatic brain injury. Top hub proteins identified within the network represent novel putative candidates involved in molecular signaling after mTBI. Our findings implicate a molecular link between mTBI signaling and neurodegeneration-associated interaction pathways. However, to validate the concept of mTBI-induced neurodegeneration involving predicted targets further experimental research is necessary.

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Supplementary Material

Peripheral microRNA alteration and pathway signaling after mild traumatic brain injury

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Supplement 1. Dysregulated miRNAs in serum, plasma and saliva after mild TBI.

Supplement 2. miRNA to unique target gene prediction based on mirDIP version 4.1.11.1 (database version 4.1.0.3): microRNA Data Integration Portal (http://ophid.utoronto.ca/mirDIP).

Supplement 3. Pathway enrichment analysis-pathDIP version 4.0.21.2 (database version 4.0.7.0): Annotated database of signaling cascades (http://ophid.utoronto.ca/pathDIP/).

Supplement 4. Protein-protein interactions retrieved by Integrated Interactions Database (IID), version 2018-11 (http://ophid.utoronto.ca/iid).

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