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Traumatic Brain Injury: Classification, Models and Markers

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Abstract

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality worldwide. Due to its high incidence rate and often long-term sequelae, TBI contributes significantly to increasing costs of medicare expenditures annually. Unfortunately, advances in the field have been stifled by patient and injury heterogeneity that pose a major challenge in TBI prevention, diagnosis and treatment. In this review, we briefly discuss the causes of TBI, followed by its prevalence, classification and pathophysiology. The current imaging detection methods and animal models used to study brain injury are examined. We discuss the potential use of molecular markers in detecting and monitoring the progression of TBI, with particular emphasis on microRNAs as a novel class of molecular modulators of injury and its repair in the neural tissue.

Keywords

Animal Models, Biomarkers, Imaging, microRNA, TBI
Causes of Brain Injury

Brain injury has been recognized as one of the leading causes of disability and death in both developed and developing countries. Depending on the cause, damage to the brain can be classified into two broad categories: traumatic and non-traumatic brain injuries. In traumatic brain injury (TBI), the damage to the brain is generally caused by external forces, either direct or transmitted, caused by falls, motor vehicle collisions, sport-related injuries, abuse/assault or pressure blasts. On the other hand, in non-traumatic brain injury, damage to the brain is mainly caused by infections, brain tumors, ischemia or stroke (Table 1). In some surgical cases, injuries to the brain tissue occur unintentionally through invasive procedures. Regardless of its cause, brain injury can result in physical, cognitive and behavioral impairments, leading to temporary or permanent dysfunction (Reis et al. 2015). The patient and injury heterogeneity associated with TBI, along with the wide spectrum of its clinical manifestations, continue to pose a significant challenge in its prevention, diagnosis, and treatment (Young et al. 2015). Therefore, improved detection methods for milder injuries, identification of reliable prognostic markers to guide clinical decision making, and development of effective therapies are critical steps towards repairing the damaged brain (Menon and Maas 2015).

Traumatic Brain Injury Statistics

In Canada, approximately 160,000 people sustain brain injuries each year, leading to 11,000 deaths and over 6,000 cases of permanent disability (“Brain Injury Association of Waterloo-Wellington; Stats” n.d., “Brain Injury Canada; Acquired Brain Injury” 2015). Nearly half of all TBI cases in Canada result from falls and motor vehicle collisions (“Brain Injury Canada; Acquired Brain Injury” 2015). A review of TBI data obtained from Canadian provinces shows
incidence rates of about 16,000 and 22,000 people per year for Ontario and British Columbia, respectively (“Brain Injury Association Sudbury & District; ABI Basics” n.d., “Northern Brain Injury Association; Brain Injury Statistics” 2014). In addition, First Nation peoples are considered as the highest risk group with a brain injury rate 4-5 times greater than that of the general population (“Northern Brain Injury Association; Brain Injury Statistics” 2014). Together, it is estimated that the direct and indirect costs associated with TBI in Canada are about 3 billion dollars annually (“Brain Injury Canada; Acquired Brain Injury” 2015), further emphasizing the need for more efficient prevention strategies.

Parallel studies in the United States estimate that approximately 1.5 million people suffer from TBI each year, where 50,000 individuals die and an additional 85,000 individuals suffer from long term disabilities (“Traumatic Brain Injury; Understanding TBI” 2006). Similar to Canada, a large percentage of brain injury cases in the U.S. are caused by falls and motor vehicle collisions (“Traumatic Brain Injury; Understanding TBI” 2006). The rate of TBI has also been increasing worldwide due to increased numbers of motor vehicle collisions in developing countries and a greater rate of falls in more developed countries as the population ages (Maas et al. 2008).

The occurrence of TBI peaks during development with nearly 30% of all cases afflicting children and youth (“Northern Brain Injury Association; Brain Injury Statistics” 2014), and it has been recorded as the most common cause of morbidity and mortality in people under the age of 45 (Langlois et al. 2006). Children are at increased risk of TBI due to physiological susceptibility during development, including a more pliable skull, larger head, weaker neck muscles, increased cervical ligament laxity, greater brain water content, less myelin, greater excitation relative to inhibition, greater cerebral blood flow and higher cerebral metabolic rate (Morrison et al. 2013).
Youth and young adults are at greater risk for TBI by virtue of their activities (e.g. sports) and risk-taking behaviors (e.g. inconsistent usage of safety devices, distracted driving, etc.).

The incidence and prevalence of TBI outnumbers those of spinal cord injury, breast cancer, multiple sclerosis and HIV/AIDS combined (“Brain Injury Canada; Acquired Brain Injury” 2015). Furthermore, it is considered as a dominant cause of death and disability, and a major health and socioeconomic concern throughout the world (Cole 2004; Reis et al. 2015; Soares de Souza et al. 2015).

**Traumatic Brain Injury Classification, Pathophysiology and Detection Methods**

The physical insult is what initiates the biochemical and pathological consequences in TBI. Thus, the clinical manifestations of TBI depend significantly on the nature of the insult (Young et al. 2015). For instance, many open head injuries are caused by objects such as bullets or knives penetrating through the skull and damaging the brain. In contrast, closed head injuries are associated with blunt, overpressure or accelerative forces (Young et al. 2015). TBI classification follows clinical severity and is assessed primarily using the Glasgow Coma Scale (GCS) (Menon and Maas, 2015; Teasdale et al. 2014; Teasdale and Jennett 1974). GCS (range 3-15) consists of the sum of three component scores (eye, motor and verbal scales) and offers a rapid assessment of brain injury severity (Table 2). A score of 13-15, 9-12, and ≤ 8 classify mild (mTBI), moderate, and severe TBI, respectively (Bodanapally et al. 2015; Teasdale et al. 2014). In addition to GCS, TBI is evaluated by various imaging modalities to determine the severity of structural damage in the brain.

TBI can be classified into primary and secondary injuries (Besenski 2002; Kubal 2012; Maas et al. 2008). Primary injuries occur at the time of the injury as a direct result of traumatic impact,
resulting in epidural or subdural hematomas, microvascular injuries, cortical contusions and axonal shearing. Secondary injuries occur over hours and days, and generally result from a complex biochemical cascade of events often manifesting as cerebral edema and elevated intracranial pressure (Besenski 2002; Faden 1996; Puntis and Smith 2017). Secondary injuries include the non-mechanical damage that may result from complex metabolic cascades set off by cell membrane disruption (Giza 2001). Subsequently, neuronal membrane deformity leads to ionic flux, release of excitatory neurotransmitters, depletion of cellular energy stores, and activation of apoptosis, resulting in neuronal death. Impairment of the cerebrovascular tissue also leads to decreased cerebral blood flow, metabolic uncoupling, as well as inflammatory responses such as activation of microglia and release of free radicals, further contributing to tissue damage during the secondary phase (Werner and Engelhard 2007). In contrast to primary injuries, secondary injuries can be decreased or delayed by supportive care with sedation, analgesia and ventilation, and by lowering intracranial pressure with intravenous osmotic agents such as mannitol or hypertonic saline, controlling cerebral metabolic rate and by cerebrospinal fluid drainage, offering opportunities to reduce their impact on brain structure and function (Haddad and Arabi 2012; Puntis and Smith 2017). Furthermore, the delayed nature of secondary injuries provides a window of opportunity for using neuroprotective agents to interrupt cell death cascades in vulnerable tissue, which has become a major focus of drug development research in TBI. However, despite promising results in preclinical testing, a wide variety of neuroprotective agents have failed to provide benefit in clinical trials (Puntis and Smith 2017; Kochanek et al. 2017).

Various imaging techniques are routinely used to help characterize TBI severity, rule out surgical lesions and provide prognostic information. Computed tomography (CT) is the most common
imaging technique as it is readily available, fast, allows access to susceptible trauma patients and quickly identifies surgical lesions that require immediate intervention (Marehbian et al. 2017) (Figure 1). Thus, CT imaging is commonly used for initial evaluation and management of intracranial problems following injury (Mechtler et al. 2014). CT uses computer-processed combination of multiple x-ray images taken from different angles to produce cross-sectional images of specific regions of the brain. It can detect cerebral edema, infarctions, hemorrhage and bone fractures. Several CT scores have been developed to aid TBI severity determination and to predict mortality, including the Marshall CT score and the Rotterdam CT Score, respectively (Bodanapally et al. 2015; Marshall et al. 1991; Maas et al. 2005).

As a complementary technique, magnetic resonance imaging (MRI) uses magnetic fields and radio waves to produce high resolution two- or three-dimensional images of the brain. It provides improved structural sensitivity over CT scanning, including better tissue contrast, fewer artifacts, and the non-use of ionizing radiation or radioactive tracers (Gallagher et al. 2007; Hollingworth et al. 2000; Smith-Bindman et al. 2012). Due to its increased sensitivity, particularly for certain types of lesions such as axonal injury which can greatly impact outcomes, MRI provides better prognostic information than CT (Marehbian et al. 2017). More frequent use of MRI, however, is hindered by its availability, cost, length of scanning time, the magnetic field that limits use of certain metallic equipment, and the inability to access the patient quickly in the event of acute deterioration. Finally, the timing of MRI may be important for identification of certain pathologies, such as vasogenic versus cytotoxic edema.

Another imaging modality occasionally used to monitor TBI is color Doppler ultrasound (CD-US). This technique uses high frequency sound waves that reflect off circulating red blood cells to gather information about cerebral blood flow. One potential use of CD-US is in screening for
post-traumatic cerebral vasospasm, which can worsen TBI outcome via cerebral infarction (Kramer et al. 2013; LaRovere et al. 2016). CD-US can be advantageous when patients have metallic implants such as pacemakers, and they are not suitable or immediate candidates for MRI. CD-US is also non-invasive and less expensive than MRI, and it does not require a special type of room setting.

Approximately 75% of all TBI are mTBI patients, including concussions (National Center for Injury Prevention and Control, 2003). Using traditional imaging modalities, such as head CT scanning, patients with mTBI usually show no abnormalities in their neural tissue (National Center for Injury Prevention and Control 2003; Rees 2003; Shin et al. 2017). While most mTBI patients become asymptomatic within days to weeks of the injury, many experience persistent cognitive, neuropsychological and somatic symptoms that are referred to as “persistent post-concussive syndrome”(Alexander 1995; Hillary et al. 2010; Johansson et al. 2009; Makley et al. 2008). Recently, it has been suggested by some that post-concussive symptoms may be more common than previously reported (Grool et al. 2016; Young and Tsao 2017; McInnes et al. 2017). The failure of conventional imaging methods to detect abnormalities in the brains of patients who subsequently suffer persistent symptoms represents a major challenge in predicting clinical outcomes in mTBI (Mechtler et al. 2014). In some cases, more advanced neuroimaging techniques, such as diffusion tensor imaging (DTI), functional MRI (fMRI), magnetoencephalography (MEG), magnetic resonance spectroscopy (MRS) and Single-Photon Emission Computed Tomography (SPECT) are utilized to better characterize the injury. These techniques have been reviewed previously (Mechtler et al. 2014; Reis et al. 2015; Shin et al. 2017).
Recent advances in neuroimaging have accelerated and improved the accuracy of TBI diagnosis and prognosis (Reis et al. 2015; Manning et al. 2017). However, these investigations are lengthy, expensive, and do not take into account the influence of the patient’s genetic background. Therefore, improved TBI care remains dependent on novel approaches to better assess TBI.

Animal Models of Traumatic Brain Injury

Over the last few decades, a number of animal TBI models have been developed to closely reflect the morphological, biochemical, molecular and behavioral changes associated with brain injury in patients. Using rodent TBI models, injury has been introduced to the brain in three complementary settings: focal impact, diffuse impact and non-impact (Table 3) (Rostami 2012).

Focal impact brain injury animal models are further divided into the weight drop (WD), fluid percussion (FP) and controlled cortical impact (CCI) models. In the WD model, the injury is produced by a free falling weight guided in a tube that is made to hit the skull, producing a contusion (Dail et al. 1981; Feeney et al. 1981; Morales et al. 2005). The severity of the injury can be adjusted by the height and mass of the weight dropped. Simulation of injury using this model is fast and easy, but limitations exist such as unintentional skull fracture, risk of a second rebound injury, and inaccuracy with regards to the impact site (Rostami 2012, Briones 2015). In the FP and CCI injury models, a craniotomy is performed between bregma and lambda. For the former, a pendulum strikes a piston of a fluid reservoir, creating a pressure pulse in the fluid that is transmitted to the exposed, intact dura, while in the latter a pneumatic or electromagnetic impact device is used to drive a rigid impactor to deliver mechanical force onto the dura (Dixon et al. 1987; Dixon et al. 1991; Lighthall 1988; McIntosh et al. 1989; McIntosh et al. 1987; Morganti-Kossmann et al. 2010; Xiong et al. 2013). All three techniques cause a deformity in the
underlying cortex, resulting in cortical tissue loss, hemorrhage, axonal injury, concussion, contusion and BBB dysfunction similar to those seen in patients (Chen et al. 1996; Schmidt and Grady 1993; Whalen et al. 1998; Xiong et al. 2013). CCI is considered a superior focal impact model, because it provides better control over factors such as the duration and velocity of impact and the depth of resulting damage in the brain, and also eliminates the risk of a rebound injury (Rostami 2012; Briones 2015). Similar to WD, FP and CCI, diffuse impact and non-impact brain injury animal models have been commonly used and well-cited in the literature (Rostami 2012). A limitation of several TBI models is the fact that injuries are commonly induced by direct contact with the brain through a craniotomy while the animal's head is immobilized, conditions which typically do not characterize human brain injury. The recently developed nonsurgical CHIMERA model (closed-head impact model of engineered rotational acceleration), which involves impact to the intact unrestrained head, overcomes this limitation (Namjoshi et al. 2014). The chimera model has been used to produce precisely controlled injuries, and allows for kinematic analysis of head movement at the time of impact, which can be correlated with behavioural, histological and biochemical outcomes (Namjoshi et al. 2017).

After injury, damage to the brain tissue can be evaluated with various markers to determine injury severity and its progression over time. For instance, the expression of cell apoptosis markers (Figure 2) can be monitored in parallel with behavioural deficits within hours-weeks of the injury to develop potential treatments for inhibiting cell death and enhancing functional recovery in the animals. While each model has its own unique advantages, it is important to note that no injury model accurately reproduces the complete spectrum of pathologies observed in human TBI. Also, animal TBI models require the use of anesthetic agents at the time of injury for ethical reasons. Since certain anesthetics have been shown to be neuroprotective, improving
functional and histological outcomes in TBI models when present at the time of injury (Rowe et al. 2013; Statler et al. 2000; Statler et al. 2006; Statler et al. 2006b), this approach may contribute to reduced clinical translation as patients are devoid of anesthetic agents at the time of injury.

Surgery is a common procedure to reduce or prevent the progression of damage caused by a hematoma, seizure or tumour in the brain. In particular, glioma evacuation generally causes lesions in the tissue surrounding the extracted tumour. Surgically-induced TBI animal models that mimic this type of damage have been developed (Frontczak-Baniewicz and Walski 2003; Jadhav et al. 2007; Jezierski et al. 2014; Rennie et al. 2013). Typically, these models involve removal of the skull overlying specific regions such as the primary motor cortex, and damaging the neural tissue to a pre-determined depth to evaluate its acute and chronic effects on the brain (Figure 3A-B). Based on our experience, a combination of various staining techniques to detect structural changes in neurons (Figure 3C-D) and biochemical assays to monitor the expression of pro- and anti-apoptotic markers (Figure 3E-F) can be used to assess the severity of damage to the brain.

In addition to these methods, *in vivo* Doppler and Optic imaging techniques can be used to establish a profile of the injury over time. Doppler imaging allows a non-invasive real time quantification of blood flow in specific regions of the brain at high resolution. The changes in blood flow in the injured brain, followed by revascularization as a repair mechanism can be readily monitored using this technology. Furthermore, *in vivo* optical imaging of fluorescent reporters such as green fluorescent protein (GFP) in transgenic mice (Fujiki et al. 2008) can be used to monitor the expression of specific proteins prior to and after injury and treatment (Figure 4). For instance, post-TBI reactive gliosis has been tracked in a spatiotemporal manner, using glial fibrillary acidic protein (GFAP)-GFP transgenic mice (Kim et al. 2012). Similar
methodologies have been used to detect the expression of nestin in the hippocampus (Yu et al. 2008; Wang et al. 2016a) and spinal cord (Matsumura et al. 2010) of Nestin-GFP transgenic mice after injury.

**Detection Markers**

There is compelling evidence that the knowledge obtained about the aberrant expression of intra- and extra-cellular proteins in the injured brain can contribute to a better understanding of the underlying mechanisms of brain injury. Furthermore, the success of post-TBI interventions heavily relies on targeting the complex molecular signaling pathways associated with the injury. To this end, several markers have been used to assess inflammation, oxidative stress, excitotoxicity and other pathophysiological mechanisms occurring within days to weeks of injury, and these biomarkers have been subjected to systematic review (Yokobori et al. 2013, Daoud et al. 2014; Mondello et al. 2017). Notably, it has been suggested that early biomarkers of structural damage such as S-100β, GFAP, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) and tau in cerebrospinal fluid (CSF) or blood may be helpful to determine whether a head CT scan is needed after mTBI (Mondello et al. 2011; Reis et al. 2015). Blood-based biomarkers of intracranial lesions in patients presenting to emergency departments after mild head injury are highly desirable in order to make informed decisions about the need for CT scans. However, recent systematic reviews indicate there is currently only sufficient evidence to support the use of serum S100B as a biomarker of injury, while there is not enough evidence to use serum or plasma GFAP, neuron specific enolase (NSE), UCH-L1, tau and neurofilament proteins in this clinical application (Mondello et al. 2017). At present, CSF remains the most reliable biofluid for providing biomarkers of brain injury (Agoston et al. 2017).
The presence of other factors such as auto-antibodies to brain-specific proteins and their breakdown products in the peripheral circulatory system is suggestive of an increased blood-brain-barrier permeability following TBI (Diamond et al. 2013). TBI patients showed nearly four times increase in anti-GFAP autoantibody levels within the first 10 days after injury (Zhang et al. 2014). Anti-GFAP autoantibodies were also elevated in TBI patients at an average of 6 months post injury compared to healthy controls (Wang et al. 2016b). Anti-N-methyl-D-aspartate (NMDA) receptor and anti-α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor are increased in the serum of children with post-traumatic headache, indicating hyper-stimulation of glutamate receptors and hypoxic ischemic brain lesions (Goryunova et al. 2007). Other markers such as anti-phospholipid correlate with vascular complications following trauma (Ngankam et al. 2011). Further research is needed in order to evaluate the usefulness of these autoantibodies as potential biomarkers of injury severity and prognosis (Reis et al. 2015). In addition, upregulation of cytokines (Galindo et al. 2011), deranged glucose profiles (Ley et al. 2011), changed levels of sex hormones (Wagner et al. 2011), and αII-spectrin breakdown products (SBDPs) are considered as useful biological signatures in assessing TBI (Forde et al. 2014; Mondello et al. 2012). Over the past decade, metabolomic profiling of biological fluids has also been used to identify markers of cerebral metabolic perturbation after TBI that correlate with injury severity or specific outcomes such as cognitive impairments (Daley et al. 2016; Oresic et al. 2016; Posti et al. 2017; Wolahan et al. 2015; Yi et al. 2017).

**MicroRNAs**

MicroRNAs (miRNAs) are short noncoding regulatory RNA molecules composed of 20-24 nucleotides that are often located within introns, and play key roles in regulating gene/protein expression (Bartel 2004, Ribbecco-Lutkiewicz et al. 2016). The human genome encodes over
3500 miRNAs (Bentwich et al. 2005, Londin et al. 2015), where individual miRNAs can target multiple genes, and a group of miRNAs can target the same gene (Lewis et al. 2005; Lim et al. 2005; Xie et al. 2005). In terms of structure, miRNAs originate from regions of RNA transcripts that fold back on themselves to form short hairpins and they function via base-pairing with complementary sequences of mRNA molecules (Bartel 2004; 2009). Once they are paired, mRNA molecules are silenced by cleavage of their strands, destabilization through shortening of their poly(A) tails and repressing translation (Bartel 2009; Fabian et al. 2010). Several laboratories have shown that miRNAs are widely expressed in the brain with many playing key roles in central nervous system (CNS) development and function (Bhalala et al. 2013; Tang et al. 2007; Wang et al. 2008). In recent years, miRNAs have been identified as a novel class of molecular modulators of TBI, based on their roles in regulation of various cellular functions in the brain (Bak et al. 2008; Bhalala et al. 2013; Coolen and Bally-Cuif 2009; Meissner et al. 2016).

**miRNAs as Biomarkers**

Detection of altered levels of specific miRNAs in peripheral blood and/or cerebrospinal fluid (CSF) after TBI suggests a potential use of miRNAs as injury biomarkers (Bian & Sun, 2011; Meza-Sosa et al. 2012; Redell et al. 2009; Yokobori et al. 2013). For instance, plasma miRNA profiles of severe TBI patients showed a reduction in miR-16 and miR-92a and an increase in miR-765 expression within 48 hours of injury, compared with those of healthy volunteers (Redell et al. 2010). In combination, these three miRNAs provided good diagnostic specificity and sensitivity, effectively distinguishing severe TBI patients from both healthy controls and patients with orthopedic rather than brain injuries. Conversely, plasma levels of miR-16 and miR-92a were increased in patients with mild TBI (Redell et al. 2010). In addition, it was
recently reported that levels of miR-93, miR-191 and miR-499 were all elevated in serum from mild, moderate and severe TBI patients. Notably, levels of all three miRNAs were related to both the severity of the injury (with severe TBI patients exhibiting significantly higher levels than mild or moderate TBI patients) and clinical outcomes several months later (Yang et al. 2016). A panel of 10 serum miRNAs upregulated in the acute phase (within 48 hours) of both mild-moderate and severe TBI patients but not in orthopedic injury patients was reported by Bhomia and colleagues (2016); four of these miRNAs were also found to be upregulated in CSF of severe TBI patients. While Bhomia et al (2016) focused on upregulated miRNAs common to both mild-moderate and severe TBI, a study by Di Pietro et al (2017) aimed to find serum miRNA biomarkers capable of discriminating between mild and severe TBI. They found that downregulation of miR-425-5p and miR-502 at early time points after injury characterized mild TBI patients, while upregulation of miR-21 and miR-335 was observed in serum from patients after severe TBI. Of interest, the ability of the miRNAs to distinguish between mild TBI, severe TBI and orthopedic injury patients was dependent on the interval between injury and serum sampling. Levels of miR-425-5p and miR-21(at 4-12 hours after injury) were also predictive of outcomes on the extended Glasgow Coma Scale 6 months later, suggesting not only diagnostic value but also a potential prognostic use of miRNAs (Di Pietro et al. 2017). Studies of blast-induced TBI in the rat have also identified plasma (or CSF) let-7i as a potential biomarker of brain injury (Balakathiresan et al. 2012), although to our knowledge this has not yet been examined in human blast injury patients. Together, these studies suggest a possible biomarker application of miRNAs, once their roles in TBI are better defined at the molecular and cellular levels (Redell et al. 2010, Hu et al. 2012), and once there is a better understanding of how type of injury and time since injury affect miRNA profiles.
miRNA alterations in animal models of brain injury

A number of studies have used microarrays to examine spatiotemporal patterns of miRNA alterations in injured cortical or hippocampal tissue in rodent TBI models (Lei et al. 2009; Liu et al. 2014; Liu et al. 2009b; Meissner et al. 2016; Redell et al. 2009). Assessment of a selected number of miRNAs in the injured mouse motor cortex shows that different miRNAs exhibit distinct expression profiles over time (Figure 5). In particular, some miRNAs such as miR-132, miR-21 and miR-30a respond within hours of the injury, with miR-21 maintaining upregulation in the injured brain compared with control for at least a week. In contrast, a number of other miRNAs examined in this study were down-regulated three days after injury and maintained lower expression levels compared with the control group after one week. Other studies using mice to model TBI show that changes in the expression of a number of miRNAs can occur as early as 1 h post injury (Sabirzhanov et al. 2014, Meissner et al. 2016). Even after mild TBI (induced by weight drop in the mouse, which resulted in no tissue damage upon histological examination), the expression of several dozen miRNAs in brain tissue was significantly altered (Chandran et al. 2017). Both the severity of the injury and time elapsed since injury influenced the pattern of miRNA changes in the brain (Chandran et al. 2017). Similarly, microarray analyses have revealed dynamic temporal regulation of miRNA expression within both the cortex and hippocampus in rat models of TBI (Bhalala et al. 2013; Lei et al. 2009; Redell et al. 2009).

The above studies demonstrate time-dependent coordinated changes in multiple miRNAs after injury. Although the function of individual miRNAs is not always known, bioinformatics analyses can suggest predicted targets of dysregulated miRNAs in the injured brain. Studies of miRNA expression in traumatically injured brain have revealed that the targets of up- or
downregulated miRNAs in injured brain are often clustered into groups linked to distinct cellular processes (Meissner et al. 2016; Reis et al. 2015; and see Figure 6). Time-course studies indicate that while many of the miRNAs affected during the acute post-injury phase are predicted to target genes involved in apoptosis, those affected in the chronic stages of injury regulate genes involved in cytoskeletal organization, intracellular trafficking and other processes related to brain repair (Bhalala et al. 2013; Bhalala, 2015; Hu et al. 2012). Importantly, changes in the expression of miRNAs after injury have been shown to coincide with changes in protein expression of some predicted targets in the damaged tissue (Redell et al. 2011). These types of studies can provide insight into both pathophysiological and endogenous repair processes occurring after injury, may help to explain the efficacy of treatments that protect or promote repair of the injured brain, and can possibly suggest novel therapeutic targets.

Similar to TBI, changes in miRNA levels have been observed following stroke and spinal cord injury (SCI). In stroke, miRNAs seem to target angiogenesis, hypoxia, endothelial cell regulation and immune response (Tan et al. 2009), and their expression profiles have been used to differentiate between ischemic and hemorrhagic stroke (Liu et al. 2010a). In SCI, over 35% of the miRNAs expressed in the spinal cord were reported to be significantly affected within the first 7 days of injury (Liu et al. 2009b). Many of the reported miRNAs target the same pathways known to be involved in other CNS injuries, including apoptosis, inflammation, cell proliferation and cell differentiation (Bhalala et al. 2013). Notably, some of these pathways are affected by different sets of miRNAs, depending on the type of injury. For instance, apoptosis is regulated by miR-29b, miR-145 and miR-497 following stroke (Dharap et al. 2009; Shi et al. 2012; Yin et al. 2010), and by miR-15b and miR-486 after SCI (Liu et al. 2010b; Moschidou et al. 2012). On the other hand, some miRNAs may play a similar role in diverse types of injury. For example, miR-
MiR-21 level has been shown to increase around the lesion site within the first 2 weeks of injury and is upregulated dramatically in the chronic stages in a murine SCI model (Bhalala et al. 2012). Similar results have also been observed within the first 7 days post injury in a rodent model of stroke (Buller et al. 2010), and miR-21 upregulation has been observed in multiple TBI models including penetrating ballistic-like brain injury (Johnson et al. 2017), CCI (Redell et al. 2010; Sandhir et al. 2014), and FPI (Lei et al. 2009). MiR-21 targets B-cell lymphoma-2 (Bcl2), phosphatase and tensin homolog (PTEN) and cell death protein 4 (CDP4) (Gabriely et al. 2008; Ge et al. 2014; Hashimi et al. 2009; Kim et al. 2009; Lei et al. 2009; Liu et al. 2009a; Lu et al. 2009; Meissner et al. 2016; Papagiannakopoulos et al. 2008; Redell et al. 2009, 2011; Sabatel et al. 2011; Sheedy, 2015; Shi et al. 2013; Van Wysberghe et al. 2011; Wickramasinghe et al. 2009; Yelamanchili et al. 2010). MiR-21 can also inhibit apoptosis by targeting PTEN, activating Ang-1/Tie-2 and Akt signaling, and promoting outgrowth of neuronal axons in TBI and stroke (Christie et al. 2010; Ding et al. 2013; Ge et al. 2015; Ge et al. 2014; Han et al. 2014; Ohtake et al. 2014; Ohtake et al. 2015; Onyszchuk et al. 2007; Weber et al. 2010; Zhao et al. 2013). Thus, the upregulation of miR-21 in damaged neural tissue may represent a potential mechanism by which the brain attempts to limit neuronal destruction in the aftermath of an injury (Bhalala, 2015). Interestingly, the miR-21 response to TBI in aged mice was shown to be attenuated, possibly contributing to worsened outcomes in older animals (Sandhir et al. 2014).

Changes in miRNA expression may play a role in the positive response to some neuroprotective therapies. While the benefits of therapeutic hypothermia remain controversial in TBI patients (Dietrich and Bramlett 2016; Yokobori and Yokota 2016), hypothermia has a long history as a therapeutic intervention in animal models of brain injury, and several miRNAs with altered
expression after TBI are downregulated under hypothermic conditions (Truettner et al. 2011).

Hypothermia dampened the upregulation of miR-874 and miR-9 observed in damaged cortex after fluid percussion injury. Since elevation of these miRNAs after injury was predicted to impair intracellular transport of proteins, disrupt membrane organization and damage the cytoskeleton, the authors suggest that the ability of hypothermia to mitigate the miRNA response to brain injury might underlie some of its therapeutic efficacy (Truettner et al. 2011).

**miRNAs as therapeutic targets**

Because of their dynamic involvement in the CNS, the inhibition and restoration of specific miRNAs has been considered a therapeutic opportunity to modulate cellular processes in TBI, SCI and stroke patients. Currently, a few options are being explored, including miRNA amplifiers, miRNA inhibitors, and artificial miRNAs (Bhalala 2015).

One strategy is to increase the levels of beneficial miRNAs in the damaged brain by introducing a plasmid containing the sequence of the pri-miRNA or pre-miRNA (precursors to the mature miRNA) into the target tissue. Another strategy is to use miRNA mimics, which are modified double-stranded RNA molecules with one strand containing the same sequence as the mature miRNA (Bhalala 2015), although mimics are not easily introduced into neurons (Zhang et al. 2013).

Another option is to use miRNA antagonism to mitigate the effect of harmful miRNAs following brain injury. miRNA inhibitors, including antagomiRs, locked nucleic acids (LNAs), and miRNA sponges (Bhalala et al. 2013; Grünweller and Hartmann, 2007; Krützfeldt et al. 2005; Wang et al. 2012), are antisense sequences that bind mature miRNA molecules and block their functions. AntagomiRs are a class of chemically engineered nuclease-resistant oligonucleotides
that prevent miRNAs from binding to a desired site on an mRNA molecule, effectively silencing them (Krützfeldt et al. 2005, Czech 2006, Yue 2011; Zhang et al. 2013); however, because of their relatively low potency, high doses are needed in vivo (Broderick and Zamore 2011). LNAs are modified oligonucleotides that contain locked ribose rings to enhance their capacity to bind to target miRNA, resulting in higher potency miRNA inhibition (Grünweller and Hartmann 2007; Stenvang et al. 2008) as well as greater nuclease resistance (Zhang et al. 2013). For longer-term inhibition of miRNAs, sponge constructs that act as competitive inhibitors of miRNAs may be useful. These are artificial RNA transcripts with multiple repeats that are complementary to the target miRNA. Integration of the sponge construct into the genome of CNS cells allows for persistent inhibition of the target miRNA (Bhalala et al. 2012; Gentner et al. 2009; Luikart et al. 2011).

Finally, the generation of artificial miRNAs designed to target multiple mRNAs may be a strategy to achieve coordinated, multi-faceted modulation of the pathophysiological response to brain injury (Bhalala et al. 2013).

While promising, relatively few studies on the ability of miRNA-based molecules to provide neuroprotection or to enhance reparative processes in the experimentally injured brain have been reported. Overexpression of a few miRNAs has been shown to attenuate neural injury in animal models of TBI (Ge et al. 2014; Ge et al. 2015; Sun et al. 2016; Sun et al. 2017). Conversely, administration of a miR-711 hairpin inhibitor after TBI resulted in improved histological and neurological outcomes in a mouse CCI model (Sabirzhanov et al. 2016). However, the utility of these tools as therapeutic agents has yet to be demonstrated.

Exosomes
Exosomes are small membranous microvesicles which carry proteins, lipids and nucleic acids, serving as a communication system between cells and tissues locally or over long distances (Chopp and Zhang 2015; Kalani et al. 2014). Many different cell types secrete exosomes, including multiple CNS cells (Kalani et al. 2014). Because they are accessible and stable in biological fluids but reflect the phenotype and condition of the parent cell, exosomes and their contents, including miRNA, have the potential to serve as biomarkers for CNS pathology (Kalani et al. 2014; Katsuda et al. 2013; Taylor and Gercel-Taylor 2014).

A handful of studies have examined the contents of exosomes released after either human TBI or experimentally induced TBI in animals. Patz et al. (2013) demonstrated that the miR-9 and miR-451 content of CSF-derived microvesicles was altered in TBI patients compared with non-injured controls. In exosomes isolated from the brains of mice subjected to CCI, the level of miR-212 was reduced, while levels of miR-7b, miR-7a, miR-21 and miR-146 were all elevated relative to sham-operated mice (Harrison et al. 2016). It is not yet known which cells might be recipients of this miRNA cargo or what impact exosome-derived miRNA might have on the progression of pathology in the injured brain.

Exosomes derived from mesenchymal stem cells have successfully been used as therapeutic agents in experimental TBI models (Kim et al. 2016; Zhang et al. 2015, 2016), although the role of miRNA in the beneficial outcomes observed following treatment with exosomes is not known. Nevertheless, it may be possible to capitalize on the ability of exosomes to act as delivery vehicles for miRNA by altering the miRNA content of exosomes to modulate specific pathophysiological pathways in TBI (Xiong et al. 2017; Yang et al. 2017).
In conclusion, TBI affects people of all ages and in all geographical areas. Despite the enormous costs of TBI to society, there are no direct therapies. Animal models of TBI may eventually aid development of TBI interventions, but must take into account the significant patient and injury heterogeneity encountered with real world injuries. Here, we review TBI prevalence, classification and pathophysiology, with emphasis on TBI animal models and emerging molecular markers. Once molecular markers are better established, they could improve current TBI diagnostic and prognostic capabilities, as well as be potential therapeutics. Molecular markers may aid TBI diagnostics by improving clinical decision making and by allowing for rapid deployment of rehabilitation services. Molecular markers may improve TBI prognostics that are essential for optimizing patient outcomes, for aiding the next wave of intervention research by stratifying patients for clinical studies, and for use in clinical audits by allowing adjustment for case mix. Also, accurate TBI prognostic tools will be invaluable to the legal guardians of brain injured patients in making informed treatment and compassionate care decisions. While identification of TBI molecular markers is a critical forward step, studies are still in their infancy.
Acknowledgements:
The authors would like to acknowledge Dorothy Fatehi, Bogdan Zurakowski and Brandon Smith for their contributions. We dedicate this manuscript to our dear friend and colleague, Dr. Mahmud Bani-Yaghoub.
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**Figure Legends**

**Figure 1:** Example of traumatic brain injury detected by MRI in a teenager, following a motor vehicle collision. A,B) CT imaging shows injury-induced hemorrhage (arrow). C,D) Brain tissue abnormalities in the left frontal lobe are observed in fluid attenuated inversion recovery (FLAIR)- and T2-based MRI axial sections. In both imaging modalities, acute blood appears dark. Notice the heterogeneous increased signal intensity consistent with evolving blood products with surrounding edema in the frontal lobe. All images courtesy of Children’s Hospital of Eastern Ontario, Ottawa, Ontario, Canada.

**Figure 2:** Caspase 3 expression in the mouse primary motor cortex in a controlled cortical impact (CCI) model. A-E) Peri-lesion cortex; A) sham, B) 3 hours, C) 1 day, D) 3 days and E) 1 week after injury. 10 week old male C57Bl/6 mice were anesthetized with isoflurane and mounted in a stereotaxic apparatus. A 5mm diameter disc centred 0.5mm posterior and 2mm lateral to Bregma was removed from the skull. An ImpactOne device (Leica Biosystems, Concord, ON) with 3mm diameter flat-tipped impactor tip was used to produce an injury 1mm deep (impact velocity was 3.25m/s with dwell time 100ms). Sections from formalin fixed brains were sectioned on a vibratome at 50 µm and processed for immunohistochemistry using anti-Caspase 3 (1:200; Cell Signaling Technology, Danvers, MA), and visualized using Vector Elite ABC reagent and Vector NovaRed (Vector Laboratories, Burlington, ON). Scale bar: 200 µm

**Figure 3:** Morphological and biochemical changes in the primary motor cortex of a surgically-induced brain injury mouse model. Injuries were induced in 8-10 week old male C57Bl/6 mice as
described in Rennie et al. (2013). Briefly, the skull overlying the motor cortex was removed, and cortical tissue was removed to a depth of 1mm. Images of whole brain in (A) sham and (B) 3 hours post-injury mice; lesion site (*). Scale bar: 1mm (A,B). Representative Golgi-Cox staining of (C) sham and (D) 3 hours post-injury brain sections show extensive tissue loss (*) in the damaged region. Here, the damage extends through layer V of the cortex. Scale bar: 200 µm (C, D). The image in (C) is a portion of an image previously published in Rennie et al. (2013).

Western blot shows the expression of pro- and anti-apoptotic proteins 3 hours, 3 days and 1 week after injury. In particular, changes in the expression of Bax1 and Bcl-xL are notable. For western blots, the protein was extracted by homogenizing the tissue in 100µl of Ripa buffer with protease inhibitors on ice using a hand held homogenizer. The homogenate was then incubated on ice for 30 to 45 minutes, and centrifuged for 20 minutes at 16 000g. 100µg of protein was loaded per well.

**Figure 4:** In vivo detection of GFP in control and transgenic GFP-expressing mice. GFP expression was monitored in the head of both A) control and B) hemizygous C57Bl/6-Tg (CAG-EGFP)131Osb/LeySopJ mice expressing GFP under the control of a beta actin promoter using an eXplore Optix imager (ART Advanced Research Technologies Inc, Saint-Laurent, Qc) with 470nm emitting laser and 1.5mm step size. Abundant levels of reporter protein can be detected in the GFP-expressing mouse.

**Figure 5:** miRNA expression in the primary motor cortex of a mechanically-induced brain injury mouse model. Surgically induced brain injury was performed in 8-10 week old male C57Bl/6 mice as described in Rennie et al. (2013). Q-RT-PCR analysis shows an upregulation of miR-
132, miR-21 and miR-30a three hours after injury. In particular, the expression of miR-21 was significantly increased in the three hour post-injury samples and remained higher three days and one week after injury, compared with the control group. In contrast to miR-21, other miRNAs showed reduction three days and one week after injury. Expression levels of miR-21 were significantly different from sham at all investigated time points; sham/3h p=0.0011, sham/3day p=0.0002, sham/1week p=0.0003. Foot note: Total RNAs were extracted from the primary motor cortex of sham and injured mice, using Tri-reagent. Taq-Man qRT-PCR experiments were performed in duplicate, using 10 ng total RNA samples from each of sham and injured mice (n=6 for each group). SnoRNA202 expression was used to normalize miRNA expression of all data points. Following normalization, the injury groups were scored against the sham group, set at 1 fold. Fold changes were calculated by mean $2^{-\Delta Ct}$, where $\Delta Ct$ is the normalized cycle number difference between the injury groups and the sham group.

Figure 6: KEGG pathways affected by miRNAs up/downregulated by brain injury. An example of bioinformatics analysis of cellular pathways and processes predicted to be affected by miRNAs modulated in injured brain. DIANA mirPath v.3 software (Vlachos et al. 2015) was used to predict KEGG pathways affected by a panel of 8 miRNAs whose expression was altered in injured mouse brain (see Figure 5 legend for experimental details). The number of miRNAs (of the 8) predicted to target genes in each pathway is shown in light grey, and the number of genes in the pathway predicted to be targeted by the altered miRNAs is shown in black. From left to right, pathways are shown in decreasing order of significance (increasing p-value), but all are significant at the p<0.05 level using the modified Fisher’s Exact test and False Discovery Rate correction built into mirPath software.
<table>
<thead>
<tr>
<th><strong>Brain injury</strong></th>
<th><strong>Traumatic brain Injury</strong></th>
<th><strong>Non-traumatic brain injury</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definition</strong></td>
<td>Damage to the brain caused by an external force.</td>
<td>Damage to the brain caused by infection, brain tumors, ischemia or stroke.</td>
</tr>
<tr>
<td><strong>Types</strong></td>
<td>Penetrating head injury</td>
<td>Closed-head injury</td>
</tr>
<tr>
<td><strong>Description</strong></td>
<td>Open head injury occurring when object penetrates the skull</td>
<td>Closed head injury occurring when the skull is not significantly damaged, but the brain is damaged</td>
</tr>
<tr>
<td><strong>Examples</strong></td>
<td>Gunshot. Knife wounds.</td>
<td>Blunt force (e.g. a fall). Overpressure (e.g. blast force). Accelerative force (e.g. car accidents). Shaking or strong rotation of the head (e.g. Shaken Baby Syndrome).</td>
</tr>
</tbody>
</table>
### Table 2: The Glasgow Coma Scale*

<table>
<thead>
<tr>
<th>Response</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>Does not open eyes</td>
<td>Opens eyes in response to painful stimuli</td>
<td>Opens eyes in response to voice</td>
<td>Opens eyes spontaneously</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Verbal</td>
<td>Makes no sound</td>
<td>Incomprehensible sounds</td>
<td>Utters inappropriate words</td>
<td>Confused, disoriented</td>
<td>Oriented, converses normally</td>
<td>N/A</td>
</tr>
<tr>
<td>Motor</td>
<td>Makes no movements</td>
<td>Extensions to painful stimuli</td>
<td>Abnormal flexion to painful stimuli</td>
<td>Flexion/Withdrawal to painful stimuli</td>
<td>Locates painful stimuli</td>
<td>Obeys commands</td>
</tr>
</tbody>
</table>

*Based on Teasdale and Jennett 1974.
**Table 3: TBI models**

<table>
<thead>
<tr>
<th>Focal Impact</th>
<th>Diffuse Impact</th>
<th>Non-Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Weight drop [1,2,3,4]</td>
<td>• Diffuse injury [14]</td>
<td>• Inertial acceleration [16,17]</td>
</tr>
<tr>
<td>• Fluid percussion injury</td>
<td>• Impact acceleration [15]</td>
<td>• Rotational TBI [18]</td>
</tr>
<tr>
<td>- Midline [5,6]</td>
<td></td>
<td>• Blast injury [19, 20]</td>
</tr>
<tr>
<td>- Lateral [7,8]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Controlled cortical impact [9, 10, 11]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Penetrating and ballistic injury [12,13]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbered citations in the above table refer to the following references: 1: (Feeney et al. 1981); 2:(Dail et al. 1981); 3:(Shapira et al. 1988); 4:(Chen et al. 1996); 5:(McIntosh et al. 1987); 6:(Dixon et al. 1987); 7:(McIntosh et al. 1989); 8:(Carbonell et al. 1998); 9:(Lighthall 1988); 10:(Dixon et al. 1991); 11:(Smith et al. 1995); 12:(Williams et al. 2005); 13:(Williams et al. 2006); 14:(Cernak et al. 2004); 15:(Marmarou et al. 1994); 16:(Gennarelli et al. 1982); 17:(Ross et al. 1994); 18:(Davidsson and Risling 2011); 19:(Long et al. 2009); 20:(Garner et al. 2009).
Figure 2

48x257mm (300 x 300 DPI)
The scientific community is mourning the loss of the young colleague, Dr. Mahmud Bani-Yaghoub (1964-2016), a Senior Research Officer and Team Leader at the National Research Council (NRC) of Canada. Mahmud was Adjunct Professor at the University of Ottawa since 2006, member of Editorial board of several journals, including American Journal of Stem Cells, and member of the Ontario Institute for Regenerative Medicine.

Mahmud was a champion of multidisciplinary science and innovation. He led many collaborative projects with national and international centres, including the Centre for Commercialization of Regenerative Medicine (CCRM).

He was a recipient of the NRC’s Outstanding Achievement Award for scientific breakthrough, NRC-Institute for Biological Sciences Outstanding Group Achievement and Industrial Partnership Awards, and NRC-Human Health Therapeutics Portfolio Leadership Award.

Mahmud made a lasting impact in advancing stem cell models and applications in regenerative medicine. His most recent achievement is the development of human stem-cell based models for accelerating the development of novel therapies for brain diseases, including a unique industrial-grade blood-brain barrier model, used in partnership with many biopharmaceutical companies. The book on ‘Fetal Stem Cells in Regenerative Medicine’ co-edited by Dr. Mahmud Bani and Dr. Dario Fauza, was published by Humana Press in the spring of 2016, a few months before his sudden departure.

Mahmud leaves a legacy of uncompromising commitment to science and innovation, outstanding scientific advances and inspirational leadership that embodies scientific entrepreneurship and innovation. NRC lost one of its rising stars, prematurely, at the peak of his career. Dr. Mahmud Bani will be missed dearly by everyone who worked with him for his boundless energy, sharp inquiring mind and genuinely caring personality.