Using Bioinformatic Tools to Identify Genes and microRNAs Associated with mild Traumatic Brain Injury Outcomes

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TiTLE: Using Bioinformatic Tools to Identify Genes and microRNAs Associated with mild Traumatic Brain Injury Outcomes

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Lay Abstract

Traumatic brain injury (TBI) is a highly prevalent neurological injury affecting millions of individuals globally. Mild TBI (mTBI), sometimes called concussion, makes up over 85% of TBI cases. A mTBI is a heterogeneous condition with acute and chronic outcomes for patients and involves complex cascades of cellular and molecular events that can lead to functional changes in genes and associated metabolites. In recent genetic studies, it has been shown that certain genotypes are associated with a higher risk of experiencing a more serious injury and a slower recovery after mTBI. These genes can be utilized as crucial biomarkers to predict how long it will take for a person to recover from a concussion. The purpose of this study was to find potential biomarkers that could help in the early detection of mTBI and the monitoring of individual patients' recovery. It was hypothesized that genes and miRNAs (and their associated proteins) involved in neuronal body, axonal and myelin integrity and regeneration would be identified as important markers of severity.

Abstract

A mild traumatic brain injury (mTBI), commonly referred to as a concussion, is when the brain experiences an abrupt acceleration and/or deceleration that sends shock waves through the brain tissue, upsetting its structure and function. A mTBI is a heterogeneous condition with acute and chronic outcomes for patients. The chronic form of mTBI can lead to a wide range of neurological, behavioral, and cognitive symptoms. Critically, this injury is not defined by a simple process or pathophysiological event but rather biomechanical and neurological brain damage that can trigger highly complex physiological cascades. These further lead to a wide range of cellular, molecular, and functional changes that alter genes and associated metabolites. These changes, if specifically characterized, could be used to predict a patient's outcome and recovery timeline. Recently, genetic studies showed that specific genotypes could increase an individual's risk of more severe injury and impaired recovery following mTBI. Consequently, an improved understanding of gene alteration and genetic changes is necessary to develop personalized diagnostic approaches which can guide the design of novel treatments. The current study proposes utilizing bioinformatic tools, biological networks, and databases to identify potential genes and microRNAs associated with the mTBI in order to aid the early diagnosis of mTBI and track recovery for individual patients. With bioinformatic techniques, we were able to identify and compare genetic and epigenetic data associated with mTBI, as well as understand the various aspects of molecular changes after brain injury. Ultimately, we analyzed and cataloged the biological pathways and networks associated with this injury. A critical search of online bioinformatics databases was performed to determine interactions between mTBI-related genes, and relevant molecular processes. The major finding was that *APOE, S100B, GFAP,*

BDNF, *AQP4*, *COMT*, *MBP*, *UCHL1*, *DRD2*, *ASIC1*, and *CACNA1A* genes were significantly associated with mTBI outcome. Those genes are primarily involved in different neurological tasks and neurological pathways such as neuron projection regeneration, regulation of neuronal synaptic plasticity, cognition, memory function, neuronal cell death and the dopaminergic pathway. This study predicted specific miRNAs linked to mTBI outcomes and candidate genes (hsa-miR-204-5p, hsa-miR-16-5p, hsa-miR-10a-5p, has-miR-218-5p, has-miR-34a-5p), and RNA-seq analysis on the GSE123336 data revealed that one miRNA found (hsa-miR-10a-5p) matched our predictions related to mTBI outcomes. Pathway analysis revealed that the predicted miRNA targets were mainly engaged in nervous system signaling, neuron projection and cell differentiation. These findings may contribute to developing diagnostic procedures and treatments for mTBI patients who are still experiencing symptoms, but validation of these genetic markers for mTBI assessment requires patient participation and correlation with advanced personalized MRI methods that show concussion related changes.

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Abbreviations:

TBI: Traumatic Brain Injurie

mTBI: Mild Traumatic Brain Injurie

CT: Computed Tomography

MRI: Magnetic Resonance Imaging

microRNAs/ miRNAs: Micro Ribonucleic Acids

RNA-seq: RNA-Sequencing

EAAs: Excitatory Amino Acids

ATP: Adenosine Triphosphate

ADP: Adenosine Diphosphate

BBB: Blood-Brain Barrier

ICP: Increased Intracranial Pressure

CNS: Central Nervous System

CSF: Cerebrospinal Fluid

DCE-MRI: Dynamic Contrast Enhanced MRI

CBF: Cerebral Blood Flow

DAI: Diffuse Axonal Injury

ROS: Reactive Oxygen Species

ETC: Electron Transport Chain

$Ca2^{+}$: Calcium

RNS: Reactive Nitrogen Species

RF: Radiofrequency

DTI: Diffusion Tensor Imaging

fMRI: functional Magnetic Resonance Imaging

BOLD: Blood-Oxygen Level Dependent

LOC: Loss of consciousness

rsMRI: Resting-state fMRI

CSF: Cerebrospinal Fluid

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

PPI: Protein-Protein Interactions

GEO: Gene Expression Omnibus

RNAs: Ribonucleic acid

mRNA: Messenger RNA

OMIM: Online Mendelian Inheritance in Man

DAVID: Database for Annotation, Visualization, and Integrated Discovery

MF: Molecular function

BP: Biological process

CC: Cellular components

CCI: Controlled Cortical Impact

DRD2: Dopamine Receptor D2

GSEA: Gene Set Enrichment Analysis

BWA: Burrows-Wheeler Alignment

MMA: Amateur Mixed Martial Art

hg38: Human genom38

FDR: False Discovery Rate

Padj: P.adjusted

DAergic: Dopaminergic

FHM: Familial Hemiplegic Migraine

FTD: Frontotemporal dementia

FADS1: Fatty Acid Desaturase 1

PTSD: Post-traumatic stress disorder

UPP: Ubiquitin-proteasome pathway

APOE: Apolipoprotein E

ANKK1: Ankyrin Repeat and Kinase Domain Containing1

ATP1A2: ATPase, Na^+/K^+ transporting, alpha 2 (+) polypeptide

AANAT: Aralkylamine N-acetyltransferase

BDNF: Brain-derived neurotrophic factor

CACNA1A: Calcium voltage-gated channel subunit alpha 1 A

COMT: Catechol-O-methyltransferase

cDNA: Complementary DNA

($O2^{\bullet-}$): Superoxide radicals

H_2O_2: Hydrogen peroxide

rmTBI: Repeated mild Traumatic Brain Injurie

NAA: N-Acetyl Aspartate

MRS: Proton Magnetic Resonance Spectroscopy

NAA/Cr: N-acetylaspartate/Creatine

NAA/Cho: N-acetylaspartate/Choline

DAMPs: Damage Associated Molecular Patterns

PRRs: Pattern Recognition Receptors

mtDNA: Mitochondrial DNA

BCL-2: B-cell lymphoma 2

PCSS: Post-Concussion Symptom Scale

SCAT: Sport Concussion Assessment Tool

SRC: Sport-Related Concussion

GCS: Glasgow Coma Scale

BESS: Balance Error Scoring System

K-D: The King Devick test

DLPFC: Dorsolateral prefrontal cortex

CRT5: Concussion Recognition Tool

HRV: Heart Rate Variability

DNA	Deoxyribonucleic Acid
GFAP	Glial fibrillary acidic protein
IGF-1B	Insulin-like growth factor 1B
H-NMR	Proton Nuclear Magnetic Resonance
IEGs	Immediate Early Genes
MBP	Myelin basic protein
MS	Mass Spectrometry
MMP4	Metalloproteinase of VMP family
MAP2K6	Mitogen-Activated Protein Kinase Kinase 6
NADH	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
Nrf2	Erythroid 2-related factor
NODAL	Nodal Growth Differentiation Factor
RUNX3	RUNX Family Transcription Factor 3
SNPs	Single Nucleotide Polymorphisms
SMAD1	SMAD Family Member 1
IL-6	Interleukin 6
IL-10	Interleukin 10
TNFa	Tumor Necrosis Factor Alpha
ENO2	Enolase 2
AQP4	Aquaporin-4
UCHL1	Ubiquitin C-Terminal Hydrolase L1

Declaration of Authorship

I, Mahnaz Tajik, declare that this thesis titled, “Using Bioinformatic Tools to Identify Genes and microRNAs Associated with mild Traumatic Brain Injury Outcomes” and the work presented in it are my own.

CHAPTER 1: INTRODUCTION

1.1 Mild Traumatic Brain Injury

Mild traumatic brain injury (mTBI), also referred to as concussion, occurs when the brain suddenly accelerates and/or decelerates inside the skull due to a traumatic force applied to the head or body. The resultant forces are transferred to the brain resulting in a range of symptoms. Depending on the severity of the injury, severe neurological conditions could result. But most TBIs are mild and lead to complete recovery. Nonetheless this condition affects millions of individuals globally, particularly the younger population [1,2]. Despite the fact that most individuals suffering from mTBI only experience a transitory interruption in brain function, postmortem investigations, more advanced imaging techniques, and biomarker studies indicate that mTBI leads to structural, pathophysiologic, and neuropathologic alterations [3-6]. An mTBI is characterized by a graded set of clinical symptoms, including dizziness, confusion, unconsciousness for thirty minutes or less, posttraumatic amnesia lasting less than 24 hours, and/or temporary neurological problems such as focal signs, seizures, or non-surgical intracranial lesions [7,8]. Most mTBI patients recover in a week to ten days. However, in some instances, the length of these symptoms may be prolonged [9,10]. The acute signs and symptoms most often reflect functional impairment as opposed to structural injury, as a result, standard structural neuroimaging studies fail to detect abnormalities [9]. Immediately following a head injury, a series of distinctive neurochemical, neurometabolic, and pathophysiological events are set in motion [11,12]. These processes initiated with neuronal cell membranes rupture and axonal stretching, leading to unregulated flux of ions via previously controlled ion channels and likely

temporary physical membrane deficiencies [12-14]. The extensive release of several neurotransmitters, including excitatory amino acids (EAAs), causes additional neuronal depolarization with potassium efflux and calcium influx [11,15,16]. In order to normalize these ionic shifts, activation of the sodium-potassium (Na^+-K^+) pump is increased to restore the neuronal membrane potential, which results in hyperglycolysis, loss of intracellular energy stores, and elevated ADP levels [12,17]. The increase in energy demand occurs in the early stages of the brain damage when cerebral blood flow is normal or reduced, resulting in an imbalance between glucose supply and demand, thus causing a cellular energy crisis [11, 14, 18]. This crisis makes the brain more susceptible to subsequent injury, increasing the likelihood of longer-lasting abnormalities. Additionally, depolarization and membrane disruption-induced calcium accumulation may hinder mitochondrial oxidative metabolism and immediately cause cell death [14]. It has been demonstrated that such intra-axonal calcium flow destabilizes neurofilaments and microtubules, affecting post-traumatic neuronal connection [14]. These changes are generally temporary and self-contained after a single mTBI, but repeated head injury may cause longer lasting pathobiological symptoms [12]. In addition to the above-mentioned acute changes, concussive brain injury leads to neuroplasticity impairment and axonal disruption, which can have a massive effect on recovery, especially in the young brain [19]. Thus, these pathophysiological dysfunctions need to be considered carefully since they result in a variety of cellular, molecular, and functional changes that modify genes and related metabolites. These changes, if specifically characterized, could be used to predict a patient's outcome and recovery timeline, particularly, to address athletes' concerns about returning to play after an injury.

Predicting mTBI outcomes is complicated and may or may not include diagnostic techniques such as magnetic resonance imaging (MRI) or computed tomography (CT). Blood

testing is not routinely performed due to a lack of consistent biomarkers. However, detailed genomic and proteomic level investigations have not been conducted. Current diagnostic protocols are limited by excessive variability. Furthermore, neither patients nor their physicians can predict recovery timelines or whether recovery will even be complete. Recently, genetic studies showed that specific genotypes including *APOE, DRD2, BDNF, COMT* could increase an individual's risk of more severe injury and impaired recovery following mTBI. Consequently, we need to improve our understanding of gene alteration, genetic changes, and the impact of proteomics as well as metabolomics to develop personalized diagnostic approaches which can guide the design of novel treatments for patients.

There are inherent drawbacks to existing diagnostic methods and clinical tools for assessing mTBI, which depend on patients' self-reported signs or general observations, as well as widely used neuroimaging techniques are not sensitive enough to identify mTBI in its early stages or to predict the duration of recovery, particularly for athletes and young children. To fill the current measurement gap, genetic biomarkers would provide a promising accessible detector of mTBI. Thus, the current study proposes utilizing bioinformatic tools, biological networks, and databases to identify potential genes and microRNAs associated with the mTBI in order to aid the early diagnosis of mTBI and track recovery for individual patients. With bioinformatics techniques, we were able to identify and compare genetic and epigenetic data associated with mTBI, as well as understand the various aspects of molecular changes after brain injury. Ultimately, we analyzed and cataloged the biological pathways and networks associated with mTBI.

1.2 Pathophysiology of mTBI

Several animal models have been used to elucidate the basic neurobiology of concussion/mTBI, and human research has progressively supported it. Biological modifications, changes in the cytoskeleton and axon [20] abnormalities in neurotransmission, and sensitivity to delayed apoptosis and chronic malfunction are all part of the neurometabolic cascade that results in concussion/mTBI [12, 21]. Some of these changes, which are still being researched and could possibly become mTBI biomarkers, are reviewed in this section.

1.2.1 Primary and Secondary Mechanisms

Molecular alterations caused by a brain injury can be categorized by occurring in a primary or secondary injury phase. The primary injury phase is the direct result of physical forces applied to the brain during the concussive event. The strain forces can cause diffuse axonal injury, extra- and intraparenchymal hemorrhages, blood-brain barrier (BBB) instability, and neuronal and glial cell damage within the brain [22, 23]. The secondary, or delayed injury, phase is more progressive than the initial mechanical damage. This phase consists of reversible systematic neuroinflammation and cellular damage, increased intracranial pressure (ICP), edema/swelling, possible microhemorrhages (infrequent), and neurochemical pathway disruptions [14, 24, 25]. It is believed that these mechanisms are linked to altered gene expression as well as the overexpression and release of a myriad of neurochemical substances that may destroy or repair cells, resulting in delayed cellular malfunction, death, or both [26]. According to some studies, these processes might offer the possibility of therapeutic interventions that can prevent progressive tissue damage and improve the regeneration of neuronal cells [26-28].

1.2.2 Blood Brain Barrier (BBB) Dysfunction

A breakdown and resultant dysfunction of the BBB has been connected to a variety of neurological conditions and the more severe forms of TBI [29]. In mTBI, BBB dysfunction can be pathogenically acute or chronic and experimental investigations revealed that BBB damage in mTBI patients might last for several years and is strongly linked to long-term neurological conditions [30]. Following primary trauma, BBB damage enhances the brain's blood vessel permeability and puts CNS proteins at risk of being exposed to peripheral immune response, which could result in antigen exposure and the formation of autoantibodies against CNS proteins [31]. Since the BBB is disrupted, circulating autoantibodies against CNS antigens can reach the brain and disrupt microglia, astrocytes, and neurons [11, 32]. It is significant to highlight that CNS autoantibodies have been associated with more than just brain injury; they have also been connected to a number of CNS diseases [33]. Studies have revealed that some CNS or/and serum biomarkers, can be employed to assess BBB dysfunctions post-injury. For example, cerebrospinal fluid (CSF)/serum albumin ratio has been utilized to assess BBB impairment in patients with severe TBI, and they continue to be the primary indicator for BBB integrity [34, 35]. Tight junction proteins, such as the integral membrane protein occludin, might be a potential biomarker for mTBI [36, 37], because when brain injury occurs, the tight junction complexes and basement membrane are disturbed, leading to increased BBB permeability and a subsequent inflammatory response [38]. A number of techniques for measuring BBB permeability have been developed using MRI and CT, with the former being most widely used for mTBI due to its noninvasive nature (i.e., lack of ionizing radiation) [39]. BBB integrity is frequently assessed quantitatively using dynamic contrast enhanced MRI (DCE-MRI) [39], which involves rapid administration of an intravenous bolus of a gadolinium-based MRI contrast agent while

producing images every few seconds. Various quantitative pharmacokinetic characteristics that represent the anatomy and function of the microcirculatory system can be determined from DCE [40]. There are a number of imaging indicators that can be utilized to detect permeability or BBB breakdown, including the volume transfer constant between the plasma and extravascular space (K^{trans}) and the leakage space (v_e) [40-43]. Studying BBB disruption following a mTBI can provide important data that will advance our knowledge of the pathophysiology of post-injury events and potentially help us improve diagnosis methods. For instance, as a result of disruption of the BBB after mTBI, damage associated molecular patterns (DAMPs) are released into the peripheral circulation, which trigger pattern recognition receptors (PRRs), leading to an increase in mitochondrial DNA (mtDNA) copy numbers in the peripheral blood [44, 45].

1.2.3 Cerebral Blood Flow (CBF) Disruption

Due to the mTBI, the cerebral autoregulatory capacity decreases, which is correlated with the activity of neurons and the rate of glucose metabolism in the brain [21, 46]. According to neuroimaging findings, reduced CBF, as measured using MRI approaches such as DCE and arterial spin labelling (ASL), is linked to neurocognition impairments and the intensity of symptoms, such as migraines [47, 48]. In a MRI study carried out by Meier et al. on concussed collegiate football players, CBF was found to be acutely decreased in the right dorsal mid-insular cortex and superior temporal sulcus [49]. At one month, the majority of people exhibited normalized CBF, however, some patients continued to have persistently low regional CBF [49]. Similar research has demonstrated that regional hypoperfusion in the frontal, pre-frontal, and temporal cortices, as well as bilateral thalami, is correlated with neuropsychological deficiencies [50, 51]. These findings indicate the potential of regional CBF identification by neuroimaging as a reliable biomarker for tracking concussion symptoms and recovery.

1.2.4 Axonal Injury

An immediate consequence of acceleration and deceleration forces generated by a blow to the head, neck, or body is localized diffuse axonal injury (DAI), in which axons shear and tear in a way related to the severity of the blow [52, 53].The mechanical damage disrupts axonal cell membrane integrity and creates diverse effects of uncoordinated, diffuse depolarization, ionic imbalances (the influx of Ca^{2+} and Na^{+}, and efflux of K^{+}), mitochondrial swelling and compaction of neurofilaments [21, 54, 55]. Following trauma, neurofilament compaction takes place as a result of phosphorylation, which affects neurofilament structure [56, 57], or proteolysis of sidearms by calpain, which causes neurofilament implosion [21, 58]. The accumulation of organelles and increased neurotransmitters caused by intra-axonal cytoskeletal abnormalities are responsible for secondary axotomy and the development of axonal bulbs [21]. Symptoms of secondary axonal disconnection can appear as quickly as 4 hours after the damage, although it has been shown that they might linger in patients with brain injury for days or even weeks [21, 59]. There are some biomarkers related to axonal injuries such as tau protein, ubiquitin carboxyl-terminal hydrolase isoenzyme L1 (UCHL1), neurofilaments (NFs), glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) [11]. Tau interacts with dynein and kinesin motor proteins to stabilize microtubules and influence axonal transport. Based on animal experiments, TBI has been connected to Aβ plaque and an elevated phosphorylated-tau (p-tau) [60]. Another study on humans performed by Rubenstein et al. found the ratio of p-tau to total tau was identified as an important diagnostic and prognostic biomarker for both acute and chronic TBI [61]. A large cohort study of 96 patients (86 with mild TBI and 10 with moderate) revealed that alteration of UCHL1 level in serum within an hour of injury was related to injury severity, based on the physician measured Glasgow coma score (GCS), and with intracranial

lesions including epidural and subdural hematoma, subarachnoid hemorrhage, diffuse axonal lesions, and Intracerebral hemorrhage that seen on neuroimaging [62]. Nonetheless, there is still a need for more investigations in this area because results are often mixed in terms of biomarker changes after mTBI.

1.2.5 Mitochondrial Dysfunction

Mitochondria are semi-autonomous double membrane-bound organelles that play an essential role in producing energy in the form of adenosine triphosphate (ATP). Mitochondrial dysfunction in traumatic brain injury can cause adverse metabolic and physiologic alterations to healthy brain function, reduces the possibility for neurological repair and increases neuronal cell death [63, 64]. Within mitochondria, excitotoxicity depolarizes the mitochondrial membrane, which initiates reactive oxygen species (ROS) production and inhibits the production of ATP (**Figure 1.1**) [65, 66]. The ROS includes superoxide radicals ($O_2^{\bullet}$–), hydroxyl radicals (-·OH) and hydrogen peroxide (H_2O_2) (**Figure 1.1**) [67]. This catalyzes the failure of the electron transport chain (ETC) and hinders processes involved with oxidative phosphorylation [63].

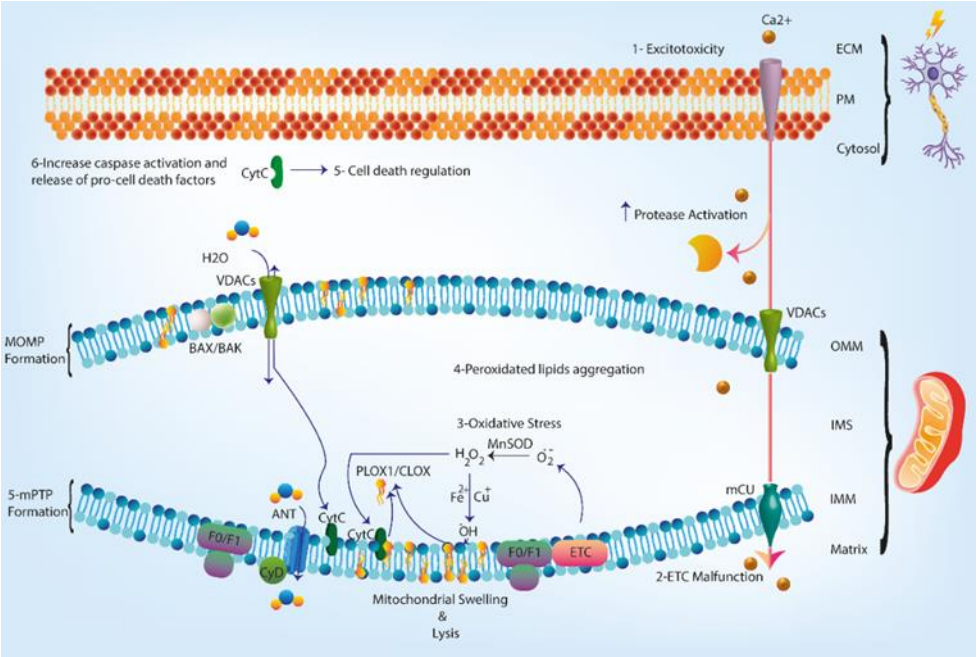


Fig 1.1. Mitochondrial mechanisms after neuronal injury and TBI. 1) Overexpression of excitatory neurotransmitters and their corresponding ion channel's activation can cause significant neuronal depolarization. Elevated the level of cytosolic Ca^{2+} is buffered and disrupted the mitochondrial membrane functions. 2) ETC breakdown triggers long-term increases in 3) oxidative stress, ROS, and RNS production in the third phase. 4) Accumulation of mitochondrial lipids, such as cardiolipin (CL), is an essential resource of oxidized free fatty acid Inflammatory Mediators. Non-enzymatic via metal ion (iron/copper) and cyt c/CL peroxidase-mediated enzyme CL peroxidation occurs when high ROS levels are determined. 5) Mitochondrial membranes instability and oxidation of CL leads to the release of pro-death factors and eventually neuronal death. **Abbreviations:** Outer Mitochondrial Membrane (OMM), Inner Mitochondrial Membrane (IMM), Intermembrane Space (IMS), Adenine Nucleotide Translocator (ANT), mitochondrial Calcium Uniporter (mCU), Voltage-Dependent Anion Channel (VDAC), Extracellular Matrix (ECM), Plasma Membrane (PM), Mitochondrial Outer Membrane Permeabilization (MOMP), mitochondrial Permeability Transition Pore (mPTP), Manganese Superoxide Dismutase (MnSOD), Iron (Fe^{2+}), Copper (Cu^{+}), cyclophilin D (CyD), Bcl-2-associated X protein BAK/BAX, ATP synthase, F0/F1.

As a consequence, efforts to restore metabolic reactions associated with the calcium cycle and cellular survival are substantially compromised [63]. Although the ETC is essential in maximizing ATP production in the presence of O_2, the triggered intracellular Ca^{2+} spike following TBI or other acute/chronic CNS injury leads to the production of reactive substances, in addition to ROS, that harm mitochondrial redox reactions such as reactive nitrogen species (RNS). During pathological conditions, these reactions can lead to overburdened healthy processes, which can exacerbate mitochondrial damage and eventually lead to ATP depletion

[67]. Mitochondria maintain vital neuronal cellular functions and metabolic homeostasis that can be severely disrupted by an mTBI. Mitochondrial function after rmTBI has been indirectly assessed by measuring energetic biomarkers [68]. For example, N-acetyl aspartate (NAA) a brain energy biomarker assessed with in vivo proton MR spectroscopy (MRS) is linked to mitochondrial function, Govindaraju et al., found reduced ratios of NAA to total creatine (i.e., NAA/Cr) and NAA to choline (i.e., NAA/Cho), are indicative of neural injury or disturbance in mTBI participants [69]. Another metabolic study done using MRS indicated NAA/Cr reduction in the peri-contusional region in 7 mTBI patients compared to 25 healthy controls [70]. More research is still needed on a variety of topics relating to mitochondrial malfunction and mTBI. The circulating blood level of mtDNA post-TBI as a diagnostic for brain mitochondrial energetics damage is one encouraging marker that might be further investigated [71] and correlated with non-invasive MRS approaches.

1.2.6 Cell death & Apoptosis

Neuronal cell death is necessary for the normal development of the CNS and is an essential process for removing dysfunctional and damaged cells [72]. Recent studies have even revealed a link between mTBI and neurological impairment caused by the apoptotic pathway, which correlates to long-lasting neuronal signs and cognitive difficulties [73]. The mechanism of cell death triggered by a brain injury can be categorized into necrosis or apoptosis. Necrosis is a passive process that occurs due to mechanical forces acting on the brain, distinguished by ion homeostasis loss and elevated neuronal Ca^{2+} that results in the swelling and rupture of cells and organelles [74]. However, apoptosis, a form of programmed cell death, is an energy-consuming biochemical process that involves the destruction of the cytoplasm and nucleus, to a state beyond repair, while maintaining the structure of other organelles [74]. A growing body of evidence

suggests that repeated mTBI can cause persistent neurodegenerative symptoms as a result of the cumulative impact on brain functions [75, 76]. In mTBI cases, microvascular damage and cellular metabolism disruption occurs, resulting in abnormalities in both vascular autoregulation and biochemical activity [76]. Repeated mTBI microarray studies discovered considerable alterations to the expression level of 78 genes involved in apoptosis, metabolism, synaptic plasticity, and stress response [76]. Meanwhile, other mTBI research has indicated that decreases in the level of BCL-2 can contribute to both apoptotic and non-apoptotic cell death in both white matter and gray matter [77]. Many clinical examinations on body fluids, such as serum and/or CSF, have demonstrated altered levels of apoptotic markers, such as caspase-3 and caspase-9, after TBI of varying degrees of severity [78-81]. Although cell death has not been known to result from a single mTBI, understanding the mechanisms involved with different apoptotic pathways may be helpful in pharmacological treatment strategies and possibly for improving brain injury assessment through the development of quantitative diagnostic biomarkers.

1.3 Current mTBI Diagnosis Techniques

1.3.1 Post-Concussion Symptom Scale (PCSS)

PCSS is a subjective self-assessment tool used to rate mTBI severity. The PCSS is an extension of the first concussion scoring questionnaire called the Rivermead Post-Concussion Symptoms Questionnaire (RPQ) [82]. PCSS includes 22 symptoms (**Table 1.1**), and each is scored from 0 to 6 (0 representing no experienced symptoms, 3 representing moderate symptoms and 6 denoting severe symptoms). Although highly subjective the PCSS is still a useful standard method for individuals to self-evaluate their symptoms and can provide important details on the

severity of a mTBI. Furthermore, it is the standard test that is done in any emergency department following concussive injury.

Table 1.1. Tested factors in the post-concussion symptoms scale (PCSS) [83].

Headache	Nausea	Vomiting	Balance problems
Trouble falling a sleep	Excessive sleep	Loss of sleep	More emotional
Irritability	Sadness	Difficulty remembering	Visual problems
Feeling foggy	Nervousness	Difficulty concentrating	Fatigue
Feeling slow	Drowsiness	Noise sensitivity	
Numbness	Dizziness	Light sensitivity	

1.3.2 Sport Concussion Assessment Tool (SCAT)

SCAT was originally designed in 2004 by the Concussion in Sport Group in Prague in order to assist patients and physicians in the identification of concussions associated with sports [84]. The SCAT5 (the current version of SCAT) is designed for use by medical professionals on patients who are 13 years of age or older and have experienced a sport-related concussion (SRC). For those under the age of 13, the SCAT5 was also developed in a child version [84]. The SCAT5 test was created in two parts: an on-field assessment and an in-office assessment.

I. The on-field assessment includes 4 parts [84]: 1) A section of ‘red flags’ including neck pain or tenderness, double vision, weakness or tingling, severe or increasing headache, seizure or convulsion, vomiting, deteriorating conscious state, increasingly restless [84], all of which are indicative of serious injury that needs immediate follow-up by hospital

medical staff; and 2) Observable symptoms, 3) Maddock memory assessment, and 4) the Glasgow Coma Scale (GCS).

II. The in-office assessment includes the following sections: 1) information about the person's history and any previous mTBIs and recovery process; 2) history of signs and injury severity; 3) cognitive screening to assess concentration, memory performance, and orientation; 4) a modified version of the Balance Error Scoring System (BESS), used to as part of a neurological examination. The cognitive assessment portion includes a delayed memory task that asks subjects to recall words or items from a list [84]. BESS is used to evaluate how concussion affects static postural stability [85].

1.3.3 King Devick Test

For a variety of reasons, disruptions in the visual system are crucial for detection of concussions. From the eyes the visual system is connected by complex pathways in the brain that cross through the frontal, parietal, and temporal lobes [86]. Cortical regions involved in saccadic function include the frontal eye fields, dorsolateral prefrontal cortex (DLPFC), supplementary motor area, posterior parietal cortex, middle temporal area and striate cortex [86-89]. These regions are in charge of organizing, starting, and carrying out synchronized saccades, like those required for reading and quick number recall [86, 87, 90, 91]. This relationship makes saccade testing a very suitable method for evaluating the neurobiological impacts of a concussion [86]. The King Devick test (K-D) examines eye movement, concentration, and language performance by having participants who quickly read numbers from a standardized card or computer program [86]. It has been established that the outcomes of this test are related to patients who have suffered concussions and have resulting substandard brain function.

There are additional methods for diagnosing concussions and mild TBI, such as the concussion recognition tool (CRT5), which non-medical practitioners can use to evaluate head injury [92]. Another metric that is infrequently used is heart rate variability (HRV), a physiological parameter that measures the variability in the time between individual heartbeats [93]. Although of potential use HRV is infrequently applied due to confounding effects of age, sex, circadian rhythm, and fitness level on the metric. Furthermore, as there are a great number of HRV models that are potentially of use the complexity of this metric makes it difficult to use as a diagnostic measure.

1.3.4 Neuroimaging Techniques

A routinely applied neuroimaging approach to assess mTBI is computed tomography (CT). However, CT is only used if someone has been unconscious following their injury with the goal of finding a brain bleed or skull fracture. These features, although indictive of serious injury, are only prevalent in less than 5% of mTBI cases.

Magnetic resonance imaging (MRI) has been an invaluable tool for mTBI assessment. As MRI uses non-ionizing radiofrequency (RF) energy, MRI is much safer than CT for patients and allows for more extensive imaging through differing contrast mechanisms. For brain imaging, the high specificity of MRI allows for tremendous grey and white matter contrast for anatomical scanning. The health of brain white matter can be predicted based on the relativistic shape of the myelin surrounding axons and the diffusivity of water along the length of the axons by using an MRI technique called diffusion tensor imaging (DTI) [94, 95]. Diffusion tensor imaging (DTI) is a MRI technique that can determine the directionality and magnitude of water molecule diffusion. This method is based on diffusive properties of water molecules which have relatively

unrestricted diffusion along the length of axons but restricted motion perpendicularly. Therefore, DTI can be used to track brain white matter tracts and assess their integrity. DTI, due to its sensitivity, can detect subtle brain changes following mTBI [96]. However, to date, DTI studies have shown conflicting results, likely because analysis has focused on comparing groups of mTBI patients to healthy age/sex-matched controls, and no two patients will have been subjected to the same injury-causing forces. More recently DTI, using a personalized analysis approach with machine learning methods, has shown a high correlation between injury and symptoms [97, 98].

In addition to structural assessment, the function of brain grey matter can be evaluated using functional magnetic resonance imaging (fMRI). The fMRI technique is based on measuring the magnetic susceptibility differences between oxygenated (diamagnetic) and deoxygenated (paramagnetic) blood, called the Blood-Oxygen Level Dependent (BOLD) signal [99- 101]. Initial studies were done to evaluate the brain doing specific tasks [102]. When task-based fMRI was established as a method to understand brain function Biswal and colleagues showed specific brain regions in the resting state were temporally synchronized [103]. This seminal work began the study of brain resting-state activation patterns. The resting-state has shown a high degree of reproducibility in test-retest reliability studies [104, 105].

Resting networks are known to be reasonably well conserved and reproducible across people over time. It is thought that resting-state fMRI (rsMRI) could reveal significant brain changes in mTBI patients [106]. The most dominant brain network is the Default Mode Network (DMN) [107]. Group assessment of the DMN has shown mixed results due to the complexities and heterogeneity of mTBI. More recently a personalized approach to rsMRI analysis has been developed and shown use in patients suffering from mTBI [108].

1.3.5 Challenges with Current Diagnostic Methods

Existing mTBI diagnosis guidelines are extremely restricted, inconsistent, and dependent on a patient's subjective self-report. Currently, the clinical assessment for mTBI provided by the World Health Organization, the Centers for Disease Control, the US Department of Defense, and the American Academy of Neurology are not sufficient for a reliable diagnosis [109]. All approaches have a lack of objective accuracy that hinders medical practitioners from recognizing and properly describing the nature of mTBI [109]. Unfortunately, a subjective clinical self-report is used to categorize the severity of brain damage (mild, moderate, severe), and arbitrary time restrictions are applied to the period of loss of consciousness (LOC) and posttraumatic amnesia [109]. Neuroimaging employs a number of variables to identify mTBI, although it misses molecular alterations in the brain brought on by head trauma. Furthermore, besides a CT to rule out bleeding and fractures, MRI (which can provide objective measures of brain changes) is rarely performed in the acute phase of suspected mTBI. Besides, to assess mTBI severity, some clinical settings and research procedures use the GCS and the duration of unconsciousness or posttraumatic amnesia, regardless of neuroimaging findings [110]. Furthermore, the clinical symptoms that are frequently present soon after a concussion are extremely unspecific and can be found in a wide variety of disorders [109]. Clinical challenges stem from the vague nature of the symptoms, compounded by the fact that the physician is relying on the concussed patient, who has neurometabolic brain disruption, to provide appropriate quantitative scores to their symptoms and a detailed history [109]. These are only a few of the difficulties and obstacles that physicians and the medical system must overcome to correctly diagnose mTBI/concussion. It is crucial for clinicians and practitioners to have the right equipment and methods to diagnose, identify, and treat mTBI adequately. Enhanced diagnostics may also facilitate decisions

regarding return to work or play. Such methods are used to describe the type of physiological malfunction, or to recognize neural impairment at a biological level. In order to improve diagnosis and prognosis techniques for detecting and grading mTBI immediately after injury are highly needed. More recently researchers have concentrated on the study of omics biomarkers as a substantial area of promise for better diagnosing and prognosing mTBI.

1.3.6 The Field of Omics for mTBI Diagnosis

Recently, methods for the high-throughput characterization and measurement of biomolecules have been developed. The term "omics" refers to the study of genomics, epigenomics, transcriptomics, proteomics, and metabolomics [111]. But other fields have also borrowed the "omics" suffix like radiology which has "radiomics". These methods enable the analysis of the kinetics, modifications, and molecular pathways in biological samples from both healthy and pathological conditions, including TBI and mTBI [112]. Omics is a quickly evolving diverse field that examines every component of the cell, tissues, and/or organism. Modern bioinformatics allows for the detailed qualitative and quantitative analysis of enormous volumes of data from small biological molecules, resulting in the highly valued knowledge of pathogenic processes [113]. Additionally, the integration of information from multiple biological domains can be facilitated by *in silico* tools and analytic methods [114]. Omics may offer a more in-depth comprehension of general molecular and cellular abnormalities by combining various TBI and mTBI pathophysiological components [112]. Furthermore, it can assist in the discovery of diagnostic and prognostic biomarkers as well as the characterization of previously unknown neuropathophysiological processes [114]. It is likely that omics technology will become an integral part of precision medicine and individualized therapy for brain injuries in the near future [115].

1.3.7 Genomics

Genomics is the most developed field of omics. The goal of genomics is to find genetic variations connected to an illness, a patient's reaction to therapy, or a patient's prognosis in the future [112]. Previous research suggests that the response, recovery rate, and outcome following a mTBI may be influenced by the individual's genetic makeup. Several genes such as *APOE, BDNF, DRD2, COMT,* and *CACNA1A* have been proposed to alter the trajectory and result of the pathogenesis of mTBI. The application of genomics is highly relevant to conditions involving any kind of head injury, including mTBI which are influenced by both genetic and external variables. High-throughput DNA sequencing is required, and cutting-edge bioinformatics is utilized to analyze the structure and function of the genomic sequence [114].

1.3.8 Transcriptomics/miRNA

Transcriptomes, estimated at about 5% of the genome, are made up of RNA molecules that carry the genetic code [116]. Transcriptional activity can be complicated by processes that cause variations in RNA molecules, like alternative splicing, RNA editing, or alternative transcription initiation and termination sites [114]. Studies have revealed that a large number of genes exhibit altered expression in inflammation, cell signaling, apoptosis and neurological pathways after TBI and mTBI [76, 117-122]. Furthermore, numerous investigations have demonstrated the possibility of miRNA profiles for prognostic and diagnostic tools to be used in a variety of biological fluids [123, 124]. Omics findings may serve as prospective therapeutic indicators for injury severity measurement and tracking, and also in the development of specific personalized medical treatment targets.

1.3.9 Bioinformatics

Bioinformatics refers to the application of computational strategies, algorithms and statistics to carry out comprehensive Omics-associated studies. It involves searching through online biological databases to evaluate gene sequences and proteins on a global level to obtain sequences or proteins that indicate differences between normal and diseased tissues, or between phenotypic traits [113]. Recently, a number of bioinformatic tools have been enhanced to manage vast omics-related data. Depending on the *in silico* platform, these tools are typically divided into three groups: web-based services, analytical software, and biological packages. The most useful platform is an online database because it is easily accessible, cost-free, and updated with new datasets. The ideal biomarkers for mTBI are those that are exclusively present in the brain but have leaked out into the blood or cerebrospinal fluid (CSF) through the damaged (i.e., more permeable) BBB.

Hypothesis

At the present time, no single marker or panel of markers is universally accepted for the diagnosis of mTBI, and limitations in diagnostic approaches lead to many cases of mTBI/concussion being misdiagnosed or not being recognized properly. On the other hand, patients who are slow to, or do not recover, will likely express a unique pattern of genes that are different from those that do recover. Recognizing these genetic profiles is very important for tracking the progression of the injury. Therefore, it was hypothesized that employing bioinformatic data mining could assist in uncovering essential genes and miRNAs associated

with brain injury. These could then be used to inform and detect engaged neurological pathways that could enhance diagnostic approaches based on biological markers.

1.3.10 Objectives

1. Utilizing bioinformatic databases, determine how many genes are correlated with mTBI
2. Using enrichment and biological pathway analysis specify mTBI candidate genes
3. Show protein-protein interaction and co-expression between mTBI candidate genes and highlight the top ten genes
4. Predict miRNAs related to mTBI genes
5. Perform RNA-sequence analysis on the GES123336 dataset to confirm miRNAs findings

Thus, in this work, the goal was to employ genomics and transcriptomics web-based analysis and the use of bioinformatic software for RNA-seq analysis, with the aim of determining the genes and miRNAs correlated with mTBI outcome. Candidate mTBI genes and miRNAs highlighted in this study are detectable in peripheral blood as a result of BBB disruption that results in the release of brain biomarkers into the bloodstream via microvesicles or exosomes. This finding will provide valuable information for better understanding recovery from mTBI, possibly allow recovery monitoring and possibly of value for developing targeted therapeutic options in the future.

CHAPTER 2: A REVIEW OF MOLECULAR AND GENETIC FACTORS FOR DETERMINING MILD TRAUMATIC BRAIN INJURY SEVERITY AND RECOVERY

2.1 Declaration

Chapter 2 is the published manuscript "A review of molecular and genetic factors for determining mild traumatic brain injury severity and recovery" by *Mahnaz Tajik, Michael D Noseworthy, Brain Disorders (2022) 100058 doi.org/10.1016/j.dscb.2022.100058*

2.2 Abstract

Mild traumatic brain injuries (mTBI) affect millions of people globally every year. The clinical presentation of this injury is highly variable, and its progression from the acute to chronic stages of injury is driven by dynamic pathophysiology. More specifically, biomechanical brain damage can trigger complex cellular, molecular, functional, genetic, and metabolomic changes. Recent research has taken aim at understanding the association between such complex changes and clinical outcomes, with the ultimate intent of identifying prognostic indicators. This is important as to date, current diagnostic protocols using patient reported symptom tracking and routine medical imaging are highly limited, often subjective, and can lead to missed diagnoses. Thus, neither patients nor their physicians can currently predict recovery timeline and whether recovery will be complete. Consequently, biological markers need to be determined that can improve diagnostic and recovery assessments following brain injuries. Possible indicator candidates, based on human and animal research, include the expression of

neuroprotective genes and microRNAs (i.e., *GFAP, BDNF, MBP*) and single nucleotide polymorphisms (i.e., *BDNF, COMT, APOE, D2R2*). However, these factors are non-specific in terms of injury location and severity. Due to the vast range of physiological, molecular and omics alterations present post-mTBI, it is clear that mTBIs are a highly complex pathophysiological problem. Thus, the purpose of this review is to provide a comprehensive understanding of post-mTBI genetic and metabolic brain changes, both at the cellular and molecular level, to understand how they can affect the symptoms and outcome of mTBIs, how these changes can be leveraged for improved acute detection of brain injury, and their potential for use in future personalized treatments.

2.3 Introduction

A mild traumatic brain injury (mTBI) is a neurological injury with variable acute clinical presentation and potential for chronic effects [125]. An mTBI is caused by biomechanical forces, such as an external blow to the head, which usually results in neurological and cognitive impairments without a predictable resolution [125]. Furthermore, an mTBI may or may not involve loss of consciousness, can lead to lingering symptoms in some cases, and mTBI-caused brain abnormalities are currently not detectable with routine clinical medical imaging [125, 126]. The severity of a TBI is categorized as either mild, moderate, or severe according to the Glasgow Coma Scale [127], with about 80% of all TBIs reported as mild [10, 128] However, even mTBIs leave many adults experiencing symptoms longer than 10 days post-concussion [10, 125, 129]. Diagnosis of an mTBI is based on a combination of neurological examinations and symptom reporting [125]; however, many of these techniques can be subjective due to patient-reported symptom severity [130].

To improve diagnosis, one focus of ongoing research has been to develop a more comprehensive understanding of the pathophysiological factors involved in brain health following head injuries. Some molecular and cellular mechanisms involved in the primary and secondary injury phases of brain damage, and focal or diffuse structural and functional injuries [14, 131] can be quantified by medical imaging techniques but they are not routinely recommended [125, 126, 131]. Thus, genetic and metabolomic factors became an active area of research to determine whether they have an influence on post-injury recovery trajectories. This review details current mTBI epidemiology, briefly outlines the molecular and physiological changes present in the primary and secondary post-mTBI injury phases and discusses in depth the omics alterations related to mTBIs that include genetic factors, polymorphisms, micro ribonucleic acids (miRNA), epigenetics and metabolomics. Due to the limited research specifically focused on mTBI associated omics factors, this review will also include discussion of moderate and severe TBI literature to provide context for future opportunities in mTBI research.

2.4 mTBI Epidemiology

Most common among children and adolescents, mTBIs have become a major public health problem and are now being referred to as the "Silent Epidemic," [132, 133] with an estimated annual incidence of approximately 42 million injuries [8, 10, 128]. According to the literature, 10% – 20% of adult mTBI patients have persistent post-concussion syndrome (PCS) weeks or months after the injury, implying that even a mild brain injury can result in long-term negative outcome and substantial disability [134, 135], putting an immense strain on the healthcare system, the economy, and society [136]. The leading causes for mTBI are falls, motor vehicle accidents, domestic violence, and sport-related head and neck injuries [8, 128, 137]. An mTBI can cause a wide range of symptoms that can appear immediately following injury or develop

days to weeks later [125, 130]. Common mTBI symptoms fall into the four categories of either cognitive, behavioural, emotional, or sleep-related and can include learning and memory difficulties, depression, sensitivity to light and sound, insomnia, dizziness, amnesia, headaches and irritability [125, 129-131]. For the majority of adult mTBI patients' symptoms will be resolved within weeks of injury; however, the remaining cases with persistent post-mTBI symptoms have a recovery time often longer than two weeks and a smaller percentage experiencing symptoms that last much longer [134, 135]. Predicting mTBI outcome is complicated due to the wide array of possible symptoms and their unpredictable resolution, and currently cannot be done solely through clinical diagnosis using subjective patient-reported symptoms and limited medical imaging [8, 125, 126, 131].

Currently, the most reliable clinical mTBI assessment is based on patient-reported symptoms captured by tests such as the Rivermead Post-concussion Symptoms Questionnaire [82], Post-Concussion Symptom Scale (PCSS) [138], and the Sport-related Concussion Assessment Tool 5th Edition (SCAT5) [84]. However, patient-reported tests are highly subjective and cause inconsistent injury severity predictions [139]. In some cases, medical imaging techniques of computed tomography (CT) and magnetic resonance imaging (MRI) are used to assess intracranial hemorrhages that may be observed in complicated mTBI or more severe forms brain injury [125, 126]. However, the current clinical diagnostic CT and MRI protocols are highly limited and provide no mTBI specific information on brain function or microstructural integrity, especially in the acute phase [126, 131, 140]. The unpredictable nature of post-mTBI symptoms and the lack of a truly quantitative diagnostic tool prevent an accurate prediction of recovery timelines and to determine if a complete recovery is possible. Therefore, an accurate diagnostic tool is vital to improve mTBI prognosis and treatment. In this review article, we

present evidence of how cellular and molecular pathways, metabolomic cascades and genetics play a role during the initial mTBI injury and how targeted alterations to these factors could influence a patient's recovery. The utilization of omics measures in mTBI assessment would remove potential biases from patient-reporting symptoms and variability between medical imaging techniques, and allow for personalized assessment, prognosis, and treatment opportunities that has not been possible to achieve through currently available clinical diagnostic methods.

2.5 Molecular and Physiological Changes Following Brain Injuries

Acceleration and deceleration forces associated with an injury event can lead to shear and stretch forces that trigger several molecular and neurometabolic changes including neurotransmitter level alterations, cytokine upregulation, biochemical mediator shifts, cell death, neuroinflammation, and genetic modifications [12, 14, 25, 74, 141]. The complexity and heterogeneity of mTBIs can cause a powerful immune response combined with acute and chronic neurovascular failures (**Figure 2.1**) [25]. Systemic alterations as a result of an mTBI disrupt many important biochemical and cellular cascades to cause blood-brain-barrier (BBB) disruption, intracellular calcium overload, reduction in glutamate transporter, which leads to glutamate excitotoxicity [142, 143], mitochondrial impairment, oxidative stress [144, 145], pro-apoptotic gene activation, inflammatory responses [146], and neuronal cell loss [147, 148]. (Table 2.1)

Molecular alterations caused by a brain injury can be categorized into either a primary or secondary injury phase. The primary injury phase is the direct result of physical forces applied to the brain during the impact. The shear and strain forces can cause diffuse axonal injuries [149,

150], extra- and intraparenchymal hemorrhages [151], BBB instability [151], and neuronal and glial cell damage within the brain (**Figure 2.1**) [25, 147, 149].

As a result of this damage, an acute rise of intracellular sodium and calcium, and efflux of potassium, disrupts cellular ionic homeostasis which causes a diffuse decrease of brain processes [14, 152]. The secondary, or delayed injury, phase can be more progressive than the initial mechanical damage (**Figure 2.1**). The secondary phase consists of reversible systematic neuroinflammation and cellular damage, increased intracranial pressure (ICP), edema (swelling), possible microhemorrhages (infrequent) and neurochemical pathway disruptions [14, 25]. Those responses can cause a substantial reduction in cerebral blood flow (CBF), ischemia and interruption of normal brain metabolism [152, 153]. The persistence of an ion imbalance accentuates any edema and triggers a further reduction of CBF [154]. Global CBF reduction impairs the process of cellular respiration by reducing tissue oxygen supply, thus creating an energy crisis from shifting mitochondrial metabolism from aerobic to anaerobic respiration [154]. In the acute and sub-acute phases, mitochondrial dysfunction contributes to the release of reactive oxygen species (ROS). Increased presence of ROS can affect the permeability of cellular membranes by initiating lipid peroxidation, deterioration of purine and pyrimidine bases, and can cause DNA strand collapse [154].

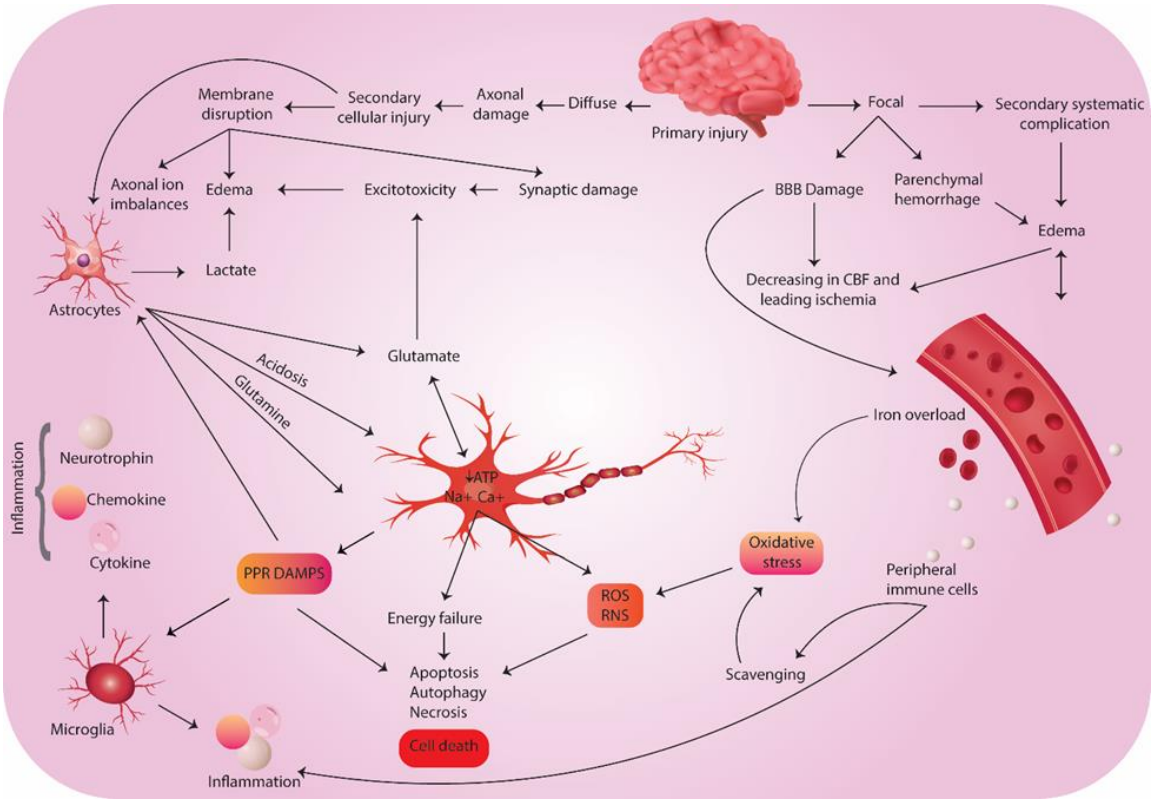


Figure 2.1. Pathophysiology of traumatic brain injury. This diagram illustrates pathological alterations throughout brain injury that cause chronic and acute damage to the neurovascular system and immune activation. After a secondary phase which is caused by diffuse and focal injuries, neurovascular damage occurs, many damage-associated molecular patterns (DAMPs) are released and lead to infiltration of gliosis and peripheral immune cell. One of the primary functions of the immune system is to remove dead cells and debris after apoptosis. The effect of immune cell dysfunction is increasing swelling and injury progression. In addition, white matter disruption and chronic behavioral deficits occur due to oxidative stress, excitotoxicity, lack of energy, and prolonged inflammation.

Experimental and clinical investigations have explored the vast, complex TBI-initiated pathophysiological effects and variability present in mTBI symptoms and outcomes [25, 155, 156]. These molecular alterations following brain injury are too complex for us to discuss in depth in this review of omics factors affecting mTBIs, thus further explanations of the molecular pathways can be found in other molecular-specific reviews [47, 141, 157]. However, there may not be extensive literature on the effects of the primary and secondary injury processes related specifically to mTBI, and more comprehensive research in this area is required.

2.6 Omics Involvement in Brain Health and mTBIs

2.6.1 Genetics Factors

Genetics affect many divergent pathogenetic pathways and influence both short- and long-term neurological and functional outcomes. Acutely post-mTBI, genetic variants (i.e., single nucleotide polymorphisms; SNPs) may regulate the severity of axonal damage, BBB disruption, inflammation, and neuronal survival, whereas long-term outcome may depend on genes responsible for neuroplasticity and neuronal regeneration [158]. Specific genetic variations can increase an individual's risk of more severe brain damage and impaired recovery [158-161]. Therefore, understanding the function of specific genes, genetic SNPs, and miRNA could translate to producing useful diagnostic biomarkers for mTBI-specific severity quantification and recovery tracking [162, 163]. According to Bennett et al. (2016), the genes involved in brain injury pathology generally belong to three broad categories: i) pro- and anti-inflammatory cytokines, which exacerbate or minimize damage; ii) neurotrophic genes, which promote repair and plasticity; and iii) catecholamine genes, which influence neurobehavioral capacity and cognitive function, and have shown diverse polymorphisms [155, 163]. All three of the aforementioned genetic categories could delay neurological healing, exacerbate impairments, and prolong recovery in mTBI patients.

Advances in genetic analysis techniques, such as microarray technology, whole-genome mapping, and genome-wide association studies have enabled the discovery of new gene targets for brain injury [164, 165]. Microarray analysis of gene expression profiling facilitates the measurement of thousands of genes and SNPs from a single RNA sample. A whole-genome microarray animal model study investigated ischemia rates in rats and found that a closed head injury altered the expression of 279 genes, including MMP4, BDNF, and Cd47, compared to the control group [117]. The researchers determined that most of the altered genes were involved in

cell proliferation, cell signaling pathways, cell differentiation, and transcription regulation [117]. Furthermore, a microarray study on humans verified 1200 genes by cDNA microarray hybridization, where 8.7% (n=104) of those 1200 genes had differential gene expression in traumatized brain tissue and were mostly involved in transcription regulation, energy metabolism, signal transduction, and intercellular adhesion and recognition [166]. There was also altered expression of immediate early genes (IEGs), such as glial fibrillary acidic protein (*GFAP*), in stress response proteins of TBI patients [166]. Whole-exome sequencing studies have indicated variants in ion channel genes such as *CACNA1A* and *ATP1A2*, which are involved with familial hemiplegic migraines and mTBI-related symptoms [167]. These results illustrated the involvement of heterozygous deleterious mutations in genes to cause neurological malfunction and a poor mTBI recovery [167]. Extensive clinical research must be performed to verify and further explore the mechanisms of gene expression changes and for patient specific TBI severity and recovery diagnostics. For example, during the disruption of the BBB following TBI, overexpression of numerous inflammatory genes have been linked to changes in BBB permeability, formation of edema, and neurological impairments [168, 169]. Thus, there is a possibility that immune system gene regulation influences a patient's clinical outcome following brain injury.

2.6.2 Polymorphisms

A single nucleotide polymorphism (SNP) is a type of abnormal, mutated genomic sequence that can significantly impact the genotype depending on the resultant mutation. Insertions, deletions, and duplications are genetic deviations that may make the body unable to perform a crucial physiological function or produce certain proteins. Genetic polymorphisms can influence any individual's clinical phenotype by changing the responsibility of encoded proteins,

modifying molecular structures, or altering gene expression [156]. Genome-wide association studies (GWAS) are currently the most comprehensive method of genetic research [170]. The goal of a GWAS is to detect all SNPs that are present across the entire genome [170]. Patients with a specific genetic profile have been shown to be more susceptible to severe TBI-related vegetative state and death [171], and therefore, these genetic profiles should be examined in mTBI cases as well. A rehabilitation study with 648 moderate and severe TBI patients indicated that the ApoE4 genotype predicted long-term functional outcomes and ApoE4 genotype carriers had poorer recovery outcomes one year after the injury [172]. There was also a gene-by-sex association, with the negative impact of the E4 allele being stronger in females than males [172]. However, some polymorphisms may also positively affect an individual's diagnosis and recovery timeline prediction and this area must be explored farther [171, 173]. Consequently, genomics research and the study of vulnerable genotypes in mTBI will be extremely valuable in demonstrating the role of genetic variables in brain injury incidence, severity, and recovery.

A polymorphism in a critical neuronal gene like brain-derived neurotrophic factor (*BDNF*) can profoundly affect brain recovery following brain injury, according to the research the Met allele leads in reduced activity-dependent *BDNF* production, and as a result less neurotrophic support for neuroplasticity [174]. *BDNF* plays a primary role in synaptic plasticity, neuronal repair and survival, and it has received attention in the context of lesion-induced plasticity and the outcome of acquired brain damage [174, 175]. Chaiaretti et al. (2003) reported that CSF and plasma *BDNF* levels increased considerably in children aged three months to sixteen years with severe TBI [176]. This elevation in BDNF levels indicated an endogenous attempt at neuroprotection against biochemical and molecular alterations generated by brain trauma, while also responding to synaptic remodeling mechanisms and protecting against neurological dysfunction and

cognitive impairments [174, 176, 177]. BDNF has many polymorphisms and the Met allele of rs6265 has been associated with poor cognitive outcome shortly following mTBI [163, 178-180]. Rs6265 is a nonsynonymous coding SNP in the pro-BDNF sequence that results in the amino acid change at codon 66 of valine to methionine [180, 181]. There have also been some studies that show the interaction of *BDNF* genetic SNPs (i.e., Met carriers (val66met): rs6265; C-carriers (T>C): rs7124442) and age on the long-term outcome of severe TBI patients including a greater risk of death [178, 182]. Furthermore, polymorphisms linked to brain damage illustrate the importance of SNPs on working memory, which has caused concentrated research on the role of catechol-O-methyltransferase (*COMT*) in the dopaminergic system [171]. COMT protein plays a vital role in working memory tasks such as executive function, decision making, inhibition, impulsivity, and emotionality [171, 183-185]. Enzymatic activity is affected by a mutation in the COMT gene that causes the Val158Met polymorphism [171, 186]. According to prior studies, individuals with the Val homozygous genotype have lower executive function and impulse control scores than those with the Met homozygous genotype [187, 188]. Thus, it appears that Val is a dominant factor in controlling COMT activity in the prefrontal cortex, which is associated with lower synaptic dopamine levels and relatively impaired prefrontal function [189]. Another noteworthy SNP related to the dopaminergic system and working memory occurs in Dopamine Receptor D2 (*DRD2*). The *DRD2* gene is located on chromosome 11 and encodes the D2 subtype of the dopamine receptor; allowing a polymorphism to this gene to influence working memory, executive function, inhibition, and impulsivity [171, 190]. Polymorphisms in *DRD2* are quite prevalent and several studies demonstrated that a *DRD2* polymorphism affects cognitive recovery after a brain injury. One of these SNPs is rs1800497 in the *ANKK1* gene which is known as DRD2 “TAQ1 A” allele [191]. The presence of the T allele

in rs1800487 has been linked to a 40% decrease in an expression of D2 receptor in the striatum and possibly other cortical brain region which found patients with a T allele genotype experiencing memory difficulties [191, 192].

Apolipoprotein E (*APOE*) is one of the most researched genes for short and long-term sequelae due to TBI [158, 160, 193, 194]. The *APOE* gene encodes a glycolipoprotein responsible for the transportation of lipids to promote repair and regeneration of neurons, as well as the construction of new cell membranes, neurites, and synapses [195, 196]. *APOE* has three allelic variants (ε2, ε3, ε4), with the most neurotoxic isoform being the ε4 allele (*APOE4*) that can cause neuropathology through proteolytic cleavage and diminished neurite branching and growth [196-199]. In a study conducted by Giarratana et al.,2020 reported *APOE4* as a risk factor for poor outcomes after repeated mTBI (rmTBI) on mice [197]. They also demonstrated that rmTBI increased phosphorylated tau levels in injured mice with *APOE4* rather than mice with *APOE3* allele, which may raise the chance of developing Alzheimer's disease [197]. Studies have indicated that *AOPE4* is present in mTBI patients who experience poor long-term recovery and deficits in neuropsychological performance due to an *APOE* polymorphism that can alter transcriptional activity in a cell-specific manner; however, there is no strong evidence linking the ε4 allele to poor outcome following mTBI [200, 201]. A GWAS of 4064 concussion cases found that *APOE4* and rs405509 were not significantly associated with concussion [165]. Overall, studying the impact of polymorphisms and SNPs on mTBI outcome could lead to targeted intervention and improved recovery through genetic profiling.

2.6.7 MicroRNA

MicroRNA (miRNA) are endogenous, short single strand, non-coding regulatory RNAs (~23 nucleotides long) that direct protein synthesis and gene expression through post-transcriptional activities [202, 203] miRNAs are processed through the mechanism of RNA interference (RNAi), which regulates their target gene's expression level and are engaged in the post-transcriptional regulation of protein expression [204]. Moreover, miRNAs have unique biogenesis, characteristics and altered expression following trauma or disease, making miRNA attractive biomarkers. In terms of detectability, circulating miRNAs are stable and readily available in biofluids such as blood, serum, plasma, CSF, saliva and urine [205-207]. These alterations may occur following a mild TBI or severe TBI. In general, the human genome encodes over 2,000 miRNAs and they regulate more than one-third of all human genes [211, 212]. miRNAs are involved in cellular procedures such as differentiation, metabolism, death or apoptosis, proliferation, and immune responses in pathophysiological conditions [213-216]. More specifically to mTBI, the highest concentration and diversity of miRNAs are found in the central nervous system (CNS), with approximately 70% of all miRNAs expressed in the brain, spinal cord and peripheral nerves [217, 218]. Due to this strong presence of miRNA in the CNS, it seems likely that miRNA dysregulation may be linked to cognitive impairment and neuropsychiatric disorders following a mTBI, but further research is required to confirm this association. Furthermore, exosomal microRNA (exomiRNA) in biofluids were evaluated as potential biomarkers for diagnosis and prognosis of neurodegenerative diseases such as Parkinson, Alzheimer (AD), and TBI [219].

Molecular Alteration	Genes	KEGG Pathways	Physiological Outcomes
Excitotoxicity	Polymorphism in Glutamate receptor genes and ion channels may influence on excitotoxicity; upregulation of genes involved in the expression of stress proteins, and nerve growth factors, can trigger excitotoxic cascades [163]	Glutamatergic synapse, Nicotine addiction	Damage to the BBB, destroys neuronal membrane integrity, edema, and cell death [25, 142]
Cell Death	TP53, BCL-2 family, TNFSF10, CASP3, ERK1/2, PARP-1, TNF, TNFSF15	Apoptosis, P53 signaling pathway, TNF signaling pathway, Alzheimer disease, TGF-beta signaling pathway, EGFR tyrosine kinase inhibitor resistance	Neuronal cell death [141].
Inflammation	IL-1 family, IL-6, TNFα, IL-10, CXCL8, CCL5, NFE2L2	Inflammatory mediator regulation of TRP channels, Toll-like receptor signaling pathway, Necroptosis, MAPK signaling pathway, Chemokine signaling pathway, TNF signaling pathway	Inflammation can lead to tissue deterioration, edema, hypoxia, and vascular damage [146].
Acidosis	ENO2, HIF1A, NOS3, NFE2L2	Glutamate and lactate pathways, TCA pathways, Oxidative stress pathway	The result of acidosis is extensive cerebral edema, BBB disruption, changed or inhibited cellular function, disruption of cell membrane and elevated permeability [148, 208].
Axonal Injury	APP, APOE, TNF, SNCA, CACNA1A, MAPT,	Alzheimer disease pathway, ALS pathway, Pathways of neurodegeneration – multiple diseases, Serotonergic synapse, Dopaminergic synapse, Neurotrophin signaling pathway, Parkinson disease	The tau protein and microtubule stability are both affected by axonal damage. It can cause tau disruption and a reduction in MT-binding capabilities. Membrane collapse and enhanced membrane permeability may also develop because of axonal damage [148, 149].
Oxidative Stress	NFE2L2, TNFα, NFKB1, CYPs, Ion channel genes, NOS3, HIF-1α	p53 signaling pathway, Pathways of neurodegeneration – multiple diseases, Parkinson disease, ALS pathway, Cellular senescence	Oxidative stress that enhances the rate of ROS-mediated lipid peroxidation, produces a deleterious effect on brain plasticity, CBF and the decreases the integrity of DNA, proteins, lipids, and membranes [144, 209].

Table 2.1. The association between molecular alterations (detailed in **Figure 2.1**) and gene expression changes after a traumatic brain injury (TBI). The effects of these alterations influence the integrity and function of neuronal cells. The genes included in this table were chosen based on their associated pathways. The genes were examined with GenBank from NCBI, and their pathways were examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [210], (https://www.genome.jp/kegg/pathway.html).

The expression of many miRNAs increases or decreases after brain injuries. One rat-model study revealed that a TBI considerably changed the expression of a significant number of mature hippocampal miRNA transcripts at 3 hours and 24 hours after injury [220]. A recent

study on human TBI patients found alterations in 17 serum miRNAs, with a severe increase of 10 miRNA upon hospital admission and their significant continuous reduction at 24 hours and 48 hours [221]. A strong correlation was found between the presence of miRNAs in the blood and mTBI and that most miRNA with differential expression were related to the regulation of genes associated with mitochondrial dysfunction and sodium, potassium, calcium, and chlorine ion channels [221]. Another human study found that patients with an mTBI had 10 circulating miRNAs altered one day post-injury and 13 miRNA altered after 15 days, but the miRNA expression in patients with a severe TBI saw 19 miRNAs altered one day post-injury and 22 miRNA altered 15 days post-injury [222]. In that study, miR-184, miR-502, miR-505, and miR-301b were selected as early candidate biomarkers for mTBI, and miR-203, miR-654-3p, and miR-655 as later candidate biomarkers for mTBI, with other promising candidate miRNA biomarkers for TBI being miR-21 and miR-335 [222]. In the exosomal microRNA study conducted by Vorn et al. (2022), a number of plasma exomiRNAs were found to be dysregulated in chronic mTBI patients compared to healthy controls [223]. This study indicated that 25 dysregulated exomiRNAs were connected with chronic mTBI and that 14 plasma exomiRNAs were related to neurological disorders via pathway analysis [223].

Overall, miRNAs are very small molecules that can pass the BBB more freely than extracellular proteins and are protected by exosomes, microvesicles and carrier proteins. Due to this, miRNAs are resistant to degradation by extracellular proteases and pH fluctuations, making them readily detectable in bodily fluids [206, 224]. These characteristics and the strong influence of miRNA on the CNS may make them reliable mTBI bioindicators. A main area of research on biofluid biomarkers for brain injuries has concentrated on identifying diagnostic blood biomarkers early after injury in order to create early diagnosis and determine the severity of

injury. Despite this, only a few reliable candidates have been found to determine the subacute or chronic sequelae of head trauma. For instance, the FDA has approved the use of *GFAP* and *UCHL1* as the first TBI biomarkers in acute TBI [225]. Studies have shown that the levels of certain blood biomarkers, such as *GFAP*, *UCHL1* and *S100B*, are significantly altered following mTBI [226-228]. However, as of now there are no remarkable genomics or metabolomics biomarkers that can demonstrate a correlation between biomarker level and mTBI severity.

2.6.8 Epigenetics

Epigenetics is the regulatory process that modifies gene expression without altering genetic sequences and defines the relationship between genetic and environmental factors to provide a link between drugs, cell physiology, toxic substances, and human conditions [229, 230]. Epigenetic changes are engaged in a diverse range of neurological diseases as a result of brain development mechanisms including neural stem cell maintenance, neuronal homeostasis plasticity and neurogenesis [231]. The study of epigenetics enhances knowledge about molecular mechanisms that exacerbate brain damage and helps discover effective therapeutic targets to improve neuronal health [232]. Mechanisms for epigenetic alteration include histone modifications, DNA methylation, DNA hydroxymethylation, editing of coding and non-coding RNA and DNA, nucleosome repositioning, and higher-order chromatin remodeling [231-233]. The role of RNAs in guiding and initiating epigenetics processes is substantial, and cell transcription acts as a history of modifications [231].

Epigenetic mTBI studies are limited but promising. An adult rat TBI model study found the activation of microglia and macrophages to be a leading cause for global cellular DNA hypomethylation and reduced 5-mc (5-mC: adding a methyl group to cytosine to form 5-

methylcytosine) one- and two-days post-injury [234]. Another study in blast-injury TBI rats showed considerable methylation changes between neurons and glial in the transforming growth factor ß, affecting genes such as *RUNX3, NODAL, MAP2K6, SMAD1*[235]. The study also found a decrease in *AANAT* (serotonin N-acetyltransferase) gene expression caused by neuronal damage triggered hypermethylation [235]. Aanat gene plays a role in depression and sleep disturbance [235]. A different rat-model study revealed histone acetylation and activation genes from promoter regions like *H3k9ac* and *H3K4me3* at P1 and *H3K9ac, H3K14ac, H3K36me3,* and *H3K4me3* at P2, as well as for the first time, an increase in the level of hippocampal IGF-1B mRNA, which could have an effect on genes such as *H3K9ac, H3K36me3,* and *H3K4me3* as they were increased three days after the controlled cortical impact (CCI) TBI [236]. Liu et al.,2014 have shown epigenetic associated alterations in miRNA early after an mTBI in the rodent hippocampus and cortex [237]. Research on rat hippocampus tissue illustrated that ten miRNAs were significantly modified one hour to seven days post-TBI, and some of them (miR-340-5p, miR-144 and miR-153) were increased significantly at different time-points after brain injury [237]. However, calcium/calmodulin-dependent serine protein kinase, nuclear factor erythroid 2-related factor (Nrf2), and α-syn predicted targets for those miRNAs were downregulated following TBI [237]. The use of telomere length (TL) as an indicator for outcomes following brain injury is also becoming an increasingly popular area of study. Telomeres play an active role in epigenetic regulation, cellular growth, and cell survival [238, 239]. Telomeres are prone to damage and shrinking due to oxidative stress, particularly in the brain, because neurons have a higher metabolism rate, limited regenerative capacity, and contain large amounts of iron and copper [240]. An animal study on concussions has demonstrated a positive linear correlation among telomere length (TL) and efficiency on a battery of behavioral

tests [239]. Shorter telomeres being involved with poorer performance and due to the connection between test battery performance and symptom severity, this study found TL to be a reliable predictor of concussion severity [239]. Telomerase and other parts of the telomeric complex might provide unique, promising therapeutic or diagnostic targets to promote neuronal survival and better outcomes after acute TBI, but further research in humans is required to validate this suggestion. Many neurological-related genes and miRNAs are altered by epigenetic mechanisms before and after transcription seem to influence brain injury outcomes. Epigenetic caused malfunctions certainly impact acute brain injuries and neurodegenerative diseases. Investigations indicated that epigenetic unbalances in DNA methylation and modification of histones primed the brain for illness and lead to poor neurological recovery [232]. The few epigenetic mTBI studies have provided unique information and insightful opportunities to better understand mTBI-caused abnormalities and how to target specific injury mechanisms. Further epigenetic research in humans will lead to better short and long-term mTBI prognosis.

2.6.9 Metabolomics

Metabolomics is an insightful field of study to understand all biochemical and pathophysiological pathways that examines molecular structures, functions and low molecular weight metabolite interactions [241, 242]. Metabolomic profiles can be used to examine in vivo enzyme activity following brain injury by determining free metabolite concentrations that can either influence localized metabolic activity or systemic metabolic activity, thereby identifying severity of the injury [243]. Mass spectrometry (MS) is one of the most popular methods for analyzing metabolomics data and is often combined with gas or liquid chromatographic separation techniques [243-245]. Another widely used method is proton nuclear magnetic resonance (H-NMR) [243, 245, 246]. The main approaches of MS and H-NMR have been used

extensively in metabolomics investigations for both targeted and non-targeted analyses [162, 245, 246]. A targeted metabolomic analysis is used to quantify a limited number of specific, known metabolites [247]. Meanwhile, a non-targeted analysis is used to identify known and unknown metabolites for a more exploratory approach, making it a semi-quantitative method [243, 247].

The study of metabolomics can provide detailed insight into brain injuries due to highly sensitive and specific capabilities of quantifying target metabolites that can indicate injury severity, track treatment responses, and monitor recovery [162, 241, 243]. There has been considerable interest in measuring protein biomarkers in blood serum, plasma, urine, and cerebrospinal fluid (CSF) to detect and assess mTBI [245]. According to the research, individuals with severe TBIs have a lower cerebral metabolic rate of oxygen and are hyperglycemic, which correlates with worse neurological outcomes and can be used as an indicator of injury severity [248]. Moreover, the brain maintains a high energy demand, further elevated by a brain injury, and releases phosphocreatine as a source of energy [249]. After an mTBI, the level of white matter creatine and phosphocreatine is increased for up to 4 months post-injury [249]. The rate of glycolysis increases to meet the elevated energy demand post-mTBI, but this leads to a rising lactate concentration in the serum, increased lactate uptake and utilization of ketones as energy in the brain [250]. The resultant lactate abundance may cause BBB damage, neuronal dysfunction, and brain edema [250]. As a consequence of ketone body failure, global membrane depolarization often occurs after an mTBI that contributes to the release of excitatory amino acids and neurotransmitters such as glutamate [251, 252]. Alterations to metabolites such as N-acetylaspartate (NAA), ATP, NADH, and ketone bodies may be promising biomarkers for detecting mitochondrial dysfunction proportional to brain

injury severity [12] or necrosis and apoptosis following an mTBI [253]. As discussed earlier, an upregulation of excitatory neurotransmitters, including phosphatidylserine translocation and glutamate, might identify apoptosis initiation via nuclear membrane lysis, DNA fragmentation, and cell membrane disintegration hours or days after brain damage [253]. Overall, metabolomics can enhance the knowledge surrounding the pathophysiological mechanisms in mTBI primary and secondary injury phases. Measuring metabolites after an mTBI can determine the pathophysiological injury response, validate clinical symptoms, and estimate prognosis.

2.6.10 Conclusions

This review discussed physiological and molecular changes post-mTBI and delved deeper into the genetic influences of mTBI severity, recovery, and assessment opportunities. Each mTBI presents a complicated, unique set of heterogenous symptoms and outcomes for each patient. Therefore, assessing brain health and function from several different perspectives allows for a more targeted understanding of cellular and molecular mechanisms, genetic predispositions, and this can in turn help inform personalized mTBI treatment plans. Genomic and metabolomic screening methods may present a promising future diagnosis methodology that will allow for personalized treatments while reducing bias and subjectivity. Several molecular and genetic pathways are involved in the secondary injury phase that make them suitable for therapeutic investigation. More extensive multi-modal studies of mTBIs using sensitive and validated omics techniques to identify and quantify robust bioindicators are required before personalized treatments are possible. However, continued progress in the fields of pathophysiology, genetics, molecular sciences, and metabolomics provide an essential framework for novel treatment methods for each patient’s unique symptoms and genotype.

CHAPTER 3: A BIOINFORMATIC STUDY OF GENETICS INVOLVED IN DETERMINING MILD TRAUMATIC BRAIN INJURY SEVERITY AND RECOVERY

3.1 Declaration

Please note that I, Mahnaz Tajik, did all of the research pertain to the bioinformatics analysis in this chapter. In this work, bioinformatics tools and online databases were used to identify genes associated with mTBI outcomes. To identify important mTBI genes and related pathways, enrichment analysis and pathway analysis were carried out. 10 mild TBI hub genes and their interactions were displayed using the CytoHubba plugin and protein-protein interaction analysis. Targets for mTBI candidate genes were identified using a miRNA prediction tools, and to verify our prediction, we performed RNA-seq analysis on publically available miRNA expression data (GSE123336) on mTBI downloaded from GEO repository. We plan to submit this paper to **Circulation: Genomic and Precision Medicine** in the coming weeks.

3.2 Abstract

Introduction: Mild traumatic brain injury (mTBI), or concussion, is a highly prevalent and devastating neurological condition. mTBI involves complex cascades of cellular and molecular events that lead to functional changes in genes and associated metabolites. The aim of this study was to identify potential biomarkers to aid the early diagnosis of mTBI and track recovery for individual patients. It was hypothesized that genes and micro ribonucleic acids (miRNAs) (and their associated proteins) involved in neuronal body, axonal and myelin integrity and regeneration would be identified as important markers of severity.

Materials and Methods: We used biological networks and bioinformatics databases to determine interactions between genes and miRNAs involved in mTBI diagnostic and molecular

components. Over 100 genes have been linked to mTBI outcomes in databases such as Gene and MalaCard and based on a review of the literature. Functional annotation analysis was applied with Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to find the genes most significantly related to mTBI and neurological function. The CytoHubba plug-in in Cytoscape was utilized to demonstrate hub genes by calculating degree value, and a string online database was used to show protein-protein interactions (PPI) between mTBI hub genes. To predict target miRNAs linked to the identified mTBI genes, 5 different miRNA prediction tools were employed. Furthermore, publicly available miRNA expression data (GSE123336) on mTBI were downloaded from the Gene Expression Omnibus (GEO) repository to compare differential miRNA expressions following brain damage with our findings. Finally, a visual network was designed and created using Cytoscape (v.3.8.2).

Results and Discussion: *APOE, S100B, GFAP, BDNF, AQP4, COMT, MBP, UCHL1, DRD2, ASIC1*, and *CACNA1A* were identified as candidate hub genes associated with mTBI outcome. Enrichment analysis revealed that these genes were implicated in neurological pathways including neuron projection regeneration and regulation of neuronal synaptic plasticity. To be used as surrogate injury markers for concussion, we predicted specific miRNAs linked to mTBI candidate genes such as hsa-miR-9-5p, hsa-miR-204-5p, hsa-miR-1908-5p, hsa-miR-16-5p, hsa-miR-10a-5p, has-miR-218-5p, has-miR-34a-5p, and has-miR-199b-5p. In addition, the result of RNA-sequencing analysis on mTBI (GSE123336) revealed that 2884 miRNAs had differential expression and that 17 miRNAs had significant changes at 0 time points and 48 hours following injury. And 2 miRNAs showed positive correlation with number of hits to the head.hsa-miR-10a-5p was one of the miRNAs found in this work that matched our predictions related to mTBI

outcome. The genes and miRNAs determined in this investigation may assist us to better understand the severity of injury and predict recovery time after mTBI.

3.3 Introduction

3.3.1 Mild traumatic brain injuries (mTBIs)

A traumatic brain injury (TBI) is a serious neurological injury that can lead to debilitating cognitive, emotional, and physical symptoms in the acute and chronic stages of recovery. The majority (~90%) of TBI are mild TBI (mTBI) [128], however the subjectivity surrounding grading injury severity and underreporting of head injuries remain substantial challenges [254-256]. Most adults recover within 10-14 days [10, 125, 129]; however, approximately 20% of adults who suffer an mTBI have symptoms lasting more than one month [9, 257, 258]. Furthermore, research has shown that older individuals with a history of mTBI may be at higher risk of neurodegenerative diseases including Alzheimer's Disease [259-261].

The stress and strain from mechanical forces applied during an mTBI event can cause shearing and compression of structures within the brain [262]. Those initial forces acutely comprise the primary injury phase, characterized by loss of axonal and blood-brain barrier (BBB) integrity, which then progresses into a secondary phase as ion imbalances and neuroinflammation gradually become exacerbated [22, 24, 263, 264]. The secondary injury phase involves a neurometabolic cascade that affects brain structure and function on a molecular level that can develop into excitotoxicity, oxidative stress and cell death [24-26, 74]. Due to the unique nature of each mTBI and person, highly sensitive and objective diagnostic tools are eminently required to aid in personalized recovery.

3.3.2 Genetic Factors

Numerous genes and molecular factors, such as *APOE, BDNF, IL-6, IL-10, TNFa, ENO2, UCHL1, GFAP*, and *S100B*, have been implicated in the pathophysiology of mild TBI and influence both short- and long-term neurological and functional outcomes [265-270]. Short-term brain health following a head injury event could be related to genetic variants that affect the severity of axonal damage, BBB disruption, inflammation, and neuronal survival, and cognitive disfunction [158, 163]. whereas long-term outcome may be determined by genes involved in neuroplasticity and neuronal regeneration [158]. As a result, identifying and characterizing the role of specific genes and micro ribonucleic acids (miRNAs) associated with mTBI could lead to the development of targeted interventions using injury specific diagnostic biomarkers. The miRNAs are small non-coding regulatory RNAs that directly regulate gene expression by preventing or increasing the translation of target messenger RNA (mRNA) [204, 271]. Moreover, miRNAs have been shown to substantially influence brain development and function making miRNA potentially useful and specific biomarkers for mTBI severity and prognosis [123, 124, 272]. Normally, the BBB provides highly specific transportation of brain-specific macromolecules such as proteins [29], however, blow to the head induced damage allows miRNAs to cross the BBB through microvesicles, exosomes, and lipoprotein carriers [272, 273]. Therefore, miRNAs that reside within the healthy brain may be more easily observed in peripheral circulation following brain injuries [272]. In the current study, we used bioinformatic workflow to systematically identify genes and their regulating miRNAs specifically associated with mTBI.

Bioinformatics analysis tools have been widely used to detect genes, miRNAs and functional pathways involved in the pathogenesis and progression of TBI [119, 166, 274, 275].

In silico studies, which rely on databases and pathway analysis software, provide valuable data from direct and indirect gene interactions to identify specific gene and miRNA alterations. However, mild TBI is a heterogeneous condition that affects numerous neurological processes in the brain, making it challenging to identify accurate and sensitive biomarkers. Currently, there are not any meaningful biomarkers that can be utilized to consistently diagnose and predict clinical outcome of mTBI. Thus, the purpose of our study was to identify potential hub genes and biological pathways associated with mTBI neurological sequelae and outcomes using multiple online databases and pathway analyses. Online miRNA bioinformatics tools were also used to predict target miRNAs correlated with hub genes and pathophysiological processes associated with mTBI. Additionally, we searched the GEO database for RNA-sequencing studies on mTBI [276] to analyze the data and compare it with our miRNA predictions. The main objective was to determine how mTBI affects expression of circulating miRNAs at various time points and after a different number of head hits, as well as to survey the biological and neuronal pathways related to these miRNAs. These results were then compared to our downloaded data to see if they were consistent with the miRNAs that we had predicted would change as a result of brain injury.

3.4 Methods

3.4.1 Literature Review

A review of the literature was performed to determine how many genes are associated with mTBI-related neurological structural and functional changes, and which genes are more significantly impacted or altered post-injury **(Figure 3.1)**. These genes were chosen by functional similarity, biological pathways, gene ontology, and phenotype related to mTBI outcome. We reviewed the physiological, molecular, and omics changes that follow brain injury, particularly mTBI, as well as earlier research on gene and miRNA changes, polymorphism, and the effect of SNPs on patient recovery. In addition, we reviewed epigenetic mechanisms that follow head trauma and their impacts on gene expression and neurological dysfunction. We included studies on humans and mammalian animal models that examined changes in blood biomarker levels following mTBI and their correlation with neurological symptoms. More information can be found in our previous review article [277].

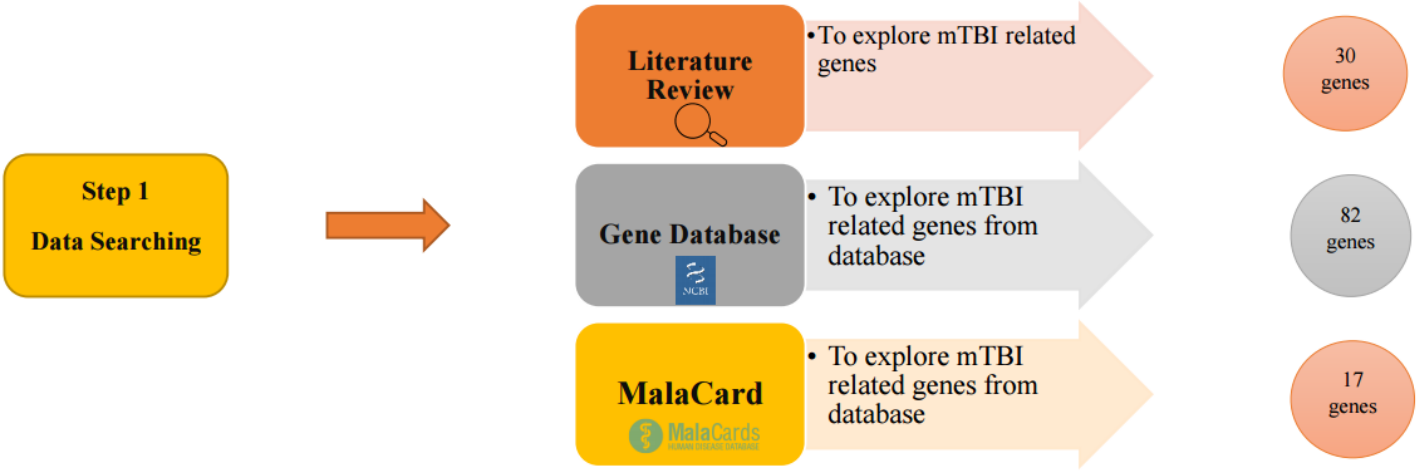


Figure 3.1. Exploring mTBI target genes using databases and a comprehensive literature review.

3.4.2 Bioinformatic Databases

Following the literature review, a critical search of online bioinformatics databases was conducted using the Gene database from NCBI (Gene) [278] and MalaCards (The human disease database) (MalaCards) [279] to determine how many genes were correlated with mTBI (**Figure 3.1**). Gene databases "supply gene-specific connections in the nexus of map, sequence, expression, structure, function, citation, and homology data" [278]. The gene identity can be determined by specifying the sequence, map position or the phenotypic characteristics of the gene [278]. These gene identification numbers are utilized across all NCBI datasets and kept up to date via annotation changes [278]. This database incorporates data and linkages to the Online Mendelian Inheritance in Man (OMIM) database, which is a continuously updated catalog of human genes, genetic disorders and traits. "Mild traumatic brain injury" was the key phrase used to start searching in the Gene database and the results were automatically displayed in Tabular format. Moreover, the results were sorted by relevance to the condition and gene weight, also human was selected as the primary organism. As determined by the Gene dataset, 82 genes and 5 miRNAs are known to be involved with mTBI in humans (a list of affiliated genes with mTBI was determined based on a Gene Weight calculation, which included multiple lines of evidence such as gene expression, protein clusters, and OMIM entries [278].

MalaCards is a comprehensive database of diseases and their annotations. MalaCards creates an electronic card for each of the 16,919 human disorders by combining and mining 44 data sources [279]. MalaCard contains disease-specific prioritized annotations, as well as inter-disease connections, based on GeneCards, GeneDecks, and their search capabilities [279]. We performed a MalaCard search to determine how many genes are associated with TBI, and 17 genes were identified as key. The relevance score for each was calculated by considering the

significance of the many resources linking the gene to the condition [279] as described in the following link [1].

3.4.3 Functional Enrichment and Pathway Analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8) was utilized to address functional annotation, visualization, and biological meanings behind the mTBI-associated genes with specific Gene Ontology (GO) [280, 281], [2]. The DAVID bioinformatics resources include a combined biological pool of knowledge and algorithms for systematically deriving biological role from an extensive list of genes and proteins [281]. GO defines the interaction across genes by annotating and categorizing the molecular function (MF), biological process (BP), and cellular components (CC) linked with a gene product [282]. This process allowed enrichment analysis of a gene collection [282], which was carried out in this study to reveal which MF, BP, and CC were disproportionately represented in our gene list.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database used to explore molecular interactions and to create a network of relationships between our candidate genes [283], [3]. To better understand the biological significance of mTBI candidate genes, our gene list was uploaded into DAVID for enrichment analysis related to GO and KEGG terms. A filter = 0.05 was used as the significance cut-off value for fold enrichment analysis in the DAVID database. Gene Set Enrichment Analysis (GSEA) focused on gene groups that shared a similar biological function, regulation, and chromosomal location [284], [4]. GSEA analysis was

[1] https://www.malacards.org/pages/info#scores
[2] https://david.ncifcrf.gov
[3] https://www.genome.jp/kegg/pathway.html
[4] http://www.gseamsigdb.org/gsea/index.jsp

performed on our candidate genes to identify biological processes or pathways that may cause neurological perturbation following mTBI (**Figure 3.2**).

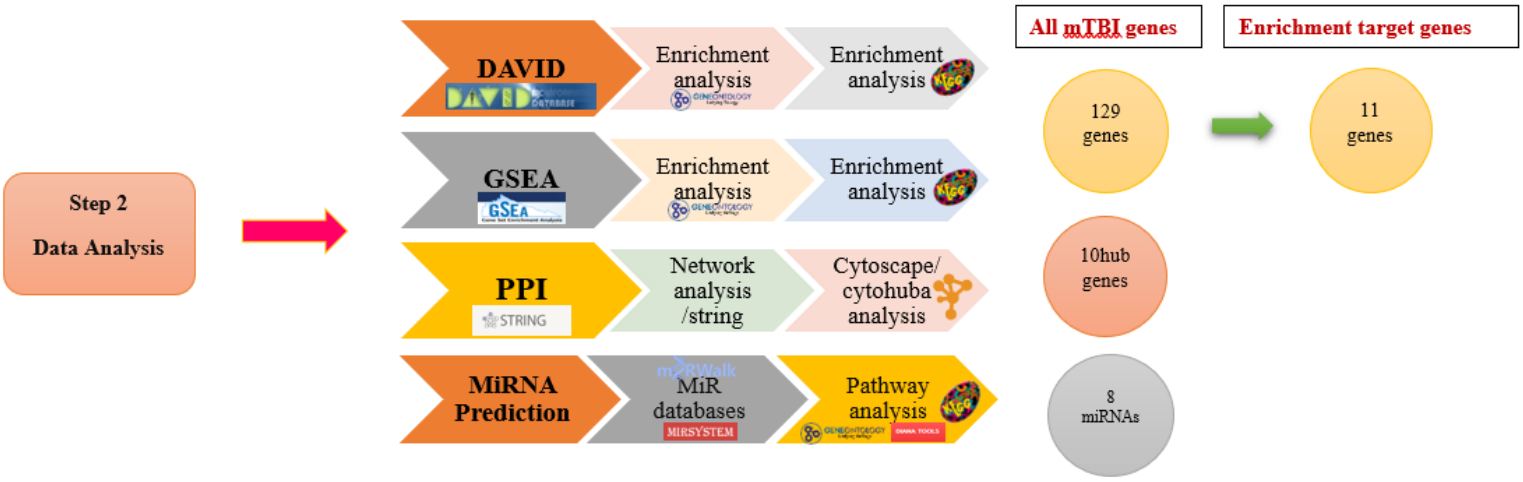


Figure 3.2. After investigating genes from step 1 (Figure 3.1), DAVID and GSEA databases in step 2 were performed to find relevant genes and biological pathways. Based on enrichment analysis, 11 genes out of 129 exhibited a significant relationship with mTBI outcomes in both datasets. Protein-protein interaction and network analysis were performed utilizing string databases, while 10 Hub genes were identified using Cytohubba. Multiple miRNA databases were used to predict miRNAs linked to mTBI candidate genes and key pathways tied to mTBI changes.

3.4.4 Protein-Protein Interaction (PPI) Network and Hub Genes

The Search Tool for the Retrieval of Interacting Genes (STRING, version 11.0) is an online database that offers unrivalled coverage and easy access to both experimental and predicted protein-protein interaction (PPI) data [285], [5]. PPI networks were built using STRING to investigate the functional interactions between mTBI candidate genes and proteins. The parameter was set to have a medium confidence score > 0.4. To visualize the network, Cytoscape (version 3.8.2) [286] with the CytoHubba plug-in [287] was used to indicate

[5] https://string-db.org

interactions between the top ten candidate genes. CytoHubba offers a user-friendly interface for network analysis based on 11 scoring or topological approaches [287] (**Figure 3.2**). Based on the degree of interaction as a primary metric of relevance, our study selected the top ten mTBI genes.

3.4.5 miRNA-target gene regulatory network

The miRNAs control gene expression by interacting with their target genes during the post-transcriptional phase. In the current study, online tools were used to first identify miRNAs that regulate mTBI candidate genes and then secondly, to construct miRNA-target gene regulatory networks using Cytoscape software (**Figure 3.2**). To predict which miRNAs, regulate mTBI candidate genes, various prediction tools were used including miRSystem, miRWalk2.0, and mirDIP. The miRSystem [288], [6] integrates with 7 well-known miRNA target gene prediction programs, and miRNAs were selected based on whether the total hit value was greater than or equal to 1. The miRWalk2.0 [7] [289] interacts with 12 different online databases to predict miRNA. With this approach miRNAs were chosen if they appeared in at least three out of the 12 miRWalk databases (miRMap [8] [290], Targetscan [9] [291], and miRDB [10] [292] The miRDIP, another comprehensive database for predicting miRNAs, was used to identify miRNAs with very high scores that were directly linked to identified mTBI-related genes [11][293] Moreover, we applied DIANA mirPath (version 3) [294] [12].

[6] http://mirsystem.cgm.ntu.edu.tw/
[7] http://mirwalk.uni-hd.de/
[8] http://cegg.unige.ch/mirmap
[9] http://www.targetscan.org/
[10] http://www.mirdb.org
[11] http://ophid.utoronto.ca/mirDIP/
[12] http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=site/page&view=software

and the miRNA pathway dictionary (miRPathDB version 2.0) [295] [13] to identify more precise miRNAs associated with the identified candidate genes based on biological pathways and gene ontology enrichment. Working with each of these databases is simple and intuitive; all that is required is the user to enter the gene list, click the "search" button, and then download the results. The only variations are the algorithms and statistical analysis that each database employs to categorize miRNAs.

3.4.6 RNA-Sequencing Data Analysis

A small RNA sequencing study of human serum and saliva prior to, during and after amateur mixed martial arts (MMA) competitions (GSE123336) [276] was downloaded from the GEO repository [14] [296] (**Figure 3.3**). GEO is a global public database that stores and openly disseminates high-throughput functional genomics data from microarray, next-generation sequencing, and other sources that are contributed by the science community [296]. The dataset (GSE123336) was based on Illumina NextSeq 500 (Homo sapiens) and consists of a total of 218 samples, including 131 serum and 87 saliva. In this study we used raw data from serum samples collected at different time points (1-week pre-injury = 7, 0 days pre-injury = 52 samples, 0 days post-injury = 52 samples, 2-3 days post-injury = 17 samples, 1-week post-injury = 3). There were two options for downloading the data: either using the SRA toolkit to download the SRA files and convert it to *fastq* files using a Linux workstation, or directly downloading the *fastq* files from the ENA repository.

[13] https://mpd.bioinf.unisb.de/overview.html
[14] http://www.ncbi.nlm.nih.gov/geo

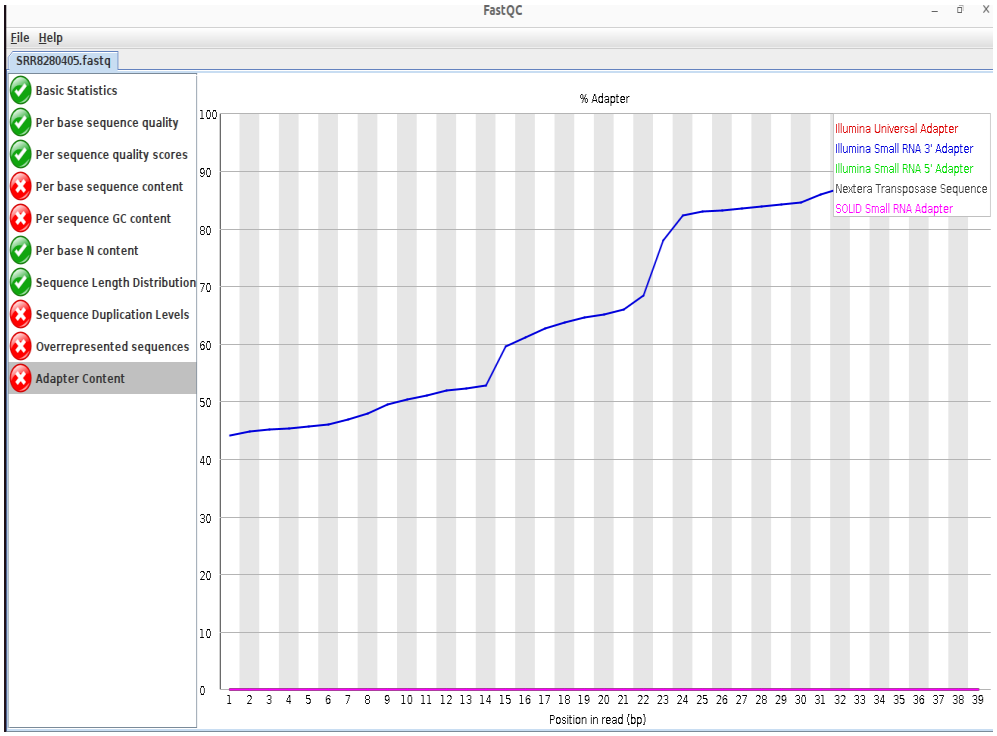
Figure 3.3. Investigating the GEO repository and downloading RNA-seq data associated with mTBI for mapping and differential expression analysis.

3.4.7 Data Processing

The original fastq files related to the GSE123336 were downloaded from ENA [15]. As a first step, fastq data were checked using fastQC tool (Babraham Bioinformatics—FastQC, a Linux-based quality control tool for high throughput sequence data) (**Figure 3.4**). The Fastq files contained 3' Illumina adapters that were trimmed using Trimmomatic software [297] [16].

[15] https://www.ebi.ac.uk/ena/browser/home
[16] http://www.usadellab.org/cms/?page=trimmomatic

(A)



(B)

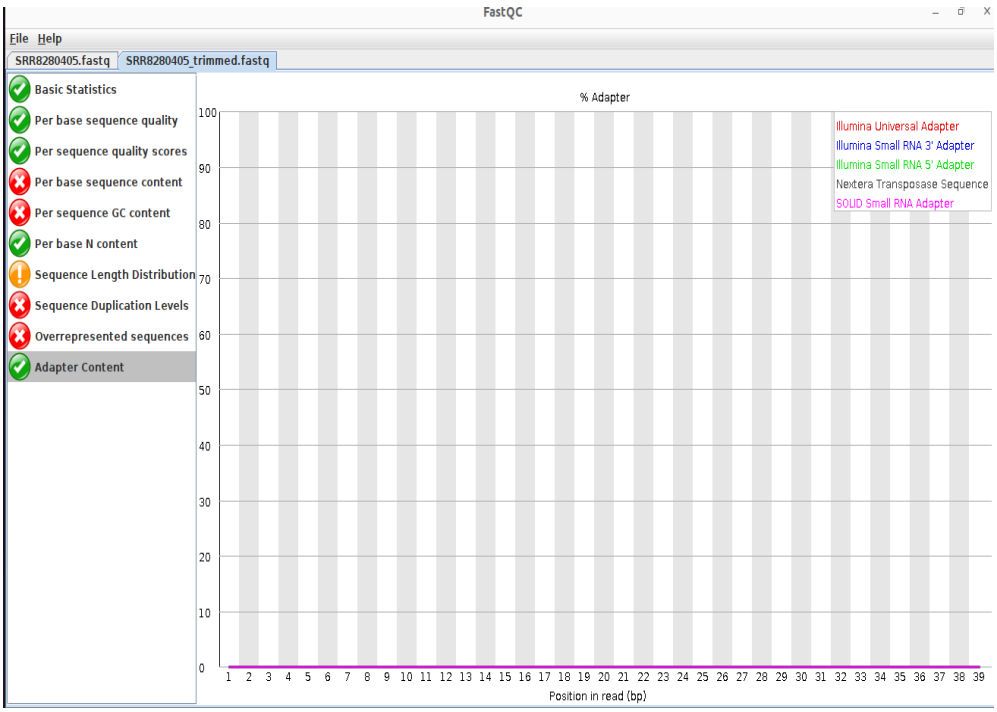


Figure 3.4. Figure (A) represents the fastq data with illumina small RNA 3' adapters (blue line). Figure (B) shows the same fastq file after trimming 3' Illumina adapters sequence with trimmomatic.

The data we utilized for analysis was single end read (i.e., the sequencer reads DNA fragments from one end to the other). Trimmomatic offers a number of useful trimming function for Illumina single-ended data. Command line-based scripts were used to specify the trimming stages to remove the adapters from sequences (see Appendix A). Subsequently, Burrows-Wheeler Alignment (BWA) software was used to perform mapping of the data against the reference genome (hg38) [17] [298]. A full index for the genome reference »hg38« was built using the function of the BWA index, then BWA *aln* algorithm was used to align short read sequence with the index. FeatureCounts were conducted for counting reads, building a count matrix, and comparing aligned reads with miRNA transcripts downloaded from the miRBase database (has.gff 3) [18] [299, 300]. As a final step, R software and the DESeq2 package were employed to identify differential miRNA expressions following mTBI at various time points and numbers of hits to the head [301]. The command line codes for BWA and FearureCount are provided in Appendix A.

3.5 Results

3.5.1 Literature Review to Identify Candidate Genes

According to existing research, neurotrophic, inflammatory and catecholamine genes are more often altered after brain trauma and serve as reliable predictors of injury severity and recovery [163]. Furthermore, they have substantial influence on biological processes associated with mTBI outcomes. To identify genes involved with mTBI, peer-reviewed journal article literature and two databases (Gene and MalaCards) were considered. As a result, 129 genes were significantly correlated with mTBI (82 genes from Gene database, 17 genes from MalaCards,

[17] https://bio-bwa.sourceforge.net
[18] https://www.mirbase.org/ftp.shtml

and 30 genes were chosen from experimental studies). In order to include genes in our study, we considered their function, expression in the blood circulation system, and most direct relationship to mTBI neuronal dysfunction and recovery. Moreover, we excluded genes that are expressed only in brain tissue, inflammatory genes, and cytokines. Based on neurological symptoms, cognitive impairment, recovery timeline and hub gene analysis, the mTBI gene list was narrowed down from 129 to 11 genes that were more associated with the outcomes of mTBI including *APOE* (Apolipoprotein E), *S100B* (s100 calcium binding protein B), *GFAP* (Glial fibrillary acidic protein), *BDNF* (Brain-derived neurotrophic factor), *AQP4* (Aquaporin-4), *COMT* (Catechol-O-methyltransferase), *MBP* (Myelin basic protein), *UCHL1* (Ubiquitin C-terminal hydrolase L1), *DRD2* (Dopamine receptor D2), *ASIC1* (Acid-sensing ion channel 1), and *CACNA1A* (Calcium voltage-gated channel subunit alpha 1 A). These genes were determined based on their functional similarities, involvement in biological pathways, phenotypes, protein interactions, and expression related to mTBI outcome.

3.5.2 Functional and Pathway Enrichment Analyses

Functional enrichment analysis was performed to identify GO terms and KEGG pathways for the 11 mTBI candidate genes (**Figure 3.5**). **Table 3.1** shows the top 5 enriched GO terms and 2 significant KEGG pathways for our candidate genes based on fold enrichment analysis. The biological processes connected with mTBI genes were considerably rich in neuronal processes, and more specifically, neuron projection regeneration, which is related to the regrowth of axons or dendrites in response to their loss or damage (Fold enrichment = 381.6, P = 4.80E-03). The neuronal cell body had the highest concentration of cellular components associated with mTBI genes, and tau protein binding was found to be the most enriched molecular function associated with mTBI genes (Fold enrichment = 279, P = 6.50E-03). Based

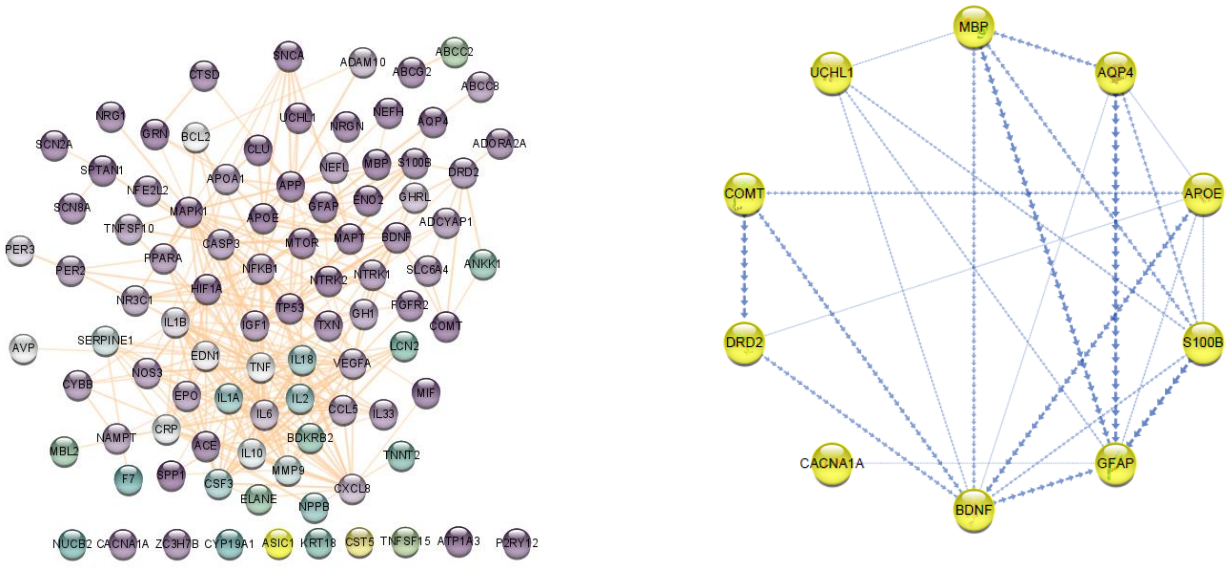
on fold enrichment analysis, the only two significantly enriched KEGG pathways were cocaine addiction (Fold-enrichment = 35.1; P = 4.90E-02) and dopaminergic synapse pathways (Fold-enrichment = 20.2; P = 6.80E-03). Furthermore, GSEA analysis showed the majority of mTBI candidate genes were involved in cognition, synaptic signaling, memory, and nervous system processes. According to the GSEA phenotype analysis, *APOE*, *BDNF*, *CACNA1A*, *COMT* and *UCHL1* were identified as the main genes associated with cognitive impairment (FDR q-value < 0.05) (**Table 3.2**).

Category	GO term	Count	Fold Enrichment	P-Value
GOTERM_BP_DIRECT	GO:0031102- neuron projection regeneration	2	381.6	4.80E-03
GOTERM_BP_DIRECT	GO:0048168- regulation of neuronal synaptic plasticity	2	190.8	9.50E-03
GOTERM_BP_DIRECT	GO:0007628- adult walking behavior	3	147.7	1.50E-04
GOTERM_BP_DIRECT	GO:0008306- associative learning	2	127.2	1.40E-02
GOTERM_BP_DIRECT	GO:0043407- negative regulation of MAP kinase activity	2	84.8	2.10E-02
GOTERM_CC_DIRECT	GO:0044297- cell body	2	52.6	3.40E-02
GOTERM_CC_DIRECT	GO:0043025- neuronal cell body	5	26.3	1.70E-05
GOTERM_CC_DIRECT	GO:0030425- dendrite	3	14.8	1.40E-02
GOTERM_CC_DIRECT	GO:0005886- plasma membrane	8	3.2	6.10E-02
GOTERM_CC_DIRECT	GO:0005737- cytoplasm	7	2.2	3.80E-02
GOTERM_MF_DIRECT	GO:0048156- tau protein binding	2	279	6.50E-03
GOTERM_MF_DIRECT	GO:0042802- identical protein binding	4	8.2	8.30E-03
KEGG_PATHWAY	hsa05030 - Cocaine addiction	2	35.1	4.90E-02
KEGG_PATHWAY	hsa04728 - Dopaminergic synapse	3	20.2	6.80E-03

Table 3.1. DAVID functional analysis for the 11 mTBI genes. The highest fold enrichment analysis represents GO terms that are strongly related to candidate genes. (Biological Process: BP. Cellular component: CC. Molecular Function: MF)

GO Terms	Genes in Overlap	P-value	FDR p-value
GOBP_COGNITION	APOE, BDNF, DRD2, ASIC1, S100B	6.48E-09	9.05E-05
GOBP_SYNSPTIC_SIGNALING	APOE, BDNF, DRD2, ASIC1, MBP, CACNA1A	1.27E-08	9.05E-05
GOBP_MEMORY	APOE, BDNF, DRD2, ASIC1	1.53E-08	9.05E-05
GOBP_NERVOUS_SYSTEM_PROCESS	APOE, BDNF, DRD2, ASIC1, S100B, MBP, AQP4	1.91E-08	9.05E-05
GOBP_REGULATION_OF_TRANS_SYNAPTIC_SIGNALING	APOE, BDNF, DRD2, ASIC1, AQP4	4.44E-08	1.40E-04
GOBP_BHAVIOR	APOE, BDNF, DRD2, ASIC1, S100B	1.85E-07	4.99E-04
GOCC_SOMATODENTRIC_COMPARTMENT	APOE, BDNF, DRD2, MBP, CACNA1A, COMT	3.10E-08	1.17E-04
GOCC_SYNAPSE	APOE, BDNF, DRD2, ASIC1, MBP, CACNA1A	4.14E-07	8.70E-04
HP_COGNITTIVE_IMPAIRMENT	APOE, BDNF, CACNA1A, COMT, UCHL1	3.40E-07	8.04E-04
HP_MENTAL_DERERIORATION	APOE, CACNA1A, COMT, UCHL1	1.29E-06	2.45E-03

Table 3.2. Gene set enrichment analysis for mTBI candidate genes. A GSEA analysis revealed that most mTBI genes involved cognitive, synaptic signaling, memory, and nervous system functions. According to this analysis, APOE, BDNF, CACNA1A, COMT, and UCHL1 are the major genes associated with cognitive impairment (P-value = 3.40E-07). (Biological Process: BP. Cellular component: CC. Human phenotype: HP)

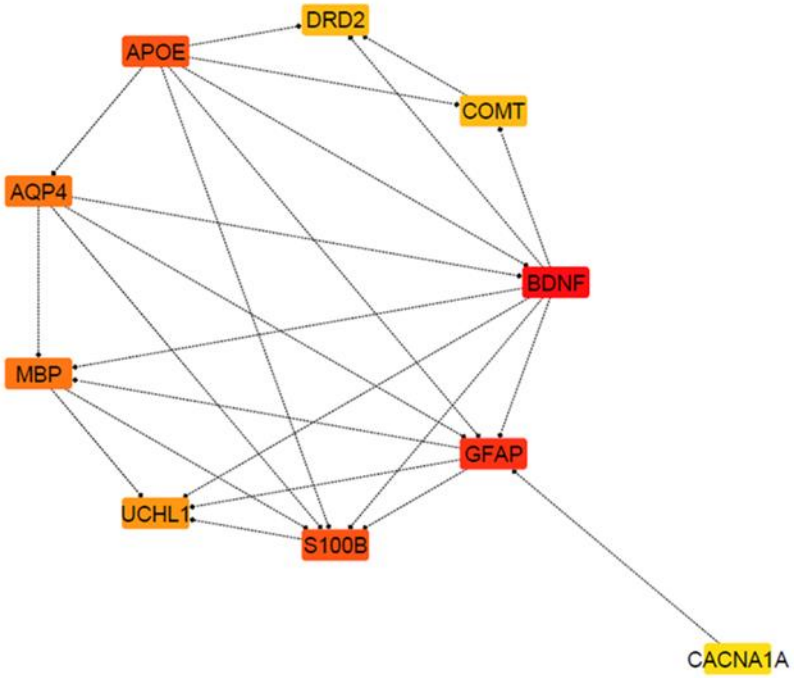


(A) (B)

Figure 3.5. (A) Potential genes related to human mTBI based on a literature review and database searches. These genes were filtered based on neural functions, and purple nodes show the genes that have more neurological functions. The protein-protein interaction network was visualized using Cytoscape v.3.8.2. (92 nodes and 327 edges). (B) 10 mTBI candidate genes according to the enrichment analysis and PPI and co-expression between them indicated by Cytoscape v.3.8.2. bold arrows represent strong interaction between candidates' genes.

3.5.3 Protein-Protein Interaction (PPI) Network Interaction and Hub Genes Analysis

By submitting the candidate genes into STRING, PPIs associated with mTBI candidate genes were obtained (**Figure 3.5**). The hub genes with CytoHubba, with the degree of interactions shown in Figure 3.5.3, which displays 10 nodes and 24 edges **Figure 3.6**. The STRING interaction network (version 11) revealed that most of the candidate genes exhibit strong interaction with each other, and enrichment analysis outcomes for the hub genes showed the role of the mTBI top genes in different neurological tasks and neurological pathways. For instance, *ASIC1, APOE, S100B, COMT* and *DRD2* play an essential role in memory function; *APOE, UCHL1, S100B, DRD2, BDNF* and *GFAP* play a key role in neuron projection development; *UCHL1, S100B, COMT, DRD2* and *APOE* are essential genes related to behavior, and *APOE, S100B, DRD2* and *BDNF* play a significant role in the regulation of cell death (these processes were selected based on their higher node degree and FDR q-value) (**Figure 3.6**).



Rank	Name	Score
1	BDNF	8
2	GFAP	7
3	APOE	6
3	S100B	6
5	MBP	5
5	AQP4	5
7	UCHL1	4
8	COMT	3
8	DRD2	3
10	CACNA1A	1

Figure 3.6. The PPI network of the 10 Hub genes candidates is clustered by CytoHubba in cytoscape software based on the degree of interactions. The Degree is indicated with the color of nodes dark colors represent higher degree and light colors indicate lower degree. Based on CytoHubba analysis BDNF shows a high degree of interaction, while CACNA1A shows a lower degree of interaction.

3.5.4 miRNA-Target Analysis

Online databases were utilized to predict target miRNAs connected to the mTBI candidate genes. Over 100 miRNAs were associated with the 11 genes according to miRSystem, miRwlk 2.0, and miRDip. To identify significant miRNA closely linked to mTBI genes the DIANA mirPath v3.0 bioinformatic tool and miRpathDB v2.0 database were used. DIANA miRPath obtained miRNA genes targets and KEGG pathways related to gene function, while miRpathDB used GO to display molecular function, cellular component, and biological process associated with mTBI selected miRNAs and their related genes. The results showed that the miRNAs of hsa-miR-9-5p, hsa-miR-204-5p, hsa-miR-1908-5p, hsa-miR-16-5p, hsa-miR-10a-5p,

has-miR-218-5p, has-miR-34a-5p, and has-miR-199b-5p were highly related to the mTBI candidate genes and thus may indicate dysfunction following mTBI. Moreover, pathway analysis revealed that predicted miRNAs targets were mainly engaged in nervous system signaling, neuron projection and cell differentiation (**Figure 3.7; Table 3.3**). To transfer nerve signals, two systems have developed. First of all, electrical signals are passed along the cell membrane within individual cells and second electrical impulses used for cell signaling are typically transformed into chemical signals that are sent by neurotransmitters [302]. Signal transmission within neurons or nervous system signaling is governed by voltage differences [302]. Therefore, an ion imbalance following brain injury may affect neuronal communication and result in neurological impairment. In the cortex, projection neuron extension or process axons to distant intracortical, subcortical, and sub-cerebral targets regulate sensory input, motor functions, and cognitive abilities [303].

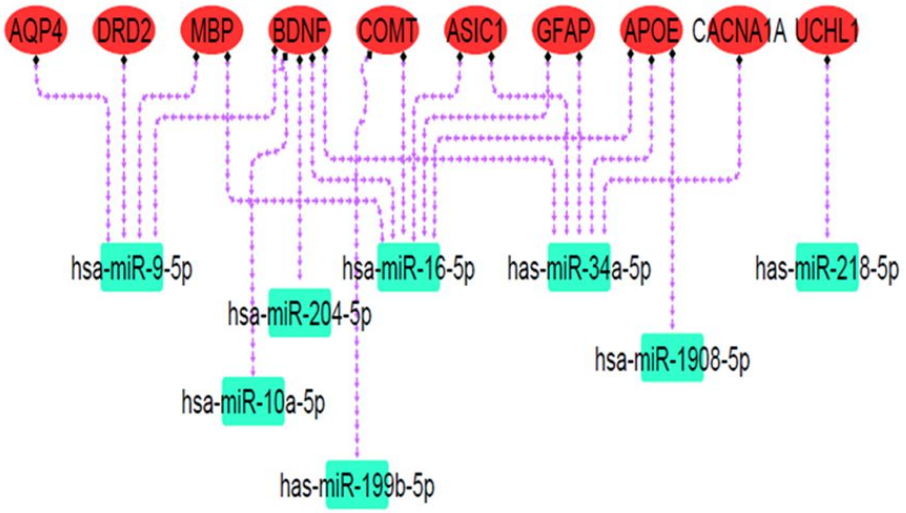


Figure 3.7. The miRNA targets regulatory network. Predicted miRNA targets are displayed in green boxes, and purple lines indicate their connections to mTBI candidate genes (red ovals). Pathway analysis revealed that these miRNAs targets were mainly engaged in nervous system signaling, neuron projection and cell differentiation.

Target miRNAs	Genes	Canonical pathways
hsa-miR-9-5p	DRD2, AQP4, MBP, BDNF,	Cell differentiation, Synaptic development, Regulation of cell differentiation
hsa-miR-204-5p	BDNF	Regulation of cell differentiation, generation of neurons
hsa-miR-1908-5p	APOE	Nervous system development, Synapse
hsa-miR-16-5p	BDNF, APOE, GFAP, COMT, MBP, ASIC1	Synapse, Regulation of protein metabolism, Regulation of metabolism process
hsa-miR-10a-5p	BDNF	Regulation of cell morphogenesis, Generation of neurons
has-miR-218-5p	UCHL1	Regulation of metabolic process, Cell differentiation, Nervous system development
has-miR-34a-5p	GFAP, BDNF, APOE, CACNA1A, ASIC1	Cell-Cell signaling, Cell death
has-miR-199b-5p	COMT	Synapse, Synaptic membrane

Table 3.3. Target miRNAs related to mTBI candidate genes. Canonical pathways selected by P-value < 0.05 as the significance cut-off value for miRNA pathway dictionary (miRPathDB version 2.0).

3.5.5 Identification of Differential Expression miRNAs in GSE123336

In the GSE123336 study, samples were taken based on the frequency of blows to the head and at various times before and after MMA competition. In light of it, we established conditions for each status (time points and number of hits) and analyzed the data based on each condition. The DESeq2 package in the RStudio software was used to identify differentially expressed miRNAs after mTBI, and the results showed that 2884 miRNAs expression profile had changed. In the first condition, the P-adjusted (P_{adj}) value from R was used to compare the 0-d post with the normal group (0 d pre & 1-week preinjury) and to identify significant miRNAs based on $P_{adj} \leq 0.05$. The findings showed that expression levels of 17 miRNAs had altered considerably immediately after injury (i.e., day 0, post), with 14 miRNAs showing upregulation and 3

displaying downregulation (**Table 3.4a; Figure 3.8**). This research revealed that hsa-miR-10a-5p was overexpressed in serum at 0 days after injury (Padj = 9.48E-06, P-value = 2.08E-08), and we anticipate that it is a critical miRNA associated with mTBI and BDNF gene expression. For the second condition, the P_{adj} value in R was utilized to compare 2-3d post-injury to the normal group (0 day and 1 week prior) to find significant miRNAs based on $P_{adj} \leq 0.05$. The results demonstrate that after 47/48 hours, similar miRNAs with a 0 day also displayed differential expression (17 miRNAs were found to have changed, with 14 indicating increases and 3 showing decreases.). Based on the analysis, there were no discernible changes in miRNA levels one week after injury compared to controls. Furthermore, the same analysis was done based on the number of hits to the head and the above time points that participants received hits during the completion, and the findings demonstrated that participants who got over 20 hits to the head had altered levels of miRNA expression, which increased the expression of some miRNAs like hsa-miR-10b-5p (P_{adj} =0.039), and although almost significant, hsa-miR-143-3p (P_{adj} =0.0082), (**Table 3.4b; Figure 3.8**). It is possible that these miRNAs may act as biomarkers in determining the severity of brain injury, although further investigation is necessary to confirm and validate this hypothesis. In terms of GO and pathway analysis, the majority of miRNAs identified were related to nervous system development, cell projection, neuronal projection, metabolic processes, and neuronal system functions (**Tables 3.4a & 3.4b**).

miRNAs ID (0 d post)	P value	P_{adj}	Predicted Targets	Pathway
hsa-miR-145-3p	2.06E-04	0.0176	BDNF	Generic Transcription Pathway, Gene expression (Transcription), regulation of metabolic process
hsa-miR-873-3p	1.12E-04	0.0118	S100B	There is no notable pathway.
hsa-miR-125b-2-3p	2.86E-08	9.78E-06	GFAP, BDNF, AQP4, DRD2, COMT, UCHL1	Synapse, regulation of nitrogen compound metabolic process, nervous system development
hsa-miR-99a-5p	3.61E-06	5.92E-04	There are no related genes	There are no notable pathways.
hsa-miR-143-3p	8.95E-11	1.22E-07	CACNA1A	Metal ion binding, cell projection, regulation of signaling, cell-cell signaling
hsa-miR-10b-5p	2.22E-09	1.51E-06	BDNF	Regulation of cell morphogenesis, regulation of cellular component organization
hsa-miR-10a-5p	2.08E-08	9.48E-06	ASIC1, BDNF	Regulation of cell morphogenesis, anatomical structure morphogenesis
hsa-miR-192-5p	1.89E-06	3.69E-04	BDNF, ASIC1, GFAP	Regulation of cellular process, regulation of metabolic process
hsa-miR-378a-3p	1.33E-05	1.81E-03	AQP4, BDNF, GFAP	Nervous system development, cell morphogenesis, regulation of cell morphogenesis, regulation of cellular component organization
hsa-miR-99b-5p	2.79E-07	7.63E-05	DRD2, COMT	Metabolic process, protein binding
hsa-miR-125a-5p	7.39E-07	1.68E-04	DRD2, CACNA1A, ASIC1, GFAP	Synapsen, negative regulation of signaling, intrinsic component of membrane
hsa-miR-24-3p	3.78E-05	4.70E-03	UCHL1, GFAP, MBP, S100B, DRD2, COMT, ASIC1	Regulation of cellular component organization, regulation of autophagy, nervous system development, neurogenesis, synapse,
hsa-miR-345-5p	3.47E-04	0.0278	GFAP, BDNF, S100B, DRD2, ASIC1, COMT	Intracellular signal transduction, neuron differentiation, synapse, cellular localization, negative regulation of signaling, neuron projection, postsynapse
hsa-miR-27b-3p	7.18E-05	8.17E-03	BDNF, DRD2, CACNA1A, ASIC1	Regulation of cell projection organization, Neuronal System, chemical synaptic transmission, metal ion binding, neurotransmitter secretion, synapse
hsa-miR-191-5p	5.16E-04	0.0391	GFAP, BDNF	Regulation of nitrogen compound metabolic process
hsa-miR-26a-5p	0.000142	0.0139	BDNF, S100B	Gene expression, Generic Transcription Pathway, regulation of cellular component organization, nervous system development
hsa-miR-184	3.90E-06	5.92E-04	AQP4	Cell projection, plasma membrane, plasma membrane bounded cell projection

Table 3.4a. The level of miRNAs that were differentially expressed at zero days and 2-3 days after injury. The miRwalk and mirsystem databases were used to investigate predicted targets, while mirpathDB was used to analyze pathways. All of these are significant based on $P_{adj} \leq 0.05$, some with very high significance.

miRNAs ID	Number of hits	P-Value	P_{adj}	Predicted genes	Pathway
hsa-miR-143-3p	Over 20	6.04E-05	0.0082	BCL2, IL6R, CACNA1A	Metal ion binding, cell projection, regulation of signaling, cell-cell signaling
hsa-miR-10b-5p	Over 20	5.74E-04	0.039	BDNF	Regulation of cell morphogenesis, regulation of cellular component organization

Table 3.4b. The level of miRNAs that were differentially expressed following number of hits to the heads. The miRWalk and miRSystem databases were used to investigate predicted targets, while miRPathDB was used to analyze pathways. According to the number of blows to the head, only two miRNAs were found to be relevant, and these are significant based on $P_{adj} \leq 0.05$.

3.6 Discussion

The discovery of genes and miRNA associated with neurological damage may aid in the diagnosis and treatment of mTBI. There are numerous pathophysiological changes that occur following mTBI that are extremely complex. Therefore, the identification of brain-specific miRNAs can help us understand the molecular alterations after a concussion, which is necessary to predict individual mTBI outcomes. This study used various bioinformatic analyses to determine hub genes and miRNAs related to brain damage and neuronal dysfunction. According to our data and pathway analyses, 11 genes were found to have a significant association with neurological function, and 8 miRNA showed strong interaction with those 11 candidate genes and were engaged in cell differentiation, nervous system development, synaptic development, generation of neurons, and cell death. The miRNA identified as closely linked to mTBI were hsa-miR-9-5p, hsa-miR-204-5p, hsa-miR-1908-5p, hsa-miR-16-5p, hsa-miR-10a-5p, has-miR-218-5p, has-miR-34a-5p, and has-miR-199b-5p. Based on the evidence obtained from the

DAVID and GSEA analyses, mTBI candidate genes were mainly enriched in neuronal projection regeneration, regulation of neuronal synaptic plasticity, cognitive function, memory, and behaviour.

To establish the differential expression of miRNAs after brain damage, we also analyzed the GSE123336 dataset from the GEO repository and compared it with our results. The results indicated significant changes in miRNA expression levels after mTBI. The hsa-miR-10a-5p was one of the miRNAs that showed significant increase immediately after injury (i.e., 0-day post injury), and we predict it as an important miRNA related to BDNF and regulation of cell morphogenesis pathway. Moreover, there was a considerable rise in hsa-miR-10b-5p and hsa-miR-143-3p at 0 day after injury, and there was a positive correlation between these changes and number of hits to the head, which means that these miRNAs could serve as a useful biomarker to measure the severity of the injury. However, more research is needed to support this claim.

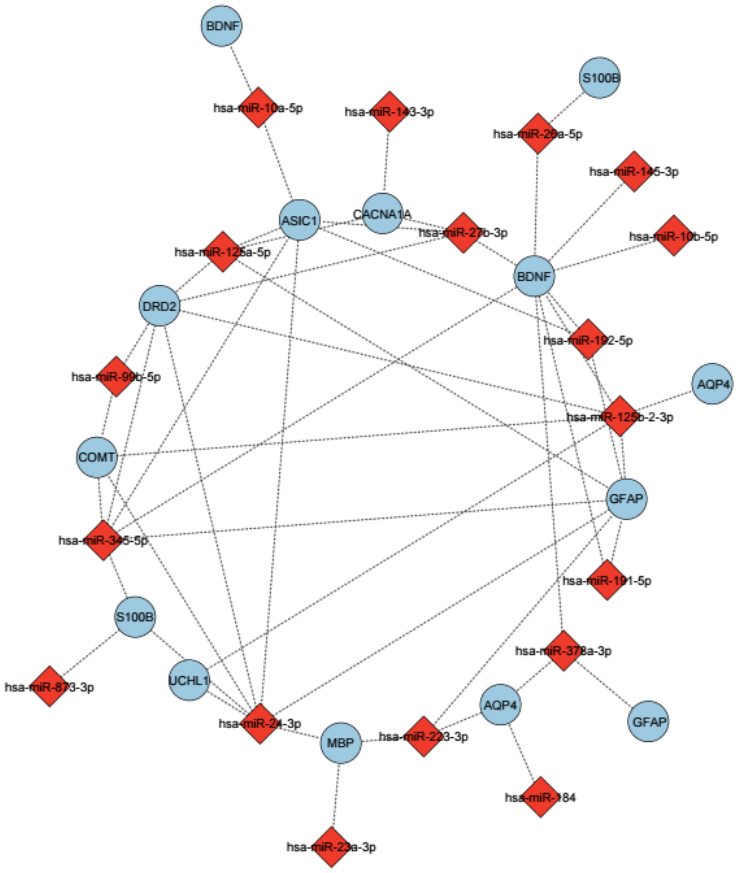


Figure 3.8. mTBI miRNAs and predicted targets. These miRNAs show differential expression based on BWA analysis. mirsystem, mirwalk databases were used to predict target genes. miRNAs are shown with red Dimond, and genes are shown with purple Circle.

Previous research has shown that the majority of mTBI patients will recover from neurological dysfunction; however, as many as 15-30% will experience long-term neurocognitive and behavioral changes [304]. Our findings suggest that mTBI candidate genes such as *APOE*, *S100B*, *GFAP*, *BDNF*, *AQP4*, *COMT*, *MBP*, *UCHL1*, *DRD2*, *ASIC1*, and *CACNA1A* may have an essential role in mTBI-related neurological disorders. As determined by the GO analysis, *GFAP* and *APOE* were the two main genes involved in neuron regrowth, *S100b* and *APOE* were involved in the regulation of neuronal synaptic plasticity, *CACNA1A*, *DRD2*,

and *UCHL1* would affect adult walking behaviour, and *ASIC1* and *DRD2* would contribute to learning.

3.6.1 APOE e4

Evidence from recent research indicates that *APOEe4* contributes to poorer recovery after brain injuries [305]. *APOE* is believed to be one of the key genes involved in neuronal cytoskeleton maintenance, neuronal synaptic transmission, neurotic dendrite, and synapse formation [306-309]. Chiang et al. identified a positive genetic link between *APOE* genotypes and brain injury outcomes and found that individuals with the *APOEe4* allele were more likely to have a poor TBI outcome [310]. Whereas other clinical trials have not found a link between the *APOEe4* and a worse result following a TBI, these variations may be related to the severity of injuries or time of sampling post-injury [267, 311].

3.6.2 GFAP

GFAP is another important protein found in the central nervous system, found within astrocytes. *GFAP* is an intermediate filament that is regulated by the *GFAP* genes and is regarded as a valuable marker for detecting brain injury. In addition to its apparent connection to mTBI-related alterations, this protein is specific to the central nervous system [312, 313] *GFAP* seems to be able to identify TBI and anticipate the necessity for neurosurgical intervention up to seven days after trauma [266]. *GFAP* elevation has been linked to astrocyte and axonal injury, and some studies have shown a significant increase in the level of *GFAP* in the serum of TBI patients [11, 314, 315]. Another study established that *GFAP* leaks from damaged cells following TBI, and the amount is related to injury severity and outcome [316]. Of significance is this group also noted that GFAP does not increase after non-brain traumas [316].

3.6.3 BDNF

As shown by hub gene analysis, another important gene associated with mTBI outcomes is *BDNF*. Based on the GO terms, *BDNF* is involved in synapse assembly, cognition, nervous system processes, memory, and the negative regulation of neuronal apoptosis. It has been considered to play a crucial role in the cellular processes that occur during TBI recoveries, such as neuronal survival, axonal sprouting, and synaptogenesis [270, 317]. *BDNF* influences the performance of existing synaptic connections as well as the development of new synaptic connections [318]. Therefore, changes in *BDNF* function, whether through synapsis or leak, are thought to influence behaviour [318, 319]. Studies show in the heterozygous condition, reduced *BDNF* actually increases body mass and aggressiveness in mice [319]. Furthermore, many studies have found that the *BDNF* polymorphism (val66met) influences cognitive function, neurodegenerative and neuroinflammatory disease following TBI, even though cognitive impairment after brain injury is multifactorial and depends on a variety of factors such as gender, age, severity, and type of brain damage [270, 320].

3.6.4 S100B

A low affinity calcium-binding protein called S100B is a hub gene, produced in glial and Schwann cells, that controls calcium homeostasis in the intracellular space [321-323]. During an astroglial injury, *S100B* is released from damaged cells into the serum or cerebrospinal fluid (CSF), causing changes in the level of calcium [323]. *S100B* levels have also been shown to increase as a result of BBB instability [324]. Previous research has shown that a high concentration of S100B during the early stages of TBI can predict a poor prognosis [265].

3.6.5 COMT

COMT is another important gene determined in this study. This gene encodes an enzyme that plays an essential part in metabolic degradation of catecholamine, dopamine, and norepinephrine called catechol-O-methyltransferase [325]. In accordance with GO, COMT is associated with learning, short-term memory, and cognitive function, and the KEGG pathway analysis revealed that COMT is one of the main genes in the dopaminergic synapse pathway. The link between COMT and cognition is well understood. However, there has not been minimal effort to understand the effect of the COMT allele on cognitive function following mTBI. Lipsky et al. found a connection between the COMT Val158Met polymorphism and a component of frontal-executive performance after TBI [187]. In this study they realized that COMT, which is likely linked with the level of endogenous dopamine, may impact the imbalances of frontal executive performance after TBI [187]. However, further research is required to determine exactly how COMT alleles affect cognitive function following a head trauma.

3.6.6 DRD2

Dopamine (DA) neurotransmission in the healthy brain controls attention, working memory, information processing speed, and cognitive performance [326, 327]. These functions are associated with frontal lobe regions that are densely projected with DA-rich striatum [328] and many of the long-term cognitive problems associated with brain damage could be explained by abnormal dopaminergic (DAergic) signaling [329]. One of the main genes involved in the DAergic pathway is DRD2 which encodes the D2 subtype of the dopamine receptor. Polymorphisms of DRD2 are quite prevalent and several studies have demonstrated that DRD2

polymorphisms affect cognitive recovery from TBI [327, 330]. One of these SNPs is rs1800497 in the ANKK1 gene which is known as a DRD2 “TAQ1 A” allele [191]. The presence of the T-allele in rs1800487 has been linked to a 40% decrease in an expression of the D2 receptor in the striatum and possibly other cortical brain regions [191, 330]. McAllister et al., 2005 found that individuals with mTBI, who were also T-allele positive, exhibited poor performance on all Continuous Performance Test measures such as the California Verbal Learning Test [330], which aims to assess important elements of cognitive psychology such proactive disruption, serial position effects, repetitive learning, and semantic structure [331].

3.6.7 CACNA1A

According to KEGG pathway analysis, CACNA1A is another gene related to the dopaminergic synapse pathway. CACNA1A encodes the α_{1A} subunit of the neuronal calcium channel [332], and polymorphism of this gene can influence the downstream effects of calcium influx into neurons during brain injury [332, 333]. In a small case study, Kors et al., found that a novel C/T substitution mutation at codon 218 in CACNA1A caused a serine to leucine switch, which resulted in delayed cerebral edema following mTBI and familial hemiplegic migraine (FHM) [332]. Another study by this group reported early seizures in two patients with mTBI who had the same mutation on the CACNA1A gene (serine/leucine) [334]. However, to understand the molecular mechanisms of the CACNA1A polymorphism, as well as its impact on mTBI outcomes and treatment, more research is needed.

3.6.8 AQP4

Aquaprin-4 (AQP4) is the most important water channel in the central nervous system, carrying the majority of water into brain cells while also controlling brain water balance [335].

AQP4 is mainly expressed in the astrocytic end foot processes near intracerebral vessels and at the ventricular interface [336]. Previous studies have shown that the presence of brain edema after TBI is one of the most important predictors of brain injury outcome [335]. Recent studies have shown that AQP4 expression is changed significantly in both clinical and trial brain injury, suggesting that genetic variations to these channels may affect the degree of edema [337-340]. In an experimental TBI study, AQP4 expression increased in the glia limitans (i.e., the outermost later of nervous tissue, just under the pia mater), but perivascular AQP4 expression decreased during the early phase when vasogenic edema was present [341]. Dardiotis et al., found that specific variations of the AQP4 gene were associated with the six-month clinical outcome following TBI [335]. The AQP4 is involved in brain edema formation following TBI. Consequently, for clinical outcome following mTBI, understanding the role of AQP4 as well as identifying potential aquaporin modulators would be promising for predicting outcome of brain injury.

3.6.9 UCHL1

One of the most well-studied protein biomarkers associated with brain injury is UCHL1. UCHL1 or neuronal-specific protein gene product 9.5 (PGP9.5) is a highly abundant protein in the brain. Approximately 5–10% of total neuronal protein is made up of this protein [342, 343]. In the brain, UCHL1 is primarily located in neuronal cells, which makes it a promising biomarker of neuronal injury [344]. UCHL1 is a multifunctional protein that is involved in cell survival, maintenance of axonal integrity, and ubiquitin-proteasome pathway (UPP) control in neurons [345- 347]. The UCHL1 protein is involved in adding or removing ubiquitin from proteins that are about to be degraded by the ATP-dependent proteasome pathway [343, 348, 349]. Because this system is responsible for removing malformed or dysfunctional proteins, UPP

failure could be a factor in aggravating axonal injury after a TBI [343]. UCHL1 is a stable protein with a low-molecular-weight that is secreted from damaged neurons, it enters the CSF, and can then be identified in the systemic circulation [350]. Researchers that examined serum biomarkers >32 hours after an injury discovered that UCHL1 was elevated in the serum of adults and children with mild to severe TBI and was directly associated to damage severity [351]. Papa et al., reported a significant increase in the level of UCHL1 following severe brain injury, and that it was detectable in body fluids such as CSF fairly early after injury (at 24, 48, and 72-hours following injury), suggesting that it might be used as a biomarker to help determine the severity of TBI [352].

3.6.10 MBP

The myelin basic protein (MBP) gene is another potential biomarker related to brain injury. This gene codes for protein that is a significant component of the myelin sheath of oligodendrocytes of the central nervous system and Schwann cells of the peripheral nervous system [353]. MBP can be released into the CSF and serum after the shearing of brain white matter that causes diffuse axonal injuries, and it has been reported to remain increased for up to 2 weeks after the injury [354, 355]. MBP, through an inflammatory response, can cause the BBB to open, allowing MBP to get into the bloodstream more easily. Berger et al., found that higher levels of MBP were linked to worse outcomes for children with brain damage [356]. Despite the fact that serum concentrations of MBP have not been evaluated in individuals who have suffered a mTBI, the potential of this protein to open the BBB [207, 357] suggested that it might be measurable following mTBI.

3.6.11 ASIC1

Following a brain injury, the pH of brain tissue decreases, and the reduction is larger in patients who have suffered a more severe injury [358, 359]. Decreased pH may increase the severity of TBI by activating acid-sensing ion channels (ASICs) [359]. ASICs are part of the degenerin/epithelial sodium channel (DEG/ENaC) superfamily which are abundantly expressed in the central nervous system [360]. Because ASICs are specifically gated by the proton, changes in the proton and lower extracellular pH during brain injury have an impact on ASICs channel activation [361, 362]. Recent studies indicate that ASIC1 has been linked to synaptic plasticity, learning, memory, and fear response [362].

3.6.12 Involved miRNA

In addition to the 11 candidate genes, our study determined 8 miRNAs to be associated with the mTBI candidate genes and neurological function. Based on computational analyses, these miRNAs might be involved in affecting mTBI pathophysiology. Previous studies indicated that some of these miRNAs were associated with brain disorders and suggested that they could be used as surrogate biomarkers [363]. Based on prediction made through a bioinformatic study of miRNA with APOE as a regulatory target, miR-1908-5p was found correlated with genes involved in bipolar disorder [363]. The location of miR-1908-5p is in the first intron of the fatty acid desaturase 1 (FADS1) gene on chromosome 11[363]. Results from pathway analysis suggested that miR-1908-5p contribute to nervous system development and neuron projection. In the GENFI cohort, miR-204-5p was shown to be significantly lower in symptomatic frontotemporal dementia (FTD) compared to pre-symptomatic mutation carriers [364]. Guedes et al., found that miR-204-5p was upregulated in individuals with post-traumatic stress disorder

(PTSD) compared to healthy controls [365]. Moreover, Weisz et al., found substantial downregulation of miR-204-5p in chronic TBI patients [366]. Surprisingly, miR-204-5p was identified to be abundant in brain, based on tissue expression patterns, and it also showed a dramatic increase 1 hour following concussion [367]. These findings suggest that this miRNA could be used as a biomarker for neuropathological disorders. MiR-9 is another interesting miRNA that has attracted the attention [368]. It has a unique expression pattern in the brain and implements activities involved in central nervous system development [368, 369]. Brain development studies indicated that MiR-9 was related to gene networks that control the proliferation of neural progenitors [369]. Therefore, the presence of this miRNA in neurological diseases is unsurprising. For instance, miR-9 was found to be downregulated in Alzheimer's disease patients [370]. Another significant miRNA in this study was miR-16-5p, which has previously been shown to be a viable potential biomarker for TBI, with the ability to distinguish between mild and severe TBI [371]. Based on GO analysis miR-16 was involved in a variety of regulatory processes that are triggered by brain injuries, including positive apoptotic regulation via BCL-2 targeting [372]. Sun et al., observed downregulation of miR-16-5p plays an important role in the faster recovery process in TBI patients via the stimulation of osteoblast proliferation and the prevention of apoptosis [373]. Furthermore, researchers revealed that within the first 24 hours after mTBI, the level of miR-16-5p was significantly higher in mTBI patients compared to severe TBI patients [374]. Furthermore, RNA-seq analysis showed that miR-10a-5p, miR-10b-5p, and miR-143-3p levels in serum rose immediately after mTBI. These results indicate that miR-10a-5p and miR-10b-5p are prominent targets for BDNF. Our analysis also shows a direct correlation between miR-10b-5p and miR-143-3p with the number of hits to the head. Therefore, these miRNAs may be a useful biomarker to determine the severity of brain injury.

The enrichment analysis has shown that miR-10a-5p and miR-10b-5p play a significant role in neuronal processes when it comes to regulating cell morphogenesis and the generation of neurons, while miR-143-3p plays an active role in cell projection and cell-cell signaling. To our knowledge, there has not been a significant amount of research on miR-10a-5p and brain injury to recommend it as a potential biomarker. Previous studies have established that miR-143-3p is linked to ischemic stroke [375], and that miR-10b-5p is considerably differently expressed in Huntington's disease [376]. Also, bioinformatic analyses revealed that miR-34a-5p miR-218-5p, and miR-199b-5p were targeted for their relationships to mTBI candidate genes, and these miRNAs are involved in pathways such as cell-cell signaling, cell death, regulation of metabolic process, cell differentiation, nervous system development. However, more research is needed to confirm the link between these miRNAs and post-mTBI symptoms.

3.7 Conclusions

In conclusion, our study identified 11 genes and 8 miRNAs that may be associated with mTBI outcomes. They are mainly involved in neurite regeneration, nervous system signaling, neurite outgrowth, and cell differentiation. The results of this study provide promising evidence towards predicting mTBI prognosis and personalized treatments but will need further research to explore their exact expression levels and influence post-mTBI.

CHAPTER 4: FUTURE PESPECTIVES & LIMITATIONS

4.1 Future Work

As a result of this study, some future work is required to achieve the process expectations set forth at the beginning. First, genes and miRNAs predicted in this research need to be confirmed with an assessment of mTBI patients. In such a study, if genetic markers can be validated for mTBI assessments, patients could be directed to specific neurological or physical treatments that could more effectively resolve symptoms and complications. A sufficient sample size is needed to ensure the validity of the biomarkers study. Additionally, collecting different types of samples (blood, saliva, and urine) would make it possible to measure the levels of circulating biomarkers in biofluids and identify the least invasive method of mTBI diagnosis and grading. It is imperative to consider various time points (before and after injury) for sampling and designing a follow-up study to better understand how genetic biomarkers influence recovery and outcome in patients.

In addition to genetic alterations, because genes directly affect metabolites, metabolomic analysis could also allow identification of post-mTBI changes. Post-mTBI metabolite profiling could be performed using high throughput methods such multi-segment injection-capillary electrophoresis-mass spectrometry.

A third focus for future work would be to relate omics research to quantitative MRI results. Using big data and machine learning MRI has recently been shown to be of use in the personalized assessment of mTBI brain changes (both functionally and structurally). Therefore, merging MRI technology with omics techniques and comparing the results could help to gain a

better understanding of how brain molecular mechanisms are altered after mTBI. By recognizing these changes, accurate diagnosis techniques can be developed for patients who suffer from mTBIs.

4.2 Limitations

The major drawback in this study was the lack of data from mTBI patients, which reduced the prognostic value of the biomarkers. Even though we had research ethics board approval for our study Covid prevented all patient recruitment for the last 2 years. Furthermore, during Covid people were unable to participate in activities that classically lead to mTBI injuries. Consequently, the project switched to an *in silico* analysis, using available data to predict biomarkers. However, another restriction was related to the insufficient amount of genetic research and genomics data on mTBI, which required us to check numerous databases. It was difficult to validate this data, when experimental results were not available; the most effective way to validate bioinformatics data is to download genomic profiles from GEO repository related to diseases and reanalyze them to compare with predicted data, but we were unable to find accurate RNA-seq or microarray datasets to match our data due to sparse availability of our required data. Only one mTBI dataset on small miRNAs was available, and we used that to verify forecasted miRNAs. Working with biomarkers has additional limitations, including the necessity for precise sample sizes to confirm biomarkers which we were unable to do because of Covid.

Appendix A

Appendix A: Code

A.1 Linux Code

A.1.1 BWA Commend

"For all the algorithms, BWA first needs to construct the FM-index for the reference genome"

Index:

- ***bwa index [-p prefix] [-a algoType] <in.db.fasta>***

bwa index '/media/Mahnaz/Samsung_T51/hg38.fa'

aln:

- ***bwa aln ref.fa short_read.fq > aln_sa.sai***
- ***bwa aln ref.fa *.fastq > *.sai***

bwa aln /media/mahnaz/Samsung_T51/hg38_fa/hg38.fa '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405_trimmed.fastq' > '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405.sai'

*Loop: for i in *_trimmed.fastq; do bwa aln /media/mahnaz/Samsung_T51/hg38_fa/hg38.fa $i > ${i%_trimmed.fastq}.sai; done*

- ***bwa samse ref.fa aln_sa.sai short_read.fq > aln-se.sam***
- ***bwa same index file file.sai*. fa > .sam***

bwa samse /media/mahnaz/Samsung_T51/hg38_fa/hg38.fa '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405.sai' '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405_trimmed.fastq' > '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405_trimmed.sam'

Note: To create a sam file (Sequence Alignment/Map), this step was done for each of the 131 samples.

- ***samtools for making bam file***

samtools view -b '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405_trimmed.sam' > '/media/mahnaz/Samsung_T51/MTBI. New. analysis/SRR8280405_trimmed.bam'

Note: To create a bam file, this step was done for each of the 131 samples.

- ***Samtools for making sorted file***

samtools sort '/media/mahnaz/Samsung_T51/MTBI. New. analysis/bwa file/SRR8280405_trimmed.bam' -o '/media/mahnaz/Samsung_T51/MTBI. New. analysis/bwa file/SRR8280405.sorted'

Note: To create a sorted file, this step was done for each of the 131 samples.

- ***featureCounts for making count matrix***

featureCounts -t miRNA -F GTF -g ID -a '/media/mahnaz/Samsung_T51/hsa.gff3' -o '/media/mahnaz/Samsung_T51/MTBI. New. analysis/bwa file/FeatureCountsTable.txt' '/media/mahnaz/Samsung_T51/MTBI. New. analysis/bwa file/SRR8280405.sorted' '/media/mahnaz/Samsung_T51/MTBI. New. analysis/bwa file/SRR8280408.sorted'….

Note: To create a count matrix, combine all 131 samples with this code and run it.

A.2 R Code:

Run DESeq2 analysis for different miRNA expression levels at different times (conditions)

- *setwd("D:\bwa_sorted_file_featureCounts")*

library(DESeq2)

- *countdata = read.table("New folder/FeatureCountsTable.txt" , header = T)*
- *rownames(countdata) = countdata$Geneid*
- *countdata = countdata[, -(1:6)]*
- *colnames(countdata) = gsub("X.media.mahnaz.Samsung_T51.bwa_sorted_file_featureCounts.", "", colnames(countdata))*
- *colnames(countdata) = gsub(".sorted", "", colnames(countdata))*
- *sample_key<-read.csv("New folder/Pheno2.new.csv")*
- *sample_key$condition = as.factor(sample_key$condition)*
- *View(sample_key)*

- *dds<-DESeqDataSetFromMatrix(countData = countdata, colData = sample_key, -design= ~condition)*
- *dds*
- *dds<-DESeq(dds)*
- *cnt = counts(dds, normalized = T)*
- *write.table(cnt,file = "cnt-miRNA.txt", row.names=T, col.names = T, sep="\t", quote = F)*

####codition_2_vs_1

- *dif = data.frame(results(dds, c("condition", "2", "1")))*
- *dif$padj = p.adjust(dif$pvalue, method = "BH")*
- *dif = dif[order(dif$padj),]*
- *write.table(dif,file = "New folder/codition_2_vs_1.txt", row.names=T, sep="\t", quote = F)*
- *dif$geneID = rownames(dif)*
- *genes.sig <- subset(dif, padj < 0.05)*
- *geneID <- unique(genes.sig$geneID)*
- *write.table(geneID,file = "New folder/ genes.sig-name (condition_2_vs_1).txt" , quote = F, row.names = F, col.names = F)*

####codition_3_vs_1

- *dif2 = data.frame(results(dds, c("condition", "3", "1")))*
- *dif2$padj = p.adjust(dif2$pvalue, method = "BH")*
- *dif2 = dif2[order(dif2$padj),]*

- *write.table(dif,file = "New folder/codition_3_vs_1.txt", row.names=T, sep="\t", quote = F)*
- *dif2$geneID = rownames(dif2)*
- *genes2.sig <- subset(dif2, padj < 0.05)*
- *geneID2 <- unique(genes2.sig$geneID)*
- *write.table(geneID2,file = "New folder/genes.sig-name (condition_3_vs_1).txt" , quote = F, row.names = F, col.names = F)*

#####**codition_4_vs_1**

- *dif3 = data.frame(results(dds, c("condition", "4", "1")))*
- *dif3$padj = p.adjust(dif3$pvalue, method = "BH")*
- *dif3 = dif3[order(dif3$padj),]*
- *write.table(dif3,file = "New folder/codition_4_vs_1.txt", row.names=T, sep="\t", quote = F)*
- *dif3$geneID = rownames(dif3)*
- *genes3.sig <- subset(dif3, padj < 0.05)*
- *geneID3 <- unique(genes3.sig$geneID)*
- *write.table(geneID3,file = "New folder/genes.sig-name (condition_4_vs_1).txt" , quote = F, row.names = F, col.names = F)*

Run DESeq2 analysis for different miRNA expression levels based on number of hits to the head (conditions)

- *setwd("/media/mahnaz/Samsung_T51/plotting bwa/BWA new_Anis")*
- *countdata = read.table("FeatureCountsTable.txt" , header = T)*

- *rownames(countdata) = countdata$Geneid*
- *countdata = countdata[, -(1:6)]*
- *colnames(countdata) = gsub("X.media.mahnaz.Samsung_T51.bwa_sorted_file_featureCounts.", "", colnames(countdata))*
- *colnames(countdata) = gsub(".sorted", "", colnames(countdata))*
- *sample_key<-read.csv("Pheno2.csv")*
- *sample_key$condition = as.factor(sample_key$condition)*
- *View(sample_key)*
- ***library("DESeq2")***
- *dds<-DESeqDataSetFromMatrix(countData = countdata, colData = sample_key, design= ~condition)*
- *dds*
- *dds<-DESeq(dds)*
- *cnt = counts(dds, normalized = T)*
- *write.table(cnt,file = "cnt2-miRNA.txt", row.names=T, col.names = T, sep="\t", quote = F)*

####codition_1_vs_0

- *dif = data.frame(results(dds, c("condition", "1", "0")))*
- *dif$padj = p.adjust(dif$pvalue, method = "BH")*
- *dif = dif[order(dif$padj),]*
- *write.table(dif,file = "codition_1_vs_0.txt", row.names=T, sep="\t", quote = F)*

- *dif$geneID = rownames(dif)*

- *genes.sig <- subset(dif, padj < 0.05)*

- *geneID <- unique(genes.sig$geneID)*

- *write.table(geneID,file = "genes.sig-name (condition_1_vs_0).txt" , quote = F, row.names = F, col.names = F)*

####codition_0_vs_2

- *dif2 = data.frame(results(dds, c("condition", "2", "0")))*
- *dif2$padj = p.adjust(dif2$pvalue, method = "BH")*
- *dif2 = dif2[order(dif2$padj),]*
- *write.table(dif,file = "codition_2_vs_0.txt", row.names=T, sep="\t", quote = F)*
- *dif2$geneID = rownames(dif2)*
- *genes2.sig <- subset(dif2, padj < 0.05)*
- *geneID2 <- unique(genes2.sig$geneID)*
- *write.table(geneID2,file = "genes.sig-name (condition_2_vs_0).txt" , quote = F, row.names = F, col.names = F)*

####condition_3_vs_0

- *dif2 = data.frame(results(dds, c("condition", "3", "0")))*
- *dif2$padj = p.adjust(dif2$pvalue, method = "BH")*
- *dif2 = dif2[order(dif2$padj),]*
- *write.table(dif,file = "codition_3_vs_0.txt", row.names=T, sep="\t", quote = F)*
- *dif2$geneID = rownames(dif2)*

- *genes2.sig <- subset(dif2, padj < 0.05)*
- *geneID2 <- unique(genes2.sig$geneID)*
- *write.table(geneID2,file = "genes.sig-name (condition_3_vs_0).txt" , quote = F, row.names = F, col.names = F)*

####condition_4_vs_0

- *dif2 = data.frame(results(dds, c("condition", "4", "0")))*
- *dif2$padj = p.adjust(dif2$pvalue, method = "BH")*
- *dif2 = dif2[order(dif2$padj),]*
- *write.table(dif,file = "codition_4_vs_0.txt", row.names=T, sep="\t", quote = F)*
- *dif2$geneID = rownames(dif2)*
- *genes2.sig <- subset(dif2, padj < 0.05)*
- *geneID2 <- unique(genes2.sig$geneID)*
- *write.table(geneID2,file = "genes.sig-name (condition_4_vs_0).txt" , quote = F, row.names = F, col.names = F)*

###condition_6_vs_7

- *dif2 = data.frame(results(dds, c("condition", "6", "7")))*
- *dif2$padj = p.adjust(dif2$pvalue, method = "BH")*
- *dif2 = dif2[order(dif2$padj),]*
- *write.table(dif,file = "codition_6_vs_7.txt", row.names=T, sep="\t", quote = F)*
- *dif2$geneID = rownames(dif2)*
- *genes2.sig <- subset(dif2, padj < 0.05)*
- *geneID2 <- unique(genes2.sig$geneID)*

- *write.table(geneID2,file = "genes.sig-name (condition_6_vs_7).txt" , quote = F, row.names = F, col.names = F)*

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