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(71) Applicant(s)
The University of Birmingham

(72) Inventor(s)
Belli, Antonio;Di Pietro, Valentina

(74) Agent / Attorney
FB Rice Pty Ltd, L 23 44 Market St, Sydney, NSW, 2000, AU

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(71) Applicant: **THE UNIVERSITY OF BIRMINGHAM** [GB/GB]; Edgbaston, Birmingham B15 2TT (GB).

(72) Inventors: **BELLI, Antonio**; Brackendene, Whinwhistle Road, East Wellow Hampshire SO51 6BN (GB). **DI PIETRO, Valentina**; 78 Christopher Road, West Midlands, West Midlands B29 6QJ (GB).

(74) Agent: **BAILEY, Jennifer**; Marks & Clerk LLP, Alpha Tower, Suffolk Street Queensway, Birmingham B1 1TT (GB).

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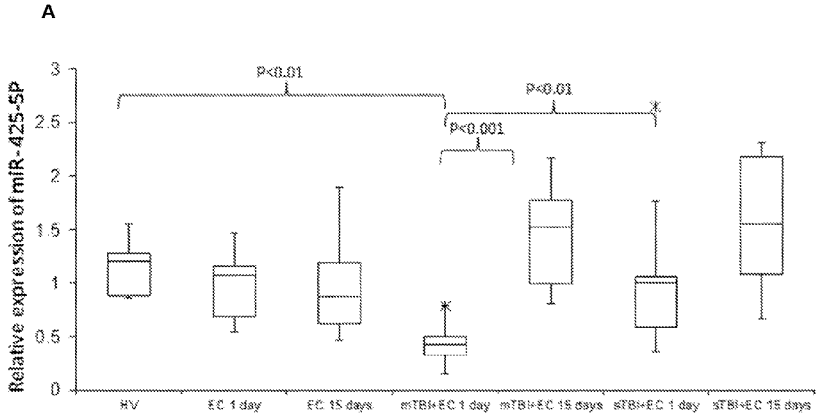
(54) Title: BIOMARKERS OF TRAUMATIC BRAIN INJURY

Figure 1



(57) Abstract: Provided is a method of diagnosing and/or monitoring traumatic brain injury (TBI) in a subject. The method comprises determining a level of at least one miRNA in a fluid sample from the subject. The miRNA may be selected from miR-425-5p, miR-502, miR-21 and miR-335. The method may involve determining whether a subject is suffering from mild-TBI or moderate-to-severe TBI. Also provided is a sensor element, a detection system, composition and a kit for diagnosing and/or monitoring TBI, and a method of determining an appropriate treatment for a subject with a suspected TBI.

Biomarkers of traumatic brain injury

Field of the invention

The present invention relates to compositions, kits, systems and methods for diagnosing and/or monitoring traumatic brain injury (TBI). More particularly, the present invention relates to the diagnosis and monitoring of TBI using miRNA biomarkers.

Background to the invention

Traumatic brain injury (TBI) is the leading cause of death and disability under the age of 45 years in Western countries. Its healthcare burden and social costs are expected to continue to rise and, by 2020, the World Health Organization projects TBI to become the third leading cause of disability worldwide.

Despite many studies, no reliable biomarkers have been found to assess the severity of TBI and predict recovery. This is especially true for mild TBI (mTBI), which remains currently difficult to assess in clinical practice. Although TBI patients are initially assessed by the Glasgow Coma Score (GCS) and neuroimaging techniques, which require costly equipment, the current diagnostic tools are lacking in the ability to precisely define and quantify the actual severity of the brain injury, thus leading to an easy detection of severe but not of mTBI, which represents the vast majority of cases (75-90%).

The correct diagnosis of mTBI is particularly important in patients, such as athletes, soldiers and children, who are at greater risk of repetitive mTBI and a catastrophic form of brain injury known as second impact syndrome (SIS) where the synergistic effects of repeated TBI result in profound damage and even death. Early diagnosis and evaluation of the severity of TBI thus becomes crucial for patients' wellbeing and ultimately saving their life.

The quest for TBI biomarkers has received significant impetus by the increased profile of sport concussion in the media. In the last few years many studies have focused on biomarkers that can support clinical decision making pitch-side or in a sports clinic. However, protein biomarkers reported in the literature lack specificity or sensitivity, or are not detectable for some time after injury. This may be due to the fact that following concussion, which is a form of TBI, brain-derived compounds are only released in very small amounts and the blood-brain barrier remains mostly closed.

MicroRNAs (miRNAs) are an abundant class of highly conserved, non-coding RNA molecules of approximately 22 nucleotides in length that induce mRNA degradation, translational repression or both *via* pairing with partially complementary sites in the 3`UTR of target genes. The human genome encodes over 2,000 miRNAs, which may target about 60% of all genes. However, despite the abundance of miRNAs, their biomolecular functions and involvement in pathology remain to be fully elucidated. They play a central role in many biological processes including cell cycle, cell metabolism, apoptosis and immune responses, and are attracting increasing interest in clinical research as potential biomarkers for the detection, identification and classification of cancers and other disease states including neurodegenerative diseases.

The present invention was devised with these issues in mind.

Summary of the invention

Disclosed herein is a method of diagnosing or monitoring traumatic brain injury (TBI) in a subject.

According to a first aspect of the present disclosure, there is provided a method for diagnosing and/or monitoring traumatic brain injury (TBI) in a subject, the method comprising detecting the presence of and/or determining a level of at least one miRNA in a sample from the subject.

The at least one miRNA (also referred to herein as “miR”) may be selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g, miR-335, miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, miR-671-3p, hsa-let-7c-5p, hsa-let-7i-5p miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, miR-424-5p, miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p; miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629. These miRNAs may be referred to herein as miRNAs of interest or target miRNAs.

In some embodiments, the at least one miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g, miR-335, hsa-miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-

624, miR-625*, and miR-671-3p. These microRNAs have been found to be biomarkers expressed in all TBI patients (mild or severe).

In some embodiments, the at least one miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g and miR-335.

In some embodiments, the at least one miRNA is selected from the group consisting of let-7c-5p, let-7i-5p, miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, and miR-424-5p; miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p.

In some embodiments, the at least one miRNA is selected from the group consisting of miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-194, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629.

For the avoidance of doubt, it will be understood that "the at least one miRNA is selected from a group of miRNAs", as used herein, means that the method in question, whether carried out for a diagnostic, prognostic, or therapeutic purpose, can be carried out with any one of the listed miRNAs or with any plurality of the listed miRNAs (e.g., two, three, four, or more of the listed miRNAs). It follows that any one or more of the listed miRNAs may be explicitly excluded. For example, where the at least one miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g and miR-335, the method may include detecting and/or assessing the level of any combination of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, and miR-let-7g to the exclusion of miR-335.

According to an aspect of the present disclosure, there is provided a method of diagnosing and/or monitoring traumatic brain injury (TBI) in a subject, the method comprising determining a level of at least one miRNA in a sample from the subject, wherein the miRNA is selected from the group consisting of: miR-425-5p; miR-502; miR-21; and miR-335.

Traumatic brain injury occurs when an external force traumatically injures the brain. There are different systems for classifying TBI based on, for example, severity, type of injury and prognosis. The most commonly used system for classifying TBI is the Glasgow Coma Scale (GCS), which grades a person's level of consciousness on a scale of 3–15 based on verbal, motor, and eye-opening reactions to stimuli. In general, a TBI with a GCS score of 13 or above is defined as mild, 9–12 as moderate and 8 or below as severe. Another system, the Mayo Classification System, has three main classifications including definite moderate-severe TBI, probable mild TBI, and possible TBI. Multiple criteria are used in each diagnosis including loss of consciousness, post-traumatic amnesia, skull fracture, and evidence of neuroradiological abnormalities including subdural haematoma, cerebral contusion, and hemorrhagic contusion. The classification of TBI using the GCS or Mayo systems will be known to those skilled in the art.

As used herein, references to “mild”, “moderate” and “severe” TBI are made in accordance with the GCS. References herein to “moderate-to-severe” TBI encompass both moderate and severe TBI in accordance with the GCS.

The diagnosis and/or monitoring of TBI using the biomarkers of the present disclosure is expected to support clinical decision making and treatment regimens in a variety of contexts, including the following situations: as part of the initial assessment by paramedics to determine whether patients should be transported to a facility with neurosurgical expertise, a major trauma centre or a local trauma unit; in the emergency department of hospitals to determine appropriate treatment, including the need for a CT brain scan; pitch-side, to assist decision making as to the removal of a player from play and assessment of the need to take a player to hospital; in sports clinics, to confirm a concussive event and enable decision making with regard to returning to play; in combat situations, to determine the need to dispatch a rescue team and evacuate a victim. Thus, subjects for whom the present disclosure may provide particular benefit include accident victims, sports players and military personnel.

In any case, but perhaps particularly where the subject is at greater risk for a TBI (e.g., where the subject is a professional athlete or enlisted in the military), a sample may be obtained from

the subject at a time prior to any known or recent trauma (e.g., near the beginning of a sporting career or prior to a military deployment) and any miRNAs of interest can be assessed at that time or later, when the subject has experienced a possible TBI. Such samples may thereby provide an internal reference standard.

In some embodiments, the subject is human.

The TBI may be mild TBI (mTBI), moderate TBI or severe TBI (sTBI). In some embodiments, the TBI is moderate-to-severe TBI (m-sTBI).

The level of the miRNA or of each miRNA in the sample may be determined quantitatively or semi-quantitatively. By "quantitatively", it will be understood that the absolute amount or concentration of the miRNA or of each miRNA in the sample is determined. The absolute amount of the miRNA or of each miRNA in the sample can then be compared to a predetermined threshold (e.g. a published literature value for expected normal levels), a known level of the same or a reference miRNA in a control sample taken from a healthy subject, or the amount of a reference miRNA in the sample taken from the subject. In some embodiments, the subject is diagnosed as having a TBI when the level of the miRNA is below the predetermined threshold, or decreased relative to a reference or control sample. In other embodiments, the subject is diagnosed as having a TBI when the level of the miRNA is increased compared to the predetermined threshold.

By "semi-quantitatively", it will be understood that the level of the or each miRNA of interest is measured relative to a reference.

The reference may be an invariant miRNA, i.e. a miRNA having an expression level that remains substantially unchanged between healthy subjects and those having a TBI. A subject may be diagnosed as suffering from a TBI if the level of the miRNA or of each miRNA of interest is increased or decreased relative to that of an invariant miRNA. Suitable invariant miRNAs include miR-331, miR-223*, miR-23a-3p and miR148b-3p. miR-23a-3p and miR148b-3p are invariant in saliva only.

In some embodiments, the level of the miRNA or of each miRNA in the sample obtained from the subject may be about 0.01 times to about 100 times, about 0.05 times to about 50 times,

about 0.1 times to about 10 times, about 0.5 times to about 5 times, about 1.0 to about 3 times, or about 1.5 times to about 2.0 times lower or higher than the level in the control sample, the reference level or the published value.

Where a device or method is employed to generate a value, we may qualify that value with the term "about" in order to capture the stated value and any variation of that value inherent to the device or method employed. Where values or ranges of values are specifically disclosed, "about" may mean plus-or-minus 10% of the stated value or range. For example, about 10 minutes may mean 9-11 minutes.

The level of the miRNA or of each miRNA of interest can be determined using methods known to those skilled in the art. In some embodiments, determining the level of the miRNA or of each miRNA of interest comprises amplifying the miRNA. In some embodiments, total miRNA may be first isolated from the sample using standard techniques, for example using the miRNeasy mini kit (Qiagen). The amount of the miRNA of interest can then be determined. In some embodiments, the level of the miRNA or of each miRNA of interest in the sample is determined using PCR (polymerase chain reaction). For example, quantitative PCR may be used for quantitative determination of the level of the miRNA or of each miRNA of interest. PCR may also be used for semi-quantitative determination, by comparing the level of the miRNA or of each miRNA of interest in the sample with that of a reference (e.g. an invariant miRNA).

Suitable techniques for miRNA detection and/or quantification, which will be known to those skilled in the art, include qPCR, miRNA assays, next-generation sequencing (NGS), and multiplex miRNA profiling assays.

In some embodiments, the level of the miRNA or of each miRNA of interest is determined using *in-situ* hybridization, for example using a probe (e.g., a labelled probe) specific for the miRNA.

The level of miRNA may be determined in a sample which was obtained from the subject immediately after injury (i.e. less than 1 hour after injury), and/or in a sample obtained at one or more time points a few hours or days after injury. Thus, changes in the miRNA level can be detected over time to enable monitoring of a TBI. In the event miRNA levels change over time, the methods described herein for monitoring TBI can be expanded to include maintaining or adjusting the subject's treatment regimen accordingly.

Depending on the specific miRNA and the type of TBI, the level of miRNA in the subject may change significantly over time. In some embodiments, it may therefore be advantageous to measure the miRNA relatively soon after injury to enable an accurate diagnosis. In some embodiments, the level of miRNA is determined in a sample obtained from the subject no more than 72 hours, no more than 48 hours, no more than 36 hours, no more than 24 hours, no more than 12 hours, no more than 6 hours, no more than 4 hours, no more than 2 hours or no more than 1 hour after injury.

The level of some miRNAs is substantially stable over time, thus allowing a diagnosis to be made a few hours, days or even weeks after injury. In some embodiments, the level of miRNA is determined in a sample obtained from the subject up to 20, 18, 15, 12, 10, 8, 5 or 2 days from injury.

In some embodiments, the level of miRNA is determined in a sample obtained from the subject immediately after injury (e.g. at T= 0h), at 4-12 hours after injury, at 48-72 hours after injury, or at 15 days after injury.

In some embodiments, the TBI is mild TBI (mTBI) or moderate-to-severe TBI (m-sTBI) and the at least one miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g, miR-335, hsa-miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, and miR-671-3p.

In some embodiments, the TBI is mild TBI (mTBI) and the miRNA is selected from the group consisting of miR-425-5p and miR-502. The subject may be diagnosed as having mTBI if the level of miR-425-5p and/or miR-502 is determined to be below a predetermined threshold, or is decreased relative to a reference.

In some embodiments, a level of miR-425-5p and/or miR-502 which is below a predetermined threshold, or decreased relative to a reference, is diagnostic of mTBI when the level is determined in a sample obtained less than 48 hours after injury.

In some embodiments, the TBI is moderate-to-severe TBI (m-sTBI) and the miRNA is selected from the group consisting of miR-21 and miR-335. The subject may be diagnosed as having moderate-to-severe TBI if the level of miR-21 and/or miR-335 is determined to be above a predetermined threshold, or increased relative to a reference.

In some embodiments, a level of miR-21 and/or miR-335 which is above a predetermined threshold, or increased relative to a reference, is diagnostic of moderate-to-severe TBI when the level is determined in a sample obtained up to 15 days after injury.

In some embodiments, the TBI is moderate-to-severe TBI (m-sTBI) and the at least one miRNA is selected from the group consisting of miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-194, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629.

The subject may be diagnosed as having m-sTBI if the level of miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-618, miR-95, miR-130a, miR-152, miR-194, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642 and/or miR-99a is determined to be above a predetermined threshold, or is increased relative to a reference.

The subject may be diagnosed as having m-sTBI if the level of miR-192, miR-429, miR-520D-3p and/or miR-629 is determined to be below a predetermined threshold, or is decreased relative to a reference.

The miRNAs may be used individually to diagnose TBI. For example, in sport concussion, miR-502 or miR-425-5p could be used to confirm that a traumatic brain injury has occurred.

Thus, a further aspect of the present disclosure provides a method of determining the severity of TBI, and the steps of this method can be repeated to monitor the subject over time. It will be appreciated that a positive result for a single miRNA (e.g. the level of a single miRNA is determined to be above/below a predetermined threshold, or is increased/decreased relative to a reference) is sufficient to determine the severity of TBI. For example, if the level of miR-425-5p is below a predetermined threshold, or decreased relative to a reference, the severity of the

TBI is determined to be mild (mTBI). However, it may be convenient to combine different miRNAs (e.g. in a test panel) to facilitate the assessment of TBI severity.

In some embodiments, the method comprises determining a level of a plurality (e.g., two or more) miRNAs in the sample. In some embodiments the two or more miRNAs are selected from the group consisting of: miR-425-5p; miR-502; miR-21; and miR-335.

In some embodiments, the method comprises determining the level of:
(i) a first miRNA selected from miR-425-5p and miR-502; and
(ii) a second miRNA selected from miR-21 and miR-335.

A subject may be diagnosed as having a TBI if the level of miR-425-5p or miR-502 is determined to be below a predetermined threshold, or is decreased relative to a reference, or the level of miR-21 or miR-335 is determined to be above a predetermined threshold, or increased relative to a reference.

In some embodiments, the TBI is mild TBI (mTBI) and the at least one miRNA is selected from the group consisting of let-7c-5p, let-7i-5p, miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miRmmiR-29a-3p, miR-29c-3p, miR-424-5p, miR-30a-5p, miR-107, miR-135b-5p, miR-199b-5p, miR-324-5p, and miR-652-3p, or a combination thereof. The subject may be diagnosed as having mTBI if there is a fold change of at least 0.5, at least 1.0, at least 1.5, at least 2.0, at least 2.5, at least 3.0, at least 3.5 or at least 4.0 in level of the microRNA compared to a reference. In some embodiments, the subject is diagnosed as having mTBI if the level(s) of the microRNA(s) is/are increased compared to a reference.

The sequences and accession numbers for miRNAs described herein are provided in Table 1:

Table 1

miRNA	sequence	miRBase Accession no.
hsa-miR-21	uagcuuaucagacugauguuga (SEQ ID No. 1)	MIMAT0000076
hsa-miR-425-5p	aaugacacgaucacucccguuga (SEQ ID No. 2)	MIMAT0003393
hsa-miR-502-5p (also known as hsa-miR-502)	auccuugcuaucugggugcua (SEQ ID No. 3)	MIMAT0002873
hsa-miR-335-5p (also known as hsa-miR-335)	ucaagagcaauaacgaaaaaugu (SEQ ID No. 4)	MIMAT0000765
hsa-miR-301b-3p (also known as hsa-miR-301b)	cagugcaaugauauugucaaagc (SEQ ID No. 5)	MIMAT0004958
hsa-miR-184	uggacggagaacugauaagggu (SEQ ID No. 6)	MIMAT0000454
hsa-miR-505-3p (also known as hsa-miR-505)	cgucaacacuugcugguuuccu (SEQ ID No. 7)	MIMAT0002876
hsa-miR-203a-3p (also known as hsa-miR-203a, hsa-miR-203)	gugaaauguuuaggaccacuag (SEQ ID No. 8)	MIMAT0000264
hsa-miR-654-3p	uaugucugcugaccaucaccuu (SEQ ID No. 9)	MIMAT0004814
hsa-miR-655-3p (also known as hsa-miR-655)	auaauacaugguuaaccucuuu (SEQ ID No. 10)	MIMAT0003331
hsa-miR-331-3p (also known as hsa-miR-331)	gccccugggccuauccuagaa (SEQ ID No. 11)	MIMAT0000760
hsa-miR-223-5p (also known as hsa-miR-223*)	cguguauuugacaagcugaguu (SEQ ID No. 12)	MIMAT0004570
hsa-miR-let-7g	ugagguaguaguuuguacaguu (SEQ ID No. 13)	MIMAT0000414
hsa-miR-126* (also known as hsa-miR-126-5p)	cauuauuacuuuugguacgcg (SEQ ID No. 14)	MIMAT0000444
hsa-miR-193a-5p	uggguuuugcgggcgagauga (SEQ ID No. 15)	MIMAT0004614
hsa-miR-144*(also known as hsa-miR-144-5p)	ggauaucaucauauacuguaag (SEQ ID No. 16)	MIMAT0004600
hsa-miR-190 (also known as hsa-miR-190a or hsa-miR-190a-3p)	cuauauaucaaacauauuccu (SEQ ID No. 17)	MIMAT0026482

hsa-miR-194 (also known as hsa-miR-194-5p)	uguaacagcaacuccaugugga (SEQ ID No. 18)	MIMAT0000460
hsa-miR-365 (also known as hsa-miR-365a-3p)	uaaugccccuaaaaauccuuau (SEQ ID No. 19)	MIMAT0000710
hsa-miR-590-3p(also known as hsa-miR-590)	uaauuuuauguauaagcuagu (SEQ ID No. 20)	MIMAT0004801
hsa-miR-624	uaguaccaguaccuuguguuca (SEQ ID No. 21)	MI0003638
hsa-miR-625*(also known as hsa-miR-625-3p)	gacuauagaacuuucccccuca (SEQ ID No. 22)	MIMAT0004808
hsa-miR-671-3p	uccgguucucagggcuccacc (SEQ ID No. 23)	MIMAT0004819
hsa-let-7c-5p	ugagguaguagguuguaugguu (SEQ ID No. 24)	MIMAT0000064
hsa-let-7i-5p (also known as hsa-let-7i)	ugagguaguaguuugugcuguu (SEQ ID No. 25)	MIMAT0000415
hsa-miR-142-3p	uguaguguuuccuacuuuaugga (SEQ ID No.26)	MIMAT0000434
hsa-miR-148a-3p (also known as hsa-miR-148a)	ucagugcacuacagaacuuugu (SEQ ID No. 27)	MIMAT0000243
hsa-miR-15b-5p (also known as hsa-miR-15b)	uagcagcacaucaugguuuaca (SEQ ID No. 28)	MIMAT0000417
hsa-miR-16-5p (also known as hsa-miR-16)	uagcagcacguaaauauuggcg (SEQ ID No. 29)	MIMAT0000069
hsa-miR-181a-5p (also known as hsa-miR-181a)	aacauucaacgcugucggugagu (SEQ ID No.30)	MIMAT0000256
hsa-miR-20a-5p (also known as hsa-miR-20;hsa-miR-20a)	uaaagugcuuauagugcagguag (SEQ ID No.31)	MIMAT0000075
hsa-miR-20b-5p (also known as hsa-miR-20b)	caaagugcucauagugcagguag (SEQ ID No.32)	MIMAT0001413
hsa-miR-221-3p (also known as hsa-miR-221)	agcuacauugucugcuggguuuc (SEQ ID No.33)	MIMAT0000278
hsa-miR-24-3p (also known as hsa-miR-24)	uggcucaguucagcaggaacag (SEQ ID No.34)	MIMAT0000080
hsa-miR-27b-3p (also	uucacaguggcuaaguucugc (SEQ ID No.35)	MIMAT0000419

known as hsa-miR-27b)		
hsa-miR-29a-3p (also known as hsa-miR-29a)	uagcaccaucugaaaucgguua (SEQ ID No.36)	MIMAT0000086
hsa-miR-29c-3p (also known as hsa-miR-29c)	uagcaccauuugaaaucgguua (SEQ ID No.37)	MIMAT0000681
hsa-miR-30a-5p (also known as hsa-miR-30a)	uguaaacauccucgacuggaag (SEQ ID No.38)	MIMAT0000087
hsa-miR-107 (also known as hsa-miR-107-10)	agcagcauuguacagggcuauca (SEQ ID No.39)	MI0000114
hsa-miR-135b-5p (also known as hsa-miR-135b)	uauggcuuuucauuccuauguga (SEQ ID No.40)	MIMAT0000758
hsa-miR-199b-5p(also known as hsa-miR-199b)	cccaguguuuagacuaucuguuc (SEQ ID No.41)	MIMAT0000263
hsa-miR-324-5p	cgcaucccccuagggcauuggugu (SEQ ID No.42)	MIMAT0000761
hsa-miR-652-3p (also known as hsa-miR-652)	aauggcgccacuaggguugug (SEQ ID No.43)	MIMAT0003322
hsa-miR-424-5p(also known as hsa-miR-424)	cagcagcaauucauguuuugaa (SEQ ID No.44)	MIMAT0001341
hsa-miR-10a (also known as hsa-miR-10-5p)	uacccuguagauccgaauuugug (SEQ ID No.45)	MIMAT0000253
hsa-miR-132 (also known as hsa-miR-132-3p)	uaacagucuacagccauggucg (SEQ ID No.46)	MIMAT0000426
hsa-miR-223 (also known as hsa-miR-223-3p)	ugucaguuugucaaauacccca (SEQ ID No.47)	MIMAT0000280
hsa-miR-143 (also known as hsa-miR-143-3p)	ugagaugaagcacuguagcuc (SEQ ID No.48)	MIMAT0000435
hsa-miR-148b (also known as hsa-miR-148b-3p)	ucagugcaucacagaacuuugu (SEQ ID No.49)	MIMAT0000759
hsa-miR-18a (also known as hsa-miR-18;hsa-miR-18a-5p)	uaaggugcaucuagugcagauag (SEQ ID No.50)	MIMAT0000072
hsa-miR-192 (also known	cugaccuaugaauugacagcc (SEQ ID No.51)	MIMAT0000222

as hsa-miR-192-5p)		
hsa-miR-429	uaauacugucugguaaaaccgu (SEQ ID No.52)	MIMAT0001536
hsa-miR-618	aaacucuacuuguccuucugagu (SEQ ID No.53)	MIMAT0003287
hsa-miR-95 (also known as hsa-miR-95-5p)	ucaauaaaugucuguugaauu (SEQ ID No.54)	MIMAT0026473
hsa-miR-130a (also known as hsa-miR-130a-3p)	cagugcaauguuaaaagggcau (SEQ ID No.55)	MIMAT0000425
hsa-miR-152 (also known as hsa-miR-152-5p)	agguucugugauacacuccgacu (SEQ ID No.56)	MIMAT0026479
hsa-miR-27b (also known as hsa-miR-27b-3p)	uucacaguggcuaaguucugc (SEQ ID No.57)	MIMAT0000419
hsa-miR-301 (also known as hsa-miR-301a-3p or hsa-miR-301a)	cagugcaauaguauugucaaagc (SEQ ID No.58)	MIMAT0000688
hsa-miR-326	ccucugggcccuuccuccag (SEQ ID No.59)	MIMAT0000756
hsa-miR-345 (also known as hsa-miR-345-5p)	gcugacuccuaguccagggcuc (SEQ ID No.60)	MIMAT0000772
hsa-miR-361 (also known as hsa-miR-361-5p)	uuaucagaaucuccaggguac (SEQ ID No.61)	MIMAT0000703
hsa-miR-422a	acuggacuuagggucagaaggc (SEQ ID No.62)	MIMAT0001339
hsa-miR-579 (also known as hsa-miR-579-3p)	uucauuugguauaaaccgcgauu (SEQ ID No.63)	MIMAT0003244
hsa-miR-642 (also known as hsa-miR-642a-5p; hsa-miR-642)	gucccucuccaaaugugucuug (SEQ ID No.64)	MIMAT0003312
hsa-miR-99a (also known as hsa-miR-99a-5p)	aacccguagauccgaucuugug (SEQ ID No.65)	MIMAT0000097
hsa-miR-520D-3p (also know as hsa-miR-520D)	aaagugcuucucuuuggugggu (SEQ ID No.66)	MIMAT0002856
hsa-miR-629 (also known as hsa-miR-629-5p)	uggguuuacguugggagaacu (SEQ ID No.67)	MIMAT0004810
hsa-miR-23a-3p (also	aucacauugccagggauuucc (SEQ ID No.68)	MIMAT0000078

hsa-miR-23a-3p (also known as hsa-miR-23a)	aucacauugccagggauuucc (SEQ ID No.68)	MIMAT0000078
hsa-miR-148b-3p (also known as hsa-miR-148b)	ucagugcaucacagaacuuugu (SEQ ID No.69)	MIMAT0000759

Conveniently the sample may be any appropriate fluid or tissue sample obtained from the subject. For example, the biological sample may comprise at least one of the group consisting of: urine, saliva, whole blood, plasma, serum, sputum, semen, faeces, a nasal swab, tears, a vaginal swab, a rectal swab, a cervical smear, a tissue biopsy, and a urethral swab. In some embodiments the sample is a fluid sample. Suitably, the sample is one that can be readily obtained from the individual, such as urine, saliva, blood and sputum. In some embodiments, the sample comprises saliva, blood, plasma or serum. It will be appreciated that in some embodiments the process of obtaining the sample does not form part of the present disclosure.

In some embodiments, the sample comprises or is constituted by serum. Not only does serum have practical advantages, but it is also free of anticoagulants such as heparin, a potential inhibitor of PCR reactions. Serum may also be less affected by haemolysis, compared to plasma.

In some embodiments, the sample is saliva. Saliva can be easily obtained from the patient (e.g. pitch-side, or in the field), without specialist training or medical equipment.

miRNAs which have been found to be indicative of mTBI in saliva include: hsa-let-7ca-5p, hsa-let-7i-5p, hsa-miR-1421-3p, hsa-miR-148a-3p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-181a-5p, hsa-miR-20a-5p, hsa-miR-20b-5p, hsa-miR-221-3p, hsa-miR-24-3p, hsa-miR-27b-3p, hsa-miR-29a-3p, hsa-miR-29c-3p, hsa-miR-340-5p, hsa-miR-424-5p; miR-30a-5p; miR-107; miR-135b-5p; miR-199b is selected from the group consisting of -5p; miR-324-5p; and miR-652-3p.

Levels of miRNAs may be used to track recovery of a subject from injury. Thus, the present disclosure encompasses monitoring the recovery of a subject from TBI, as an alternative or in addition to the initial diagnosis.

In some embodiments, the method comprises monitoring TBI and the level of the at least one miRNA is determined in a sample obtained from the subject at least 2, at least 3, at least 5, at

least 7, at least 10 or at least 14 days after injury. In some embodiments, the level of the at least one miRNA is determined in a sample obtained from the subject 15 days after injury. In some embodiments, the level of the at least one miRNA is determined in at least two samples obtained at different time intervals after injury, thus allowing recovery to be monitored. For example, miRNA levels could be determined at 7 and 14 days following injury, or at 5, 10 and 15 days following injury. A return of miRNA levels to normal may be indicative of recovery of the subject from the TBI.

In some embodiments, a subject is determined to have recovered from mTBI if the level of miR-425-5p and/or miR-502 is no longer below a predetermined threshold or no longer decreased relative to a reference.

In some embodiments, a subject is determined to have recovered from moderate-to-severe TBI if the level of miR-21 and/or miR-335 is no longer above a predetermined threshold or no longer increased relative to a reference.

The diagnosis of a subject as suffering from a TBI, and in particular diagnosis of mild TBI or moderate-to-severe TBI, may facilitate in the determination of an appropriate treatment. The present disclosure thus provides a test that enables healthcare workers, such as physicians, clinicians, paramedics, and even non-medical personnel (e.g. teachers, sports coaches, military personnel) to decide on appropriate action for a subject suspected of having a TBI. A subject determined as having a TBI may therefore receive the most appropriate treatment as a result of a diagnosis being made. The method of the present disclosure may thus further comprise directing appropriate therapy to a subject diagnosed with a TBI.

A subject diagnosed with TBI may be further evaluated, e.g. by CT scanning. In some embodiments, the subject is admitted to hospital. In some embodiments, if moderate-to-severe TBI can be ruled out, the subject may not need to be admitted to hospital for evaluation. A subject diagnosed with moderate-to-severe TBI may be admitted to hospital, or a specialist centre with neurotrauma expertise.

A subject diagnosed with a TBI (particularly mTBI) outside a hospital environment, for example, at a sporting event, during combat or during play, may be removed from play or combat immediately. The subject may subsequently be started on a graduated return to play or combat.

In a further aspect, there is provided a method for determining whether it is appropriate to administer to a subject a therapy for alleviating TBI, the method comprising:
determining a level of at least one miRNA in a sample from the subject; and
determining whether or not it is appropriate to administer a therapy for alleviating TBI, based on the level of the at least one miRNA.

It will be appreciated that the step of administering the therapy to the subject does not form a part of the claimed method, unless specifically stated.

In some embodiments the method may further comprise administering to the subject an appropriate treatment. In some embodiments, the treatment may comprise a therapy for alleviating TBI. Accordingly, the present disclosure features methods of diagnosing and treating TBI in a subject, the method comprising the steps of (a) obtaining a sample (e.g., a sample of blood, plasma, urine, or saliva) from the subject; (b) detecting one or more miRNAs (selected from those described herein); diagnosing the patient as having a TBI when the level(s) of the miRNA(s) differ from a reference standard (as described herein); and administering a treatment for the TBI.

In a further aspect, the present disclosure provides a method of determining an appropriate treatment to a subject suspected of suffering from a TBI, the method comprising identifying whether or not the subject has a TBI by determining a level of at least one miRNA in a sample from the subject.

If a subject is identified as having a TBI, an appropriate treatment may include one or more of the following: further evaluating the subject, for example by further tests (e.g. verbal, cognitive, motor and/or optical tests), CT and/or MRI scanning; removing the subject from activity (e.g. the activity during which the TBI was incurred); admitting the subject to hospital or a specialist clinic; surgery; and administering a therapy for alleviating TBI to the subject.

The therapy for alleviating TBI may include neuroprotective drugs, e.g. drugs to treat cerebral swelling such as mannitol and hypertonic saline, and/or other neuroprotective measures, such as avoidance of hypertensive resuscitation and the use of sedation.

In some embodiments, the subject may be subsequently monitored to track their recovery, for example in a hospital or clinic setting.

According to a further aspect of the present disclosure, there is provided a method of detecting and/or determining a level of a target miRNA in a subject, the method comprising the steps of (a) obtaining a sample from the subject; and (b) detecting and/or determining the level of the target miRNA in the sample by contacting the sample with a probe that is specific for the target miRNA.

The sample may be any appropriate fluid or tissue sample obtained from the subject, as defined above. In some embodiments, the sample is blood, serum, plasma, urine or saliva.

In some embodiments, the method may comprise determining the level of two or more target miRNAs in the sample.

According to a further aspect of the present disclosure, there is provided a therapy for alleviating TBI for use in a method of treating a subject in need thereof, wherein said subject is identified as having a TBI by determining a level of at least one miRNA in a sample from the subject.

The step of determining the level of the target miRNA may comprise contacting the sample with a substrate functionalized with the probe, for example a chip comprising the probe. The substrate or chip may conveniently include multiple probes, each specific for a different target miRNA.

The subject may have suffered an injury, in particular a head injury. The subject may be suspected as having a TBI. In some embodiments, the sample is obtained no more than 72 hours, no more than 48 hours, no more than 36 hours, no more than 24 hours, no more than 12 hours, no more than 6 hours, no more than 4 hours, no more than 2 hours or no more than 1 hour after injury.

In some embodiments, the method further comprises treating the subject. The treatment may include one or more of the following: further evaluating the subject, for example by further tests (e.g. verbal, cognitive, motor and/or optical tests), CT and/or MRI scanning; removing the subject from activity (e.g. the activity during which the TBI was incurred); admitting the subject

to hospital or a specialist clinic; and administering a therapy for alleviating TBI to the subject. In some embodiments, the treatment comprises administering an effective amount of a neuroprotective drug.

Thus, in yet a further aspect the present disclosure provides a method of treating TBI, the method comprising:

determining a level of at least one miRNA in a sample from the subject; and

if the level of the at least one miRNA is indicative of mTBI, administering a treatment appropriate for mTBI; or

if the level of the at least one miRNA is indicative of m-sTBI, administering a treatment appropriate for m-sTBI.

It will be appreciated by those skilled in the art that different treatment pathways may be used for mTBI and m-sTBI.

An appropriate treatment for mTBI may include: removing the subject from activity; treatment *in situ* or in the community; further evaluating the subject in hospital without overnight admission (typically mTBI patients are discharged with promptly with head injury advice); or admission to hospital for a period of observation (typically 1-2 days). The subject may be further evaluated using tests (e.g. verbal, cognitive, motor and/or optical tests). CT scanning is generally only required if certain indications are present, including suspected skull fracture, post-traumatic seizure, focal neurological defecit, repeated vomiting, a GCS score of less than 13 on initial assessment (less than 14 for children, or less than 15 for infants under 1 year), in accordance with NICE guidelines.

An appropriate treatment for m-sTBI may include: MRI or CT scanning (particularly within 1 hour of injury); admission to hospital (which may include admission to intensive care and/or transfer to a specialist clinic or major trauma centre with neurosurgical facilities); neuromonitoring; surgery; administering a therapy for alleviating TBI, such as administering neuroprotective drugs, e.g. drugs to treat cerebral swelling such as mannitol and hypertonic saline, and/or other neuroprotective measures, such as avoidance of hypertensive resuscitation and the use of sedation.

Thus, the present disclosure mayenable subjects with a TBI to be quickly stratified into mTBI or m-sTBI, so that they may receive the most appropriate treatment.

According to a further aspect of the present disclosure, there is provided a detection system for diagnosing and/or monitoring TBI, the detection system comprising a sensor element comprising a substrate functionalized with a probe specific for a target miRNA The detection system can further comprise a detection device that is capable of detecting the binding of a target miRNA to the probe.

According to a yet further aspect of the present disclosure, there is provided a sensor element for use in a detection system for diagnosing and/or monitoring TBI, the sensor element comprising a substrate functionalized with a probe specific for a target miRNA.

The sensor element may further comprise a sample addition zone for receiving a sample (e.g. a fluid sample) thereon.

The probe is capable of selectively binding the miRNA of interest. The substrate may be functionalized with a plurality of probes. The probes may all be the same, or two or more different probes may be provided. For example, in some embodiments, the substrate may be functionalized with a first probe specific for a first miRNA, and a second probe specific for a second miRNA. The first and second probes may be grouped together, for example on different portions of the sensor element.

In a further aspect of the present disclosure, there is provided a composition for use in a method of diagnosing and/or monitoring traumatic brain injury (TBI) in a subject, the composition comprising a probe specific for a target miRNA. The composition may comprise any one of the listed miRNAs or with any plurality of the listed miRNAs (e.g., two, three, four, or more of the listed miRNAs).

In some embodiments, the target miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g, miR-335, miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, miR-671-3p, hsa-let-7c-5p, hsa-let-7i-5p, miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p,

miR-29a-3p, miR-29c-3p, miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p, miR-424-5p, miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629.

In some embodiments, the target miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g, miR-335, hsa-miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, and miR-671-3p. These microRNAs have been found to be biomarkers expressed in all TBI patients (mild or severe).

In some embodiments, the target miRNA is selected from the group consisting of miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-502, miR-21, miR-let-7g and miR-335.

In some embodiments, the target miRNA is selected from the group consisting of let-7c-5p, let-7i-5p, miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, and miR-424-5p; miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p.

In some embodiments, the target miRNA is selected from the group consisting of miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-194, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629.

In some embodiments, the target miRNA is selected from the group consisting of miR-425-5p, miR-502, miR-21 and miR-335.

The probe may comprise a biological molecule such as a protein (e.g. an antibody) or a nucleic acid. In some embodiments, the probe comprises a nucleic acid. The nucleic acid may comprise a sequence which is at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identical to a sequence which is the complement of the full-length sequence of the target miRNA. In some embodiments, the nucleic acid comprises a sequence which is 100% identical to a sequence which is the complement of the sequence of the target

miRNA (i.e. the receptor comprises a nucleic acid sequence which is the exact complement of the target miRNA sequence).

The probes may be attached to a surface of the substrate by any suitable means, such as by coupling chemistry known to those skilled in the art. In some embodiments, each probe is attached to a surface of the substrate via a linker. In some embodiments, the probe comprises a moiety for immobilizing the probe on the substrate, or for attaching the probe to a linker immobilized on the substrate.

Alternatively or in addition, the probe may comprise a detectable label. The detectable label may be, for example, radioactive, fluorescent, luminescent, or antibody-based (e.g., it may constitute a conventional tetrameric antibody or a detectable fragment thereof).

The substrate of the sensor element may be formed from any suitable material. In some embodiments, the substrate comprises or is formed from metal, plastic, glass, silica, silicon, graphite, graphene, or any combination thereof. In some embodiments, the substrate comprises multiple layers. For example, a substrate may be prepared by forming a surface or layer of graphene on a layer of silicon carbide or silica. The graphene surface may be chemically modified, for example to graphene-oxide (GO) or graphene-amine (GA). Methods for forming graphene layers, such as epitaxial growth and sublimation growth, will be known to those skilled in the art.

Conveniently, probes comprising or constituted by a nucleic acid can be attached to a GO surface via a linker, using an amide coupling reagent (e.g. (O-(7-azabenzotriazole-1-yl)-N,N,N,N'-tetramethyluronium hexafluorophosphate (HATU)). A sensor element comprising a surface functionalized with a nucleic acid probe can then be used to selectively detect its complementary miRNA.

Suitable linkers may comprise an aniline moiety (or a derivative thereof), a benzoic acid moiety (or a derivative thereof) or an ethendiamine moiety (or a derivative thereof). An aniline linker may be formed by attaching a nitrobenzene molecule (or derivative) to a graphene surface (e.g. using a diazonium salt), and reducing the nitrobenzene to aniline. The amine group of the aniline may then be used to attach to the probe. Similarly, a diazonium salt (e.g. 4-benzoic acid diazonium tetrafluoroborate) can be used to attach a benzoic acid or benzoic acid derivative to a

graphene surface. An ethanediamine moiety may be attached to carboxylated graphene or graphene oxide.

The sensor element may be comprised within a test strip. The test strip may be disposable.

The detection device may be configured to detect the binding of a target miRNA to the receptor by any suitable means known to those skilled in the art, for example by detecting changes in electrical impedance, hydrogen ion concentration, or conformational changes resulting from hybridisation.

The detection device may further include a user interface to output data to a user.

In some embodiments, the detection device includes a database of treatment information. The device may be capable of identifying suitable treatment options from the database depending on the levels of the or each miRNA of interest. The treatment information may be provided to the user via the user interface.

Conveniently, the detection device may be portable, e.g. hand-held. The detection device may comprise a data storage unit for storing miRNA levels and other information relating to the subject. In some embodiments, the device comprises a data communication means for communicating data to other devices. For example, the device may communicate data wirelessly through WiFi, 3G, 4G, Bluetooth, or through a mobile app. This may conveniently enable the data to be easily accessed by medical professionals if necessary.

It is thus envisaged that the detection device of the present disclosure provides an affordable, portable, point of care means for diagnosing and monitoring TBI non-invasively. The device may be used by ambulance crews, the military, schools, sports clubs and healthcare professionals, enabling the correct assessment and triage of patients suspected to have a TBI.

In a further aspect there is provided a kit for use in the present methods. The kit may comprise at least probe (e.g. a protein, such as an antibody, or a nucleic acid) which is capable of selectively binding the miRNA of interest. In some embodiments, the kit comprises an array comprising a plurality of probes. In some embodiments, the at least one probe is a primer for carrying out PCR. The kit may further comprise instructions for use, for example instructions for

use in the diagnosis and/or monitoring of TBI. The kit may further comprise suitable buffers and reagents, such as amplification primers and enzymes (e.g. DNA polymerase, reverse transcriptase for conversion of miRNA to cDNA).

In another aspect the present invention provides a method of

i) diagnosing mild traumatic brain injury (mTBI) in a subject, the method comprising determining the level of miR-502 in a fluid sample obtained from the subject up to 48 hours from injury; and/or

ii) monitoring mTBI in a subject, the method comprising determining the level of miR-502 in a fluid sample obtained from the subject at least 2 days after injury,

wherein the fluid sample comprises serum.

In yet another aspect the present invention provides the use of a sensor element in a detection system in a method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) of the present invention, the sensor element comprising a substrate functionalized with at least one probe specific for miR-502, wherein the probe comprises a nucleic acid, and wherein the nucleic acid comprises a sequence which is at least 80% identical to a sequence which is the complement of SEQ ID NO: 3.

In a further aspect the present invention provides the use of a detection system in a method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) of the present invention, the detection system comprising:

- a sensor element as described herein; and
- a detection device that is capable of detecting the binding of a target miRNA to the probe.

In yet a further aspect the present invention provides the use of a kit in a method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) in a fluid sample from a subject of the present invention, the kit comprising at least one probe specific for miR-502, wherein the fluid sample comprises serum, wherein the probe comprises a nucleic acid, and wherein the nucleic acid comprises a sequence which is at least 80% identical to a sequence which is the complement of the sequence of SEQ ID NO: 3.

In another aspect the present invention provides a method of treating mild traumatic brain injury (mTBI) in a subject that has been identified using a method of the present invention as having mTBI by determining the level of at least one miRNA in a fluid sample from the subject obtained from the subject up to 48 hours from injury, wherein the fluid sample comprises serum, and wherein the at least one miRNA comprises miR-502, the method comprising administering a neuroprotective drug or a sedative.

In yet another aspect the present invention provides the use of a neuroprotective drug or a sedative in the manufacture of a medicament for treating mild traumatic brain injury (mTBI) in a subject that has been identified using a method of the invention as having a mTBI by determining a level of at least one miRNA in a fluid sample from the subject obtained from the subject up to 48 hours from injury, wherein the fluid sample comprises serum, and wherein the at least one miRNA comprises miR-502.

In a further aspect the present invention provides there is provided the use of a composition in the method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) in a subject of the present invention, the composition comprising at least one probe specific for miR-502, wherein the probe comprises a nucleic acid, and wherein the nucleic acid comprises a sequence which is at least 80% identical to a sequence which is the complement of the sequence of SEQ ID NO: 3.

In yet a further aspect the present invention provides there is provided the use of the sensor element, detection system, kit, or composition of the present invention, wherein the sensor element, detection system, kit or composition further comprises at least one further probe specific for a target miRNA, wherein the miRNA is selected from the group consisting of:

(a) miR-425-5p;

(b) miR-425-5p, miR-21, and miR-335, or any combination thereof; and

(c) miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-21, miR-let-7g, miR-335, miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, miR-671-3p, hsa-let-7c-5p, hsa-let-7i-5p, miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p, miR-424-5p, miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-27b, miR-301, miR-326, miR-345,

miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629, or any combination thereof.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each of the appended claims.

It will be appreciated that statements made herein in relation to any aspect of the invention may equally apply to any other aspect of the invention, as appropriate.

Detailed description of the invention

Embodiments of the invention will now be described by way of example and with reference to the accompanying Figures:

Figure 1: miR-425-5p and miR-502 expression in 3 different categories of trauma and HV

MiR-425-5p and miR-502 expression in 10 HV, 10 mTBI+EC (1 day), 10 mTBI+EC (15 days), 10 EC (1 day), 10 EC (15 days), 10 sTBI+EC (1 day) and 10 sTBI+EC (15 days) patients, detected by qRT-PCR analysis. MiR-425-5p expression was found to be remarkably decreased in mTBI+EC (1 day) compared to HV ($p<0.01$), mTBI+EC (15 dys) ($p<0.001$) and sTBI+EC (1day) ($p<0.01$) (Figure 1A). MiR-502 expression was found to be remarkably decreased in mTBI+EC (1 day) compared to HV ($p<0.05$), mTBI+EC (15 dys) ($p<0.01$) and sTBI+EC (1day) ($p<0.05$) (Figure 1B).

Figure 2: miR-21 and miR-335 expression in 3 different categories of trauma and HV

MiR-21 and miR-335 expression in 10 HV, 10 mTBI+EC (1 day), 10 mTBI+EC (15 days), 10 EC (1 day), 10 EC (15 days), 10 sTBI+EC (1 day) and 10 sTBI+EC (15 days) patients, detected by qRT-PCR analysis. MiR-21 expression was found to be significantly up-regulated in sTBI+EC (1 day and 15 days) compared to HV ($p<0.01$) (Figure 2A). MiR-335 expression was found to be

remarkably up-regulated in sTBI+EC (1 day) compared to HV ($p<0.001$), EC (15 days) ($p<0.001$) and mTBI+EC (1 day) ($p<0.05$) (Figure 2B).

Figure 3: Time course of miR-425-5p (Figure 3A) and miR-502 (Figure 3B) expression in 3 different categories of trauma and HV

MiR-425-5p and miR-502 expression in 30 HV, 30 mTBI+EC, 30 EC, 30 sTBI+EC patients at different time points from injury (T0, T4-12h, T48-72h, 15days) detected by qRT-PCR analysis. MiR-425-5p expression was found to be remarkably decreased in mTBI+EC at T0 and T4-12h compared to HV, sTBI+EC and EC ($p<0.05$). MiR-502 expression was found to be remarkably

decreased in mTBI+EC at T0 and T4-12h compared to HV, sTBI+EC and EC ($p<0.05$). P values were determined by Tukey's post-hoc test.
* significantly different from HV

Figure 4: Time course of miR-21 (Figure 4A) and miR-335 (Figure 4B) expression in 3 different categories of trauma and HV

MiR-21 and miR-335 expression in 30 HV, 30 mTBI+EC, 30 EC, and 30 sTBI+EC patients at different time points from injury (T0, T4-12h, T48-72h, 15days) detected by qRT-PCR analysis. MiR-21 expression was found to be significantly up-regulated in sTBI+EC at T4-12h, T48-72h and 15 days compared to HV ($p<0.01$). MiR-335 expression was found to be remarkably up-regulated in sTBI+EC at T0, T4-12h, T48-72h and 15 days compared to HV and mTBI+EC ($p<0.001$) but not EC only. P values were determined by Tukey's post-hoc test.
* significantly different from HV

Examples

As miRNAs are emerging as promising biomarkers in a range of different pathologies, the present inventors sought to explore their role in TBI.

Example 1

Materials and methods

Patients and samples collection

Study participants were recruited from the Surgical Reconstruction and Microbiology Research Centre (SRMRC) at Queen Elizabeth Hospital of Birmingham (UK) as part of Brain Biomarkers after Trauma (The Golden Hour Study) study (Ethics Ref. 13/WA/0399).

First, we performed screening of 754 miRNAs in 5 mTBI with extra-cranial injury (EC) patients, 5 sTBI+EC injury patients and healthy volunteers (HV) at 1 day and 15 days from injury with the aim to select specific candidate biomarkers able to discriminate mild from severe TBI and predict the recovery of mTBI after 15 days. Based on this information (Table 2), it was then possible to confirm the results study in an enlarged cohort of patients of 40 individuals grouped in 4 different categories: HV (n=10), EC (n=10), mTBI+EC (n=10), sTBI+EC (n=10). Healthy

volunteers were consented and enrolled in the RECOS study. EC injury patients had radiographically-confirmed fractures, no head trauma, no infection, no history of neurological or psychiatric disorders and no alcohol or drug dependency. Mild TBI with EC included those with a non-penetrating head trauma and Glasgow Coma Scale (GCS) score >13. Severe TBI with EC included patients with GCS score of 8 or below. All patients were gender and age matched to HVs.

Sample processing

Peripheral blood samples were taken at 1 day and 15 days from injury in each patient. The blood samples were processed for serum isolation within 2h after withdrawal. Whole blood was left to stand for about 30' at room temperature before being centrifuged at 3000 rpm for 10' at 4°C. Serum was divided into aliquots and stored at -80°C until analysis.

RNA isolation, reverse transcription and miRNAs profiling by TaqMan Low Density Array (TLDA)

Initial screening (discovery set) were performed on 5 mTBI+EC and 5 sTBI+EC patients, which were compared to HV at the two different time points (1 day and 15 days from the injury). The serum of these patients was used to profile the transcriptome of 754 miRNAs. Serum samples were centrifuged at 2000 rpm for 10' to pellet and remove any circulating cell or debris. MiRNAs were extracted from 400µl of serum samples by using Qiagen miRNeasy mini kit (Qiagen, GmbH, Hilden, Germany), according to Qiagen supplementary protocol for purification of small RNAs from serum and plasma and finally eluted in 30 µl volume of RNase free water. The concentration and purity of the resulting RNA was determined with a ND-1000 UV-Vis Spectrophotometer (NanoDrop). 20 ng of serum RNAs was retrotranscribed and pre-amplified, according to the manufacturer's instructions. Pre-amplified products were loaded onto TLDAs, TaqMan Human MicroRNA Array v3.0 A and B (Applied Biosystems LifeTechnologies™). PCRs on TLDAs were performed on 7900HT Fast RealTime PCR System (Applied Biosystem, LifeTechnologies™).

Data analysis

To obtain an accurate miRNA profiling, we used the global median normalization method. Similar to microarray analysis, Ct values from each sample were normalized to the median Ct of the array. Moreover, by computing the Pearson correlation among the Ct medians and means of each array and Ct of each miRNA, we identified two miRNAs that showed an expression profile close to the median and mean of TLDAs, i.e., miR-331 and miR-223*. These miRNAs were also

confirmed to be among the most stable in TLDAs by applying two different methods: DataAssistv.3software (AppliedBiosystem Life Technologies™) and geNorm Algorithm. Accordingly, miR-331 and miR-223* were used as reference genes for validation by single TaqMan assays. Expression fold changes were calculated by the 2−ΔΔCT method. Differentially expressed miRNAs (DE miRNAs) were identified by Significance of Microarrays Analysis (SAM) computed by Multi experiment viewer v4.8.1, applying a two-class unpaired test among ΔCts and using a p-value based on 100 permutations; imputation engine: K-nearest neighbours (10 neighbours); false discovery rate<0.15 was used as correction for multiple comparisons. We accepted as reliable only DE miRNAs concordant by using all endogenous controls.

Single TaqMan Assays

Ten differentially expressed miRNAs were chosen from the arrays as potential candidate biomarkers with the aim to discriminate mild from severe TBI and to monitor the recovery of mild. These candidates were used to validate the data in an enlarged cohort of 30 patients (validation set) grouped in 3 different categories (mTBI+EC, sTBI+EC and EC only) and 10 controls (HV) at two different time points (1 and 15 days from injury) by single TaqMan assays (AppliedBiosystems, Life Technologies™). Samples were extracted and retrotrascribed as described above and RT-qPCR analysis was performed in Bio-Rad iQ5 Real-time PCR Detection System (Bio-Rad, CA, USA). Expression fold changes were calculated by the 2-ΔΔCT method.

Statistical analysis

The data were check for normal distribution and transformed to perform parametric tests. Comparisons across groups at each time and within groups over time were performed by the one-way analysis of variance and Tukey's post-hoc test on transformed data. A receiver operating characteristic analysis was utilised to calculate sensitivity and specificity of each biomarker in diagnosing either mTBI or sTBI expressed as area under the curve (AUC). All analyses were carried on SPSS v.20 (IBM). Differences were considered as statistically significant at p-value < 0.05.

RESULTS

Expression profiles by TaqMan Low Density Arrays (TLDA)

From 754 screenable miRNAs of TLDA, we identified ten circulating miRNAs at 1 day and 13 at 15 days in mTBI+EC, 19 at 1 day and 22 at 15 days in sTBI+EC differentially expressed (Table 2). From this list, hsa-miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625* and miR-671-3p were excluded for further analysis because they were expressed in most of the patients, hence not suitable candidate biomarkers for mild or severe trauma only. However, the above microRNAs can identify TBI of any severity and are therefore useful TBI biomarkers. On the other hand, miR-184, miR-301b, miR-502 and miR-505 uniquely and differentially expressed in mTBI+EC at 1 day, were selected as early candidate biomarkers of mTBI. In addition, miR-203, miR-425-5p, miR-654-3p and miR-655 differentially expressed at 15 days post mTBI+EC were selected as candidate biomarkers able to track the recovery of mTBI.

Finally, two miRNAs, miR-21 and miR-335, constantly expressed at both time points in sTBI+EC, were selected for further studies.

Table 2: Fold change of microRNAs differentially expressed in 5 mTBI+EC (1 and 15 days) and 5 sTBI+EC (1 and 15 days) patients, compared to 5 HV and detected by TLDA.

mTBI+EC 1 day VS HV		**mTBI+EC 15 days VS HV**		**sTBI+EC 1 day VS HV**		**sTBI+EC 15 days VS HV**	
hsa-miR-184	0.1383	hsa-miR-193a-5p	24.901	hsa-let-7g	0.3250	hsa-miR-130a	14.979
hsa-miR-190	0.1278	hsa-miR-194	20.946	hsa-miR-10a	11.953	hsa-miR-152	9.8921
hsa-miR-425-5p	**0.0798**	hsa-miR-203	4.1763	hsa-miR-132	8.1875	hsa-miR-190	0.0277
hsa-miR-502	**0.0538**	hsa-miR-365	3.7087	hsa-miR-143	26.193	hsa-miR-193a-5p	13.655
hsa-miR-505	7.7696	**hsa-miR-425-5p**	**3.0166**	hsa-miR-148b	20.095	hsa-miR-194	12.301
hsa-miR-126*	0.1570	hsa-miR-654-3p	0.0878	hsa-miR-18a	26.806	**hsa-miR-21**	**12.662**
hsa-miR-144*	0.2758	hsa-miR-655	0.0756	hsa-miR-190	0.0634	hsa-miR-27b	18.977
hsa-miR-590-3p	0.3842	hsa-miR-671-3p	4.0584	hsa-miR-192	0.2245	hsa-miR-301	21.954
hsa-miR-624	0.0836	hsa-miR-126*	0.2978	hsa-miR-193a-5p	29.785	hsa-miR-326	93.099
hsa-miR-301b	0.0435	hsa-miR-144*	0.2645	**hsa-miR-21**	**7.1654**	**hsa-miR-335**	**45.050**
		hsa-miR-590-3p	0.3035	hsa-miR-223	4.6799	hsa-miR-345	37.699
		hsa-miR-624	0.1643	**hsa-miR-335**	**37.192**	hsa-miR-361	37.295
		hsa-miR-625*	0.1521	hsa-miR-365	5.5771	hsa-miR-422a	77.373
				hsa-miR-429	0.1294	hsa-miR-579	4.7203
				hsa-miR-618	29.527	hsa-miR-642	41.515
				hsa-miR-95	20.788	hsa-miR-671-3p	8.4425
				hsa-miR-144*	0.2759	hsa-miR-99a	12.218
				hsa-miR-624	0.0304	hsa-miR-144*	0.2905
				hsa-miR-625*	0.0981	hsa-miR-520D-3p	0.2579
						hsa-miR-590-3p	0.4188
						hsa-miR-625*	0.1575
						hsa-miR-629	0.2793

Single TaqMan assay for candidate biomarkers of mTBI

In order to validate these findings, we subsequently tested the expression of selected miRNAs in three separate and independent groups (10 mTBI + EC, 10 sTBI + EC, 10 EC) at the two chosen time points (1 and 15 days from injury) by using single TaqMan assays. The results were compared to 10 HV. The fold changes were calculated by the 2-ΔΔCT method, using miR-331 and miR-223* as reference genes.

Among the candidate biomarkers of mTBI at both time points (miR-184, miR-301b, miR-502, miR-505, miR-203, miR-425-5p, miR-654-3p and miR-655), two showed interesting results and were significantly and differentially expressed in the three different categories compared to HV. In particular, miR-425-5p and miR-502 showed a similar trend (Fig 1). They were both significantly downregulated in mTBI+EC (mean of 0.387±0.201 and 0.314±0.146) respectively at 1 day from injury compared to HV ($p<0.001$), EC ($p<0.001$) and sTBI+EC ($p<0.001$). At 15 days from mild injury, miR425-5p and miR-502 returned back to normal levels (0.886±0.310 and 1.157±0.258). The expression of miR-425-5p and miR-502 was also found similar to HV in EC samples at both 1 day and 15 days from injury, thus suggesting that these two biomarkers are differentially expressed in brain injury patients only. Moreover, neither of them showed any significant difference in sTBI+EC compared to HV at both time points. Therefore, miR-425-5p and miR-502 could be considered the most promising candidate biomarkers for the early diagnosis and monitoring of mTBI at 15 days after trauma. AUCs for these biomarkers are shown in Table 3.

Single TaqMan assay for candidate biomarkers of sTBI

miR-21 and miR-335 were analysed as potential biomarkers of sTBI since they both appeared upregulated at both time points of sTBI+EC in the initial screening. They showed to be strong candidates in the second dataset of patients as well (Fig 2). Mir-21 was significantly upregulated at both time points in sTBI with EC (7.106±4.192 and 4.012±1.577) with respect to HV (p,0.001), EC ($p<0.001$) and mTBI ($p<0.001$). No significant differences were found in the remaining categories compared to HV. miR-335 showed upregulation in sTBI+EC and at both time points (16.824±14.195 and 12.324±8.931, respectively). On day 1, there this group were significantly different from controls ($p=0.001$) and mTBI+EC ($p=0.031$) but not EC. Interestingly, a significant upregulation in EC patients was found at 1 day (7.951±4.870), but not at 15 days from injury (1.260±0.531). For this reason, on day 15, miR335 was significantly higher in the sTBI+EC group with respect to HV ($p=0.002$), EC ($p=0.007$) and mTBI+EC ($p=0.001$). miR-335 did not

show any significant difference in mTBI+EC at both time points compared to HV. AUCs for these biomarkers are also shown in Table 3.

Table 3: Area under the curve

Variable(s)	Area	Asymptotic 95% Confidence Interval	
		Lower Bound	Upper Bound
miR-502 1 day	.993	.972	1.000
miR-502 15 days	Not Significant	-	-
miR-425-5p 1 day	.994	.977	1.000
miR-425-5p 15 days	Not Significant	-	-
miR-21 1 day	.979	.934	1.000
miR-21 15 days	.975	.929	1.000
miR-335 1day	.758	.592	.921
miR-335 15 days	.957	.884	1.000

Discussion

The present study investigated if changes in the levels of miRNAs can be applied to the diagnosis of TBI, and evaluating its severity. Four miRNAs were identified as being differentially expressed in TBI; miR-425-5p, miR 502, miR-21 and miR-335.

miR-425-5p showed significant results at day 1 in mTBI+EC compared to the HV and similar results were obtained in all other categories. Its downregulation within the first 24h from the mild injury and the its return back to normal levels after 15 days, makes miR-425-5p a suitable candidate biomarker of mild trauma.

miR-502 was also found to be differentially expressed in mTBI+EC. The trend of this miRNA was very similar to miR-425-5p. It showed specificity for brain injured patients and could also be used to track recovery, since it returns back to normal value after 15 days form the mild injury.

Following sTBI, 2 miRNAs (miR-21, miR-335) were noted to be expressed at both 1 and 15 days and were thus selected as potential biomarkers for sTBI. miR-21 and miR-335 were significantly up-regulated at both time points when compared with controls in sTBI+EC. Therefore, the overexpression confirmed the results of the array and showed the potential of these molecules as biomarkers of sTBI.

The selected panel of miRNAs have the potential to diagnose TBI accurately and enable the stratification of patients according to severity which, in turn, allows the delivery of the most appropriate treatment.

Example 2

Patients and sample collection

Study participants were recruited from the Surgical Reconstruction and Microbiology Research Centre (SRMRC) at Queen Elizabeth Hospital of Birmingham (UK) as part of SIR (The Steroids and Immunity from injury through to Rehabilitation) study (Ethics Ref. 11/SW/0177), ReCoS (The REpetitive COncussion in Sport) study (Ethics Ref. 11-0429AP28) and Golden Hour study (Ethics Ref. 13/WA/0399). Written informed consent was received from participants or valid proxy (family or a professional not directly involved in the study) prior to inclusion in the study.

The second dataset of samples used serum samples from a total of 120 individuals grouped in 4 different categories: HV (n=30), EC (n=30), mTBI+EC (n=30), sTBI+EC (n=30) and blood was taken at different time points (T0-1h, T4-12h, T48-72h, 15 days) in each patient. Healthy volunteers were consented and enrolled in the ReCoS study. EC injury patients had radiographically-confirmed orthopaedic fractures, no head trauma, no infection, no history of neurological or psychiatric disorders and no alcohol or drug dependency. Mild TBI with EC included those with a non-penetrating head trauma and Glasgow Coma Scale (GCS) score ≥13. Severe TBI with EC included patients with GCS≤8.

Sample processing

The blood samples were processed for serum isolation within 2h after withdrawal. Whole blood was left to stand for about 30' at room temperature before being centrifuged at 3000 rpm for 10' at 4°C. Serum was divided into aliquots and stored at -80°C until analysis.

RNA isolation, data analysis, assays and statistical analysis were carried out as described in Example 1.

RESULTS

Single TaqMan assay for candidate biomarkers of mTBI

In order to validate the findings of Example 1, the expression of selected miRNAs in 3 separate and independent groups (30 mTBI + EC, 30 sTBI + EC, 30 EC) was measured at different time points (T0, T4-12h, T48-72h and 15 days from the injury) by using single TaqMan assays. The results were compared to 10 HV. The fold changes were calculated by the 2-ΔΔCT method, using miR-331 and miR-223* as reference.

Among the candidate biomarkers of mTBI at both time points (miR-184, miR-502, miR-505, miR-301b, miR-203, miR-425-5p, miR-654-3p and miR-655), only two showed interesting results and were significantly and differentially expressed in the 3 different categories compared to HV. Specifically, miR-425-5p and miR-502 showed a similar trend (Figure 3). They were both significantly downregulated in mTBI+EC, miR-425-5p at T0-1h ($p=0.01845$), and at T4-12h ($p=0.01962$) respectively compared to HV, or EC and sTBI+EC ($p<0.05$), and miR-502 at T0-1h and at T4-12h compared to HV ($p=0.02538$ and $p=0.03718$ respectively), or EC and sTBI+EC ($p<0.01$). After 48h from mild injury, miR425-5p and miR-502 returned back to normal levels. The expression of miR-425-5p and miR-502 was also found in EC group at a comparable level to HV, thus suggesting that these two biomarkers are downregulated in brain injury patients only. Moreover, neither of them showed any significant downregulation in sTBI+EC compared to HV at all time points. Therefore, miR-425-5p and miR-502 could be considered the most promising candidate biomarkers for the early diagnosis and monitoring of mTBI. AUCs for these biomarkers at the most relevant time points, are shown in Table 4.

Single TaqMan assay for candidate biomarkers of sTBI

MiR-21 and miR-335 were analysed as potential biomarkers of sTBI since they both appeared upregulated at both time points of sTBI+EC in the initial screening. They showed to be strong candidates in the second dataset of patients also (Figure 4). Mir-21 was significantly upregulated in sTBI with EC at all time points after 4h from injury with respect to HV, EC and mTBI+EC ($p=0.00306$ at T4-12h, $p=0.00844$ at T48-72h and $p=0.00077$ at 15days). No

significant differences were found in the remaining categories compared to HV. MiR-335 showed upregulation in sTBI+EC and at all time points compared to HV (p=0.00109 at T0-1h, p=0.00284 at T4-12h, p=0.00012 T48-72h and p=0.01284 at 15days) and mTBI+EC but not significant upregulation was found compared to EC. AUCs for these biomarkers are also shown in Table 4.

Table 4: Area under the curve of the four candidate biomarkers of TBI at different time points. Only most relevant time points are shown.

Variable(s)	Area	Sig	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
miR-425-5p T0				
mTBI vs HV	1	.002	1	1
mTBI vs sTBI	.911	.020	.778	1.000
mTBI vs EC	.950	.01	.860	1.000
miR-502 T0				
mTBI vs HV	1	.001	1.000	1.000
mTBI vs sTBI	.990	.001	.960	1.000
mTBI vs EC	.990	.001	.940	1.000
miR-21 T4-12h				
sTBI vs HV	.961	.003	.880	1.000
sTBI vs mTBI	.960	.001	.870	1.000
sTBI vs EC	.900	.004	.740	1.000
miR-335 T0				
sTBI vs HV	.990	.000	.960	1.000
sTBI vs mTBI	.780	.023	.590	.960
sTBI vs EC	.500	1	.240	0.780

5

Discussion

This study validated the previous finding that changes in the levels of miRNAs can be applied to the diagnosis of TBI, and evaluating its severity. The study confirmed that the following four miRNAs are differentially expressed in TBI; miR-425-5p, miR 502, miR-21 and miR-335.

miR-425-5p showed significant results at T0 and T4-12h in mTBI+EC compared to the HV and similar results were obtained in all other categories. Its downregulation return back to normal levels after T48-72h, confirms that miR-425-5p a suitable candidate biomarker of mild trauma.

miR-502 was also confirmed to be differentially expressed in mTBI+EC. The trend of this miRNA was very similar to miR-425-5p. It showed specificity for brain injured patients and could also be used to track recovery, since it returns back to normal value after T48-72h form the mild injury.

Following sTBI, 2 miRNAs (miR-21, miR-335) were noted to be expressed at all time points analysed and were thus confirmed as potential biomarkers for sTBI. miR-21 and miR-335 were significantly up-regulated when compared with controls in sTBI+EC. Therefore, the overexpression confirmed the potential of these molecules as biomarkers of sTBI.

Example 3

Saliva samples were collected from concussed professional athletes after 2-3 days from injury and the microRNAs present in the saliva was analysed. The athletes were diagnosed clinically as having mTBI.

Materials and methods

MicroRNAs were analysed using nCounter technology (nanoString Technologies®), which uses molecular "barcodes" and microscopic imaging to detect and count up to several hundred unique transcripts in one hybridization reaction. Each colour-coded barcode is attached to a single target-specific probe corresponding to a microRNAs of interest.

Analysis was carried out according to the manufacturer's protocol which includes the following steps:

Hybridization: The technology employs two ~20-base probes per microRNA that hybridize in solution. A reporter probe carries the signal, while a capture probe allows the complex to be immobilized for data collection.

Purification and Immobilization: After hybridization, the excess probes are removed and the probe/target complexes are aligned and immobilized in the nCounter Cartridge.

Data Collection: Sample cartridges are placed in a digital analyzer instrument for data collection. Colour codes on the surface of the cartridge are counted and tabulated for each target molecule.

RESULTS

Table 5 below is a list of microRNAs which were found to be significantly and differentially expressed in concussed athletes compared to healthy volunteers. The table shows the fold change in expression of the microRNAs in the patients compared to the control group. The fold changes were calculated using miR-23a-3p and miR-148b-3p as reference genes.

Table 5

microRNA	Fold change
hsa-let-7c-5p	2.80
hsa-let-7i-5p	1.82
hsa-miR-142-3p	1.41
hsa-miR-148a-3p	1.72
hsa-miR-15b-5p	2.30
hsa-miR-16-5p	2.32
hsa-miR-181a-5p	1.54
hsa-miR-20a-5p+hsa-miR-20b-5p[§]	2.16
hsa-miR-221-3p	2.90
hsa-miR-24-3p	1.94
hsa-miR-27b-3p	3.45
hsa-miR-29a-3p	4.03
hsa-miR-29c-3p	1.44
hsa-miR-424-5p	2.88
hsa-miR-30a-5p	2.16
hsa-miR-107	1.72
hsa-miR-135b-5p	1.97
hsa-miR-199b-5p	1.88
hsa-miR-324-5p	5.61
hsa-miR-652-3p	3.43

[§] the values for miR-20a-5p and miR-20b-5p were combined due to the nCounter technology not being able to distinguish between them.

Discussion

This study shows that microRNAs present in saliva are an indicator of concussion/mTBI. This is significant because saliva is more easily obtained than blood, and thus detection of salivary microRNAs offers a rapid and convenient means for diagnosing TBI, particularly pitch-side.

Claims

1. A method of

i) diagnosing mild traumatic brain injury (mTBI) in a subject, the method comprising determining the level of miR-502 in a fluid sample obtained from the subject up to 48 hours from injury; and/or

ii) monitoring mTBI in a subject, the method comprising determining the level of miR-502 in a fluid sample obtained from the subject at least 2 days after injury,

wherein the fluid sample comprises serum.

2. The method of claim 1, wherein the method further comprises comparing the level of miR-502 with a predetermined threshold or a reference level.

3. The method of claim 2, wherein the reference level is the level of a reference miRNA in a control sample taken from a healthy subject or the level of a reference miRNA in a sample taken from the subject.

4. The method of any one of claims 1 to 3, further comprising determining the level of at least one further miRNA, wherein the at least one further miRNA is selected from the group consisting of:

(a) miR-425-5p;

(b) miR-425-5p, miR-21, and miR-335, or any combination thereof;

(c) miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-21, miR-let-7g, miR-335, miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, miR-671-3p, hsa-let-7c-5p, hsa-let-7i-5p miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, miR-424-5p, miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p; miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629, or any combination thereof;

(d) miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-21, miR-let-7g, miR-335, hsa-miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, and miR-671-3p, or any combination thereof; and

(e) miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-21, miR-let-7g and miR-335, or any combination thereof.

5. The method of claim 4, comprising determining the level of two, three, four, or more miRNAs.

6. The method of any one of claims 1 to 5, wherein the subject is diagnosed as having mTBI if the level of miR-502 is determined to be below a predetermined threshold or decreased relative to a reference.

7. The method of any one of claims 1 to 6, wherein the method comprises determining the level of:

(i) miR-502; and

(ii) a second miRNA selected from miR-21 and miR-335.

8. The method of claim 7, wherein the subject is diagnosed as having a TBI of any severity if the level of miR-502 is determined to be below a predetermined threshold, or is decreased relative to a reference, and the level of miR-21 or miR-335 is determined to be above a predetermined threshold, or is increased relative to a reference.

9. The method of any one of claims 1 to 8, wherein the fluid sample comprising serum was obtained from the subject:

(a) no more than 48 hours after injury; or

(b) no more than 24 hours after injury.

10. Use of a sensor element in a detection system in the method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) according to any one of claims 1 to 9, the sensor element comprising a substrate functionalized with at least one probe specific for miR-502, wherein the probe comprises a nucleic acid, and wherein the nucleic acid comprises a sequence which is at least 80% identical to a sequence which is the complement of SEQ ID NO: 3.

11. The use of claim 10, wherein the substrate is formed from metal, plastic, glass, silica, silicon, graphite or graphene, or any combination thereof.

12. Use of a detection system in the method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) according to any one of claims 1 to 9, the detection system comprising:

- a sensor element described in claim 10 or claim 11; and
- a detection device that is capable of detecting the binding of a target miRNA to the probe.

13. Use of a kit in the method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) in a fluid sample from a subject according to any one of claims 1 to 9, the kit comprising at least one probe specific for miR-502, wherein the fluid sample comprises serum, wherein the probe comprises a nucleic acid, and wherein the nucleic acid comprises a sequence which is at least 80% identical to a sequence which is the complement of the sequence of SEQ ID NO: 3.

14. A method of treating mild traumatic brain injury (mTBI) in a subject that has been identified using the method of any one of claims 1 to 9 as having mTBI by determining the level of at least one miRNA in a fluid sample from the subject obtained from the subject up to 48 hours from injury, wherein the fluid sample comprises serum, and wherein the at least one miRNA comprises miR-502, the method comprising administering a neuroprotective drug or a sedative to the subject.

15. Use of a neuroprotective drug or a sedative in the manufacture of a medicament for treating mild traumatic brain injury (mTBI) in a subject that has been identified using the method of any one of claims 1 to 9 as having a mTBI by determining a level of at least one miRNA in a fluid sample from the subject obtained from the subject up to 48 hours from injury, wherein the fluid sample comprises serum, and wherein the at least one miRNA comprises miR-502.

16. Use of a composition in the method for diagnosing and/or monitoring mild traumatic brain injury (mTBI) in a subject according to any one of claims 1 to 9, the composition comprising at least one probe specific for miR-502, wherein the probe comprises a nucleic acid, and wherein the nucleic acid comprises a sequence which is at least 80% identical to a sequence which is the complement of the sequence of SEQ ID NO: 3.

17. Use of:

the sensor element described in any one of claims 10 to 12;
the detection system described in any one of claims 10 to 12;
the kit described in claim 13;
the medicament described in claim 15; or

the composition described in claim 16,

wherein the use further comprises at least one further probe specific for a target miRNA, wherein the miRNA is selected from the group consisting of:

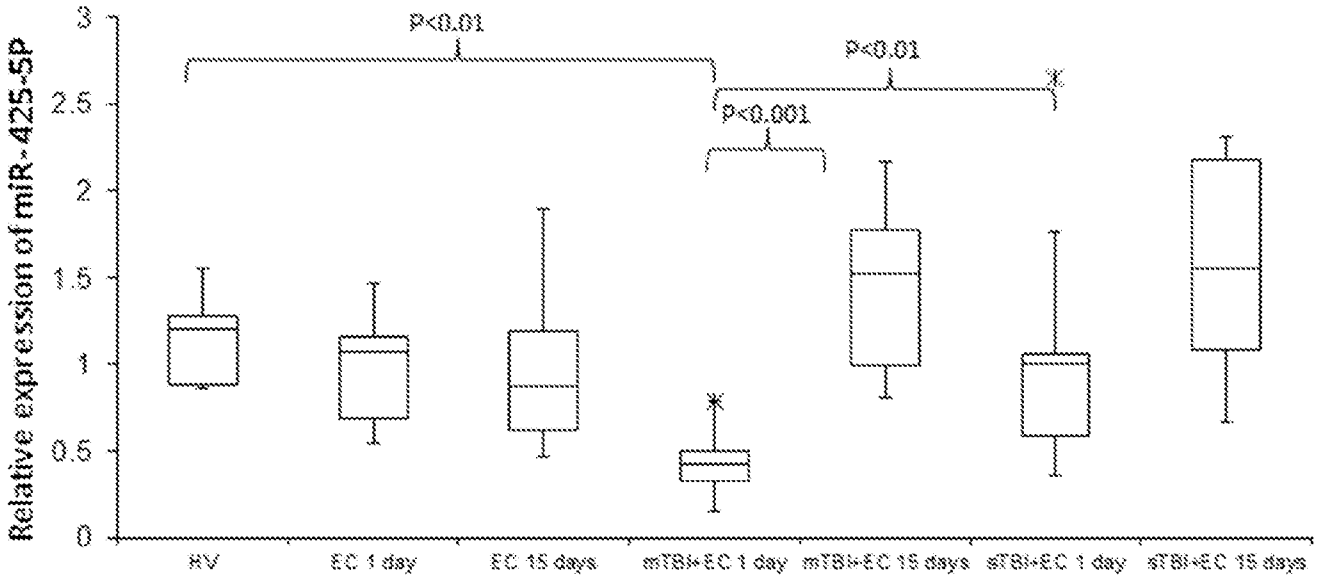
(a) miR-425-5p;

(b) miR-425-5p, miR-21, and miR-335, or any combination thereof; and

(c) miR-505, miR-203, miR-654-3p, miR-655, miR-184, miR-301b, miR-425-5p, miR-21, miR-let-7g, miR-335, miR-126*, miR-193a-5p, miR-144*, miR-190, miR-194, miR-365, miR-590-3p, miR-624, miR-625*, miR-671-3p, hsa-let-7c-5p, hsa-let-7i-5p, miR-142-3p, miR-148a-3p, miR-15b-5p, miR-16-5p, miR-181a-5p, miR-20a-5p, miR-20b-5p, miR-221-3p, miR-24-3p, miR-27b-3p, miR-29a-3p, miR-29c-3p, miR-30a-5p; miR-107; miR-135b-5p; miR-199b-5p; miR-324-5p; miR-652-3p, miR-424-5p, miR-10a, miR-132, miR-223, miR-143, miR-148b, miR-18a, miR-192, miR-429, miR-618, miR-95, miR-130a, miR-152, miR-27b, miR-301, miR-326, miR-345, miR-361, miR-422a, miR-579, miR-642, miR-99a, miR-520D-3p and miR-629, or any combination thereof.

Figure 1

A


Relative expression of miR-425-5P
P<0.01
P<0.01
P<0.001
3
2.5
2
1.5
1
0.5
0
HV
EC 1 day
EC 15 days
mTBI+EC 1 day
mTBI+EC 15 days
sTBI+EC 1 day
sTBI+EC 15 days

B

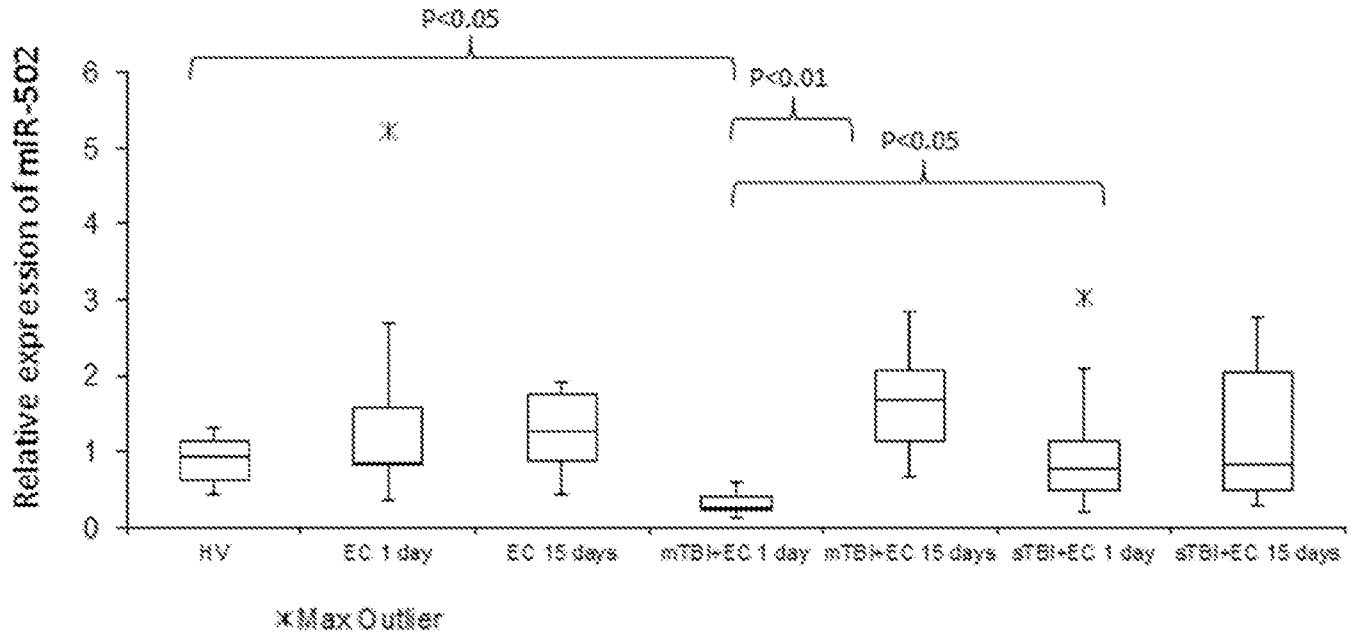
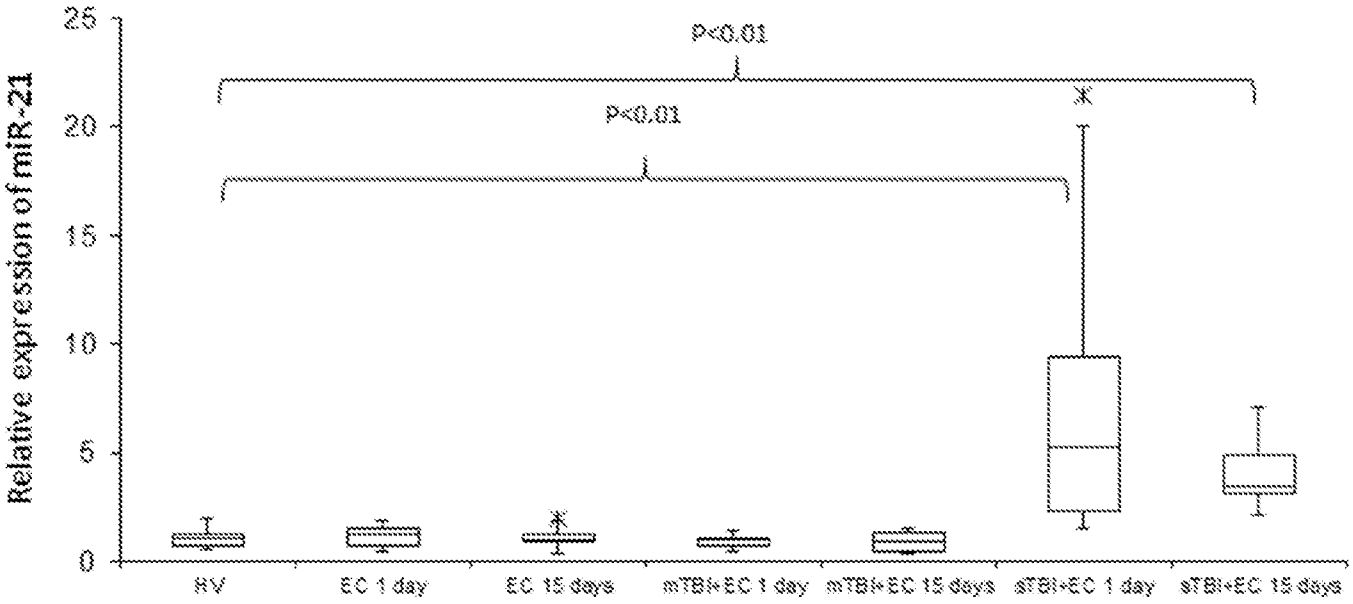

Relative expression of miR-502
P<0.05
P<0.01
P<0.05
6
5
4
3
2
1
0
HV
EC 1 day
EC 15 days
mTBI+EC 1 day
mTBI+EC 15 days
sTBI+EC 1 day
sTBI+EC 15 days
Max Outlier

Figure 2

A


Relative expression of miR-21
25
20
15
10
5
0
P<0.01
P<0.01
RV
EC 1 day
EC 15 days
mTBI+EC 1 day
mTBI+EC 15 days
sTBI+EC 1 day
sTBI+EC 15 days

B

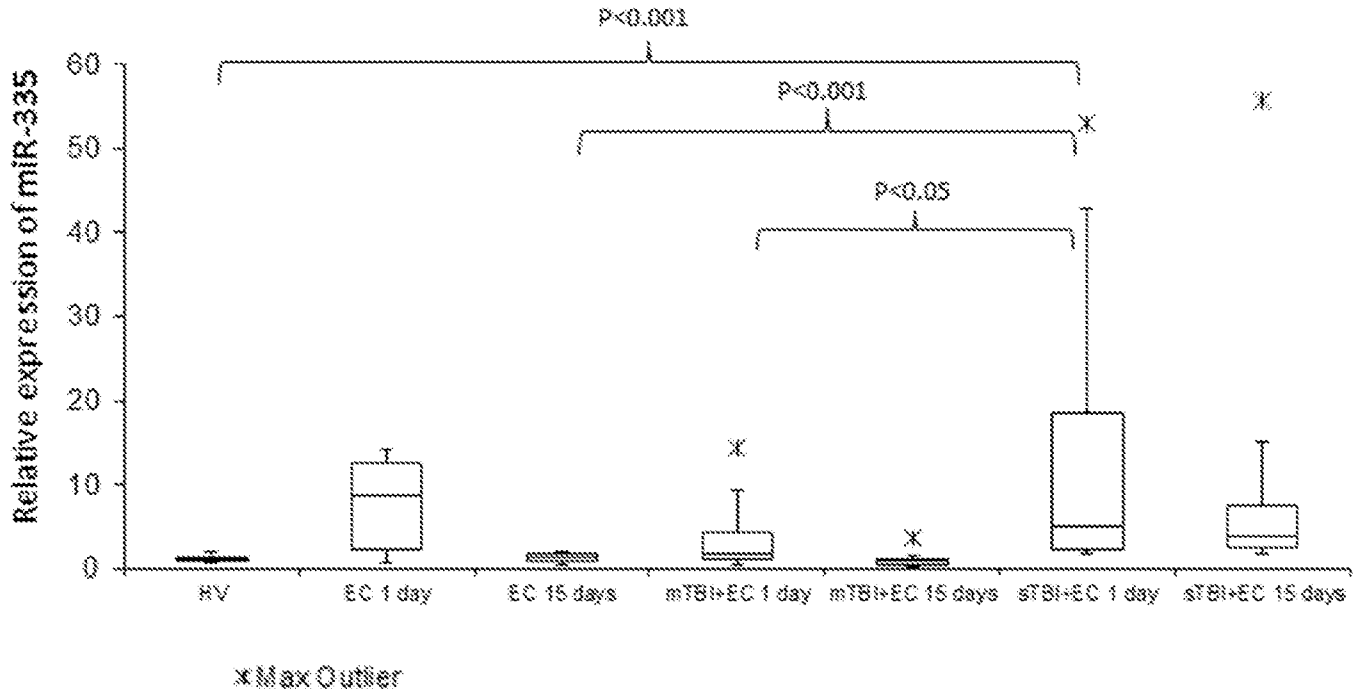

Relative expression of miR-335
60
50
40
30
20
10
0
P<0.001
P<0.001
P<0.05
RV
EC 1 day
EC 15 days
mTBI+EC 1 day
mTBI+EC 15 days
sTBI+EC 1 day
sTBI+EC 15 days
Max Outlier

Figure 3A

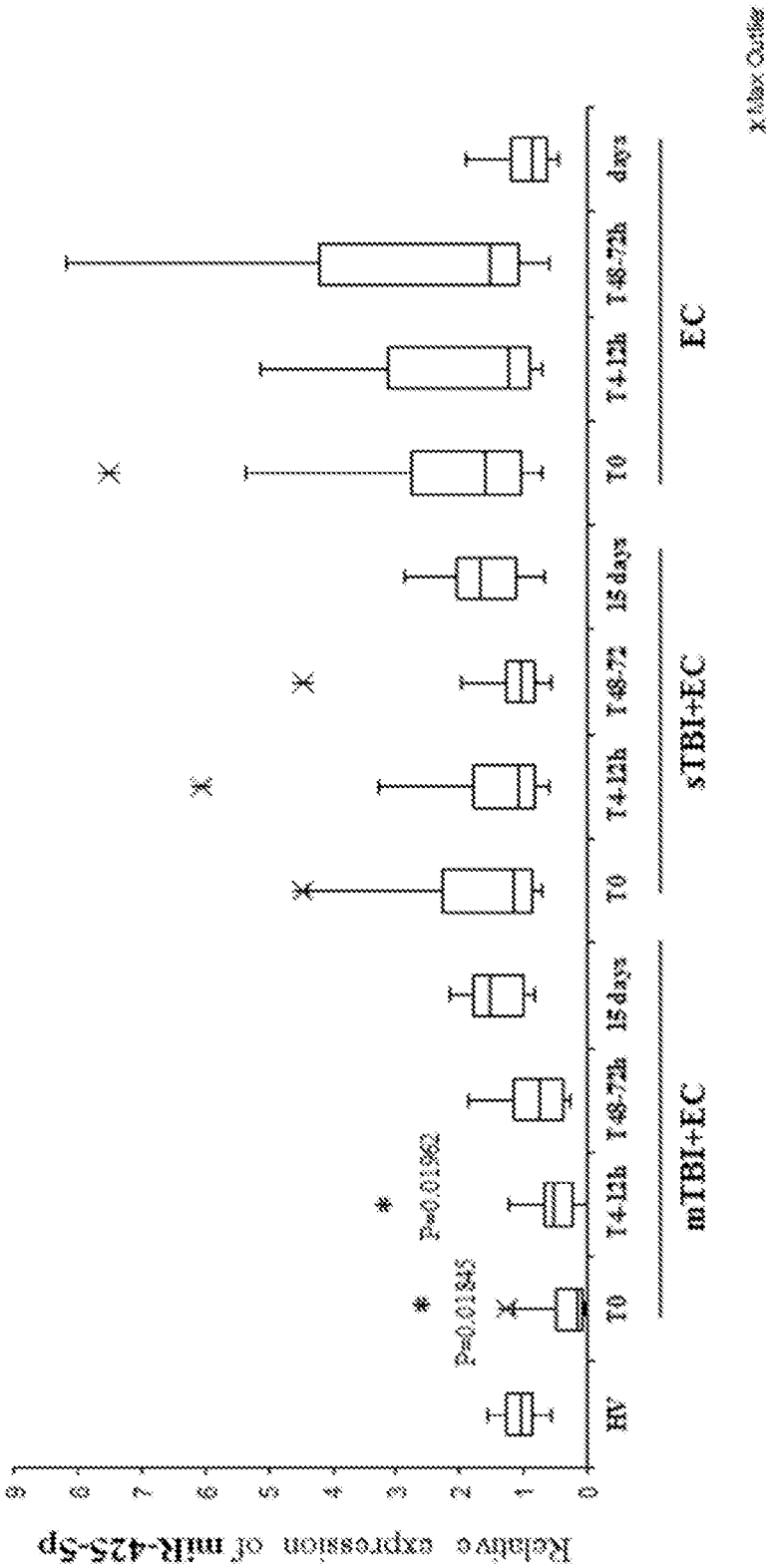

x Max Outlier
Relative expression of miR-425-5p
RV
T0
T4-12h
T48-72h
15 days
T0
T4-12h
T48-72
15 days
T0
T4-12h
T48-72h
4wks
mTBI+EC
sTBI+EC
EC
P=0.0183
P=0.01862

Figure 3B

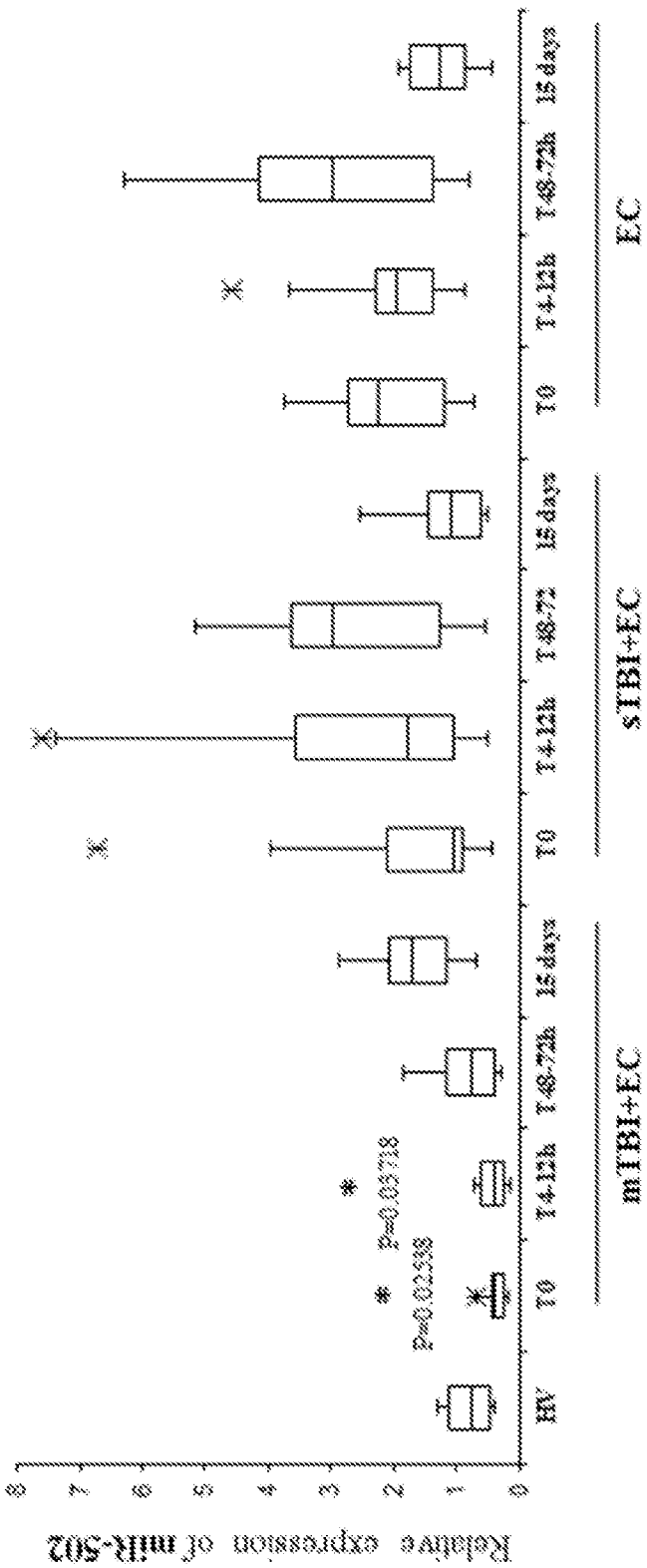

Relative expression of miR-502
HV
T0
T4-12h
T48-72h
15 days
T0
T4-12h
T48-72
15 days
T0
T4-12h
T48-72h
15 days
mTBI+EC
sTBI+EC
EC
P=0.05718
P=0.02538

Figure 4A

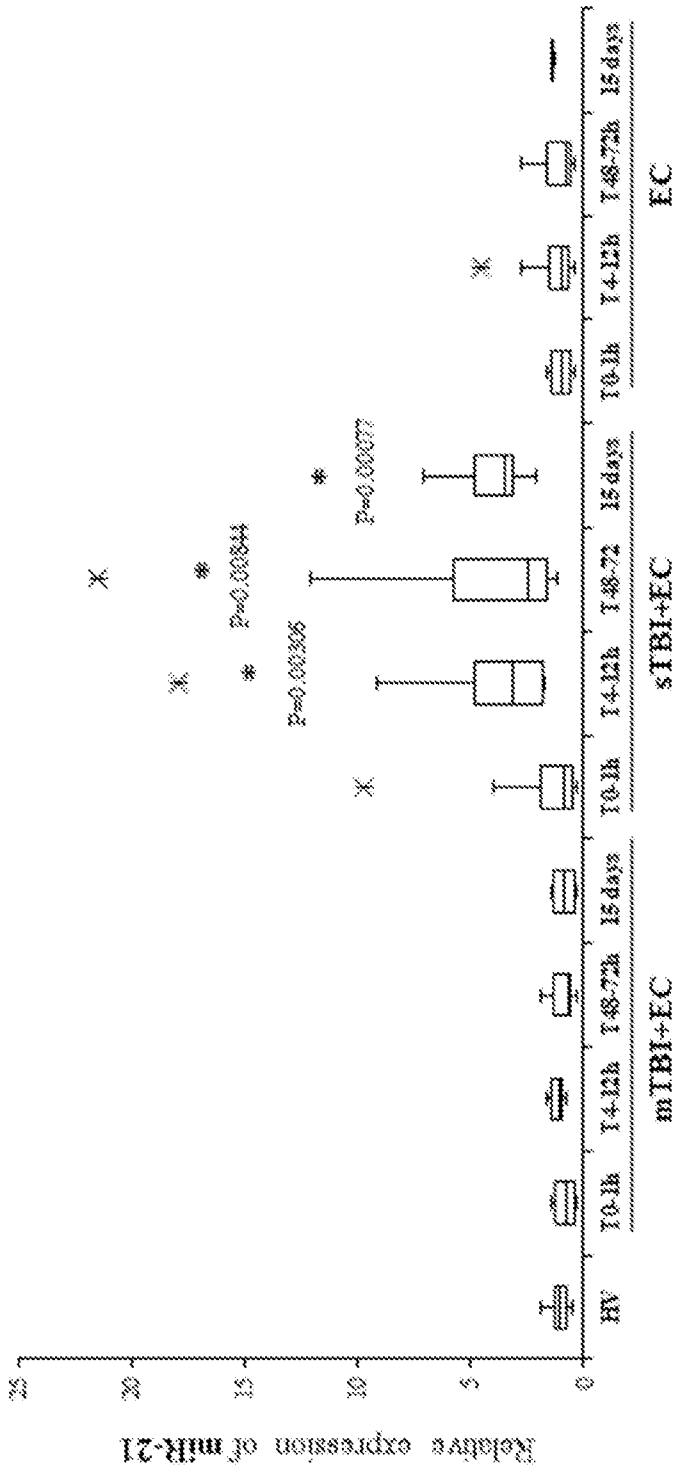
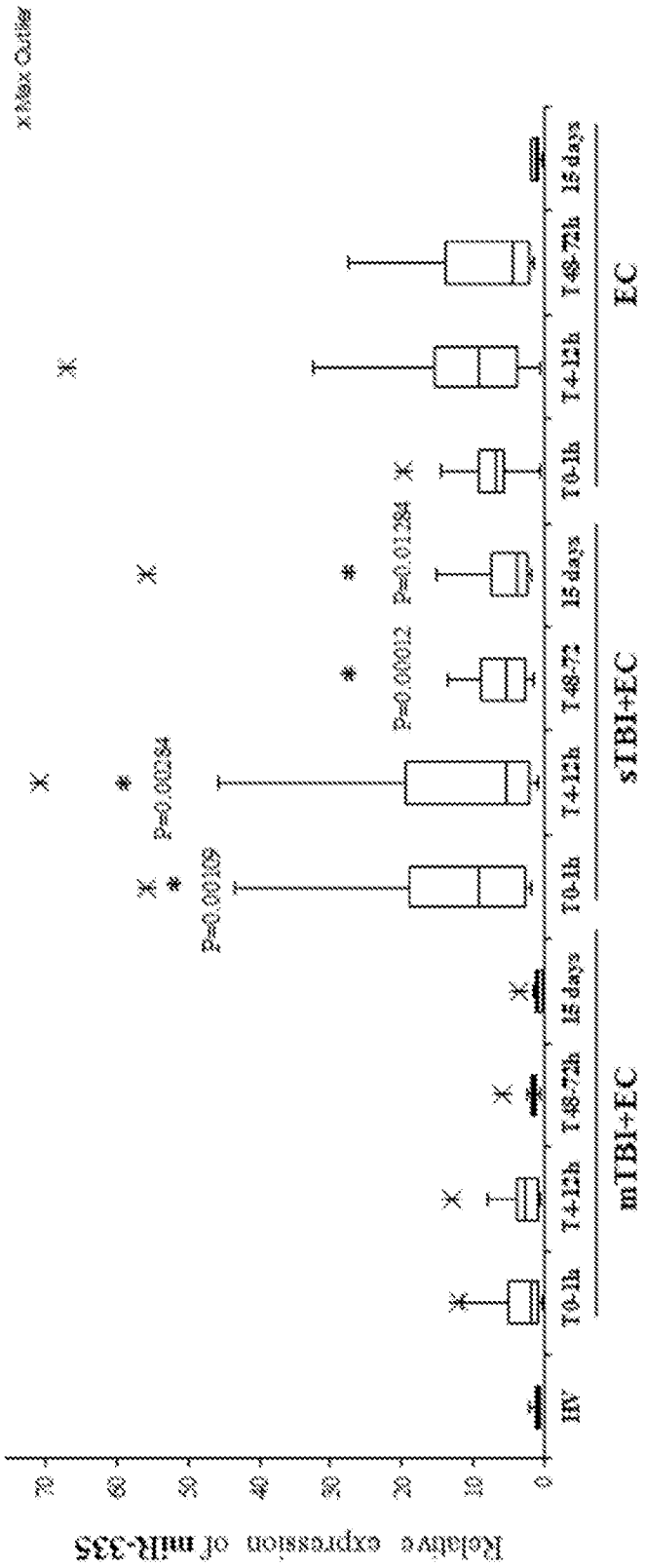

Relative expression of miR-21
HV
T0-1h
T4-12h
T48-72h
16 days
mTBI+EC
T0-1h
T4-12h
T48-72
16 days
sTBI+EC
T0-1h
T4-12h
T48-72h
16 days
EC
P=0.0006
P=0.0084
P=0.0077

Figure 4B


x Max, Outlier
P=0.00308
P=0.00284
P=0.00012
P=0.01284
Relative expression of miR-335
0
20
40
60
80
100
HV
T0-1h
T4-12h
T48-72h
15 days
mTBI+EC
T0-1h
T4-12h
T48-72
15 days
sTBI+EC
T0-1h
T4-12h
T48-72h
15 days
EC