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## (54) Title: IL-10, S100B AND H-FABP MARKERS AND THEIR USE IN DETECTING TRAUMATIC BRAIN INJURY

Single analytes and panels to rule out CT-  
SCAN in mTBI

Geneva-Sevilla only molecules		
S100B	Pat -/+	111/22
	Mann U	0.000
	SP%	18.0
H-FABP	Pat -/+	111/22
	Mann U	0.013
	SP%	27.9
IL-10	Pat -/+	111/22
	Mann U	0.000
	SP%	27.0

Geneva-Sevilla panels with IL-10		
Panel IL-10+S100B+H-FABP+age	Pat -/+	111/22
	Pos	3
	SP%	56.8
Panel IL-10+H-FABP+age	Pat -/+	111/22
	Pos	2
	SP%	43.2
Panel S100B+IL-10+age	Pat -/+	111/22
	Pos	2
	SP%	49.5
Panel S100B+H-FABP+IL-10	Pat -/+	111/22
	Pos	3
	SP%	47.7
Panel S100B+IL-10	Pat -/+	111/22
	Pos	2
	SP%	38.7
Panel H-FABP+IL-10	Pat -/+	111/22
	Pos	2
	SP%	43.2
Panel IL-10+age	Pat -/+	111/22
	Pos	1
	SP%	27

Fig. 1

(57) Abstract: The invention relates to IL-10, S100B or H-FABP as biomarkers or a combination of these biomarkers and their use in detecting traumatic brain injury or mild traumatic brain injury (mTBI).

IL-10, S100B AND H-FABP MARKERS AND THEIR USE IN DETECTING TRAUMATIC BRAIN INJURY

The invention relates to biomarkers and novel biomarkers, their use in diagnostics of brain injury or brain related injuries, in particular mild traumatic brain injury (mTBI), and methods as well as devices for the detection of same in an individual.

### **Background of the Invention**

Brain injuries have a high incidence worldwide. In particular mild traumatic brain injury (mTBI) has a significant incidence in the world and is responsible for high health cost. In contrast to severe TBI, mTBI is not obvious to detect and thus usually a computer tomography (CT) scan is performed before significant brain injury can be ruled in or out.

So far, it is still a challenge identifying which patients with a number of neurological injuries and in particular a mild traumatic brain injury (mTBI), can be safely sent home. Computer tomography (CT) scan is thus the main tool today to detect a cerebral lesion in these patients. However, many of the scans are negative and cost-intensive. Therefore, in any clinical decision rules for mild TBI, defined as presenting with a Glasgow Coma Score (GCS) (Jennet and Teasdale, 1978) of 13-15, rapid and reliable identification of patients with intracranial lesions is critical to avoid post-traumatic complications and minimize secondary brain damages (Graham, et al., 1998). Several studies aimed to first screen all mild TBI-patients with a simple blood test to reduce the number of unnecessary CT scans and discharge patients faster have been reported (Berger et al., 2007; Poli-de-Figueiredo et al., 2006). In the last years, especially S100B was extensively investigated as potential promising marker for mTBI and is highly promoted by companies. Nevertheless, its clinical utility remains controversial. In mild TBI adults, S100B below a cut-off of 0.10 µg/L was described to allow a maximal reduction of only 30% in CT scans. S100B failed to be a relevant prognostic marker for paediatric TBI patients, estimated only as adjunct in determining children with low-risk TBI (Tavarez et al., 2012).

A mild traumatic brain injury (mTBI, also called concussion, minor head trauma, and minor brain/head injury) is a type of closed head injury, defined as the result of a blunt trauma or acceleration/deceleration forces causing a brief change in mental status (confusion, disorientation or loss of memory) or loss of consciousness for less than 30 minutes. Usually, loss of consciousness is very brief and ranges between a few seconds to minutes. Mild TBI remains the biggest percentage of all closed head, brain injury cases admitted to the hospitals. Currently, the primary criterion for evaluating patients with TBI in clinical setting is the Glasgow Coma Scale (GCS), which assesses the level of consciousness following TBI. A mild traumatic brain injury is most likely to be diagnosed only when there is a change in the mental status at the time of injury or hospital admission (the person is dazed, confused, or loses consciousness, GCS score 13-15). In US 10% of head injury patients are classified at admission as having severe (GSC below 8), 10% as moderate (GCS 9-12), and remaining 80% as mild TBI (GCS 13-15) (Narayan RK, Michel ME, et al, J Neurotrauma., 2002). Similar proportions are indicated by World Health Organization in Europe, that estimated 70 to 90% of treated head injuries are classified to present as mild (Cassidy JD et al, 2004, Journal of Rehabilitation Medicine). It remains a public health problem as 10% of patients with mTBI can suffer long-term disabilities such as headache, fatigue, difficulty thinking, memory problems, attention deficits, mood swings, sleep disorders, frustration and even epileptic events (Jallo and Narayan, 2000; Narayan et al, 2002). Due to the complicated etiology it remains challenging to identify which patients with mTBI can be safely sent home without the need for treatment intervention (Jagoda et al., 2008). Currently to counter-act possible post-traumatic complications and secondary brain damages mTBI patients are further diagnosed with tools such as computerized tomography (CT) scans and magnetic resonance imaging (where available) (Graham et al., 1998). In the group of patients with mTBI only 3 to 19% present with an abnormal CT result revealing an acute intracranial lesion in patients (Jagoda et al., 2008; Bazarian et al., 2006; Borg et al., 2004). The other 80%+ of these scans show normal head CT, indicating no complications from injury, and as such are not cost effective and are time-consuming for both patient and medical staff.

The use of biomarkers has been proposed as a means to reduce the amount of unnecessary CT scans (Berger et al. 2007; Poli-de-Figueiredo et al., 2006) and for use in decentralized sites where access to CT equipment is absent. However so far no biomarker-assay is available which gives test results capable of properly classifying the majority of patients and therefore useful in serial screening.

As described the method of choice today is a CT scan due to the insufficient reliability and high percentage of false negative results with known biomarkers for TBI detection. One such known biomarker is S100B.

In recent years, S100B has been extensively investigated as a potential promising blood marker for mTBI (Ruan S et al., 2009; Goyal et al. 2013). Nevertheless, its clinical utility remains controversial. In mTBI adults, S100B below a cut-off of 0.1µg/L was described to allow a maximal reduction of 30 % in CT scans (ref from researchers). S100B failed to be a relevant prognostic marker for pediatric TBI patients, estimated only as adjunct in determining children with low-risk TBI (Tavarez et al., 2012; Filippidis et al., 2010).

S100B is a low-molecular-weight (9–13 kDa), non-ubiquitous Ca<sup>2+</sup>-modulated protein implicated in e.g. regulation of enzyme activities, dynamics of cytoskeleton elements, cell growth and differentiation and Ca<sup>2+</sup> homeostasis (Donato R., 2003). In the central nervous system (CNS) it is primarily found and secreted by glial cells (Donato R., 2003). Due to its involvement in calcium homeostasis it has neuroprotective function e.g. prevents mitochondrial failure and cell death in the absence of glucose by increasing cellular calcium concentrations (Barger et al, 1995) or promote neurite outgrowth and astrocytic proliferation (Reeves et al, 1994). Significantly increased S100B levels are associated with severe TBI and may reflect ongoing structural damage and cell death after injury (Ingebrigtsen et al. 2002, Missler et al, 1999).

In an injury like mTBI one cannot risk a significant percentage of false negatives in view of the detrimental consequences if a patient exhibiting mTBI would be allowed to leave the hospital and suffer serious complications, or even death thereafter due to a wrong diagnosis. Thus the cut-offs defined for such tests need to be biased towards very high specificity (close to 100%) which can result in a very low sensitivity in consequence. This limitation has made the known individual biomarkers for mTBI not feasible for routine diagnostics in a clinical setup.

In view of the cost pressure in healthcare and the high cost of a CT scan it is highly desirable and a long felt need to find alternative, reliable and cost-effective routes of classifying a potential mTBI patient.

Thus one object underlying the present application is to provide for alternative or new feasible biomarkers for the detection or/and classifying of any brain related traumatic state and in particular for mTBI, and for assays and devices useful and reliable therefore

and in mTBI diagnostics which can be used in a clinical or non-clinical context, or to improve known approaches to neurological or mTBI screening and analysis.

### **Summary of the Invention**

In one aspect the invention provides a method, composition, kit, assay for the classifying or detection of brain injuries or disorders or diseases like TBI, transient ischemic attack, brain tumors, seizures, epilepsy, cerebral abscess, encephalopathies and multiple sclerosis by use of the biomarker IL10 or a combination of markers like IL10, fatty-acid-binding protein (FABP), glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neuromodulin (GAP43), neurofilament protein H (NFH), neurofilament protein M (NFM), neurofilament protein L (NFL), S100B, Tau, spectrin breakdown products, ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1) and vascular cell adhesion protein 1 (VCAM).

In another aspect the present invention provides the biomarker IL10, or a combination of a selection of blood brain biomarkers IL10, optionally combined to the known (and not sufficiently specific) S100B for reliable detection of brain injury like a disease or disorder selected from the group consisting of TBI, transient ischemic attack, brain tumors, seizures, epilepsy, cerebral abscess, encephalopathies and multiple sclerosis in a sample and in particular for mTBI detection. In particular these biomarkers were combined and adjusted in panels yielding specificity above 50% at a fixed sensitivity of 95% or 100%. This will allow clinicians and also on site medical emergency staff to better manage the detected diseases or disorders and in particular mild TBI patients and therefore potentially reduce CT scans but also the consequences associated with a delayed diagnosis of brain injury.

In another aspect the invention concerns a combination of at least two biomarkers wherein the biomarkers are selected from IL10, S100B, GFAB, HFABP, and UCHL-1 or fragments, variants or mutants thereof.

In another aspect the invention concerns devices, e.g. biomarker panels, to detect early traumatic brain injury (TBI) lesions better or complement S100B to rule out CT scans in mild TBI patients.

In other aspects the invention relates to methods and devices for the detection of a medical condition in a patient like TBI and mTBI.

In another aspect the invention relates to a method and devices making use of an algorithm to detect a medical condition of an individual in a sample.

### **Brief Description of the Figures**

Fig. 1 shows the results of the specificity comparison of IL-10 (27%), H-FABP (27%) and S100b (18%). The sensitivity has been set at 100%. This confirms previous results on H-FABP that has a better performance than S100b to rule-out negative CT patients but also the capacity of IL-10 to over perform S100b. When molecules are combined into panels of three molecules, the specificity increases up to 57% (S100b, HFABP, IL-10 and age) and up to 49% when two molecules are combined (S100b, IL-10 and age).

Fig. 2 shows the result of IL10 (specificity 27%) for a 100% sensitivity in a cohort of mTBI patients.

Fig. 3 shows the result of IL10 (specificity 28%) for a 100% sensitivity in a cohort of mTBI patients.

Fig. 4 shows the result of S100b (specificity 28%) for a 100% sensitivity in a cohort of mTBI patients.

Fig. 5 shows the result of the combination of S100b and IL10 (specificity 39%) for a 100% sensitivity in a cohort of mTBI patients.

Fig. 6 shows the result of the combination of HFABP and IL10 (specificity 43%) for a 100% sensitivity in a cohort of mTBI patients.

Fig. 7 shows the result of the combination of S100b and HFABP (specificity 46%) for a 100% sensitivity in a cohort of mTBI patients.

Fig. 8 shows the result of the combination of S100b, HFABP and IL-10 (specificity 57%) for a 100% sensitivity in a cohort of mTBI patients.

### Detailed Description of the Invention

The invention relates to a method for screening for a disease or disorder selected from the group consisting of TBI, transient ischemic attack, brain tumors, seizures, epilepsy, cerebral abscess, encephalopathies and multiple sclerosis in a specimen (sample) comprising the steps of using a sample under suitable conditions and detecting IL10 or at least two biomarkers under suitable conditions wherein the biomarkers are selected from the group consisting of IL10, S100B, H-FABP, fatty-acid-binding protein (FABP), glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neuromodulin (GAP43), neurofilament protein H (NFH), neurofilament protein M (NFM), neurofilament protein L (NFL), S100B, Tau, spectrin breakdown products, ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1 and, vascular cell adhesion protein 1 (VCAM).

The invention has the potential to make a world broad impact on the clinical practice in the management of brain injuries and in particular mTBI. The invention is feasible to provide for a diagnostic panel of markers that can be easily used and are reliable and safe.

The results described below on IL10, S100B, GFAP, UCHL-1 and H-FABP panels of at least two of these molecules highlight that a point of care test (POCT) or an array can be readily used for diagnostic purposes. Traumatic brain injury (TBI) is the leading cause of death and disability in adults younger than 40 years and in children worldwide. Accurate determination of the initial brain damage after brain injury is crucial in establishing a neurologic prognosis and to balance risks and benefits of treatment options. The invention advantageously provides for such a tool and method.

In a preferred embodiment the invention relates to a method wherein the biomarkers are selected from IL10, S100B, GFAP, UCHL-1 and H-FABP or fragments, variants or mutants thereof. Particularly useful is the combination of two or three markers.

Unexpectedly and surprisingly the inventors could show that by a combination of at least two markers of the invention a reliable and easy to use method can be provided to reliably analyse a specimen and thereby rule out brain injury complications in an individual characterized by mTBI and thereby to avoid costly CT scans or even transportation to a centre capable of performing such.

In particular the present invention shows that blood IL-10 alone or in combination with

other known TBI biomarkers, e.g. H-FABP, S100b, GFAP, UCHL-1 and clinical data (GCS, age) will allow clinicians to better manage mild TBI patients and therefore potentially reduce CT scans (specificity around 60% for a sensitivity of 100%).

It was not predictable that the markers of the invention and the particular selection of certain markers or/and the combination of certain markers could be applied to provide for a reliable and specific method to assay for the brain injuries or disorders or diseases or medical complications as described herein and particularly TBI and mTBI. The invention will have not only a positive impact on cost in such analysis and specifically mTBI analysis but represents also an easy to use and fast method in this medical area.

In particularly the invention is advantageous in that it provides for an improved specificity (>50%) for 100% sensitivity to rule out CT scans in brain injuries or other complications and mild TBI patients using a panel of IL10, or at least two markers within IL10, S100B, GFAP, HFABP and UCHL-1.

A direct comparison of available state of the art markers with the invention makes it apparent how advantageous the invention is: with a panel of at least 2 new biomarkers according to a preferred embodiment of the invention, whereby sensitivity is 100%, 56% of the mTBI could be directly discharged compared with 18% for individual S100B marker analysis.

Another advantageous embodiment is a combination with the molecule markers as described herein with other markers like age or GCS. In particular such an application and use of the markers according to the invention in a particular patient group characterized by GCS score of 13 or more or 15 or more, or in a particular age group, e.g. 60 years or more, 65 years or more, 70 years or more will yield very positive and highly reliable test results. More so, in this manner the invention positively achieves that reliable test results can be achieved by the use of less molecular markers which does not only have technical advantages but is also advantageous from a cost point of view.

The invention provides the unexpected advantage that a brain injury and the particular medical complications as described herein can be identified and screened for not only in a fully equipped hospital but by use of a simple test device anywhere and without the use of qualified medical personnel.

In the following certain terms of the invention will be defined in more detail.



“Brain injury” is any state of a patient or individual which is the cause of sudden impact on the head or the individual. A particular brain injury is TBI or mTBI.

“TBI” in the sense of the invention is any brain injury caused by a traumatic incident as described above with reference to the prior art.

“Identification” or “identify” or “classify” in the sense of the invention is the analysis of a sample of an individual to assess whether the individual has a brain injury and particularly TBI or mTBI; the identification of e.g. TBI and mTBI can be verified by use of a CT scan or MRI analysis.

“Diagnostic method” or “diagnostic” in the sense of the invention is any useful method with a suitable sequence of method steps for the detection, visualization and/or quantification of the test result generally known in the art.

“Assay” in the sense of the invention is any method generally known in the art to detect TBI or mTBI like ELISA or any other standard methods for detection of biomarkers.

“Device” in the sense of the invention is a combination of the biomarkers or panel of biomarkers according to the invention that can be used to perform an assay for TBI or mTBI detection. Examples are carrier plates, test stripes, biochip arrays or the like known in the art.

“Marker” or “biomarker” or “molecular marker” or “molecular biomarker” in the sense of the invention is any useful biomarker to detect in a sample of preferably blood, plasma, saliva, tears, CSF or urine a brain injury, preferably traumatic brain injury (TBI) or other disorders as described below; preferably the combination of at least two markers is suitable to detect mild TBI (mTBI); the markers are used in a suitable assay setup wherein preferably the selectivity is set to 100% and the specificity is preferably more than 40%, even more preferred more than 50%, more than 55%, more than 58%, 60% or 70%.

As a marker in the sense of the invention qualifies any marker of glial cells, neuronal cells, or vascular cells. Preferred markers of the invention are:

IL10 (Interleukin-10)

Fatty-acid-binding proteins (FABPs)

Glial fibrillary acidic protein (GFAP)  
Neuron-specific enolase (NSE)  
Neuromodulin (GAP43)  
Neurofilament protein H (NFH)  
Neurofilament protein M (NFM)  
Neurofilament protein L (NFL)  
S100B  
TAU  
Spectrin breakdown products  
Ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1)  
Vascular cell adhesion protein-1 (VCAM)

In addition to the “markers” as described above it is also within the scope of the invention that one, two, three or even more markers can be combined with defining the patient or individual by age or GCS score. Age and GCS can thus be denoted as “marker” in the sense of the invention. Such markers like age and GCS can also be used in the sense of the invention to define a patient subgroup or subgroup of individuals. A preferred age group is below 50, 60, 70 or more than 50, 60, 65, 70 years of age. A GCS of 13 to 15 can preferably be used to define a patient subgroup and can be used in combination with any of the other markers defined herein. In such a manner the individuals can be stratified and patient groups can be formed both to adapt and increase the test performance or to reduce the markers needed to achieve a reliable test result and preferably to adjust the features of the detection method or the components of a test kit.

A marker “panel” in the sense of the invention is a combination of at least two biomarkers, preferably two or three markers, used in combination in a suitable setup or device.

“Sensitivity” in the sense of the invention refers to the assay result of true positives in the analysis of TBI or mTBI. Preferably the sensitivity in the analysis according to the invention is set to 95% to 100%, or 100% (i.e. no false negative diagnoses).

“Specificity” in the sense of the invention is the so-called true negative rate in an assay to identify TBI or mTBI. The specificity is preferably targeted to be at least 50% and preferably higher, e.g. 58%, 60%, 65%, 70%.

A “sample” or “specimen” in the sense of the invention is any fluid or tissue useful for performing an assay or detection method to identify TBI, preferably mTBI. Preferably the

sample is a blood, plasma or urine sample taken from an individual. The sample is treated according to generally known procedures to keep or make them feasible for the marker analysis according to the invention.

In the following preferred embodiments of the invention will be described.

In a preferred embodiment the invention relates to a method wherein the sample is blood, plasma, saliva, tears, CSF, or urine. A blood sample is a very easy way of sample collection and thus the method according to the invention will be readily performed with simple means.

The method according to the invention is can be applied to all disorders, diseases or medical complications as described herein and is particularly useful for TBI and in particular for mild TBI (mTBI).

In an alternative embodiment the invention concerns a composition comprising or consisting of at least two markers useful for TBI detection in a sample of an individual wherein the markers are selected from the group consisting of IL10, fatty-acid-binding protein (FABP), glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neuromodulin (GAP43), neurofilament protein H (NFH), neurofilament protein M (NFM), neurofilament protein L (NFL), S100B, Tau, spectrin breakdown products, ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1) and vascular cell adhesion protein 1 (VCAM).

Said composition advantageously is comprising or consisting of two or three markers wherein the markers are selected from IL10, S100B, GFAP, HFABP and UCHL1 or fragments, variants or mutants thereof.

In an alternative embodiment the invention relates to a kit comprising or consisting of two or three markers of any of the above markers.

Furthermore, the invention relates to an assay device comprising or consisting of two or three markers of any of the above markers. Preferably the Assay device comprises or consists of a biochip, biomarker panel on a carrier, or test strip.

In addition to the above described embodiments it will be possible to combine the method and device of the invention with non-marker observations on the patient as part of a decision matrix, e.g. brain injury score, pupilar dilation, cognitive tests etc. which will lead to a reliable decision making of hospitalization of an individual or liberating him.

The invention encompasses also further subgroups of marker combinations being advantageous in terms of the test results that can be achieved. These subgroups are a combination of two, three, four or five markers selected from the group consisting of IL10 combined with either of FABP, GFAP, NSE, GAP43, NFH, NFM, NFL, S100B and VCAM,. In particular preferred is a combination of two markers of IL10, FABP, GFAP, NSE, GAP43, NFH, NFM, NFL, S100B and VCAM, , more preferably a combination of IL10, S100B, VCAM and H-FABP, or IL10, FABP and GFAP, or IL10, UCHL-1, VCAM and H-FABP, or GFAP, VCAM and H-FABP, or UCHL-1, VCAM and H-FABP, or IL10, S100B, VCAM and UCHL-1, or S100B, IL10 and H-FABP, or IL10, NSE, GAP43 and NFH, or NFM, NFL and S100B, or IL10, FABP, GFAP and NSE, or GAP43, NFH and NFM, or NFL, S100B and UCHL-1, or IL10, GAP43, NFH and NFM, or NFL, S100B and UCHL-1, or IL10, GFAP, NSE and GAP43.

Surprisingly, it was shown that the combination of a least two out of IL-10, H-FABP, S100b and age in panels gives rise to increased specificity around 60% for a sensitivity of 100% to rule out CT scans in mild TBI patients.

The invention achieves an improved specificity (>50%) for 100% sensitivity to rule out CT scans in mild TBI patients using a panel of two or 3 markers within IL-10, S100b, HFABP and age.

The invention will be described in more detail in the following examples which are meant to be illustrative without any restriction and which represent preferred embodiments of the invention.

As will be apparent from the experimental part describing the invention, the invention has the advantage that it achieves very reliable test results. Accordingly in preferred embodiments it provides for a method, composition, kit or assay wherein the sensitivity is more than 95%, preferably 100%, and the specificity is more than 50%, preferably more than 55%, more preferably more than 60%, and more preferably more than 70%.

In an alternative embodiment the invention provides for a method of or a system for analyzing in a specimen a medical condition wherein a medical device as described herein is applied under appropriate conditions, making use of any of the biomarkers described herein for the analysis of any of the disorders or diseases of the invention and making use of an algorithm wherein the test results are further defined by way of the algorithm, e.g. quantified.

The steps of such a method or system are in a preferred embodiment as follows:

The method and system are capable to analyze at least two test results in a sample of an individual, useful for the diagnosis of a medical condition like brain injury, with the system comprising:

(a) at least two databases comprising:

- (i) a first test result collected from a first diagnostic test;
- (ii) a second test result collected from a second diagnostic test different than the first diagnostic test;
- (iii) optionally, subsequent test result(s) collected from subsequent diagnostic test(s) different from the previous diagnostic test(s)
- (iv) optionally, secondary subject observations or measurements;
- (v) one or more diagnostic cut-offs associated with the first diagnostic test, with the second diagnostic test, with subsequent diagnostic tests, and with the subject observations or measurements, wherein such cut-offs collectively integrate to assess probability of brain injury status.

(b) one or more processors operatively encoded to automatically:

- (i) apply an interpretation algorithm to generate a subject result coordinate based on the database of test results.
- (ii) optionally apply a second interpretation algorithm to generate a probability of error in the subject result coordinate.

### Examples

The following examples are meant to illustrate the invention in more detail without to be construed as limiting in any sense.

The blood IL10, H-FABP and S100B content of patients presenting or not cerebral lesions on CT scan with a Glasgow Coma Scale > 13 and within 6 hours after the onset of TBI were quantified and compared using ELISA analysis. The study population comprised a total of 97 individuals.

ELISA was performed using IL10 from Mesoscale (US), H-FABP from Hycult (NL) and S100B from Abnova (TW). Each plasma sample were assayed in duplicate and distributed randomly on the plates.

Protein levels were initially expressed in relative fluorescence unit (RFU) and

concentrations were calculated using a calibration curve obtained on the same plate with the recombinant proteins. Statistical analyses were performed using IBM SPSS Statistics software version 19.0.0 (IBM Corporation, NY, USA). To assess the ability of proteins to discriminate between different populations, non-parametric tests were performed. A Wilcoxon matched pairs test was performed for age and sex matched data and a Mann-Whitney for non-matched data. For data containing more than two groups, a one-way ANOVA Kruskal-Wallis test was used. Receiver Operating Characteristic (ROC) curves analysis was performed and cut-off (CO) points obtained from the curves. Optimal threshold values were chosen to provide the highest specificity for 100% sensitivity. Multivariate logistic regression analysis was used to compare the values of plasma S100B, H-FABP and IL10 levels as CT scan rule out markers.

In these experiments the inventors succeeded in providing a panel (i.e. a small set of two or three) of biomarkers or biomolecules that could be useful in a clinical setting. We could show that each member of the panel provides a different angle to the diagnosis and taken together they lead to a more accurate prediction. Each member of the panel fulfils several criteria: firstly it must have a predictive power itself, i.e. it must be able to distinguish the disease types to a certain extent. Secondly it must be easy to measure with high reproducibility. Thirdly, it should have a central role in the biological processes that were found by the network analysis.

IL10, or IL10 in combination with either of S100B or H-FABP were identified as particularly useful in such a panel analysis according to the invention. We developed PanelomiX toolbox, which is able to extract optimal panels from a small number of molecules and provides a simple, easy to interpret set of threshold rules for disease type classification. The rule-based classifier just counts the number of molecules whose quantity passes specific threshold values. It mimics the way many clinical scores are built and is therefore easy to understand by people working in a clinical environment. Briefly, the optimized cut-off values were obtained by iterative permutation-response calculations using all available parameters. Each cut off value was changed iteratively by quantiles of 2% increment, and specificity was determined after each iteration until a maximum of specificity was achieved for 100% sensitivity.

## Results

The figure 1 (left) below shows the results of the specificity comparison of IL-10 (27%), H-FABP (27%) and S100b (18%). The sensitivity has been set at 100%. This confirms previous results on H-FABP that has a better performance than S100b to rule-out negative CT patients but also the capacity of IL-10 to over perform S100b.

When molecules are combined into panels of three molecules, the specificity increases up to 57% (S100b, HFABP, IL-10 and age) and up to 49% when two molecules are combined (S100b, IL-10 and age).

### Single analytes and panels to rule out CT-SCAN in mTBI

Geneva-Sevilla only molecules		
<b>S100B</b>	Pat -/+	111/22
	Mann U	0.000
	SP%	18.0
<b>H-FABP</b>	Pat -/+	111/22
	Mann U	0.013
	SP%	27.9
<b>IL-10</b>	Pat -/+	111/22
	Mann U	0.000
	SP%	27.0

Geneva-Sevilla panels with IL-10		
<b>Panel IL-10+S100B+H-FABP+age</b>	Pat -/+	111/22
	Pos	3
	SP%	56.8
<b>Panel IL-10+H-FABP+age</b>	Pat -/+	111/22
	Pos	2
	SP%	43.2
<b>Panel S100B+IL-10+age</b>	Pat -/+	111/22
	Pos	2
	SP%	49.5
<b>Panel S100B+H-FABP+IL-10</b>	Pat -/+	111/22
	Pos	3
	SP%	47.7
<b>Panel S100B+IL-10</b>	Pat -/+	111/22
	Pos	2
	SP%	38.7
<b>Panel H-FABP+IL-10</b>	Pat -/+	111/22
	Pos	2
	SP%	43.2
<b>Panel IL-10+age</b>	Pat -/+	111/22
	Pos	1
	SP%	27

The results described here above on IL10 alone or in combination with at least one of S100B and H-FABP panels of at least two of these molecules or a combination of three as shown highlight that they can be easily used in a POCT or an array for serial diagnosis.

Accordingly, the surprising and unexpected advantage is an improved specificity (>50%) for 100% sensitivity to rule out CT scans in mild TBI patients using a panel of two or three markers within S100B, HFABP and IL10.



### Claims

1. Method for screening for a disease or disorder selected from the group consisting of TBI, transient ischemic attack, brain tumors, seizures, epilepsy, cerebral abscess, encephalopathies and multiple sclerosis in a sample comprising the steps of using a sample under suitable conditions and detecting an IL10 biomarker under suitable conditions or a combination of at least two biomarkers wherein the biomarkers are selected from the group consisting of IL10, S100B, H-FABP, fatty-acid-binding protein (FABP), glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neuromodulin (GAP43), neurofilament protein H (NFH), neurofilament protein M (NFM), neurofilament protein L (NFL), Tau, spectrin breakdown products, ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1) and vascular cell adhesion protein 1 (VCAM).
2. Method according to claim 1 wherein one or more biomarkers according to claim 1 is combined with a marker which is age or a defined GCS, preferably 13 to 15.
3. Method according to claim 1 wherein one or more biomarkers according to claim 1 is used for screening a defined age or age group or a defined GCS group.
4. Method according to claim 2 or 3 wherein the age group has an age of less than 50, less than 60, or more than 60, more than 70, or more than 80. Method according to claim 2, 3 or 4 wherein the GCS is at least 13 or at least 15.
5. Method according to any of claims 1 to 4 wherein the biomarker is selected from IL10, S100B, GFAP, HFABP, and UCHL-1 or fragments, variants or mutants thereof.
6. Method according to any of claims 1 to 5 wherein the sample is blood, plasma, urine, saliva, tears (lachrymal fluid), or CSF.

7. Method according to any of the preceding claims wherein the TBI is mild TBI (mTBI).
8. Composition comprising or consisting of at least two markers useful for the detection of a disease or disorder selected from the group consisting of TBI, transient ischemic attack, brain tumors, seizures, epilepsy, cerebral abscess, encephalopathies and multiple sclerosis in a sample of an individual wherein the markers are selected from the group consisting of IL10, S100B, H-FABP, fatty-acid-binding protein (FABP), glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neuromodulin (GAP43), neurofilament protein H (NFH), neurofilament protein M (NFM), neurofilament protein L (NFL), S100B, Tau, spectrin breakdown products, ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1) and vascular cell adhesion protein 1 (VCAM).
9. Composition comprising or consisting of two or more markers wherein the markers are selected from IL10, S100B, GFAP, HFABP and UCHL-1 or fragments, variants or mutants thereof.
10. Composition according to claim 9 wherein the TBI is mild TBI (mTBI).
11. Kit comprising or consisting of two or more markers according to claim 1.
12. Assay device comprising or consisting of two or more markers according to claim 1.
13. Assay device according to claim 12 which device comprises or consists of a biochip, biomarker panel, a carrier, or a test strip.
14. Method, composition, kit or assay according to any of the preceding claims wherein the sensitivity is more than 95%, preferably 100%, and the specificity is more than 50%, preferably more than 55%, more preferably more than 60%, and more preferably more than 70%.
15. A method according to claim 1, which makes use of an algorithm.

Single analytes and panels to rule out CT-  
SCAN in mTBI

Geneva-Sevilla only molecules		
<b>S100B</b>	Pat -/+	111/22
	Mann U	0.000
	SP%	18.0
<b>H-FABP</b>	Pat -/+	111/22
	Mann U	0.013
	SP%	27.9
<b>IL-10</b>	Pat -/+	111/22
	Mann U	0.000
	SP%	27.0

Geneva-Sevilla panels with IL-10		
<b>Panel IL-10+S100B+H-FABP+age</b>	Pat -/+	111/22
	Pos	3
	SP%	56.8
<b>Panel IL-10+H-FABP+age</b>	Pat -/+	111/22
	Pos	2
	SP%	43.2
<b>Panel S100B+IL-10+age</b>	Pat -/+	111/22
	Pos	2
	SP%	49.5
<b>Panel S100B+H-FABP+IL-10</b>	Pat -/+	111/22
	Pos	3
	SP%	47.7
<b>Panel S100B+IL-10</b>	Pat -/+	111/22
	Pos	2
	SP%	38.7
<b>Panel H-FABP+IL-10</b>	Pat -/+	111/22
	Pos	2
	SP%	43.2
<b>Panel IL-10+age</b>	Pat -/+	111/22
	Pos	1
	SP%	27

Fig. 1

Fig. 2

IL-10  
GCS 15 + 1 s  $\leq 6h$   
100% SE for 27%SP

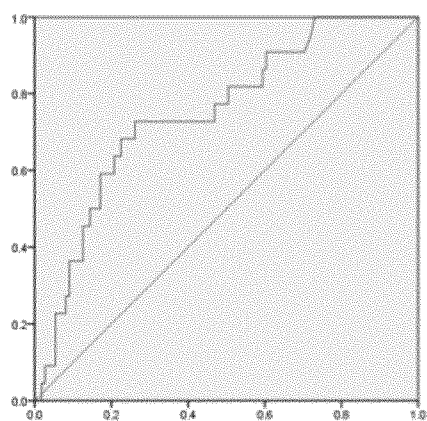


Fig. 3

H-FABP  
GCS 15 + 1 s  $\leq 6h$   
100% SE for 28%SP

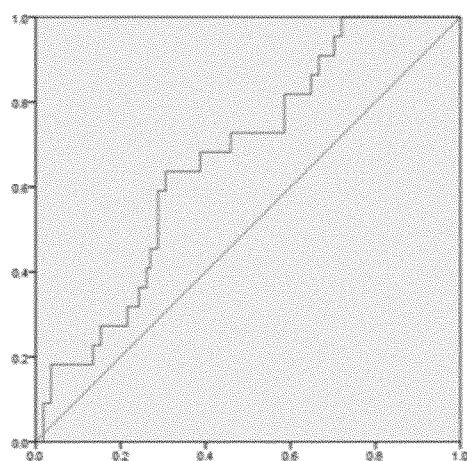


Fig. 4

S100B  
GCS 15 + 1 s  $\leq 6h$   
100% SE for 18%SP

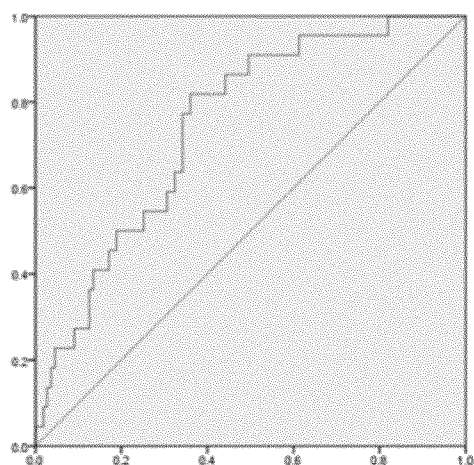


Fig. 5

panel S100B IL-10  
GCS 15 + 1 s  $\leq 6h$   
100% SE for 39%SP

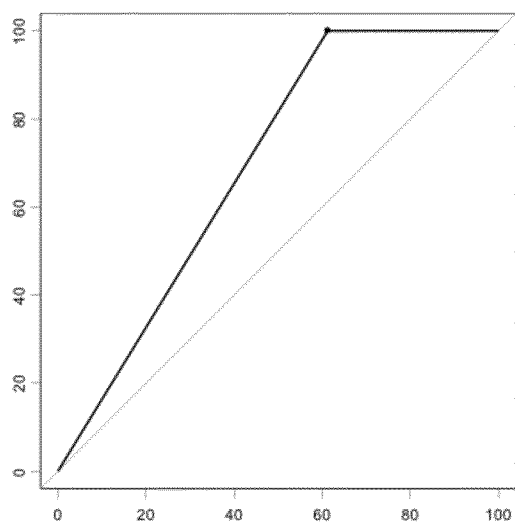


Fig. 6

panel HFABP IL-10  
GCS 15 + 1 s  $\leq$  6h  
100% SE for 43%SP

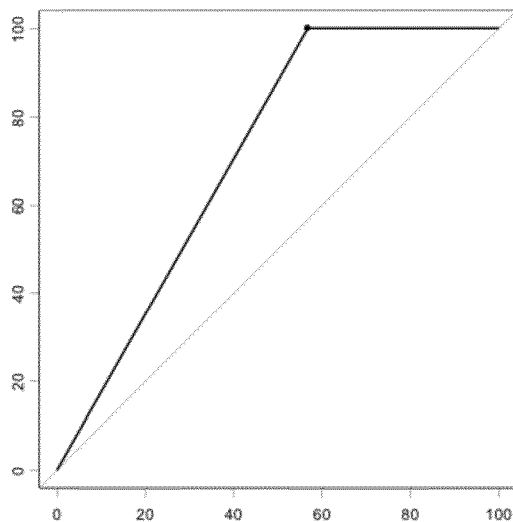




Fig. 7

panel age S100B HFABP  
GCS 15 + 1 s  $\leq$  6h  
100% SE for 46%SP

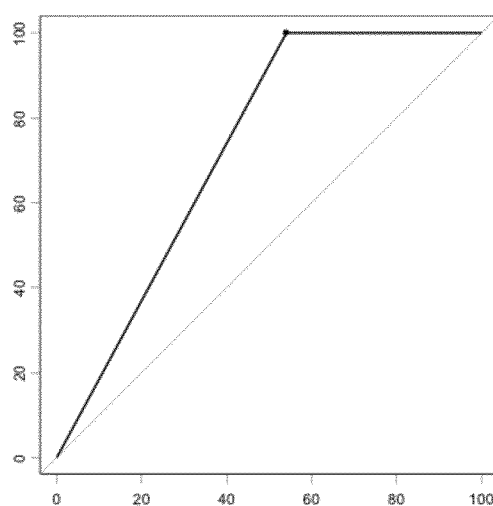
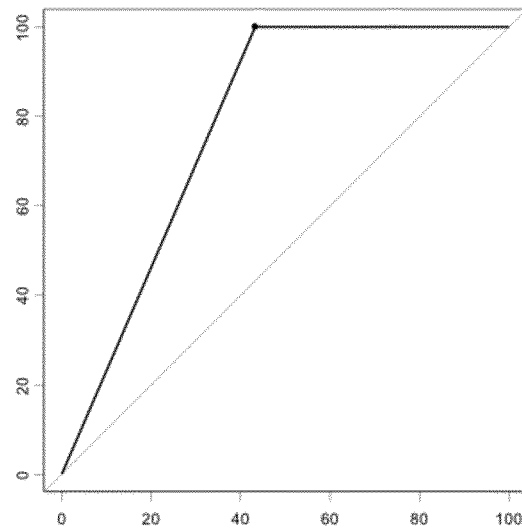


Fig. 8

panel age S100B HFABP IL-10  
GCS 15 + 1 s  $\leq 6h$   
100% SE for 57%SP



## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2018/065090

A. CLASSIFICATION OF SUBJECT MATTER  
INV. G01N33/68  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, INSPEC, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DASH P K ET AL: "Biomarkers for the Diagnosis, Prognosis, and Evaluation of Treatment Efficacy for Traumatic Brain Injury", NEUROTHERAPEUTICS, ELSEVIER INC, US, vol. 7, no. 1, 1 January 2010 (2010-01-01) , pages 100-114, XP026824397, ISSN: 1933-7213 [retrieved on 2010-01-01]	1-3,5-15
Y	the whole document in particular: abstract par. "S100B"; page 102 par. "Fatty Acid Binding Proteins"; page 103 par. "Anti-inflammatory cytokines"; page 104 page 111, left-hand column, line 2 - line -/--	4



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 July 2018

Date of mailing of the international search report

27/07/2018

Name and mailing address of the ISA/

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Tuynman, Antonin

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/065090

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	9	
X	----- TOSHIAKI HAYAKATA ET AL: "CHANGES IN CSF S100B AND CYTOKINE CONCENTRATIONS IN EARLY-PHASE SEVERE TRAUMATIC BRAIN INJURY :", SHOCK, vol. 22, no. 2, 1 August 2004 (2004-08-01) , pages 102-107, XP055489344, US ISSN: 1073-2322, DOI: 10.1097/01.shk.0000131193.80038.f1	1-3,5,6, 8-15
Y	the whole document in particular: abstract page 102, right-hand column, line 3 - line 14 par. "CSF and serum analysis"; page 103	4,7
X	----- PELSERS M M A L ET AL: "Fatty acid-binding proteins as plasma markers of tissue injury", CLINICA CHIMICA ACTA, ELSEVIER BV, AMSTERDAM, NL, vol. 352, no. 1-2, 1 February 2005 (2005-02-01), pages 15-35, XP027648936, ISSN: 0009-8981 [retrieved on 2005-02-01]	1,5,6, 8-15
Y	the whole document in particular: abstract page 27, paragraph 5.6 par. "Reference limits"; page 19 - page 20 par. "Assays for FABPs"; page 18 - page 19	4,7
X	----- SINGH KAVITA ET AL: "Longitudinal changes in the DTI measures, anti-GFAP expression and levels of serum inflammatory cytokines following mild traumatic brain injury", EXPERIMENTAL NEUROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 275, 26 July 2015 (2015-07-26), pages 427-435, XP029357341, ISSN: 0014-4886, DOI: 10.1016/J.EXPNEUROL.2015.07.016 bridging par.; page 427 - page 428 ----- -/--	1-7,14

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2018/065090

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FLÁVIA MAHATMA SCHNEIDER SOARES ET AL: "Interleukin-10 Is an Independent Biomarker of Severe Traumatic Brain Injury Prognosis", NEUROIMMUNOMODULATION, vol. 19, no. 6, 1 January 2012 (2012-01-01), pages 377-385, XP055489312, ISSN: 1021-7401, DOI: 10.1159/000342141 the whole document abstract</p> <p>-----</p>	1-7,14
X	<p>SIMON J I SANDLER ET AL: "Clinical applications of biomarkers in pediatric traumatic brain injury", CHILD'S NERVOUS SYSTEM, SPRINGER, BERLIN, DE, vol. 26, no. 2, 10 November 2009 (2009-11-10), pages 205-213, XP019780252, ISSN: 1433-0350 the whole document in particular: par. "Using paired or multiple biomarkers"; page 210</p> <p>-----</p>	1,5-14
X	<p>KUMAR RAJ G ET AL: "Principal components derived from CSF inflammatory profiles predict outcome in survivors after severe traumatic brain injury", BRAIN, BEHAVIOR AND IMMUNITY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 53, 17 December 2015 (2015-12-17), pages 183-193, XP029452074, ISSN: 0889-1591, DOI: 10.1016/J.BBI.2015.12.008 the whole document abstract</p> <p>-----</p>	1-6,8-15
X	<p>WO 2016/055148 A2 (UNIVERSITÉ DE GENÈVE [CH]; FUNDACIÓ HOSPITAL UNI VALL D HEBRON INST DE) 14 April 2016 (2016-04-14) the whole document claims 1-15</p> <p>-----</p>	1-15

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP2018/065090

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-15(partially)  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

Continuation of Box II.2

Claims Nos.: 1-15(partially)

The present application and claims relate to an extremely large number of possible methods and products for screening for a variety of neurological diseases using a variety of (panels of) biomarkers. Technical support and a sufficient disclosure in the sense of Article 6 and 5 PCT is only present for the biomarkers IL-10, S100B or H-FABP alone or in combination in conjunction with TBI (including mTBI).

The claims however try to seek protection for combinations of at least two biomarkers including combinations where the previously mention IL-10, S100B or H-FABP are absent, namely combinations of at least two markers of fatty-acid-binding protein (FABP), glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neuromodulin (GAP43), neurofilament protein H (NFH), neurofilament protein M (NFM), neurofilament protein L (NFL), Tau, spectrin breakdown products, ubiquitin carboxyl terminal hydrolase-L1 (UCH-L1) and vascular cell adhesion protein 1 (VCAM). And this is not the only problem: the application tries to seek protection for the screening or detection of a broad variety of neurological diseases, like transient ischemic attack, brain tumors, seizures, epilepsy, cerebral abscess, encephalopathies and multiple sclerosis without providing any data. This is simply not credible or plausible. There are serious doubts based on plenty verifiable facts that a marker or combination thereof indicative for one neurological disease is not indicative for other neurological diseases. There are also serious doubts based on plenty verifiable facts that a marker or combination thereof claimed to be indicative for one neurological disease without any data, turned out not to be marker at all. Therefore there is neither technical support nor a sufficient disclosure for such combinations in conjunction with the diseases claimed.

The non-compliance with the substantive provisions is to such an extent, that the search must be performed taking into consideration the non-compliance in determining the extent of the search of the claims (PCT Guidelines 9.19 and 9.23), i.e. in determining what can be meaningfully searched.

The applicant has therefore been invited to file a statement indicating the subject-matter to be searched within the time limit indicated in the present communication.

In this statement the applicant has agreed with the examiner that the subject-matter to be searched is to be performed on biomarkers IL-10, S100B or H-FABP alone or in combination in conjunction with the disease TBI (including mTBI). Therefore the present search has been limited to biomarkers IL-10, S100B or H-FABP alone or in combination in conjunction with the disease TBI (including mTBI).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2018/065090

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016055148 A2	14-04-2016	AU 2015330355 A1	20-04-2017
		CA 2963748 A1	14-04-2016
		CN 107003317 A	01-08-2017
		EP 3204778 A2	16-08-2017
		JP 2017535763 A	30-11-2017
		WO 2016055148 A2	14-04-2016
-----			