HAPTOGLOBIN FOR USE IN TREATING TRAUMATIC BRAIN INJURY (TBI)

FIELD OF THE INVENTION

The present invention relates to haptoglobin (Hp) and optionally hemopexin (Hx) for use in the treatment of traumatic brain injury (TBI) and in particular in the treatment or prevention of a secondary brain injury in a subject suffering from a TBI.

BACKGROUND OF THE INVENTION

Traumatic brain injury (TBI) is a major health issue and contributor to mortality, disability, and health expenditure. TBI is an intracranial injury, resulting from injury to the head through an external force such as following a fall, vehicle crash or a blow to the head with an object. The pathomechanism in TBI is extremely complex and involves a broad spectrum of physiological, cellular and molecular pathways.

In general, TBI can be divided into two discrete stages, primary and secondary injury (Prasetyo, 2020). The primary brain injury is an irreversible process in the form of the physical damage to the brain that occurs at the moment of exposure to the external force and causes the brain to be displaced within the skull. This can involve contusion and laceration, diffuse axonal injury, brain swelling and intracranial hemorrhage, and invariably results in immediate cell death. The secondary brain injury occurs gradually as a consequence of a complex cascade of cellular and molecular alterations resulting in further brain damage and involves a dynamic interplay between ischemia, inflammation, and cytotoxic processes (Enriquez and Bullock, 2004). Such processes may be initiated at the time of primary injury and may endure hours and days or longer. Although the forces initiating the primary injury generally take less than 100 milliseconds to occur, the resulting pathophysiological events are much more prolonged and progressive and may ultimately be the deciding factors in the patient's recovery. Evidence of secondary brain injury has been found at autopsy in 70-90% of all fatal TBI patients. For patients who survive the initial injury, morbidity and mortality will be determined by secondary injury processes (Prasetyo, 2020).

TBI is often associated with intracranial hemorrhage and subsequent release of harmful blood components and their breakdown products into the cellular environment. Following the primary injury, neuroinflammation increases rapidly, suggesting that neuroinflammation plays some role in the TBI process. The products of iron dysregulation and blood-related toxicity caused by breakdown of blood components have been stipulated to be the major driver of the TBI-induced detrimental neuroinflammatory environment. Scavengers of these toxic products in the blood include the plasma proteins haptoglobin (Hp) and hemopexin (Hx). Studies suggest a role for Hp and Hx in maintaining healthy brain function by controlling iron homeostasis. The pathways and cellular processes involved in mediating this neuroprotection however are still unknown.

The current in depth understanding of iron regulation is a direct consequence of numerous studies in neurodegenerative diseases. The understanding of iron regulation in the central nervous system (CNS) has become apparent and is highlighted in neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD). In AD, elevated iron is observed in post-mortem brains (Duce et al., 2010), as well as in both plaques and tangles in the hippocampus (Quintana et al., 2006). Similarly, there is growing evidence to suggest that iron accumulate in the brain after TBI, by magnetic resonance imaging (MRI) in patients with mild TBI (Raz et al., 2011; Nisenbaum et al., 2014). The increase of unbound iron caused by brain injury can also be detrimental as it can give rise to toxic reactive species. Therefore, iron homeostasis is tightly regulated, as iron dyshomeostasis in either direction can compromise cell viability and cause severe cellular damage (Wang and Pantopoulos, 2011).

In TBI, the causation of primary injuries, such as a strike from an object or an elevated fall, may potentially cause hemorrhage to the brain. However, iron accumulation may not only be a result of heme iron contaminant from blood. Furthermore, a cascade of secondary injuries observed following in TBI can also mediate an elevation of intracellular iron levels. After the initial trauma, these secondary injuries (secondary brain injury) inter alia involve excitotoxicity, oxidative stress and neuroinflammation, all of which contribute to the pathogenesis of neurodegenerative diseases. The similarities between neurodegenerative diseases and TBI suggest that iron dyshomeostasis may also be a substantial contributor to the pathogenesis of TBI.

Processes such as oxidative stress, generated by reactive oxygen species (ROS), inflammation and excitotoxicity greatly exacerbate neural injury, and neurons, as well as astrocytes, microglia and endothelial cells, are differentially affected and responsive to these factors. After hemolysis, cell-free hemoglobin undergoes oxidation to methemoglobin, which through several hemichrome intermediates, finally degrades into a denatured globin protein and the redox-active heme moiety. Because of a combination of redox activity and lipophilicity, free heme is toxic in many ways, including covalent modification of substrates, intercalation in the lipid bilayer, and lipid peroxidation, which perturbs membrane homeostasis to cause cellular dysfunction and cell death (Gutteridge and Smith, 1988).

Recent clinical and experimental evidence suggests that Hp is involved in an intrinsic molecular defensive mechanism that is focused on controlling free iron. One of the critical mechanisms involved in deactivation of cell-free hemoglobin in the mammalian body during a hemorrhagic event is formation of highly stable complexes of hemoglobin with Hp an endogenous hemoglobin-binding protein present in blood plasma and almost absent within the brain itself (Schaer et al., 2006) and subsequent clearance of the Hp-hemoglobin complexes primarily by tissue macrophages and circulating monocytes, and likely by other cell types such as astrocytes and microglial cells that are mediated via CD163 (Zhang et al., 2012). Early studies have shown that serum Hp concentrations are increased in patients with severe head injuries and that the serum Hp level could potentially be a predictive biomarker of the hemorrhagic brain-lesion severity (Auer and Petek, 1978). In both physiological and pathophysiological conditions, Hp is synthesized mainly by hepatocytes and then released to the peripheral circulation (Yang et al., 2013). However, some studies suggest that Hp may be expressed within brain cell types. Increased Hp immunoreactivity and upregulation of Hp mRNA in reactive astrocytes have been shown in an experimental ischemia model, suggesting de novo Hp synthesis in the brain (Lee et al., 2002).

Hp is not the only protein that is expressed to deal with iron. Hx neutralizes the redox toxicity of heme by formation of the heme-Hx complex, which prevents heme from generating free radical reactions (Hvidberg et al., 2005). Hx is an abundant plasma protein that is widely expressed by neurons and glia (Morris et al., 1993). Oxidative stress generated by neutrophils and other phagocytic cells is exacerbated by heme from hemoglobin. Under *in vitro* conditions designed to mimic brain damage after bleeding and oxidative stress from inflammation, Hahl et al. (Hahl et al., 2013) addressed the mechanisms of neuroprotection by Hx in SH-SY5Y neuroblastoma cells and

showed that Hx can protect brain neurons immediately after neural damage and the ensuing inflammatory conditions, by heme sequestration, safe heme delivery, and induction of heme oxygenase-1 and the amyloid precursor protein. While the extracellular antioxidant role of Hx (Gutteridge and Smith, 1988) is maintained, the intracellular free heme and iron are kept at safe levels in neurons.

Hp and Hx were reported for use in treating or preventing an adverse secondary neurological outcome following a hemorrhagic stroke, where the stroke is accompanied by extravascular erythrolysis and release of cell-free hemoglobin (Hb) and/or heme into the cerebral spinal fluid (CSF), by exposing the CSF of the stroke victim to Hp and/or Hx for a period of time sufficient to allow the Hp and/or Hx to form a complex with, and thereby neutralize, the cell-free Hb and/or heme (WO 2020/234195 A1 and WO 2022/162218 A1).

Galea et al. (2023) focusses on the development of intrathecal haptoglobin supplementation for the treatment of aneurysmal subarachnoid hemorrhage. The patients treated, however, did not suffer from TBI.

Robicsek et al. (2019) discusses various potential mechanisms of secondary brain injury. While Robisek et al. mentions that haptoglobin has a heme scavenging property, as well as neuroprotective, immunomodulatory, and antioxidant functions, the reference does not teach or suggest treating secondary brain injury in a patient suffiering from TBI by administering haptoglobin.

US 2023/0181688 A1 aims at treating plasma protein imbalances or depletion in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a protein composition comprising haptoglobin. US 2023/0181688 A1 does not disclose or suggest treating secondary brain injury.

Buehler et al. (2020) is a review which discusses key concepts of Hb toxicity and perspectives on the use of haptoglobin as a therapeutic protein.

TBI is often associated with intracranial hemorrhage, however, it involves a pathogenesis, which is considerably more complex, involving a broad spectrum of physiological, cellular and molecular

pathways. Despite numerous efforts to pharmacologically intervene in secondary brain injury following the initial trauma, none have demonstrated clinical efficacy (Prasetyo, 2020; Galgano et al., 2017). Secondary brain injury is thought to account for the development of many of the neurological deficits observed after TBI, and their delayed nature suggests that there is a window for the therapeutic intervention to prevent progressive tissue damage and improve functional recovery after injury. Due to the extended injury period, which inter alia involves excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, apoptotic cell death, axonal degeneration, and neuroinflammation, for treatment to be effective, efficient therapeutic agents are needed over acute, subacute and/or chronic period of the TBI. Ideally, such therapeutic agents should be effective during the early stages of TBI, that is during acute and subacute phase and suitable for administration in a manner that minimizes patient discomfort and risk of complications. Thus, there is an unmatched clinical need for *in vivo* treatment of TBI.

SUMMARY OF THE INVENTION

The above-mentioned need is addressed by the present invention. It has been found by the present inventors that haptoglobin (Hp) alone or in combination with hemopexin (Hx) can be used for the treatment of TBI and in particular for the treatment or prevention of a secondary brain injury following the initial trauma in a subject suffering from a TBI. Specifically, it has been surprisingly found by the present inventors that Hp, alone or in combination with Hx, administered for example during the acute or subacute phase of the TBI, that is after the initial trauma, can treat or prevent secondary brain injury. The result of the studies undertaking by the present inventors highlight these neuroprotective effects of Hp in preventing and/or improving secondary brain injury particularly at early timepoints after the initial trauma of the TBI. These results identify Hp, alone or in combination with Hx, as a new therapeutic for reducing cellular damage in TBI. For example, the present inventors surprisingly found in a mouse model for TBI a significant reduction in lesion size associated with significant improvement in motor function and reduced levels in astrocyte and microglia reactivity (indicating a reduced neuroinflammatory response), at 24 hours and 7 days after the initial trauma of the TBI, when Hp was administered alone or in combination with Hx, for example 30 min and 5 days after the initial trauma. The present inventors thus demonstrate for the first time a significant reduction in lesion size, improved behavioral outcome and reduced neuroinflammatory response following Hp treatment alone or together with Hx in a TBI model.

Furthermore, it has been unexpectedly found by the present inventors that the neuroprotective treatment using Hp and optionally Hx following the initial trauma (the primary brain injury) in a subject suffering from TBI is particularly effective when administered shortly after the initial trauma, that is for example such that a first dose of the Hp and optionally Hx is administered within less than 48 hours, or 24 hours, after the initial trauma. Additionally, it has been surprisingly found by the present inventors that the Hp and optionally Hx can be administered intravenously, rendering treatment much more patient friendly and reducing the risk of administration-related issued compared to administration directly into the brain or the spine of a TBI patient.

Thus, a first aspect of the present invention relates to haptoglobin (Hp) for use in the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI). The treatment may optionally further include the administration of hemopexin (Hx). This includes that the Hp and optionally Hx may preferably be administered intravenously, preferably by injection or infusion and may optionally be administered simultaneously, sequentially or separately, said modes of administration being encompassed by the term “co-administered”.

A second aspect of the present invention relates to haptoglobin (Hp), and optionally hemopexin (Hx), for use in the neuroprotective treatment of a traumatic brain injury (TBI) in a subject; or to Hp for use in the neuroprotective treatment in a subject suffering from a TBI. According to a third aspect the present invention relates to Hp, and optionally Hx, for use in the reduction of progressive brain damage in a subject suffering from a TBI. In a fourth aspect the present invention relates to Hp, and optionally Hx, for use in the treatment or prevention of intracranial lesions in a subject suffering from a TBI. In a fifth aspect the present invention relates to Hp, and optionally Hx, for use in the treatment or prevention of intracranial lesion formation and/or progression in a subject suffering from a TBI. In a sixth aspect the present invention relates to Hp, and optionally Hx, for use in the treatment or prevention of neuroinflammation in a subject suffering from a TBI. In a seventh aspect the present invention relates to Hp, and optionally Hx, for use in the treatment of impaired motor function in a subject suffering from a TBI. In an eighth aspect the present invention relates to Hp, and optionally Hx, for use in treating or preventing one or more adverse effects associated with iron accumulation in the brain parenchyma of a subject suffering from a TBI. In a ninth aspect the present invention relates to Hp, and optionally Hx, for use in treating or preventing iron-mediated toxicity in the brain parenchyma of a subject suffering from a TBI.

The present invention therefore relates to the following embodiments:

[1] Haptoglobin (Hp) for use in the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI).

[2] The haptoglobin for use according to item 1, wherein the haptoglobin is administered intravenously, preferably by injection or infusion.

[3] The haptoglobin for use according to item 1 or 2, wherein the haptoglobin is administered as a bolus injection.

[4] The haptoglobin for use according to item 1 or 2, wherein the haptoglobin is administered as a continuous intravenous infusion, preferably over 0.5 to 24 hours, or over 1 to 18 hours, or over 2 to 12 hours.

[5] The haptoglobin for use according to any of the preceding items, wherein the subject is at risk of developing a secondary brain injury.

[6] The haptoglobin for use according to any of the preceding items, wherein the traumatic brain injury (TBI) is accompanied by a secondary brain injury.

[7] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury is within the brain parenchyma and/or the meninges.

[8] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury is within the brain parenchyma.

[9] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury involves, or results from, excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, brain iron accumulation, apoptosis, ferroptosis, necrosis, axonal degeneration, neuroinflammation, dysfunction of cerebral blood flow, cerebral ischemia, cerebral hypoxia, cerebral hypotension, dysfunction of brain metabolism, cerebral hypo- or hyper-carbia, cerebral hypo- or hyper-glycemia, cerebral hypo- or hyper-thermia, cerebral edema (e.g. vasogenic cerebral edema and/or cytotoxic cerebral edema), raised intracranial pressure, breakdown of the blood–brain barrier and/or seizures; preferably excitotoxicity, oxidative stress and/or neuroinflammation and optionally mitochondrial dysfunction, lipid peroxidation, apoptosis, ferroptosis, necrosis, axonal degeneration, cerebral ischemia, cerebral hypoxia, cerebral hypotension, cerebral hypo- or hyper-carbia, cerebral hypo- or hyper-glycemia, cerebral hypo- or hyper-thermia, cerebral edema, raised intracranial pressure, breakdown of the blood-brain barrier and/or seizures.

[10] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury is selected from the group consisting of formation and/or progression of intracranial lesions, cerebral edema (e.g. cytotoxic, vasogenic or ionic) and cerebral edema-related damage, neuroinflammation-related damage, impaired motor function, impaired cognitive function, neurotoxicity, apoptosis, ferroptosis and/or necrosis of brain cells, nitric oxide depletion-related damage, oxidative tissue injury, iron-mediated damage, ischemia-related damage (including delayed ischaemic neurological deficit (DIND), delayed cerebral ischaemia (DCI), damage from cerebral vasospasm, and/or damage from cerebral vasoreactivity), blood-brain barrier impairment-related damage, and spreading depolarization, or a combination thereof; or wherein the secondary brain injury comprises one or more of the conditions of said group.

[11] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury comprises, or consists of, intracranial lesions.

[12] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury comprises, or consists of, formation and/or progression of intracranial lesions.

[13] The haptoglobin for use according to item 11 or 12, wherein the intracranial lesions are monitored using magnetic resonance imaging (MRI) or a computed tomography (CT) scan, preferably using MRI optionally by determining lesion volume.

[14] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury comprises, or consists of, neuroinflammation-related damage.

[15] The haptoglobin for use according to item 14, wherein neuroinflammation comprises gliosis, preferably astrogliosis and/or microgliosis.

[16] The haptoglobin for use according to item 14 or 15, wherein neuroinflammation is assessed by monitoring gliosis, preferably astrogliosis and/or microgliosis.

[17] The haptoglobin for use according to any of items 14 to 16, wherein astrogliosis is monitoring by testing for an astrocyte marker protein, preferably GFAP, and/or wherein microgliosis is monitoring by testing for a microglia/macrophage marker protein, preferably IBA1.

[18] The haptoglobin for use according to item 17, comprising determining the marker protein or proteins in blood serum or cerebrospinal fluid (CSF).

[19] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin is for use in reducing glial reactivity, particularly astrocyte and/or microglia reactivity, in the subject suffering from the TBI (following the primary brain injury).

[20] The haptoglobin for use according to any of the preceding items, wherein the secondary brain injury comprises, or consists of, impaired motor function (neuromotor impairment).

[21] The haptoglobin for use according to any of the preceding items, wherein a secondary brain injury in a subject suffering from a traumatic brain injury (TBI) means the injury following the primary brain injury (the initial trauma) of the TBI; and/or wherein the secondary brain injury is caused by the TBI; and/or wherein the TBI is a primary brain injury preceding the secondary brain injury; and/or wherein the secondary brain injury occurs progressively after an initial trauma or primary brain injury as a direct consequence thereof.

[22] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises exposing the brain parenchyma of the subject to the haptoglobin (Hp) administered to the subject.

[23] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises exposing the brain parenchyma of the subject to a therapeutically effective amount of the haptoglobin (Hp) administered to the subject.

[24] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI).

[25] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering at least one dose of haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI).

[26] The haptoglobin for use according to any of the preceding items, wherein the treatment with haptoglobin is started within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI).

[27] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering a primary dose of haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI).

[28] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering the haptoglobin to the subject during the acute or subacute phase of the traumatic brain injury (TBI).

[29] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering the haptoglobin to the subject during the acute phase of the traumatic brain injury (TBI).

[30] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering at least one dose of the haptoglobin to the subject during the acute or subacute phase of the traumatic brain injury (TBI).

[31] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering at least one dose of the haptoglobin to the subject during the acute phase of the traumatic brain injury (TBI).

[32] The haptoglobin for use according to any of the preceding items, wherein the treatment is started during the acute or subacute phase of the traumatic brain injury (TBI).

[33] The haptoglobin for use according to any of the preceding items, wherein the treatment is started during the acute phase of the traumatic brain injury (TBI).

[34] The haptoglobin for use according to any of items 28 to 33, wherein the acute phase is defined as the first 24 hours (day 0) after the initial trauma (primary brain injury) of the TBI and the subacute phase is defined as the time period between one day and three weeks after the initial trauma (primary brain injury) of the TBI.

[35] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering the haptoglobin within less than 48 hours, preferably 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI).

[36] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering at least one dose of the haptoglobin within less than 48 hours, preferably 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, after the initial trauma (the primary injury) of the traumatic brain injury (TBI).

[37] The haptoglobin for use according to any of the preceding items, wherein the treatment is started within less than 48 hours, preferably within 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, after the initial trauma (the primary injury) of the traumatic brain injury (TBI).

[38] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering a primary dose of the haptoglobin within less than 48 hours, preferably 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, after the initial trauma (the primary injury) of the traumatic brain injury (TBI).

[39] The haptoglobin for use according to any of the preceding items, comprising exposing the subject to the haptoglobin for 2 to 56 days, preferably 3 to 28 days, more preferably 5 days to 21 days, more preferably 7 days to 14 days, after the initial trauma (the primary injury) of the traumatic brain injury (TBI).

[40] The haptoglobin for use according to item 27 or 38, wherein the treatment comprises administering a second dose of the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, or one day, or 12 hours, after the primary dose.

[41] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin is administered at least once a week, preferably two times per week, or three times per weeks, or every other day.

[42] The haptoglobin for use according to any of items 1 to 40, wherein the haptoglobin is administered every 24 hours, or every 18 hours, or every 12 hours, or every 8 hours, or every 6 hours.

[43] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin is administered at least two times a week, for a period of one week, or two weeks, or three weeks, or four weeks, or longer.

[44] The haptoglobin for use according to any of items 1 to 38, wherein the treatment consists of administering one dose of the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma (the primary injury) of the TBI.

[45] The haptoglobin for use according to any of items 1 to 38, wherein the treatment consists of administering one dose of the haptoglobin within less than 2 days, or one day, or 12 hours after the initial trauma (the primary injury) of the TBI.

[46] The haptoglobin for use according to any of the preceding items, wherein a traumatic brain injury (TBI) is an injury to the brain caused by an external force.

[47] The haptoglobin for use according to any of the preceding items, wherein treatment or prevention of a secondary brain injury (following the primary brain injury/initial trauma of a TBI) means reduction of progressive brain damage.

[48] The haptoglobin for use according to any of the preceding items, wherein the use is in the neuroprotective treatment of the traumatic brain injury (TBI) in the subject.

[49] The haptoglobin for use according to any of the preceding items, wherein the traumatic brain injury (TBI) is moderate TBI or severe TBI.

[50] The haptoglobin for use according to item 49, wherein TBI severity is assessed using the Glascow Coma Scale (GCS); optionally wherein moderate TBI is characterized by a GCS of 9 to 12 and severe TBI is characterized by a GCS of <9.

[51] The haptoglobin for use according to any of the preceding items, wherein the traumatic brain injury (TBI) is accompanied by an intracerebral hemorrhage (ICH), an epidural hemorrhage, a subdural hemorrhage and/or a subarachnoid hemorrhage.

[52] The haptoglobin for use according to item 51, wherein the intracerebral hemorrhage (ICH) is an intraparenchymal hemorrhage (IPH) or an intraventricular hemorrhage (IVH).

[53] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) is human Hp.

[54] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) is exogenous Hp.

[55] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) is selected from the group consisting of Hp1-1 tetramers (comprising, or consisting of, two haptoglobin alpha1 chains and two haptoglobin beta chains), Hp1-2 multimers (comprising, or consisting of, haptoglobin alpha1 chains, haptoglobin alpha2 chains and haptoglobin beta chains), Hp2-2 multimers (comprising, or consisting of, haptoglobin alpha2 chains and haptoglobin beta chains), and a combination thereof.

[56] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) comprises, or consists of, at least 50 % (e.g. wt.-% or mol.-%), or 55 %, or 60 %, or 70 %, or 80 %, or 85 %, or 90 %, or 93 %, or 95 %, or 97 %, or 99 %, Hp2-2 multimers; and optionally no more than 50 % (e.g. wt.-% or mol.-%), or 40 %, or 30 %, or 20%, or 15%, or 10 %, or 7 %, or 5 %, or 3 %, or 1 %, Hp1-1 tetramers, or Hp1-2 multimers, or a combination of Hp1-1 tetramers and Hp1-2 multimers.

[57] The haptoglobin for use according to any of the preceding items, wherein the alpha chain fraction of the haptoglobin (Hp) comprise, or consist of, at least 60 % (e.g. wt.-% or mol.-%), or 70 %, or 80 %, or 85 %, or 90 %, or 93 %, or 95 %, or 97 %, or 99 %, haptoglobin alpha2 chain; and optionally no more than 40 % (e.g. wt.-% or mol.-%), or 30 %, or 20%, or 15%, or 10 %, or 7 %, or 5 %, or 3 %, or 1 %, haptoglobin alpha1 chain.

[58] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin comprises, or consists of, haptoglobin alpha1 chains and haptoglobin beta chains.

[59] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin comprises, or consists of, haptoglobin alpha1 chains, haptoglobin alpha2 chains and haptoglobin beta chains.

[60] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin comprises, or consists of, haptoglobin alpha2 chains and haptoglobin beta chains.

[61] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) has a molecular composition corresponding to a mixture of Hp2-2 phenotype and Hp1-2 phenotype, optionally wherein said mixture of Hp2-2 phenotype and Hp1-2 phenotype comprises, or consists of, 35-75 % Hp2-2 phenotype and 25-65 % Hp1-2 phenotype; preferably 40-70 % Hp2-2 phenotype and 30-60 % Hp1-2 phenotype; more preferably 44-64 % Hp2-2 phenotype and 36-56 % Hp1-2 phenotype; or 49-59 % Hp2-2 phenotype and 41-51 % Hp1-2 phenotype; or 50-58 % Hp2-2 phenotype and 42-50 % Hp1-2 phenotype; or 51-57 % Hp2-2 phenotype and 43-49 % Hp1-2 phenotype; or 52-56 % Hp2-2 phenotype and 44-48 % Hp1-2 phenotype; or 53-55 % Hp2-2 phenotype and 45-47 % Hp1-2 phenotype, e.g. 54 % Hp2-2 phenotype and 46 % Hp1-2 phenotype.

[62] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) comprises, or consists of, 30-100 % Hp2-2 multimers, 0-60 % Hp1-2 multimers, and 0-40 % Hp1-1 tetramers; preferably 40-70 % Hp2-2 multimers, 20-55 % Hp1-2 multimers, and 1-25 % Hp1-1 tetramers; more preferably 44-64 % Hp2-2 multimers, 26-46 % Hp1-2 multimers, and 3-17 % Hp1-1 tetramers; even more preferably 48-60 % Hp2-2 multimers, 30-42 % Hp1-2 multimers, and 5-15 % Hp1-1 tetramers; or 50-58 % Hp2-2 multimers, 32-40 % Hp1-2 multimers, and 6-14 % Hp1-1; or 51-57 % Hp2-2 multimers, 33-39 % Hp1-2 multimers, and 7-13 % Hp1-1; or 52-56 % Hp2-2 multimers, 34-38 % Hp1-2 multimers, and 8-12 % Hp1-1; or 53-55 % Hp2-2 multimers, 35-37 % Hp1-2 multimers, and 9-11 % Hp1-1; optionally wherein the % is wt.-% or mol.-%.

[63] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) comprises, or consists of, Hp2-2 multimers and Hp1-2 multimers, and optionally Hp1-1 tetramers.

[64] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) comprises, or consists of, Hp2-2 multimers.

[65] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) comprises, or consists of, Hp1-1 tetramers, or Hp1-2 multimers, or a combination thereof.

[66] The haptoglobin for use according to any of the preceding items, wherein the Hp1-1 tetramer comprises, or consists of, two haptoglobin alpha1 chains and two haptoglobin beta chains.

[67] The haptoglobin for use according to any of the preceding items, wherein the Hp1-2 multimer comprises, or consists of, one or more haptoglobin alpha1 chains, optionally one or more haptoglobin alpha2 chains, and two or more haptoglobin beta chains; optionally wherein in the multimer the number of haptoglobin beta chains is equivalent to the number of alpha chains.

[68] The haptoglobin for use according to any of the preceding items, wherein the Hp1-2 multimer comprises, or consists of, one or more haptoglobin alpha1 chains, one or more haptoglobin alpha2 chains, and two or more haptoglobin beta chains; optionally wherein in the multimer the number of haptoglobin beta chains is equivalent to the number of alpha chains.

[69] The haptoglobin for use according to any of the preceding items, wherein the Hp2-2 multimer comprises, or consists of, three or more haptoglobin alpha2 chains and three or more haptoglobin beta chains; optionally wherein in the multimer the number of haptoglobin beta chains is equivalent to the number of alpha chains.

[70] The haptoglobin for use according to any of items 55 to 70, wherein the haptoglobin alpha1 chain is selected from the group consisting of alpha1F chain, alpha1S chain, or a combination of alpha1F chain and alpha1S chain; optionally wherein haptoglobin alpha1F chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:3, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 97.5%, 98%, 98.7%, or 99% sequence identity thereto; and/or optionally wherein haptoglobin alpha1S chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:4, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 97.5%, 98%, 98.7%, or 99% sequence identity thereto.

[71] The haptoglobin for use according to any of items 55 to 71, wherein the haptoglobin alpha2 chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:5, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 97.8%, 98%, 98.5% 99% or 99.3% sequence identity thereto.

[72] The haptoglobin for use according to any of items 55 to 71, wherein the haptoglobin beta chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:6, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 98.3%, 98.7%, 99%, 99.1% or 99.5% sequence identity thereto.

[73] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) is plasma derived.

[74] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin (Hp) is a recombinant protein.

[75] The haptoglobin for use according to any of the preceding items, wherein the treatment further comprises administration of hemopexin (Hx).

[76] The haptoglobin for use according to item 75, wherein the hemopexin (Hx) is human Hx.

[77] The haptoglobin for use according to item 75 or 76, wherein the hemopexin (Hx) is exogenous Hx.

[78] The haptoglobin for use according to any of items 75 to 77, wherein the hemopexin (Hx) is plasma derived.

[79] The haptoglobin for use according to any of items 75 to 78, wherein the hemopexin (Hx) is a recombinant protein.

[80] The haptoglobin for use according to any of items 75 to 79, wherein the hemopexin comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:8, or an amino acid sequence having at least 75%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.3%, 99.5% or 99.7% sequence identity thereto.

[81] The haptoglobin for use according to any of items 75 to 80, wherein the hemopexin comprises, or consists of, amino acid residues 24-462 of the NCBI Reference Sequence NP_000604.1 (SEQ ID NO:7), or an amino acid sequence having at least 75%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.3, 99.5% or 99.7% sequence identity thereto.

[82] The haptoglobin for use according to any of items 75 to 81, wherein the treatment comprises exposing the brain parenchyma of the subject to the hemopexin (administered to the subject).

[83] The haptoglobin for use according to any of items 75 to 82, wherein the treatment comprises exposing the brain parenchyma of the subject to a therapeutically effective amount of the hemopexin (administered to the subject).

[84] The haptoglobin for use according to any of items 75 to 83, wherein the hemopexin (Hx) is administered in the same manner as specified for the haptoglobin in any of items 2 to 74.

[85] The haptoglobin for use according to any of items 75 to 84, wherein the hemopexin (Hx) is administered in the same manner as specified for the haptoglobin in any of items 22-31, 35-36 and 38-45.

[86] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin is administered as part of a pharmaceutical composition optionally comprising one or more pharmaceutically acceptable additives (excipients), preferably selected from carriers, stabilizers (stabilizing agents), buffering agents, isotonifiers, surface active agents including non-ionic detergents, diluents, binders, thickeners, lubricants, preservatives, antioxidants, and combinations thereof.

[87] The haptoglobin for use according to any of items 75 to 86, wherein the hemopexin is administered as part of a pharmaceutical composition optionally comprising one or more pharmaceutically acceptable additives (excipients), preferably selected from carriers, stabilizers (stabilizing agents), buffering agents, isotonifiers, surface active agents including non-ionic detergents, diluents, binders, thickeners, lubricants, preservatives, antioxidants, and combinations thereof.

[88] The haptoglobin for use according to item 86, wherein the pharmaceutical composition consists of the haptoglobin and the optional one or more pharmaceutically acceptable additives.

[89] The haptoglobin for use according to item 87, wherein the pharmaceutical composition consists of the hemopexin and the optional one or more pharmaceutically acceptable additives.

[90] The haptoglobin for use according to any of items 1 to 87, wherein the treatment comprises administering to the subject a pharmaceutical composition comprising the haptoglobin and optionally hemopexin (Hx).

[91] The haptoglobin for use according to any of items 1 to 87, wherein the treatment comprises administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of the haptoglobin and optionally hemopexin (Hx).

[92] The haptoglobin for use according to any of items 75 to 91, wherein the haptoglobin and the hemopexin (Hx) are administered to the subject simultaneously.

[93] The haptoglobin for use according to any of items 75 to 92, wherein the haptoglobin and the hemopexin (Hx) are comprised in one pharmaceutical composition, optionally comprising one or more pharmaceutically acceptable additives (excipients), preferably selected from carriers, stabilizers (stabilizing agents), buffering agents, isotonifiers, surface active agents including non-ionic detergents, diluents, binders, thickeners, lubricants, preservatives, antioxidants, and combinations thereof.

[94] The haptoglobin for use according to any of items 86 to 90, wherein the pharmaceutical composition comprises a therapeutically effective amount of haptoglobin (Hp) and optionally hemopexin (Hx).

[95] The haptoglobin for use according to any of items 86 to 91, wherein one dose of the pharmaceutical composition comprises a therapeutically effective amount of haptoglobin (Hp) and optionally hemopexin (Hx).

[96] The haptoglobin for use according to any of items 86 to 95, wherein the pharmaceutical composition consists of the haptoglobin (Hp), the hemopexin (Hx), and the one or more pharmaceutically acceptable additives.

[97] The haptoglobin for use according to any of the preceding items, wherein the haptoglobin is administered in liquid form.

[98] The haptoglobin for use according to any of items 75 to 97, wherein the hemopexin is administered in liquid form.

[99] The haptoglobin for use according to any of items 86 to 98, wherein the pharmaceutical composition is in liquid form.

[100] The haptoglobin for use according to any of items 1 to 96, wherein the haptoglobin is administered in solid form.

[101] The haptoglobin for use according to any of items 75 to 96 and 100, wherein the hemopexin is administered in solid form.

[102] The haptoglobin for use according to any of items 83 to 96 and 100-101, wherein the pharmaceutical composition is in solid form.

[103] The haptoglobin for use according to any of items 86 to 98, wherein the pharmaceutical composition is administered in the same manner as specified for the haptoglobin in any of items 2 to 74.

[104] The haptoglobin for use according to any of items 75 to 89, wherein the haptoglobin and the hemopexin are administered to the subject separately.

[105] The haptoglobin for use according to item 104, wherein the haptoglobin and the hemopexin are administered to the subject sequentially.

[106] The haptoglobin for use according to any of the preceding items, wherein the subject is human.

[107] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering a therapeutically effective amount of haptoglobin and optionally hemopexin.

[108] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering at least one dose comprising a therapeutically effective amount of haptoglobin and optionally hemopexin.

[109] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises exposing the brain parenchyma of the subject to the haptoglobin and optionally hemopexin administered to the subject.

[110] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises exposing the brain parenchyma of the subject to a therapeutically effective amount of the haptoglobin and optionally hemopexin administered to the subject.

[111] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering haptoglobin to the subject in a dosage of at least 1 ng, or 10 ng, or 100 ng, or 1 µg, or 10 µg, or 100 µg, or 1 mg, or 5 mg, or 10 mg, or 30 mg, or 200 mg, or at least 400 mg, per kg of body weight of the subject per dosage unit.

[112] The haptoglobin for use according to any of the preceding items, wherein the treatment comprises administering haptoglobin to the subject in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, or 100 µg to 500 mg, or 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject per dosage unit.

[113] The haptoglobin for use according to any of items 86-88 and 90-112, wherein the pharmaceutical composition comprises haptoglobin in a dosage of at least 1 ng, or 10 ng, or 100 ng, or 1 µg, or 10 µg, or 100 µg, or 1 mg, or 5 mg, or 10 mg, or 100 mg, or 200 mg, or at least 400 mg, per kg of body weight of the subject.

[114] The haptoglobin for use according to any of items 86-88 and 90-113, wherein the pharmaceutical composition comprises haptoglobin in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, or 100 µg to 500 mg, or 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject.

[115] The haptoglobin for use according to any of items 75-87 and 89-114, wherein the treatment comprises administering hemopexin to the subject in a dosage of at least 1 ng, or 10 ng, or 100 ng, or 1 µg, or 10 µg, or 100 µg, or 1 mg, or 5 mg, or 10 mg, or 30 mg, or 200 mg, or at least 400 mg, per kg of body weight of the subject per dosage unit.

[116] The haptoglobin for use according to any of items 75-87 and 89-114, wherein the treatment comprises administering hemopexin to the subject in a dosage of 1 ng to 1000 mg, or 10 ng

to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, or 100 µg to 500 mg, or 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject per dosage unit.

[117] The haptoglobin for use according to any of items 86-87 and 89-116, wherein the pharmaceutical composition comprises hemopexin in a dosage of at least 1 ng, or 10 ng, or 100 ng, or 1 µg, or 10 µg, or 100 µg, or 1 mg, or 5 mg, or 10 mg, or 100 mg, or 200 mg, or at least 400 mg, per kg of body weight of the subject.

[118] The haptoglobin for use according to any of items 86-87 and 89-117, wherein the pharmaceutical composition comprises hemopexin in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, or 100 µg to 500 mg, or 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject.

[119] The haptoglobin for use according to any of items 82-84 and 86-114, wherein the pharmaceutical composition comprises from 0.001 g to 10 g, or 0.01 g to 5 g, haptoglobin.

[120] The haptoglobin for use according to any of items 86-87 and 89-119, wherein the pharmaceutical composition comprises from 0.001 g to 10 g, or 0.01 g to 5 g, hemopexin.

[121] The haptoglobin for use according to any of the preceding items, wherein the treatment additionally comprises administering to the subject a further agent for treating or preventing a secondary brain injury in a subject suffering from a TBI.

[122] The haptoglobin for use according to any of the preceding items, wherein a biofluid of the subject, preferably the cerebrospinal fluid (CSF) and/or serum, is monitored to assess treatment progress.

[123] The haptoglobin for use according to item 122, wherein one or more TBI biomarkers (e.g. selected from Hp-related proteins (HPR), heme-binding protein 1 (HEBP1), heme-binding protein 2 (HEBP2), ferritin heavy chain (FTH1), ferritin light chain (FTL), transferrin receptor protein-1 (TFRC1) and combinations thereof, and optionally glial fibrillary acidic protein (GFAP), ubiquitin carboxylterminal hydrolase L1 (UCH-L1), S100B, neuron-specific enolase (NSE), neurofilament protein light (NfL), and/or total tau) in a biofluid of the subject, preferably CSF and/or serum, are monitored, preferably by liquid chromatography mass spectrometry (LC-MS);

optionally wherein, during the acute and/or subacute phase of the TBI the secondary brain injury is marked by an increase in HPR level, an increase in HEBP2 level, a decrease in

HEPB1 level, an increase in FTH1 level, an increase in FTL level, and/or a decrease in TFRC1 level in the CSF and/or plasma of the subject; and

optionally wherein treatment progress is marked by a decrease in HPR level, a decrease in HEBP2 level, an increase in HEPB1 level, a decrease in FTH1 level, a decrease in FTL level, and/or an increase in TFRC1 level in the CSF and/or plasma of the subject.

[124] The haptoglobin for use according to any of the preceding items, wherein treatment progress is monitored using MRI, CT, PET, SPECT, transcranial Doppler (TCD), or a combination thereof.

[125] The haptoglobin for use according to any of the preceding items, wherein treatment progress is monitored using standard electroencephalogram (EEG) or quantitative electroencephalography (qEEG), pupilometers, infrared scanner, magnetoencephalography (MEG), vision and oculomotor assessment using an eye movement tracking device (such as saccadometers or electrooculography), or a combination thereof.

[126] The haptoglobin for use according to any of the preceding items, wherein treatment progress is monitored using one or more glial, preferably astroglial, metabolic biomarkers (e.g. GFAP, or IBA1, and/or brain-specific isoform of the glycolytic enzyme aldolase, ALDOC).

[127] Haptoglobin (Hp) for use in the neuroprotective treatment of a traumatic brain injury (TBI) in a subject; optionally wherein the use is characterized as defined in any of items 1 to 126.

[128] Haptoglobin (Hp) for use in the neuroprotective treatment in a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[129] Haptoglobin (Hp) for use in the reduction of progressive brain damage in a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[130] Haptoglobin (Hp) for use in the treatment or prevention of intracranial lesions in a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[131] Haptoglobin (Hp) for use in the treatment or prevention of intracranial lesion formation and/or progression in a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[132] Haptoglobin (Hp) for use in the treatment or prevention of neuroinflammation in a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[133] Haptoglobin (Hp) for use in the treatment of impaired motor function in a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 1226.

[134] Haptoglobin (Hp) for use in treating or preventing one or more adverse effects associated with iron accumulation in the brain parenchyma of a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[135] Haptoglobin (Hp) for use in treating or preventing iron-mediated toxicity in the brain parenchyma of a subject suffering from a traumatic brain injury (TBI); optionally wherein the use is characterized as defined in any of items 1 to 126.

[136] Use of haptoglobin (Hp) for the manufacture of a medicament i) for the neuroprotective treatment of a traumatic brain injury (TBI) in a subject, or ii) for the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI), or iii) for the reduction of progressive brain damage in a subject suffering from a traumatic brain injury (TBI); optionally wherein the treatment is characterized as defined in any of items 1 to 126 and/or wherein the medicament comprises haptoglobin, and optionally hemopexin (Hx), as defined in any of items 1 to 126.

[137] Method of neuroprotective treatment of a traumatic brain injury (TBI) in a subject, comprising administering haptoglobin (Hp), and optionally hemopexin (Hx), as defined in any one of items 1 to 126 to the subject; optionally wherein the treatment is characterized as defined in any of items 1 to 126.

[138] Method of treating or preventing a secondary brain injury in a subject suffering from a traumatic brain injury (TBI), comprising administering haptoglobin (Hp), and optionally hemopexin (Hx), as defined in any one of items 1 to 126 to the subject; optionally wherein the treatment is characterized as defined in any of items 1 to 126.

[139] Method for the reduction progressive brain damage in a subject suffering from a traumatic brain injury (TBI), comprising administering haptoglobin (Hp), and optionally hemopexin (Hx), as defined in any one of items 1 to 126 to the subject; optionally wherein the treatment is characterized as defined in any of items 1 to 126.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic representation of the experimental design. Five independent cohorts of mice (n=10-12) were included in this longitudinal study. TBI was induced by a controlled-cortical impact (CCI) on the right parietal cortex of the mice brain. 30 min and 5 days post-TBI, compounds were administered alone or in a combination. Brains were analyzed by MRI and DigiGait analysis as well as biochemical analysis at 24 h, 48 h and 7 days post TBI (i.e. post initial trauma in the form of the CCI) as depicted in the diagram.

Figure 2: *In vivo* characterization of haptoglobin (Hp) and hemopexin (Hx) levels at 24 h and 7 days after TBI (that is, after the initial trauma in the form of controlled-cortical impact [CCI]). Representative images (n=6 for each group and timepoint) showing Hp and Hx protein levels in WT SHAM and TBI mice brains treated with 400 mg/kg of Hp or Hx alone or in combination 24 h and 7 days after TBI (i.e. after the CCI) as assessed by western blot analysis. Presence of Hp and Hx was confirmed in Hp and Hx or combination treated mice brains at 24 h after the CCI and diminished 7 days after the CCI. The observed molecular weight for Hp and Hx was 43 and 75 kDA, respectively.

Figure 3: Administration of 400 mg/kg of kg hemopexin (Hx) post controlled-cortical impact (CCI) showed unchanged left forelimb function, as determined using DigiGait™, compared to control (placebo). TBI-placebo mice exhibit deficits in their left forelimb, prominent at 24 h post CCI; and returned to SHAM levels at 48 h and 7 days after CCI as compared to SHAM mice in parameters of propulsion(s) (**A**), swing(s) (**B**) and stride(s) (**C**) duration as detected by DigiGait™ system. No significant changes were detected between TBI-Hx group and TBI-placebo group for these parameters (n=6-12). In assessing time taken to brake (**D**), TBI-Hx group had decreased brake duration as compared to their SHAM and placebo counterparts at 24 h after CCI. Data represents mean + SEM.

Figure 4: Administration of 400 mg/kg of haptoglobin (Hp) post controlled-cortical impact (CCI) improves neurological function compared to control (placebo), as determined using DigiGait™ 24 h after CCI in left forelimb function. Following CCI, TBI-placebo mice exhibited deficits in their left forelimb, prominent at 24 h, which returned to SHAM levels at 48 h and 7 days after TBI as compared to SHAM mice in parameters of propulsion(s) (**A**), swing(s) (**B**) and stride(s) (**C**) duration

as detected using the DigiGait™ system. Significant behavioural improvement was detected in TBI-Hp group compared to TBI-placebo group (n=6-8) 24 h after CCI (**A**), (**B**) and (**C**). When assessing time taken to brake (**D**), TBI-Hp mice showed a significant decrease in brake duration at 48 h after CCI as compared to their SHAM and placebo group counterparts. Data represents mean + SEM.

Figure 5: Administration of 200 mg/kg of haptoglobin (Hp) and 200mg/kg hemopexin (Hx) in combination post CCI improves neurological function compared to control (placebo), as determined using DigiGait™ 24 h after TBI in left forelimb function. Following CCI, TBI-placebo mice exhibited deficits in their left forelimb, prominent at 24 h, which returned to SHAM levels at 48 h and 7 days after TBI as compared to SHAM mice in parameters of propulsion(s) (**A**), swing(s) (**B**) and stride(s) (**C**) duration as detected by DigiGait™ system. Significant behavioural improvement detected between TBI-Hx+Hp group and TBI-placebo group (n=6-8) 24 h after CCI (**A**), (**B**) and (**C**). When assessing time taken to brake (**D**), TBI-Hx+Hp mice showed a significant decrease in brake duration at 48 h after CCI as compared to their SHAM and placebo group counterparts. Data represents mean + SEM.

Figure 6: MRI images reveal a statistically smaller lesion volume in Hp-treated mice alone or in combination with Hx at 24 h and 7 days post controlled-cortical impact (CCI) as compared to the PBS-treated mice. Representative T2-MRI images of SHAM, TBI-PBS, TBI-Hp, TBI-Hx and TBI-Hx+Hp mice showing lesion area at 24h and 7 days after CCI (**A**). Quantification of MRI analysis showed that Hp and Hp+Hx-treated mice had significantly reduced lesion volumes at 24 h and 7 days post CCI as compared to PBS-treated mice (**B**).

Figure 7: Hp treated mice exhibit reduced GFAP expression in the ipsilateral striatum at 7 days post controlled-cortical impact (CCI) as compared with PBS-treated mice 24 h after TBI. (**A**) GFAP protein expression was detected in the ipsilateral cortex and ipsilateral striatum of WT brains by western blot analysis (n=3). GFAP levels were normalized to β-actin. For densitometry calculations, GFAP/β-actin ratio was then determined from these values and was calculated as a fold change relative to SHAM control as shown in (**B**). Data is expressed as mean + SEM, *$p < 0.05$, **$p < 0.01$. IC = ipsilateral cortex, IS = ipsilateral striatum.

Figure 8: Compound treated mice exhibit a reduced GFAP immunostaining compared to PBS treated mice 24 h post controlled-cortical impact (CCI). (**A** and **C-F**) High resolution of GFAP (grey) staining near the lesion area. (**B**) Representative diagram indicates brain regions (marked by grey box) assessed for GFAP immunostaining. GFAP intensity is quantified in (**G**) showing reduced GFAP immunoreactivity in the Hx, Hp and Hp+Hx-treated mice as compared to the PBS-treated mice 24 h after CCI suggesting a reduced neuro-inflammatory response in the presence of Hp and/or Hx. Data represent mean + SEM (n=4).

Figure 9: Hp and/or Hx treated mice exhibit ramified microglial morphologies with IBA-1 immunostaining 24 h post TBI (i.e. post CCI). 24 h after CCI, compound treated mice displayed non-activated ramified morphology of microglia with IBA1 staining (**D**, **E** and **F**) as compared to PBS-treated mice (B). (**C**) Representative diagram indicates brain regions (marked by grey box) assessed for IBA-1 immunostaining. IBA1 expression was reduced in the compound treated mice 24h after TBI as quantified in (**G**) (n=4 mice). Data represent mean + SEM.

Figure 10: (**A**) Increased expression of CSF Hp (α and β chains) was detected at 1 day post controlled-cortical impact (CCI), with a significant upregulation in Hp-related protein (HPR) (**B**) identified at day 0 to day 7 post CCI as compared to the control group. (**C**) This correlated with an upregulation in scavenger receptor cysteine-rich type 1 (CD163) protein at day 4 to day 10 post CCI. A significant upregulation in heme-binding protein 2 (HEBP2) levels was detected at day 1 to day 10 post CCI (**D**); while HEBP1 levels in the human CSF TBI samples was reduced as compared to the control group (**E**). Hx was detected at a low level in the human TBI CSF as compared to the control group (**F**). A marked increase in the expression of iron-regulatory proteins ferritin light chain (FTL) and ferritin heavy chain (FTH1), which was associated with a reduced transferrin receptor protein-1 (TFRC1) level at similar time points, was observed (**G**, **H**, and **I**).

DETAILED DESCRIPTION

Treatment or prevention of secondary brain injury

The present invention is based, at least in part, on the unexpected finding of the present inventors that haptoglobin (Hp) can be used in the neuroprotective treatment of a traumatic brain injury (TBI), and in particular, that Hp is effective in treating or preventing secondary brain injury that arises in

the minutes, hours and days after the initial trauma, that is after exposure to the external force acting on the head of a subject and resulting in TBI. Thus, the present inventors found that, following the initial trauma to the head resulting in TBI, haptoglobin can be used for the reduction of progressive brain damage.

Haptoglobin and hemopexin (Hx), which previously were reported for use in treating or preventing an adverse secondary neurological outcome following a hemorrhagic stroke by exposing the cerebral spinal fluid (CSF) of a stroke victim to Hp and/or Hx to complex and neutralize cell-free hemoglobin (Hb) and/or heme released into the CSF, have not been reported for use in the treating TBI. TBI, although often associated with intracranial hemorrhage, involves a pathogenesis, which is considerably more complex, including a broad spectrum of physiological, cellular and molecular pathways and events, including excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, apoptosis, ferroptosis, necrosis, axonal degeneration, and neuroinflammation. Without wishing to be bound by any theory, the present inventors have found, surprisingly, that treatment with Hp, alone or in combination with Hx, is beneficial on multiple fronts in the complex cascade of physiological, cellular and molecular events that results from the structural damage suffered in the initial trauma resulting in TBI (e.g. the structural damage suffered in the initial impact of the head), particularly when administered early after this initial trauma, and is thus useful in treating, reversing and/or preventing such physiological, cellular and/or molecular events, thereby reducing progressive brain damage.

Therefore, in a first aspect the present invention relates to haptoglobin (Hp) for use in the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI). The term “for use in the” as used herein is to be understood to be synonymous with “for use in a method of”. The terms “traumatic brain injury” and “TBI” are used herein interchangeably and are known to those skilled in the art, preferably referring to an intracranial injury caused by an external force, in particular an intracranial injury resulting an injury to the head through an external force, such as a physical impact, e.g. following a fall, vehicle crash or a blow to the head with an object. According to a particular embodiment of the present invention, a TBI is an injury to the brain caused by an external force. For example, a traumatic brain injury may result when the brain is subjected to an external physical force that results in progressive neuronal cell damage and/or cell death.

An external physical force giving rise to the initial trauma of a traumatic brain injury (TBI) may induce, for example, skull fracture, meningeal injury, parenchymal injury, and/or vascular injury. A traumatic brain injury may manifest as either blunt trauma (closed injury from initial trauma) or an open trauma (open injury from initial trauma). Accordingly, the Hp according to the invention may be for use in treating a TBI comprising a blunt initial trauma, as well as a TBI comprising a penetrating initial trauma. The term “initial trauma” or “primary trauma” as used herein in the context of a TBI preferably refers to a trauma to the head (a “trauma to the head” being injury/damage to the head [such as to scalp, skull, meninges, brain and/or cerebral vasculature] resulting from exposure to an external force) that occurs at the moment of, and is directly caused by, exposure to the external force that results in a TBI.

According to the present invention, the haptoglobin (Hp) is for use in the treatment or prevention of a secondary brain injury in a subject suffering from a TBI. TBI can be divided into two discrete stages, primary and secondary injury (Prasetyo, 2020). The term “primary brain injury” as used herein refers to an irreversible process in the form of the physical damage (injury) to the brain that occurs at the moment of, and is directly caused by, exposure to an external force that results in a TBI. Primary brain injury involves damage to brain tissue directly resulting from the exposure to an external force giving rise to a TBI and may include contusion(s), laceration(s), focal lesion(s), diffuse axonal injury, brain swelling and/or intracranial hemorrhage, and invariably results in immediate cell death.

The term “secondary brain injury” in the context of a TBI is known to the skilled person. The term "secondary brain injury", as used herein, generally refers to a (secondary) injury to brain tissue that follows the primary brain injury, the primary brain injury being the brain injury (brain damage) that occurs at the moment of exposure to and is directly caused by an external force that results in a TBI. Thus, the term “secondary brain injury” as used herein may for example refer to any brain injury that directly follows the primary brain injury of a TBI, that is, it preferably refers to the brain injury that directly follows the initial trauma of the TBI. The secondary brain injury arises in the minutes, hours and days after the initial trauma (the primary brain injury), that is after exposure to the external force resulting in a TBI. Thus, additionally, the term “secondary brain injury” as used herein may for example refer to the brain injury also caused by the external force acting on the head of the subject and resulting in the TBI, but occurring progressively after the initial trauma or primary brain injury as a direct consequence thereof. According to one embodiment of the present

invention, “treatment or prevention of a secondary brain injury” (following the primary brain injury/initial trauma of a TBI) means reduction of progressive brain damage.

According to a particular embodiment of the present invention, the haptoglobin is for use in the neuroprotective treatment of a traumatic brain injury (TBI) in the subject. The terms “neuroprotective” and “neuroprotection” in the context of the present invention generally refer to the protection (preservation) of function and/or structure of neurons and/or prevention of neuronal cell death (i.e. protection of neurons from injury or degeneration). Thus, a “neuroprotective treatment” in the present context preferably refers to a treatment that is aimed at protection (preservation) of function and/or structure of neurons and/or prevention of neuronal cell death (thus preferably protecting neurons from injury and/or degeneration following an initial trauma resulting in a TBI) in a subject suffering from a TBI.

Secondary brain injury (after exposure to the external force that results in a TBI) may be caused by a cascade of cellular and molecular events initiated as a consequence of the primary brain injury, such as mass effect (i.e. compression, and consequently injury, of areas of brain tissue or brain structures surrounding a focal lesion or contusion, e.g. due to leaking of blood or cerebrospinal fluid, or edema), physical disruption, the physiological response to a hematoma, like inflammation, and/or the release of blood and blood components, thus preferably involving a dynamic interplay between ischemia, inflammation, and cytotoxic processes. As such, the term “secondary brain injury” as used herein preferably refers to a process that occurs gradually as a consequence of a complex cascade of cellular and molecular alterations resulting in further brain damage following the primary brain injury. Secondary brain injury may comprise functional and/or structural impairment of brain cells (such as neurons and glia cells) and cerebral cell death.

According to one embodiment of the present invention, the subject suffering from traumatic brain injury (TBI) is at risk of developing a secondary brain injury. According to another embodiment of the present invention, the TBI of the subject is accompanied by a secondary brain injury. According to a specific embodiment of the present invention, the secondary brain injury is within the brain parenchyma and/or the meninges. According to another specific embodiment of the present invention, the secondary brain injury is within the brain parenchyma.

The secondary brain injury may for example be the result of excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, brain iron accumulation (e.g. through blood hemoglobin and iron release into the brain parenchyma), cerebral cell death (apoptosis, ferroptosis [i.e. programmed cell death dependent on iron and characterized by the accumulation of lipid peroxides] and/or necrosis), axonal degeneration, neuroinflammation, dysfunction of cerebral blood flow, cerebral ischemia, cerebral hypoxia, cerebral hypotension, dysfunction of brain metabolism, cerebral hypo- or hyper-carbia, cerebral hypo- or hyper-glycemia, cerebral hypo- or hyper-thermia, cerebral edema (e.g. vasogenic cerebral edema and/or cytotoxic cerebral edema), raised intracranial pressure, breakdown of the blood–brain barrier and/or seizures, preferably of at least excitotoxicity, oxidative stress and/or neuroinflammation. According to one embodiment the secondary brain injury is brain damage involving, or resulting from, excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, brain iron accumulation (e.g. through blood hemoglobin and iron release into the brain parenchyma), apoptosis (apoptotic cell death), ferroptosis, necrosis, axonal degeneration, neuroinflammation, dysfunction of cerebral blood flow, cerebral ischemia, cerebral hypoxia, cerebral hypotension, dysfunction of brain metabolism, cerebral hypo- or hyper-carbia, cerebral hypo- or hyper-glycemia, cerebral hypo- or hyper-thermia, cerebral edema (e.g. vasogenic cerebral edema and/or cytotoxic cerebral edema), raised intracranial pressure, breakdown of the blood–brain barrier and/or seizures. According to a preferred embodiment the secondary brain injury involves (or is the result of) excitotoxicity, oxidative stress and/or neuroinflammation, and optionally mitochondrial dysfunction, oxidative stress, lipid peroxidation, brain iron accumulation, apoptotic cell death, necrosis, axonal degeneration, dysfunction of cerebral blood flow, cerebral ischemia, cerebral hypoxia, cerebral hypotension, dysfunction of brain metabolism, cerebral hypo- or hyper-carbia, cerebral hypo- or hyper-glycemia, cerebral hypo- or hyper-thermia, cerebral edema, raised intracranial pressure, breakdown of the blood–brain barrier and/or seizures.

Illustrative examples of secondary brain injury include formation and/or progression of intracranial lesions, cerebral edema and cerebral edema-related damage, neuroinflammation-related damage, impaired motor function, impaired cognitive function, neurotoxicity, cerebral cell death (apoptosis, ferroptosis and/or necrosis of brain cells) such as neuronal cells death and glial cell death, nitric oxide depletion-related damage, oxidative tissue injury, iron-mediated damage (damage associated with accumulation of iron in the brain), ischemia-related damage (such as from cerebral vasospasm, impaired cerebral vasoreactivity, delayed ischemic neurological deficit [DIND], and/or

delayed cerebral ischemia [DCI]), blood–brain barrier impairment-related damage, and spreading depolarization.

According to one embodiment of the present invention, the secondary brain injury is selected from the group consisting of formation and/or progression of intracranial lesions, cerebral edema and cerebral edema-related damage, neuroinflammation-related damage, impaired motor function, impaired cognitive function, neurotoxicity, cerebral cell death (apoptosis, ferroptosis and/or necrosis of brain cells) such as neuronal cells death and glial cell death, nitric oxide depletion-related damage, oxidative tissue injury, iron-mediated damage (damage associated with accumulation of iron in the brain), ischemia-related damage (such as from cerebral vasospasm, impaired cerebral vasoreactivity, delayed ischemic neurological deficit [DIND], and/or delayed cerebral ischemia [DCI]), blood–brain barrier impairment-related damage, and spreading depolarization, or a combination thereof. According to a specific embodiment of the present invention, the secondary brain injury is selected from the group consisting of formation and/or progression of intracranial lesions, cerebral edema and cerebral edema-related damage, neuroinflammation-related damage, impaired motor function, cerebral cell death (apoptosis, ferroptosis and/or necrosis of brain cells), iron-mediated damage (damage associated with accumulation of iron in the brain), blood–brain barrier impairment-related damage, or a combination thereof. According to another specific embodiment of the present invention, the secondary brain injury is selected from the group consisting of formation and/or progression of intracranial lesions, cerebral edema and cerebral edema-related damage, neuroinflammation-related damage, impaired motor function, iron-mediated damage (damage associated with accumulation of iron in the brain), or a combination thereof. According to yet another specific embodiment of the present invention, the secondary brain injury is selected from the group consisting of formation and/or progression of intracranial lesions, neuroinflammation-related damage, impaired motor function, or a combination thereof.

Secondary brain injury in the form of cerebral edema may for example refer to any cerebral edema that occurs after the primary brain injury, but as a consequence of the initial trauma and/or the primary brain injury. Secondary brain injury in the form of cerebral edema-related damage preferably refers to any brain injury that is not part of an edema itself and, in part or completely, results from cerebral edema, that is, brain injury associated with cerebral edema. Cerebral ischemia-related damage preferably refers to brain injury that is, in part or completely, caused by

or the direct result of, cerebral ischemia, that is, brain injury associated with ischemia. Impaired motor function preferably refers to neuromotor impairment. Iron-mediated damage in the context secondary brain injury preferably refers to brain injury that is, in part or completely, caused by or the direct result of, accumulation of iron in the brain, that is, brain injury associated with accumulation of iron in the brain, e.g. through blood hemoglobin and iron release into the brain parenchyma and/or through other mechanisms. Ferroptosis refers to programmed cell death dependent on iron and characterized by the accumulation of lipid peroxides.

According to a preferred embodiment the secondary brain injury comprises, or consists of, intracranial lesions. According to another preferred embodiment the secondary brain injury comprises, or consists of, formation and/or progression of intracranial lesions. The term "intracranial lesion" is known to the skilled person and preferably refers to a lesion occurring in the skull (i.e. in the cranium), that is a localized pathological change in the tissue within the skull (i.e. a localized damage or abnormal change in the tissue within the skull). According to one embodiment the term "intracranial lesion" refers to a brain lesion (i.e. brain mass lesion). According to another embodiment the term "intracranial lesion" refers to a lesion that is located within the brain parenchyma. Intracranial lesions may be visualized or monitored, for example, using magnetic resonance imaging (MRI) or a computed tomography (CT) scan, preferably using MRI optionally by determining lesion volume.

According to a preferred embodiment the secondary brain injury comprises, or consists of, neuroinflammation. The term "neuroinflammation" is known to the skilled person. As used herein "neuroinflammation" in the context of TBI and secondary brain injury preferably refers to inflammation (i.e. a short and/or long-term inflammatory response) within the brain following exposure to an external force that results in TBI. The term "neuroinflammation-related damage" preferably refers to brain injury that is, in part or completely, caused by or the direct result of, neuroinflammation, that is, brain injury associated with neuroinflammation. According to one embodiment, neuroinflammation comprises gliosis, e.g. astrogliosis and/or microgliosis. Neuroinflammation may be assessed, for example, by monitoring gliosis, e.g. astrogliosis and/or microgliosis. Astrogliosis can be determined, for example, by testing for an astrocyte marker protein, such as glial fibrillary acidic protein (GFAP). Microgliosis can be determined, for example, by testing for a microglia/macrophage marker protein, such as Ionized calcium-binding adapter molecule 1 (IBA1). Such a marker protein or marker proteins may be determined for example in

the blood or cerebrospinal fluid (CSF). According to a particular embodiment of the present invention, the haptoglobin is for use in reducing glial reactivity, particularly astrocyte and/or microglia reactivity, in the subject suffering from the TBI.

According to a preferred embodiment the secondary brain injury comprises, or consists of, impaired motor function. The term “impaired motor function” or “neuromotor impairment” is known in the art and preferably refers to a partial or total loss of function of a body part, such a limb or limbs. Impaired motor function may be monitored, for example, by assessing gait, balance, strength, reaction time, etc.

The traumatic brain injury (TBI) may be categorized, for example as mild TBI, moderate TBI or severe TBI. According to one embodiment of the present invention, the TBI is mild TBI. According to another embodiment of the present invention, the TBI is moderate TBI. According to yet another embodiment, the TBI is severe TBI. Severe traumatic brain injury can for example occur from lacerations, skull fractures, and conversely, even in the absence of external signs of head injury. There are different ways to assess the severity of TBI in a subject. For example, behavioral assays (e.g. Glasgow Coma Scale (GCS) index), neuroimaging (e.g. CT or MRI), and/or blood-based biomarkers (e.g. GFAP or UCH-L1) may be used (Berwick et al., 2022). They may be combined, since each can provide unique and complementary information.

The Glasgow Coma Scale (GCS) provides a practical method for assessing and classifying TBI patients for clinical care and research. Patients are assessed and assigned a numerical score for each of the three components of the scale - based on eye opening (E), verbal response (V), and motor response (M) (Teasdale and Jennett, 1974). The summing up of these separate subscale scores leads to a single sum, or total, score (e.g. GCS 8). Based on this sum score a patient can then be categorized as suffering from mild TBI (GCS 13–15), or moderate TBI (GCS 9–12), or severe TBI (GCS <9). The GCS reflects the depth of impaired consciousness and is best obtained following initial resuscitation (oxygenation, rehydration and support of blood pressure) but prior to use of sedating drugs, neuromuscular blocking agents, or endotracheal intubation. Thus, according to one embodiment of the present invention, the TBI severity in a subject is assessed inter alia using the Glascow Coma Scale (GCS); optionally whereby mild TBI is characterized by a GCS of 13-15, moderate TBI is characterized by a GCS of 9 to 12, and severe TBI is characterized by a GCS of <9.

TBI is often accompanied by an intracranial hemorrhage such as an intracerebral hemorrhage (ICH), an epidural hemorrhage, a subdural hemorrhage and/or a subarachnoid hemorrhage. According to one embodiment of the present invention, the traumatic brain injury (TBI) is accompanied by an intracranial hemorrhage. According to another embodiment of the present invention, the traumatic brain injury (TBI) is accompanied by an intracerebral hemorrhage (ICH), an epidural hemorrhage, a subdural hemorrhage and/or a subarachnoid hemorrhage. In this context, an intracerebral hemorrhage (ICH) may be for example be an intraparenchymal hemorrhage (IPH) or an intraventricular hemorrhage (IVH).

The subject is not particularly limited as long as it is suffering from a TBI. According to a specific embodiment the subject suffering from TBI sustained the initial trauma resulting in TBI within the last 100 days, preferably within the last 50 days, more preferably within the last 28 days, e.g. within the last 25 days, or 20 days, or 15 days, or 14 days, or 13 days, or 12 days, or 11 days, or 10 days, or 9 days, or 8 days, or 7 days, or 6 days, or 5 days, or 4 days, or 3 days, or 2 days, or 1 day. According to a preferred embodiment the subject is human.

Haptoglobin

The term “haptoglobin” or “Hp” is known those skilled in the art and may refer to a tetrameric or multimeric glycoprotein that can form a complex with cell-free hemoglobin (Hb) to neutralize the biological activity of cell-free Hb. Herein, “haptoglobin” and “Hp” are used interchangeably.

In humans, genetically, the Hp gene can be present in the form of three alleles, Hp1F, Hp1S, and Hp2, which control the formation of six Hp phenotypes, 1F-1F, 1S-1S, 1F-1S, 2-1F, 2-1S, and 2-2. Due to the lack of functional difference between Hp1F and Hp1S, which differ only in two amino acids, only two alleles, Hp1 and Hp2, are often considered, which manifest themselves as three phenotypes: homozygous Hp1-1 and Hp2-2, and heterozygous Hp1-2 (also referred to as Hp2-1) depending on the combination of inherited allelic variants (Naryzny, SN and Legina OK, 2021). In Western populations, it is estimated that the distribution of Hp1-1 is about 16%, Hp1-2 is about 48%, and Hp2-2 is about 36%.

Produced for example by hepatocytes, a main function of Hp is to prevent iron loss due to hemolysis of erythrocyte, through its affinity and binding capacity for free hemoglobin (Hb). In vivo, Hp is synthesized as a single-chain polypeptide precursor (Pre-Hp1 or Pre-Hp2), which is post-translationally cleaved into an amino-terminal α chain and a carboxy-terminal β chain, that is, the α and β-subunits of the native protein. The polypeptide precursor may include an amino-terminal 18 residue signal sequence before the α chain, and/or an intervening polypeptide between the α and β-regions (Misumi et al., 1983), which in humans may be a single amino acid (e.g. Arg84 α1-chain and Arg143 α2-chain). In vivo, post-translational events generally also result in the proteolytic removal of the signal sequence and the incorporation of core oligosaccharide side chains into the β-region by membrane-associated enzyme systems (Haugen et al., 1981).

The basic unit of Hp is usually composed of an alpha (α) chain and a beta (β) chain linked together with a disulfide bridge, forming an αβ-heterodimer. Two such basic units, linked by a disulfide bond via their respective α chains can form a tetrameric Hp found in most mammals. In the human population, in addition to the shorter alpha chain variant of for example approximately 9 kDa found in most mammals, a variant with a long α chain is also present. This alpha chain variant appears to have arisen by an early intragenic duplication, presumably originating from an unequal crossing over of two basic alleles, resulting in an Hp with a longer alpha (α) chain of for example approximately 16 kDa. The short and long α chains are designated as alpha1 (α1) chain and alpha2 (α2) chain, respectively. Since during the intragenic duplication the cysteine forming the intermolecular disulfide bond between the α chains is also duplicated, humans carrying the long variant (α2) allele usually exhibit a multimeric Hp phenotype (Naryzny, SN and Legina OK, 2021). That is, for both α1 chain and α2 chain, a cysteine close to the N-terminus is usually present and may form a disulfide bridge to another α chain, a cysteine close to the C-terminus is usually present and may form a disulfide bridge to a β chain, and additionally only for α2 chains, a central cysteine is usually present and may form a third disulfide bridge thereby interacting with an additional αβ unit (α1β or α2β) to form a multimer which goes beyond a tetrameric $(\alpha\beta)_2$ structure.

Thus, the Hp1-1 phenotype typically presents itself at the protein level as a single form in the configuration of a tetramer containing two alpha1 (α1) chains and two beta (β) chains, also referred to as a “Hp1-1 dimer” or “Hp1-1 homodimer” due to the two (α1β) basic units making up the tetramer. That is, ordinarily such Hp1-1 protein may be a tetramer composed as follows: (α1β)2. The Hp1-2 phenotype is generally characterized by haptoglobin proteins that comprise, apart from

beta chains, alpha1 chains and alpha2 chains, and thus may form multimers such as linear polymers. That is, for the Hp1-2 phenotype, typically such a linear Hp1-2 protein (polymer) may be composed as follows: (α1β)2 (α2β)n, where n = 0, or 1, or 2, or 3, or 4, or 5, or 6, or 7, or 8, etc. The Hp2-2 phenotype is generally characterized by haptoglobin proteins that are composed of alpha2 chains and beta chains and thus can form multimers, in particular cyclic polymers. That is, for the Hp2-2 phenotype, typically a Hp2-2 protein may be a cyclic multimeric protein comprising, or consisting of, three or more (α2β) basic units, i.e. ordinarily such a cyclic Hp2-2 protein (polymer) may be composed as follows: (α2β)n, where n = 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, etc. The beta chain is mainly responsible for binding Hb, thus the different phenotypes have similar Hb binding affinity.

The α1 chain comprises two variants determining the existence of subtypes within Hp phenotypes Hp1-2 and Hp1-1, polymorphic variants α1F and α1S. As used herein “α1F” and “α-1S” may refer to α1 isoforms differing in amino acid sequence at the positions 52 and 53 in relation to mature human α1 isoform amino acid sequence (Naryzny, SN and Legina OK, 2021). As used herein, “α1F” and “α1S” may for example refer to α1 isoforms differing in amino acid sequence only at the positions 52 and 53 when using mature human α1 isoform amino acid sequence as a reference, where the chain α1F contains aspartic acid and lysine, while chain α1S contains an asparagine and a glutamic acid in these positions. These different isoforms share essentially equivalent functional characteristics as for example in hemoglobin affinity, binding and clearance.

The canonical sequence of α2-β precursor of human haptoglobin (human haptoglobin isoform 1 preproprotein) may correspond to isoform 1 in the UniProt entry P00738 (NCBI accession numbers NP_005134). The sequence of P00738 isoform 1 (Pre-Hp2) may be as shown in SEQ ID NO: 1. The sequence of a α1-β precursor of an isoform of human haptoglobin (human haptoglobin isoform 2 preproprotein) may correspond to isoform 2 in the UniProt entry P00738 (NCBI accession numbers NP_001119574.1). In the UniProt and NCBI entries this sequence differs from canonical Isoform 1 in that amino acids 38-96 are missing. The sequence of isoform 2 (Pre-Hp1) may be as shown in SEQ ID NO: 2. The α1F chain of human haptoglobin may have an amino acid sequence as shown in SEQ ID NO: 3. The α1S chain of human haptoglobin may have an amino acid sequence as shown in SEQ ID NO: 4. The α2 chain of human haptoglobin may have an amino acid sequence as shown in SEQ ID NO:5. The β chain of human haptoglobin may have an amino acid sequence as shown in SEQ ID NO:6.

As used herein, the term “haptoglobin” is to be understood to encompass all phenotypes (including all isoforms) of Hp. The haptoglobin may be homologous (so long as it consists essentially of Hp of the same isoform) or heterologous (including combinations of different Hp isoforms, including Hp1-1, Hp1-2 and Hp2-2). Optionally, the haptoglobin for use according to the present invention may be a mixture of two or more phenotypes and/or isoforms. It should be understood that the composition of Hp may ultimately depend on the phenotype of the source. For example, if a pooled plasma sample is used to extract/purify Hp, more than one isoform of Hp may be isolated. Suitable methods for determining Hp isoforms present in an isolate will be familiar to one skilled in the art.

The term “haptoglobin” or “Hp” as used herein includes functional analogues of naturally occurring Hp (such as naturally occurring human Hp), as well as naturally occurring Hp. The term "functional analogue" is intended to mean an agent that shares substantially identical biological activity as a naturally occurring (native) Hp, for example, such a biological activity being any ability of native human haptoglobin useful in the treatment of TBI (i.e. the treatment or prevention of secondary brain injury), e.g. including the ability to form a complex with a cell-free Hb to neutralize its biological activity. According to one embodiment the biological activity is at least the ability of the functional analogue to form a complex with a cell-free Hb to neutralize its biological activity.

The haptoglobin may include human and non-human variants. Preferably, the haptoglobin is a human haptoglobin or a haptoglobin having an amino-acid sequence derived from human. Particularly, where the Hp is intended for administration to a human subject, it is generally preferable that the haptoglobin is a (native) human haptoglobin or a close functional analog of (native) human haptoglobin. However, it is to be understood that a non-human isoform of haptoglobin may be used where the intended subject is a human, as long as the non-human isoform of haptoglobin retains a functionality of human haptoglobin such as the ability to bind to human Hb. Illustrative examples of non-human isoforms of haptoglobin will be familiar to persons skilled in the art, illustrative examples of which include haptoglobin derived from bovine, equine or porcine. Preferably, the haptoglobin is a human haptoglobin, such as a protein disclosed under NCBI accession numbers NP_005134 and NP_001119574.1 or UniProt accession number P00738. Various variants are available at that UniProt entry. Thus, the term “human haptoglobin” as used herein may include a protein as disclosed in such NCBI and UniProt entries, as well as functional analogues which are closely related thereto on the sequence level with an amino acid

sequence identity of at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, etc. to amino acid sequences shown in these NCBI and UniProt entries (including e.g. SEQ ID NO: 1-6 disclosed herein).

The Hp for use according to the present invention may be naturally occurring (native) Hp (e.g., derived from plasma) or it may be produced as a recombinant protein. For example, the Hp may be plasma-derived Hp, or recombinant Hp, or a combination thereof. Thus, as used herein, the term “haptoglobin” may for example mean a haptoglobin protein that has been isolated or otherwise at least partially purified from a natural source (e.g., plasma), or a recombinantly produced haptoglobin protein, that comprises, consists of, amino acid residues as shown in SEQ ID NO:3, 4, 5 and/or 6, or an amino acid sequence having at least 80% sequence identity thereto.

The Hp, for example, may comprise, or consist of, plasma derived Hp. In one embodiment, the Hp is plasma derived. For example, the Hp may include an at least partially purified haptoglobin recovered from human plasma. In some embodiments, the haptoglobin comprises, or consists of, naturally occurring human plasma haptoglobin. Suitable naturally occurring forms of Hp are known to those skilled in the art, illustrative examples of which include those disclosed in Koch et al., 2002 and Kasvosve et al., 2010. According to one embodiment, the haptoglobin comprises, or consists of, plasma derived Hp. Protocols for isolating Hp from natural sources of Hp (e.g., plasma) will be familiar to those skilled in the art; an illustrative example of which is described in WO2014/055552.

Where haptoglobin is purified from native source, a person skilled in the art will understand that it may be desirable to reduce the level of active virus content (virus titre) and other potential infectious agents (for example, prions) in the solution. This may be particularly desirable where the starting material is derived from blood plasma. Methods of reducing the virus titre in a solution will be known to persons skilled in the art. Examples include pasteurization (for example, incubating the solution at 60 °C for 10 hours in the presence of high concentrations of stabilizers such as glycine (e.g. 2.75 M) and sucrose (e.g. 50%) and/or other selected excipients or salts), dry heat treatment, virus filtration (passing the solution through a nano-filter; e.g., 20 nm cutoff) and/or subjecting the solution to treatment with a suitable organic solvent and detergent for a period of time and under conditions to inactivate virus in the solution. Solvent detergent has been used for over 25 years to inactivate enveloped viruses particularly in plasma-derived products. Thus it may be carried out using various reagents and methods known in the art (see, for example, US4540573 and US4764369). Suitable solvents include tri-n-butyl phosphate (TnBP) and ether.

In some embodiments, the solvent is about 0.3%, and preferably TnBP. Suitable detergents include non-ionic detergents such as polysorbate (Tween) 80, polysorbate (Tween) 20, Triton X-100, Octyl glucoside (OPG) (typically at about 1%). The selection of treatment conditions including solvent and detergent concentrations depend in part on the characteristics of the source of haptoglobin with less pure sources generally requiring higher concentrations of reagents and more extreme reaction conditions. A preferred detergent is polysorbate 80 and a particularly preferred combination is polysorbate 80 and TnBP. A preferred detergent is polysorbate 20 and a particularly preferred combination is polysorbate 20 and TnBP. A preferred detergent is OPG and a particularly preferred combination is OPG and TnBP. The source of haptoglobin may be stirred with solvent and detergent reagents at a temperature and for a time sufficient to inactivate any enveloped viruses that may be present. For example, the solvent detergent treatment may be carried out for about 4 hours at 25 °C. The solvent detergent chemicals are subsequently removed by for example adsorption on chromatographic media such as C-18 hydrophobic resins or eluting them in the drop-through fraction of ion exchange resins under conditions which adsorb the protein of interest.

The virus inactivation step can be performed at any suitable stage of the purification process. In an embodiment disclosed herein, the viral inactivation step comprises pasteurization or treatment with an organic solvent and detergent. In another embodiment disclosed herein, the virus inactivation step comprises virus filtration. Where virus filtration is used, the addition of a free amino acid (e.g., arginine) prior to the filtration step can significantly improve the flux rate and recovery of haptoglobin through the filter. In an embodiment disclosed herein, a solution comprising haptoglobin is subject to a viral inactivation step prior to purification of haptoglobin. An advantage of employing a virus inactivation step such as solvent detergent treatment prior to purification is that it allows for the removal of the organic solvent and detergent from the treated solution by utilizing conditions that promote binding of the haptoglobin to a resin and removal of the organic solvent and detergent with the flow-through (drop-through) fraction.

In a further embodiment, the haptoglobin (Hp) comprises, or consists of, a recombinant Hp. Hp may be suitably produced as a recombinant protein in a microorganism, and may be isolated and, if desired, further purified. Microorganisms suitable for the production of recombinant Hp will be familiar to those skilled in the art, illustrative examples of which are bacteria, yeast or fungi, eukaryotic cells (eg mammalian or insect cells), or recombinant viral vectors (e.g. adenovirus, poxvirus, herpesvirus, shimki forest fever virus, baculovirus, bacteriophage, Sindbis virus or

Sendai virus). Bacteria suitable for producing recombinant peptides will be familiar to those skilled in the art, illustrative examples of which include *E. coli*, *B. subtilis*, or any other bacteria capable of expressing a peptide sequence. Illustrative examples of suitable yeast types for producing recombinant peptides include Candida (*Candida*), blood Chiapas Pastoris (*Pichia pastoris*), *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, or any other yeast capable of expressing peptides. Such methods are well known in the art. Methods for isolating and purifying recombinantly produced peptide sequences are also well known in the art and include, for example, gel filtration, affinity chromatography, and ion exchange chromatography.

To facilitate isolation of recombinant Hp as described herein, a fusion polypeptide in which a peptide sequence of Hp or a functional analog thereof is translationally fused (covalently bound) to a heterologous polypeptide allowing isolation by affinity chromatography can be manufactured. Illustrative examples of suitable heterologous polypeptide are a His-tag (for example, His 6: 6 histidine residues), a GST- tag (glutathione transferase -S-), or the like. For the production of recombinant Hp, phage libraries and/or peptide libraries are also suitable, e.g., generated by binding chemistry or obtained by high-throughput screening techniques for the most diverse structures. Illustrative examples of recombinant Hp are NCBI accession number in NP_005134.1 and NP_001119574.1 (as described by Morishita K et al., 2018) and UniProt accession number P00738.

A recombinant haptoglobin may be prepared by recombinant methodologies known in the art. For example, a nucleic acid molecule comprising a nucleic acid sequence encoding the haptoglobin protein (or a precursor thereof) can be transfected into a suitable host cell capable of expressing said nucleic acid sequence, incubating said host cell under conditions suitable for the expression of said nucleic acid sequence and recovering said protein. Suitable methods for preparing a nucleic acid molecule encoding the recombinant haptoglobin are also known in the art, based on knowledge of the genetic code, possibly including optimizing codons based on the nature of the host cell (e.g. microorganism) to be used for expressing and/or secreting the recombinant haptoglobin protein. Suitable host cells are also be known in the art, illustrative examples of which include prokaryotic cells (e.g. *E. coli*) and eukaryotic cells (e.g. *P. pastoris*, Chinese hamster ovary (CHO) cell lines CHO-K1 and CHO-S, as described in WO2016/054072; NS0 hybridoma cells and HEK293 cells, as described in WO2012/050874). Reference is made to Ausubel et al., 2002.

The Hp for use may be provided in an isolated and/or purified form that is enriched, concentrated or otherwise has a specific activity, amount or concentration that is greater than the activity, amount or concentration of the haptoglobin in the starting material from which it is derived. In one embodiment, a formulation of purified haptoglobin, e.g. in liquid form, is derived from plasma.

The Hp for use may be endogenous Hp (e.g. obtained and purified from the blood of the subject) or exogenous Hp. According to one embodiment of the present invention, the Hp for use is exogenous Hp. The term “exogenous” in this context refers to Hp which does not originate from the subject to be treated with the Hp.

The haptoglobin for use in accordance with the present invention may for example be selected from the group consisting of Hp1-1 tetramers (each tetramer comprising, or consisting of, two haptoglobin alpha1 chains and two haptoglobin beta chains), Hp1-2 multimers (e.g. comprising, or consisting of, haptoglobin alpha1 chains, haptoglobin alpha2 chains and haptoglobin beta chains), Hp2-2 multimers (comprising, or consisting of, haptoglobin alpha2 chains and haptoglobin beta chains), and a combination thereof. Thus, in one embodiment, Hp is selected from the group consisting of Hp1-1 tetramers (“homodimers”), Hp1-2 multimers, Hp2-2 multimers, and combinations thereof. Optionally, the Hp comprises, or consists of, Hp2-2. Optionally, the Hp comprises, or consists of, Hp1-2. Further optionally, the Hp comprises, or consists of, Hp1-1. The haptoglobin for use according to the present invention may for example be a mixture of Hp1-1 and Hp 1-2, or a mixture of Hp 1-2 and Hp 2-2.

Preferably, the haptoglobin for use according to the present invention comprises Hp2-2. For example, the haptoglobin for use according to the present invention may be a mixture of Hp2-2 and Hp 1-2; or the haptoglobin for use according to the present invention may be a mixture of Hp2-2 and Hp 1-1; or the haptoglobin for use according to the present invention may be a mixture of Hp2-2, Hp1-2 and Hp 1-1; or the haptoglobin for use may consist of Hp2-2.

According to a particular embodiment of the present invention, the Hp for use comprises, or consists of, at least 50 % (e.g. wt.-% or mol.-%), or 55 %, or 60 %, or 70 %, or 80 %, or 85 %, or 90 %, or 93 %, or 95 %, or 97 %, or 99 %, or 99.5 %, or 99.9 %, Hp2-2 multimers; and optionally no more than 50 % (e.g. wt.-% or mol.-%), or 40 %, or 30 %, or 20%, or 15%, or 10 %, or 7 %, or 5 %, or 3 %, or 1 %, or 0.5 %, or 0.1 %, Hp1-1 tetramers, or Hp1-2 multimers, or a combination of

Hp1-1 tetramers and Hp1-2 multimers. According to another particular embodiment, the alpha chain fraction of the haptoglobin for use comprise, or consists of, at least 60 % (e.g. wt.-% or mol.-%), or 70 %, or 80 %, or 85 %, or 90 %, or 93 %, or 95 %, or 97 %, or 99 %, or 99.5 %, or 99.9 %, haptoglobin alpha2 chain; and optionally no more than 40 %, or 30 %, or 20%, or 15%, or 10 %, or 7 %, or 5 %, or 3 %, or 1 %, or 0.5 %, or 0.1 %, haptoglobin alpha1 chain. As used herein the "%" may for example refer to wt.-% or mol.-%.

According to a particular embodiment of the present invention, the haptoglobin (Hp) comprises, or consists of, haptoglobin alpha2 chains and haptoglobin beta chains. According to another specific embodiment, the haptoglobin comprises, or consists of, haptoglobin alpha1 chains, haptoglobin alpha2 chains and haptoglobin beta chains. According to yet another specific embodiment, the haptoglobin comprises, or consists of, haptoglobin alpha1 chains and haptoglobin beta chains. According to yet another specific embodiment, the haptoglobin comprises, or consists of, Hp2-2 multimers. According to yet another specific embodiment, the haptoglobin (Hp) comprises, or consists of, Hp2-2 multimers and Hp1-2 multimers, and optionally Hp1-1 tetramers. According to yet another specific embodiment, the Hp comprises, or consists of, Hp1-1 tetramers, or Hp1-2 multimers, or a combination thereof; or comprises, or consists of, Hp1-1 tetramers, Hp1-2 multimers and Hp2-2 multimers.

According to certain specific embodiments of the present invention, the haptoglobin (Hp) has a molecular composition corresponding to a mixture of Hp2-2 phenotype and Hp1-2 phenotype, optionally wherein said mixture of Hp2-2 phenotype and Hp1-2 phenotype comprises, or consists of, 35-75 % Hp2-2 phenotype and 25-65 % Hp1-2 phenotype; preferably 40-70 % Hp2-2 phenotype and 30-60 % Hp1-2 phenotype; more preferably 44-64 % Hp2-2 phenotype and 36-56 % Hp1-2 phenotype; or 49-59 % Hp2-2 phenotype and 41-51 % Hp1-2 phenotype; or 50-58 % Hp2-2 phenotype and 42-50 % Hp1-2 phenotype; or 51-57 % Hp2-2 phenotype and 43-49 % Hp1-2 phenotype; or 52-56 % Hp2-2 phenotype and 44-48 % Hp1-2 phenotype; or 53-55 % Hp2-2 phenotype and 45-47 % Hp1-2 phenotype, e.g. 54 % Hp2-2 phenotype and 46 % Hp1-2 phenotype.

According to other specific embodiments of the present invention, the haptoglobin (Hp) comprises, or consists of, 30-100 % Hp2-2 multimers, 0-60 % Hp1-2 multimers, and 0-40 % Hp1-1 tetramers; preferably 40-70 % Hp2-2 multimers, 20-55 % Hp1-2 multimers, and 1-25 % Hp1-1 tetramers;

more preferably 44-64 % Hp2-2 multimers, 26-46 % Hp1-2 multimers, and 3-17 % Hp1-1 tetramers; even more preferably 48-60 % Hp2-2 multimers, 30-42 % Hp1-2 multimers, and 5-15 % Hp1-1 tetramers; or 50-58 % Hp2-2 multimers, 32-40 % Hp1-2 multimers, and 6-14 % Hp1-1; or 51-57 % Hp2-2 multimers, 33-39 % Hp1-2 multimers, and 7-13 % Hp1-1; or 52-56 % Hp2-2 multimers, 34-38 % Hp1-2 multimers, and 8-12 % Hp1-1; or 53-55 % Hp2-2 multimers, 35-37 % Hp1-2 multimers, and 9-11 % Hp1-1; optionally wherein "%"refers to wt.-% or mol.-%.

According to yet other specific embodiments of the present invention, the haptoglobin (Hp) comprises, or consists of, 35-75 % Hp2-2 multimers and 25-65 % Hp1-2 multimers; preferably 40-70 % Hp2-2 multimers and 30-60 % Hp1-2 multimers; more preferably 44-64 % Hp2-2 multimers and 36-56 % Hp1-2 multimers; or 49-59 % Hp2-2 multimers and 41-51 % Hp1-2 multimers; or 50-58 % Hp2-2 multimers and 42-50 % Hp1-2 multimers; or 51-57 % Hp2-2 multimers and 43-49 % Hp1-2 multimers; or 52-56 % Hp2-2 multimers and 44-48 % Hp1-2 multimers; or 53-55 % Hp2-2 multimers and 45-47 % Hp1-2 multimers, e.g. 54 % Hp2-2 multimers and 46 % Hp1-2 multimers; optionally wherein "%"refers to wt.-% or mol.-%.

A Hp1-1 tetramer may for example comprise, or consist of, two haptoglobin alpha1 chains and two haptoglobin beta chains. A Hp1-2 multimer may for example comprise, or consist of, one or more haptoglobin alpha1 chain, optionally one or more haptoglobin alpha2 chains, and two or more haptoglobin beta chains, optionally wherein in the multimer the number of haptoglobin beta chains is equivalent to the number of alpha chains in the multimer. Or a Hp1-2 multimer may for example comprise, or consist of, one or more haptoglobin alpha1 chains, one or more haptoglobin alpha2 chains, and two or more haptoglobin beta chains; optionally wherein in the multimer the number of haptoglobin beta chains is equivalent to the number of alpha chains. A Hp2-2 multimer may for example comprise, or consist of, three or more haptoglobin alpha2 chains and three or more haptoglobin beta chains, optionally wherein in the multimer the number of haptoglobin beta chains is equivalent to the number of alpha chains.

The Hp may for example be a functional analog of native Hp. In one embodiment, the functional analogue has at least 85% or greater sequence identity with the amino acid sequence of the alpha and/or beta chain of native Hp (e.g. human plasma haptoglobin). According to a particular embodiment, in the haptoglobin for use according to the present invention, the haptoglobin alpha1 chain is selected from the group consisting of alpha1F chain, alpha1S chain, or a combination of

alpha1F chain and alpha1S chain. According to a specific embodiment, haptoglobin alpha1F chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:3, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 97.5%, 98%, 98.7%, or 99% sequence identity thereto; and/or haptoglobin alpha1S chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:4, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 97.5%, 98%, 98.7%, or 99% sequence identity thereto. According to another specific embodiment, in the haptoglobin for use according to the present invention, the haptoglobin alpha2 chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:5, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 97.8%, 98%, 98.5% 99% or 99.3% sequence identity thereto. According to yet another specific embodiment, in the haptoglobin for use according to the present invention, the haptoglobin beta chain comprises, or consists of, an amino acid sequence as shown in SEQ ID NO:6, or an amino acid sequence having at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 98.3%, 98.7%, 99%, 99.1% or 99.5% sequence identity thereto.

Reference to “at least 80%” means, for example, after best alignment or best fit analysis, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity. Thus, in one embodiment, the sequence has at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity (or sequence homology) with the amino acid sequence of the respective alpha and/or beta chain of native Hp (e.g. human Hp).

In the present context, the terms "percentage sequence identity", "% sequence identity", "percentage identity" and "% identity" between two amino acid (peptide) sequences preferably means the percentage of identical amino acid residues in corresponding positions in the two optimally aligned sequences. No conservative substitutions are considered as part of identity. Neither N- or C-terminal extensions or insertions should be construed as reducing sequence identity or homology. To determine the "percentage identity" of the two amino acid sequences, the sequences are aligned together. To achieve an optimal match, gaps can be introduced into the sequence (i.e. deletions or insertions which can also be placed at the sequence ends). Amino acid residues in the corresponding positions are then compared. When a position in the first sequence is occupied by the same amino acid residue that occupies the corresponding position in the second

sequence, the molecules are identical in that position. The percentage identity between two sequences may for example be a function of the number of identical positions divided by the sequences [i.e. % identity = (number of identical positions / total number of positions) x 100].

Thus, the terms "percentage sequence identity", "% sequence identity", etc. as used herein may refer to the extent that sequences are identical or structurally similar on an amino acid-by-amino acid basis over a window of comparison. For example, a "percentage of sequence identity", may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For example, "sequence identity" is the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software Engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software.

The percentage identity can be obtained by using mathematical algorithms. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul et al. 1997. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel et al. (1994-1998) in: Current Protocols in Molecular Biology, John Wiley & Sons Inc. A non-limiting example of an algorithm used for comparing two sequences is incorporated in the BLASTp program of Altschul (Altschul SF et al., 1990). Those programs can also be used to achieve alignments even in the presence of one or more gaps for insertions. For this purpose, the BLASTp program can be used with default parameters. When using the BLAST program, the BLOSUM62 matrix is often employed.

Optionally, the functional analog of Hp is a functional fragment of native Hp (e.g. human haptoglobin from plasma). A functional fragment of native Hp can be of any suitable length so long

as the fragment retains the ability to treat or prevent secondary brain injury. According to a particular embodiment a functional fragment is a fragment of any suitable length so long as the fragment retains the ability to form complexes with cell-free Hb and neutralize its biological activity.

Alternatively, the functional analog may be a polypeptide having an amino acid sequence that differs from the naturally occurring (native) Hp molecule (i.e., comparator). A functional analogue differs from the amino acid sequence of the alpha and/or beta chain of a native Hp by one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9 or more) amino acid substitutions having different amino acid sequences, wherein the difference does not abrogate or completely abolish the ability of the analog to form complexes with cell-free Hb and neutralize its biological activity. In some embodiments, the functional analog comprises an amino acid substitution that enhances the ability of the analog to form a complex with a cell-free Hb, as compared to native Hp. In one embodiment, the functional analog has an amino acid sequence that differs from the amino acid sequence of the alpha and/or beta chain of a native Hp by one or more conservative amino acid substitutions. As used herein, the term “conservative amino acid substitution” refers to altering the amino acid identity at a given position and replacing it with an amino acid of approximately equivalent size, charge and/or polarity. Examples of naturally conservative substitutions of amino acids include the following groups of eight substituents (designated by the general one-letter code): (1) M, I, L, V; (2) F, Y, W; (3) K, R, (4) A, G; (5) S, T; (6) Q, N; (7) E, D; and (8) C, S. In one embodiment, a functional analog comprises amino acid substitutions and/or other modifications relative to native Hp to increase the stability of the analog or increase the solubility of the analog. Functional analogs may be naturally occurring polypeptides or may be produced synthetically by chemical synthesis using methods known to those skilled in the art.

Hemopexin

The haptoglobin for use according to the present invention may be administered together with hemopexin (Hx). It was found by the present inventors that Hp can be administered together (that is not necessarily simultaneously, but in the same treatment cycle) with hemopexin, and that if done so the dose of haptoglobin can be reduced without abolishing the effects and/or their extent observed with haptoglobin alone in treating TBI. The term “hemopexin” or “Hx” is known to those skilled in the art and may refer to a single chain glycoprotein that binds heme with a high affinity

(for example with a KD of < 1 pM), thereby forming a heme-hemopexin complex. Herein, “hemopexin” and “Hx” are used interchangeably.

Hx, also referred to as p-1 B-glycoprotein, has been described as the first line of defense in the body against heme toxicity at least in part due to its ability to bind heme with high affinity, for example at an equimolar ratio, and its function as a heme specific carrier from the bloodstream to the liver. Hemopexin has also been reported to possess serine protease activity and several other functions (Lin et. al. 2016), such as anti- and pro-inflammatory activities, the ability to inhibit cellular adhesion and binding of certain divalent metal ions. Hx was reported as a single peptide chain folding into two four-bladed β-propeller domains, resembling two thick disks that lock together at about an 90° angle and are joined by an interdomain linker (Paoli et al. 1999). The heme, which is released into the blood as the result of intra- and extravascular hemolysis, may be bound between the two four-bladed β-propeller domains of Hx in a pocket formed by the interdomain linker peptide.

Hemopexin is a glycoprotein containing for example about 20% carbohydrates, including sialic acid, mannose, galactose, and/or glucosamine. Human hemopexin may comprise as many as six disulphide bridges. Endogenous hemopexin may control the adverse effects of free heme under physiological steady-state conditions, while having little effect in maintaining steady-state heme levels under pathophysiogical conditions, such as those associated with hemolysis, where a high level of heme leads to the depletion of endogenous hemopexin, causing heme-mediated oxidative tissue damage.

The amino acid sequence of the human hemopexin precursor may correspond to the amino acid sequence of UniProt ID P02790 and of NCBI Reference Sequence NP_000604.1 (SEQ ID NO:7), while mature human hemopexin may for example have a sequence corresponding to amino acid residues 24-462 of the amino acid sequence of UniProt ID P02790 and of NCBI Reference Sequence NP_000604.1 (SEQ ID NO:7), that is SEQ ID NO:8. It is to be understood that the term "hemopexin" or “Hx”, as used herein, includes all phenotypes (including all isoforms) of Hx. The Hx may be homogenous (insofar as it consists essentially of an Hx of the same isoform) or heterogeneous (insofar as it comprises a combination of different Hx isoforms, including human and non-human isoforms of Hx). Suitable methods for determining Hx isoforms that are present

in an isolate will be familiar to persons skilled in the art, illustrative examples of which include high performance size exclusion chromatography (HPLC-SEC assay) and Hx ELISA.

The term “hemopexin” or “Hx” as used herein includes functional analogues of naturally occurring Hx (e.g. naturally occurring human Hx), as well as naturally occurring Hx. The term "functional analogue" is intended to mean an agent that shares substantially identical biological activity as a naturally occurring (native) Hx, for example as long as the biological activity is at least the ability of the analogue to form a complex with heme to neutralize it.

The hemopexin may include human and non-human variants. Preferably, the hemopexin is a human hemopexin or a hemopexin having an amino-acid sequence derived from human. Particularly, where the Hx is intended for administration to a human subject, it is generally preferable that the hemopexin is a (native) human hemopexin or a close functional analog of (native) human hemopexin. However, it is to be understood that a non-human isoform of hemopexin may be used where the intended subject is a human, as long as the non-human isoform of hemopexin retains a functionality of human hemopexin such as the ability to bind to human heme. Illustrative examples of non-human isoforms of hemopexin will be familiar to persons skilled in the art, illustrative examples of which include hemopexin derived from bovine, equine or porcine. Preferably, the hemopexin is a human hemopexin, such as a protein disclosed under the UniProt ID P02790 or the NCBI accession number NP_000604.1. The term “human hemopexin” as used herein may include a protein as disclosed in such NCBI and UniProt entries, as well as functional analogues which are closely related thereto on the sequence level with an amino acid sequence identity of at least 80%, 85%, 90%, 93%, 95%, 96%, 97%, etc. to amino acid sequences shown in these NCBI and UniProt entries (including e.g. SEQ ID NO: 7-8 disclosed herein).

The Hx to be used in the treatment as defined herein above according to the present invention may be a naturally occurring (native) Hx (e.g., plasma derived) or it may be produced as a recombinant protein. For example, the Hx may be plasma-derived Hx, or recombinant Hx, or a combination thereof. Thus, as used herein, the term “hemopexin” may for example mean a hemopexin protein that has been isolated or otherwise at least partially purified from a natural source (e.g., plasma), or a recombinantly produced hemopexin protein, that comprises, consists of, amino acid residues as shown in SEQ ID NO:8, or an amino acid sequence having at least 80% sequence identity thereto.

The terms “at least partially purified”, “isolated”, “purified” and the like are used herein to mean that the protein of interest (e.g. haptoglobin and/or hemopexin) is provided in an isolated and/or purified form; that is, e.g. separated, isolated or purified from their natural environment, and are provided in a substantially pure or homogeneous form. Such proteins will typically be free or substantially free of material with which they are naturally associated, such as other polypeptides or nucleic acids with which they are found in their natural environment, or the environment in which they are prepared (e.g. cell culture) when such preparation is by recombinant DNA technology practiced *in vitro* or *in vivo*. For example, an at least partially purified protein of interest may comprise no more than 50% impurities (of total protein). Thus, in an embodiment disclosed herein, the purified haptoglobin and/or hemopexin comprises, consists or consists essentially of no more than 50%, preferably no more than 45%, preferably no more than 40%, preferably no more than 35%, preferably no more than 30%, preferably no more than 25%, preferably no more than 20%, preferably no more than 15%, preferably no more than 10%, or preferably no more than 5% impurities (of total protein) or preferably no more than 1% impurities (of total protein).

The Hx, for example, may comprise, or consist of, plasma derived Hx. In one embodiment, the Hx is plasma derived. For example, the Hx may include an at least partially purified hemopexin recovered from human plasma. In some embodiments, the hemopexin comprises, or consists of, naturally occurring human plasma hemopexin. Suitable methods of purifying hemopexin from natural sources such as plasma will be familiar to a person skilled in the art, an illustrative example of which is described in WO2014/055552. Where hemopexin is purified from native source such as blood plasma, a person skilled in the art will understand that it may be desirable to reduce the level of active virus content (virus titre) and other potential infectious agents (for example, prions) in the solution. This may be particularly desirable where the starting material is derived from blood plasma. Methods of reducing the virus titre in a solution will be known to persons skilled in the art. Further details in this regard are described elsewhere herein in the context of haptoglobin and apply mutatis mutandis to hemopexin.

In another embodiment, the hemopexin comprises, or consists of, a recombinant Hx. Hx may be suitably produced as a recombinant protein in a microorganism, which may be isolated and, if desired, further purified. Microorganisms suitable for the production of recombinant Hx will be familiar to those skilled in the art, illustrative examples of which are bacteria, yeast or fungi,

eukaryotic cells (eg mammalian or insect cells), or recombinant viral vectors (eg, adenovirus, poxvirus, herpesvirus, shimki forest fever virus, baculovirus, bacteriophage, Sindbis virus or Sendai virus). Bacteria suitable for producing recombinant peptides will be familiar to those skilled in the art, illustrative examples of which include *E. coli*, *B. subtilis*, or any other bacteria capable of expressing a peptide sequence. Illustrative examples of suitable yeast types for producing recombinant peptides include Candida (*Candida*), blood Chiapas Pastoris (*Pichia pastoris*), *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, or any other yeast capable of expressing peptides. Such methods are well known in the art. Methods for isolating and purifying recombinantly produced peptide sequences are also well known in the art and include, for example, gel filtration, affinity chromatography, and ion exchange chromatography. An illustrative example of recombinant hemopexin is described in WO2016/054072.

To facilitate isolation of recombinant Hx as described herein, a fusion polypeptide in which a peptide sequence of Hx or a functional analog thereof is translationally fused (covalently bound) to a heterologous polypeptide allowing isolation by affinity chromatography can be manufactured. Illustrative examples of suitable heterologous polypeptide are a His-tag (for example, His 6: 6 histidine residues), a GST- tag (glutathione transferase -S-), or the like. For the production of recombinant Hx, phage libraries and/or peptide libraries are also suitable, e.g., generated by binding chemistry or obtained by high-throughput screening techniques for the most diverse structures. Illustrative examples of Hx are NCBI accession number NP_000604.1 and UniProt accession number P02790. Further details regarding the preparation of recombinant protein are described elsewhere herein in the context of haptoglobin and apply mutatis mutandis to Hx.

The hemopexin may be provided in an isolated and/or purified form that is enriched, concentrated or otherwise has a specific activity, amount or concentration that is greater than the activity, amount or concentration of the hemopexin in the starting material from which it is derived. In one embodiment, a formulation of purified hemopexin, e.g. in liquid form, is derived from plasma.

The Hx to be used in the treatment as defined herein above may be endogenous Hx (e.g. obtained and purified from the blood of the subject) or exogenous Hx. According to one embodiment of the present invention, the Hx to be used in the treatment as defined herein above is exogenous Hx. The term “exogenous” in this context refers to Hx which does not originate from the subject to be treated with the Hx.

In one embodiment disclosed herein, the hemopexin comprises, or consists of, amino acid residues 24-462 of NCBI Reference Sequence NP_000604.1 (SEQ ID NO:7), or an amino acid sequence having at least 75%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.3%, 99.5% or 99.7% sequence identity thereto. It is to be noted that amino acid residues 1-23 of SEQ ID NO:7 encode a signal sequence. Thus, in one embodiment, the hemopexin comprises, or consists of, amino acid residues 24-462 of SEQ ID NO:7 (i.e. SEQ ID NO:8), or an amino acid sequence having at least 75%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.3%, 99.5% or 99.7% sequence identity thereto. Amino acid residues 24-462 of SEQ ID NO:7 are shown in SEQ ID NO:8.

In another embodiment, the hemopexin is a hemopexin comprising, or consisting of, an amino acid sequence as shown in SEQ ID NO:8 (i.e. amino acid residues 24-462 of SEQ ID NO:7), or an amino acid sequence having at least 75%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, 99%, 99.3%, 99.5% or 99.7% sequence identity thereto.

Reference to "at least 75%" includes e.g. 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99.1, 99.2, 99.3, 99.4, 99.5, 99. 7 or 100% sequence identity, for example, after optimal alignment or best fit analysis. Thus, in an embodiment disclosed herein, the hemopexin comprises, or consists of, an amino acid sequence having at least 75%, preferably at least 80%, preferably at least 85%, preferably at least 90%, preferably at least 93%, preferably at least 95%, preferably at least 96%, preferably at least 97%, preferably at least 98%, preferably at least 99%, preferably at least 99.3%, preferably at least 99.5%, or preferably at least 99.7% sequence identity to the amino acid sequence shown in SEQ ID NO:8. The term "percent sequence identity" or "% sequence identity" is to be understood as defined elsewhere herein. Sequence identity with respect to the hemopexin, as herein described, may relate to the percentage of amino acid residues in the candidate sequence that are identical with the residues of the corresponding peptide sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percentage homology, and not considering any conservative substitutions as part of the sequence identity, while neither N- or C- terminal extensions, nor insertions shall be construed as reducing sequence identity or homology.

In some embodiments disclosed herein, the functional analog of hemopexin is a heme-binding fragment of naturally occurring (native) hemopexin. A heme-binding fragment of hemopexin, also referred to interchangeably herein as a “heme-binding fragment”, is a portion of the native hemopexin molecule (human or non-human) that retains at least part of the functional activity of the parent molecule to bind to heme. Heme binding can readily be determined by using methods known to persons skilled in the art, an illustrative example of which is the method described by Lipiski et al. Modifications to the method by Lipiski et al. (2013) can also be used. In one embodiment, the hemopexin binds to heme with a binding affinity having an equilibrium-dissociation constant (KD) of 1x10-12 or less.

Administration

Generally, the route and mode of administration of the haptoglobin (Hp) are not particularly limited as long as ensuring an effective treatment of the subject suffering from TBI. According to one embodiment of the present invention, the treatment comprises exposing the brain parenchyma of the subject to the haptoglobin (Hp), and optionally hemopexin (Hx), administered to the subject. According to another embodiment of the present invention, the treatment comprises exposing the brain parenchyma of the subject to a therapeutically effective amount of the Hp, and optionally Hx, administered to the subject.

Preferably, the haptoglobin is administered parenterally, more preferably by injection or infusion. Thus, for example, the haptoglobin may be administered systemically, for example by intravenous administration, such as intravenous injection or infusion. According to a preferred embodiment of the present invention, haptoglobin (Hp) is administered intravenously, for example by injection or infusion. It was surprisingly found by the present inventors that Hp, alone or in combination with hemopexin (Hx), can be administered intravenously to effectively treat a TBI in a subject and particular to effectively treat or prevent a secondary brain injury in a subject suffering from a TBI. According to one embodiment of the present invention, the Hp is administered as a bolus injection. According to another embodiment the Hp is administered as a continuous intravenous infusion, preferably over 0.5 to 24 hours, or over 1 to 18 hours, or over 2 to 12 hours.

The haptoglobin (Hp) may be administered as a single dose or as multiple doses. The treatment may comprise administering the haptoglobin to the subject during the acute or subacute phase of

the traumatic brain injury (TBI). Thus, according one embodiment of the present invention, the treatment comprises administering the haptoglobin to the subject during the acute phase of the TBI. According to another embodiment of the present invention, the treatment comprises administering the haptoglobin to the subject during the subacute phase of the TBI. According to a specific embodiment of the present invention, the treatment comprises administering at least one dose of the haptoglobin to the subject during the acute or subacute phase of the TBI. According to another specific embodiment of the present invention, the treatment comprises administering at least one dose of the haptoglobin to the subject during the acute phase of the TBI. According to yet another specific embodiment of the present invention, the treatment is started during the acute or subacute phase of the traumatic brain injury (TBI). According to yet another specific embodiment of the present invention, the treatment is started during the acute phase of the traumatic brain injury (TBI). In the context of the present invention the acute phase of a TBI may be defined, for example, as the first 24 hours (day 0) after the initial trauma (primary brain injury) of the TBI and the subacute phase as the time period between one day and three weeks after the initial trauma (primary brain injury) of the TBI.

According to one embodiment of the present invention, the treatment comprises administering the Hp within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI). According to another embodiment of the present invention, the treatment comprises administering at least one dose of haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma (the primary brain injury) of the TBI. According to yet another embodiment of the present invention, the treatment with haptoglobin is started within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma (the primary brain injury) of the TBI. According to a specific embodiment of the present invention, the treatment comprises administering a primary dose of haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma of the TBI. According to a further specific embodiment of the present invention, the treatment comprises administering a second dose of the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, or one day after the primary dose. Further doses may be administered e.g. with a similar dosing interval.

It was found by the present inventors that the use of Hp, alone or in combination with hemopexin (Hx), is particularly effective in treating TBI, and specifically secondary brain injury, when

administered early after the initial trauma. According to a preferred embodiment of the present invention, the treatment comprises administering the haptoglobin within less than 48 hours, or 36 hours, after the initial trauma (the primary brain injury) of the traumatic brain injury (TBI). Thus, the treatment may comprise administering at least one dose of the haptoglobin within less than 48 hours, or 36 hours, after the initial trauma of the TBI. According to another preferred embodiment of the present invention, the treatment comprises administering the haptoglobin within less than 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, or 30 minutes after the initial trauma of the TBI. Thus, the treatment may comprise administering at least one dose of the haptoglobin within less than 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, or 30 minutes after the initial trauma of the TBI. According to yet another preferred embodiment of the present invention, the treatment is started within less than 48 hours, preferably within 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, or 30 minutes after the initial trauma of the TBI.

According to a specific embodiment of the present invention, the treatment comprises administering a primary dose of the haptoglobin within less than 48 hours, preferably 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, or 30 minutes after the initial trauma (the primary injury) of the traumatic brain injury (TBI). According to a further specific embodiment of the present invention, the treatment comprises administering a second dose of the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, or one day after the primary dose. Further doses may be administered with a similar dosing interval.

Duration of treatment and dosing interval for the haptoglobin are not particularly limited as long as ensuring effective treatment of the subject suffering from traumatic brain injury (TBI) and may be determined by those skilled in the art for example based on a number of clinical factors such as the severity of the TBI, subject (patient) related factors such as age and body weight, the symptoms, the patient's sensitivity to the haptoglobin and/or the response to the treatment.

The dosage of Hp may be adjusted to provide an optimal therapeutic response. For example, several divided doses may be administered daily, weekly, or in other suitable time intervals, or the dosage may be proportionally reduced according to the urgency of the situation. Optionally, the haptoglobin may be administered three times a week, for a period of one month or longer. Alternatively, the haptoglobin may be administered for a period of, for example, one to three

months. According to a specific embodiment of the present invention, the treatment comprises exposing the subject to the haptoglobin for 2 to 56 days, preferably 3 to 28 days, or 5 days to 21 days, or 7 days to 14 days after the initial trauma (the primary injury) of the TBI.

In specific embodiments, the haptoglobin is administered, twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four weeks to every month, from every 10 days to every two weeks, or from two to three times a week, etc. According to other specific embodiments of the present invention, the haptoglobin is administered at least once a week, preferably two times per week, or three times per weeks, or every other day. According to yet another specific embodiment of the present invention, the haptoglobin is administered every 24 hours, or every 18 hours, or every 12 hours, or every 8 hours, or every 6 hours. According to yet another specific embodiment of the present invention, the haptoglobin is administered at least two times a week, for a period of one week, or two weeks, or three weeks, or four weeks, or longer. Or the haptoglobin, for example, may be administered according to a repeat pattern of two 48-hour and one 72-hour dosing intervals.

According to an alternative specific embodiment of the present invention, the treatment consists of administering one dose of the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days after the initial trauma (the primary injury) of the TBI. According to another alternative specific embodiment of the present invention, the treatment consists of administering one dose of the haptoglobin within less than 2 days, or 36 hours, or 24 hours, or 12 hours, or 8 hours, or 6 hours, or 4 hours, or 2 hours, or 1 hour, or 30 min after the initial trauma of the TBI.

Dosage size is not particularly limited as long as it ensures an effective treatment of the subject while minimizing adverse effects. It may be affected by subject (patient) related factors such as age and body weight, the severity of the TBI and the specific symptoms, other concurrently administered drugs and the route of administration. Thus, the dosage of haptoglobin to be administered may vary according to the nature and severity of the TBI and its symptoms, the physical condition of the subject, the therapeutic regimen (e.g., whether a second therapeutic agent is used), and the selected route of administration; the appropriate dosage may be readily determined by a person skilled in the art.

The treatment may, for example, comprise administering a therapeutically effective amount of haptoglobin (Hp). Or the treatment may, for example, comprise administering at least one dose comprising a therapeutically effective amount of Hp. As used herein, the term “therapeutically effective amount” refers to an amount or concentration of Hp, sufficient to reduce or prevent one or more symptoms of TBI, in particular one or more symptoms of a secondary brain injury, for example, an amount or concentration of Hp sufficient to treat or prevent formation and/or progression of intracranial lesions; and/or treat or prevent neuroinflammation-related damage; and/or treat or prevent impaired motor function; and/or neutralize adverse biological effects resulting from accumulation of iron in the brain. It will be understood by those skilled in the art that a therapeutically effective amount of a peptide may vary depending on several factors, illustrative examples of which include the route of administration, the health and physical condition of the subject being treated, the taxonomic group of the subject being treated, the severity of the TBI and the symptoms associated with it. Combinations of any are included.

A therapeutically effective amount of Hp will typically fall within a relatively wide range that can be determined by one of ordinary skill in the art. For example, the therapeutically effective amount of Hp may range from e.g. 0.00005 g to 50 g, from 0.0005 g to 40 g, from 0.001 g to 30 g, from 0.005 g to 20 g, from 0.01 g to 15 g, or from 0.05 g to 10 g, per subject. The Hp to be administered may be in a concentration of e.g. from 2 µM to 20 mM, from 2 µM to 5 mM, from 2 µM to 300 µM, from 5 µM to 100 µM, 5 µM to 50 µM, or 5 µM to 30 µM, and the administrated amount may be e.g. from 1 to 50 mL, from 1 to 40 mL, from 1 to 30 mL, or from 3 to 20 mL, for example from 5 to 10 mL, per subject. Alternatively, a therapeutically effective amount of Hp may be based on the body weight of the subject. For example, the therapeutically effective amount of Hp may be as specified below. In other embodiments, the haptoglobin may be administered in a dosage of from 5 to 10 mL at a concentration of from 1 to 1000 mg/dL per subject.

The treatment may for example comprise administering haptoglobin to the subject in a dosage of at least 1 ng, or at least 10 ng, or at least 100 ng, or at least 1 µg, or at least 10 µg, or at least 100 µg, or at least 1 mg, or at least 5 mg, or at least 10 mg, or at least 30 mg, or at least 200 mg, or at least 400 mg, per kg of body weight of the subject per dosage unit. According to one embodiment of the present invention, the treatment comprises administering haptoglobin to the subject in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, 100 µg to 500 mg, 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200

mg, per kg of body weight of the subject per dosage unit. The Hp may be administered as a composition, said composition for example comprising Hp in a dosage of at least 1 ng, or at least 10 ng, or at least 100 ng, or at least 1 µg, or at least 10 µg, or at least 100 µg, or at least 1 mg, or at least 5 mg, or at least 10 mg, or at least 100 mg, or at least 200 mg, or at least 400 mg, per kg of body weight of the subject. According to an exemplary embodiment of the present invention, such a composition comprises haptoglobin in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, 100 µg to 500 mg, 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject. According to another exemplary embodiment of the present invention, such a composition comprises from 0.001 g to 10 g, or 0.01 g to 5 g, haptoglobin. It is to be understood that the dosage may for example also include a range defined by any of two values mentioned herein above alone or in the context of other ranges.

The haptoglobin may be administered alone or in combination with hemopexin. Moreover, the treatment may additionally comprise administering to the subject a further agent for treating a TBI.

The haptoglobin may be administered to the subject in a dosage form of, for example, a lyophilized formulation or an aqueous solution. The dosage form, that is a pharmaceutical composition, may further comprise one or more optional pharmaceutically acceptable additives. Pharmaceutically acceptable additives (excipients) are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to, carriers, buffers, diluents, binders, disintegrants, surface active agents, thickeners, lubricants, preservatives, antioxidants. The dosage forms to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes. Further details are provided herein below in the context of pharmaceutical compositions.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of the haptoglobin will be determined by the extent of the TBI and the nature of the underlying symptoms, the form, route and site of administration, and the age and condition of the particular subject being treated, etc., and that a physician may ultimately determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance with normal clinical practice.

In some embodiment of the haptoglobin for use according to the present invention, the treatment further comprises administration of hemopexin (Hx). Thus, in such embodiments, the treatment may comprise exposing the brain parenchyma of the subject to the hemopexin administered to the subject (for example a therapeutically effective amount of Hx).

If the treatment further involves administration of hemopexin, the haptoglobin and the hemopexin may, for example, be administered simultaneously, sequentially or separately, said modes of administration being encompassed by the term “co-administered”. Thus, in one embodiment the treatment further comprises administration of hemopexin (Hx), and the haptoglobin and the hemopexin are administered simultaneously. According to another embodiment the treatment further comprises administration of hemopexin, and the haptoglobin and the hemopexin are administered sequentially (that is, in close temporal proximity to each other). According to another embodiment the treatment further comprises administration of hemopexin, and the haptoglobin and the hemopexin are administered separately (that is, not necessarily in close temporal proximity to each other, however preferably within the same dosing interval).

Where the treatment further comprises administration of hemopexin, in certain embodiments the details regarding administration provided in the context of haptoglobin apply mutatis mutandis to the administration of hemopexin. For example, in some embodiments, route and mode of administration of hemopexin are as defined herein for haptoglobin. According to a preferred embodiment, the hemopexin is administered intravenously. Or in certain embodiments, the duration of treatment and dosing interval for hemopexin are the same as defined herein for haptoglobin.

Similarly, in certain embodiments, the dosage size for hemopexin is the same as defined herein for haptoglobin. In certain specific embodiments, for example, the treatment comprises administering hemopexin to the subject in a dosage of at least 1 ng, or at least 10 ng, or at least 100 ng, or at least 1 µg, or at least 10 µg, or at least 100 µg, or at least 1 mg, or at least 5 mg, or at least 10 mg, or at least 30 mg, or at least 200 mg, or at least 400 mg, per kg of body weight of the subject per dosage unit. In certain other specific embodiments the treatment comprises administering hemopexin to the subject in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, 100 µg to 500 mg, 1 mg to 400 mg, or

5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject per dosage unit. In yet other specific embodiments, the hemopexin may be administered as a composition, said composition for example comprising Hx in a dosage of at least 1 ng, or at least 10 ng, or at least 100 ng, or at least 1 µg, or at least 10 µg, or at least 100 µg, or at least 1 mg, or at least 5 mg, or at least 10 mg, or at least 100 mg, or at least 200 mg, or at least 400 mg, per kg of body weight of the subject. According to certain exemplary embodiments of the present invention, such a composition comprises Hx in a dosage of 1 ng to 1000 mg, or 10 ng to 900 mg, or 0.1 µg to 800 mg, or 1 µg to 700 mg, or 10 µg to 600 mg, 100 µg to 500 mg, 1 mg to 400 mg, or 5 mg to 300 mg, or 10 mg to 200 mg, per kg of body weight of the subject. According to other exemplary embodiments of the present invention, such a composition comprises from 0.001 g to 10 g, or 0.01 g to 5 g, hemopexin. It is to be understood that the dosage may for example also include a range defined by any of two values mentioned herein above alone or in context of other ranges.

The haptoglobin may be administered in liquid or solid form. This means the haptoglobin may be administered as part of a liquid composition (e.g. as an aqueous solution), or as a solid powder or the like. The hemopexin, if included in the treatment, may be administered in liquid or solid form. According to a preferred embodiment the haptoglobin is administered in liquid form, that is in a liquid composition. Hemopexin, if included in the treatment, according to a preferred embodiment is administered in liquid form, that is in a liquid composition.

Means to monitor treatment progress of a subject suffering from TBI are known to those skilled in the art. According to one embodiment of the haptoglobin for use according to the present invention a biofluid of the subject, e.g. the cerebrospinal fluid (CSF) and/or blood (blood, plasma, and/or serum), is monitored to assess treatment progress. In particular, one or more TBI biomarkers (e.g. selected from Hp-related proteins (HPR), heme-binding protein 1 (HEBP1), heme-binding protein 2 (HEBP2), ferritin heavy chain (FTH1), ferritin light chain (FTL), transferrin receptor protein-1 (TFRC1) and combinations thereof, and optionally glial fibrillary acidic protein (GFAP), ubiquitin carboxylterminal hydrolase L1 (UCH-L1), S100B, neuron-specific enolase (NSE), neurofilament protein light (NfL), and/or total tau) may be monitored in a biofluid of the subject, preferably CSF and/or blood, for example by using liquid chromatography mass spectrometry (LC-MS).

The present inventors found that Hp-related proteins (HPR), heme-binding protein 1 (HEBP1), heme-binding protein 2 (HEBP2), ferritin heavy chain (FTH1), ferritin light chain (FTL), and

transferrin receptor protein-1 (TFRC1) correlate well with both TBI severity and treatment progress. Particularly, the present inventors found in the mouse model that during the acute and/or subacute phase of the TBI, the secondary brain injury is marked by an increase in HPR level, an increase in HEBP2 level, a decrease in HEPB1 level, an increase in FTH1 level, an increase in FTL level, and a decrease in TFRC1 level, rendering these proteins suitable TBI biomarkers that can be monitored in the CSF and/or blood of the subject. Therefore, according to a specific embodiment of the present invention treatment progress is marked by a decrease in HPR level, a decrease in HEBP2 level, an increase in HEPB1 level, a decrease in FTH1 level, a decrease in FTL level, and/or an increase in TFRC1 level in the CSF and/or blood of the subject.

According to another embodiment of the haptoglobin for use according to the present invention, treatment progress is monitored using MRI, CT, PET, SPECT, transcranial Doppler (TCD), or a combination thereof. According to yet another embodiment, treatment progress is monitored using standard electroencephalogram (EEG) or quantitative electroencephalography (qEEG), pupilometers, infrared scanner, magnetoencephalography (MEG), vision and oculomotor assessment using an eye movement tracking device (such as saccadometers or electrooculography), or a combination thereof. According to yet another embodiment treatment progress is monitored using one or more glial, preferably astroglial, metabolic biomarkers (e.g. GFAP, or IBA1, and/or brain-specific isoform of the glycolytic enzyme aldolase, ALDOC) in a biofluid of the subject.

Pharmaceutical Compositions

Pharmaceutical compositions (therapeutic formulations) of the haptoglobin for use according to the invention can be prepared for storage and eventual administration as lyophilized formulations or aqueous solutions by mixing the polypeptide having the desired degree of purity with optionally one or more pharmaceutically acceptable additives (excipients), such as carriers, stabilizers, buffering agents, isotonifiers, surface active agents including non-ionic detergents, diluents, binders, thickeners, lubricants, preservatives, antioxidants, and other miscellaneous additives. See, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be nontoxic to the recipients (subject) at the dosages and concentrations employed. Similarly, pharmaceutical compositions of the hemopexin as described herein above, or a combination of haptoglobin and hemopexin, can be prepared as lyophilized formulations or aqueous solutions by

mixing the polypeptide having the desired degree of purity with optionally one or more pharmaceutically acceptable additives as described above.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They can be present at concentrations ranging from about 2 mM to about 50 mM. Suitable buffering agents include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid- monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid- disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used.

Preservatives can be added to retard microbial growth. They can be added in amounts ranging e.g. from 0.2 %-1 % (w/v). Suitable preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, and iodide), hexamethonium chloride, and alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Isotonifiers can be added to ensure isotonicity of liquid compositions and include polhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol,

ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (e.g., peptides of 10 residues or fewer); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophylic polymers, such as polyvinylpyrrolidone; monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccacharides such as raffinose; and polysaccharides such as dextran. Stabilizers can be present in the range from 0.1 to 10,000 weights per part of weight active protein.

Surface active agents such as non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), pluronic polyols, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.). Non-ionic surfactants can be present e.g. in a range of about 0.05 mg/ml to about 1.0 mg/ml, or in a range of about 0.07 mg/ml to about 0.2 mg/ml.

Preservatives may include for example octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol.

Additional miscellaneous excipients include bulking agents (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), salt-forming counter-ions (e.g. sodium); metal complexes (e.g. Zn-protein complexes), and cosolvents.

According to certain embodiments, the pharmaceutical composition comprises as active component either both, the haptoglobin as well as hemopexin, or alternatively comprises only the haptoglobin without hemopexin depending on whether hemopexin is included in the treatment or not and the herein disclosed mode of administration (e.g. simultaneously or separately).

Thus, according to one embodiment the haptoglobin for use according to the present invention is part of a pharmaceutical composition comprising, or consisting of, the haptoglobin and optionally one or more pharmaceutically acceptable additives, such as those specified above. According to a specific embodiment the haptoglobin for use according to the present invention is part of a pharmaceutical composition consisting of the haptoglobin and optionally one or more pharmaceutically acceptable additives, such as those specified above. According to another embodiment the haptoglobin for use according to the present invention is part of a pharmaceutical composition comprising, or consisting of, the haptoglobin, hemopexin, and optionally one or more pharmaceutically acceptable additives, such as those specified above. According to another specific embodiment the pharmaceutical composition consists of the haptoglobin, hemopexin, and optionally one or more pharmaceutically acceptable additives, such as those specified above. According to a specific embodiment the pharmaceutical composition comprises water.

According to another embodiment the hemopexin as specified herein above is part of a pharmaceutical composition comprising, or consisting of, the hemopexin and optionally one or more pharmaceutically acceptable additives, such as those specified above. According to a specific embodiment the pharmaceutical composition comprises water.

Consequently, according to a particular embodiment, the treatment as specified herein comprises administering to the subject a pharmaceutical composition comprising the haptoglobin, and optionally hemopexin (Hx). According to another particular embodiment, the treatment comprises administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of the haptoglobin, and optionally hemopexin (Hx).

According to one embodiment, the pharmaceutical composition comprises a therapeutically effective amount of haptoglobin (Hp), and optionally hemopexin (Hx). According to another embodiment, one dose of the pharmaceutical composition comprises a therapeutically effective amount of haptoglobin (Hp) and optionally hemopexin (Hx). Hemopexin, if included in the treatment, may also be formulated into a separate pharmaceutical composition comprising e.g. a therapeutically effective amount of Hx (when administered in combination with Hp), optionally such that one dose of the composition comprises a therapeutically effective amount of Hx.

The pharmaceutical composition (therapeutic formulation) herein may also contain a further therapeutic agent in addition to haptoglobin and the optional hemopexin. Examples of suitable further therapeutic agents may be further active ingredients suitable to treat one or more symptoms associated with TBI.

The pharmaceutical composition is preferably formulated to be administered intravenously, preferably by injection or infusion.

The pharmaceutical composition may be a solid composition or a liquid composition. According to one embodiment the pharmaceutical composition is a solid composition. According to a preferred embodiment the pharmaceutical composition is a liquid composition, preferably an aqueous composition. Thus, the pharmaceutical composition comprising haptoglobin and/or hemopexin may be a liquid formulation the composition of which may depend on the intended use. For instance, where such a liquid composition is to be administered to a subject in need thereof as a neat composition (i.e., without further dilution), the haptoglobin and/or hemopexin content would typically be suitable for direct administration, having regard, for example, to factors such as the dosage required and the volume to be administered. As an example, the haptoglobin and/or hemopexin content may be optimized such that it is high enough so to minimize the volume of the liquid formulation to be administered to the subject, having regard to the desired therapeutic dose, and low enough so as to minimize the viscosity of the liquid formulation to allow for administration without further dilution. Thus, in an embodiment disclosed herein, the haptoglobin and/or hemopexin content is optimized so as to minimize the viscosity of the liquid formulation such that it is suitable for administration without further dilution. Suitable viscosities will be familiar to persons skilled in the art and is likely to depend on factors such as the route and/or volume of administration.

In some embodiments, the pharmaceutical compositions are substantially free of other components with which haptoglobin and/or hemopexin (if hemopexin is used) may be associated when derived from natural sources (e.g., other plasma-derived proteins) or produced recombinantly. Thus, in one embodiment, the pharmaceutical compositions may comprise less than 20% of total protein, preferably less than 10% of total protein, and more preferably less than 5% of total protein of other components with which they are normally associated (i.e., impurities). In one embodiment, the pharmaceutical composition comprises a haptoglobin content of at least

90%, preferably at least 95%, preferably at least 97%, preferably or at least 98%, preferably at least 99% or more preferably at least 99.5% by weight of total protein. In one embodiment where the pharmaceutical composition comprises hemopexin, the pharmaceutical composition comprises a hemopexin content of at least 90%, preferably at least 95%, preferably at least 97%, preferably or at least 98%, preferably at least 99% or more preferably at least 99.5% by weight of total protein. In one embodiment where the pharmaceutical composition comprises haptoglobin and hemopexin, the pharmaceutical composition comprises a combined haptoglobin and hemopexin content of at least 90%, preferably at least 95%, preferably at least 97%, preferably at least 98%, preferably at least 99% or more preferably at least 99.5% by weight of total protein.

In particular embodiments, especially where the pharmaceutical composition is in liquid form, the level of purity of haptoglobin and/or hemopexin in the pharmaceutical compositions is determined using immunonephelometry. Suitable methods of performing immunonephelometry will be familiar to a person skilled in the art. The level of purity of haptoglobin and/or hemopexin in the composition can be determined by measuring the haptoglobin and/or hemopexin content by immunonephelometry on a BNII instrument (Siemens Healthcare, Malvern, PA, USA) or similar. The total protein content of the liquid formulation can be determined by the Bradford method or UV spectrometry at 280nm. Then the percentage purity of haptoglobin and/or hemopexin can be calculated by dividing the haptoglobin and/or hemopexin content by the total protein content and multiplying by 100. The percentage purity of other trace proteins contained in a stable liquid formulation of haptoglobin and/or hemopexin can be determined in an analogous manner. Where the haptoglobin and/or hemopexin has been purified from human plasma, illustrative examples of trace proteins that may be present include albumin, alpha-1-acid glycoprotein, alpha-1-antitrypsin, alpha-2-macroglobulin, apolipoprotein A-I, antithrombin-III, ceruloplasmin, haptoglobin, immunoglobulin A (IgA), immunoglobulin G (IgG) and transferrin.

Where the pharmaceutical composition is formulated as a liquid formulation and this liquid formulation is to be administered to a subject in need thereof as a neat composition (i.e., without further dilution), suitable dosages of haptoglobin, or hemopexin, or a combination thereof, will be familiar to persons skilled in the art and are likely to depend on factors such as the nature and severity of the TBI and its symptoms to be treated, the age, weight and gender of the subject to be treated, the presence of any other underlying conditions and combinations of the foregoing.

According to some embodiments, the pharmaceutical composition is formulated as a liquid composition comprising a haptoglobin content of between 0.1 mg/mL and 500 mg/mL, preferably between 1 mg/mL and 400 mg/mL, or 5 mg/mL and 300 mg/mL, or 10 mg/mL and 200 mg/mL, or 50 mg/mL and 150 mg/mL, etc. According to some embodiments, the pharmaceutical composition is formulated as a liquid formulation comprises a hemopexin content of between 0.1 mg/mL and 500 mg/mL, preferably between 1 mg/mL and 400 mg/mL, or 5 mg/mL and 300 mg/mL, or 10 mg/mL and 200 mg/mL, or 50 mg/mL and 150 mg/mL, etc. Thus, in some embodiments the pharmaceutical composition is formulated as a liquid composition, comprising at least 0.1 mg/mL, or 0.5 mg/mL, or 1 mg/mL, or 2 mg/mL, or 5 mg/mL, or 10 mg/mL, or 20 mg/mL, or 30 mg/mL, or 50 mg/mL, or 100 mg/mL and/or no more than 500 mg/mL, or 400 mg/mL, or 350 mg/mL, or 300 mg/mL, or 250 mg/mL, or 200 mg/mL, or 150 mg/mL, or 120 mg/mL, haptoglobin; and/or at least 0.1 mg/mL, or 0.5 mg/mL, or 1 mg/mL, or 2 mg/mL, or 5 mg/mL, or 10 mg/mL, or 20 mg/mL, or 30 mg/mL, or 50 mg/mL, or 100 mg/mL and/or no more than 500 mg/mL, or 400 mg/mL, or 350 mg/mL, or 300 mg/mL, or 250 mg/mL, or 200 mg/mL, or 150 mg/mL, or 120 mg/mL, hemopexin. In an embodiment where the composition is in solid form, the above contents may be equivalent but in mg per gram of composition instead.

In other embodiments, the pharmaceutical composition can be prepared as a concentrate, wherein the concentrate is to be diluted for administration. One of the advantages of preparing the pharmaceutical composition as a concentrate is that it minimizes the volume for storage. The concentrate can be diluted prior to or during administration to the subject, as desired. Suitable concentrations of purified hemopexin that can be prepared as a concentrate will be familiar to persons skilled in the art. In an embodiment disclosed herein, the pharmaceutical composition is a liquid composition concentrate comprising a haptoglobin and/or hemopexin content of at least 250 mg/mL.

In yet other embodiments, the pharmaceutical composition is in liquid form and comprises at least 1 pM, or 5 pM, or 10 pM, or 20 pM, or 50 pM, or 100 pM, etc. and no more than 20 mM Hp, or 10 mM Hp, or 5 mM Hp, or 1 mM Hp, or 500 µM Hp, or 100 µM Hp, or 50 µM Hp, or 10 µM Hp, or 1 µM Hp, 500 nM, 100 nM, or 10 nM or 1 nM, or 500 pM, or 300 pM, etc. haptoglobin; and/or 1 pM, or 5 pM, or 10 pM, or 20 pM, or 50 pM, or 100 pM, etc. and no more than 20 mM Hp, or 10 mM Hp, or 5 mM Hp, or 1 mM Hp, or 500 µM Hp, or 100 µM Hp, or 50 µM Hp, or 10 µM Hp, or 1 µM Hp, 500 nM, 100 nM, or 10 nM or 1 nM, or 500 pM, or 300 pM, etc. hemopexin.

The haptoglobin content or the hemopexin content of a composition may be measured for example by UV absorbance spectroscopy, or by immunonephelometry; if the composition is in solid form, dissolution in a suitable solvent first is required. Illustrative examples of UV absorbance spectroscopy and immunonephelometry are described elsewhere herein.

Further aspects

According to a second aspect the present invention relates to haptoglobin (Hp) for use in the neuroprotective treatment of a traumatic brain injury (TBI) in a subject, or for use in the neuroprotective treatment in a subject suffering from a traumatic brain injury (TBI). According to a third aspect the present invention relates to haptoglobin (Hp) for use in the reduction of progressive brain damage in a subject suffering from a traumatic brain injury (TBI). According to a fourth aspect the present invention relates to haptoglobin (Hp) for use in the treatment or prevention of intracranial lesions in a subject suffering from a traumatic brain injury (TBI). According to a fifth aspect the present invention relates to haptoglobin (Hp) for use in the treatment or prevention of intracranial lesion formation and/or progression in a subject suffering from a traumatic brain injury (TBI). According to a sixth aspect the present invention relates to haptoglobin (Hp) for use in the treatment or prevention of neuroinflammation in a subject suffering from a traumatic brain injury (TBI). According to a seventh aspect the present invention relates to haptoglobin (Hp) for use in the treatment of impaired motor function in a subject suffering from a traumatic brain injury (TBI). According to an eighth aspect the present invention relates to haptoglobin (Hp) for use in treating or preventing one or more adverse effects associated with iron accumulation in the brain parenchyma of a subject suffering from a traumatic brain injury (TBI). According to a ninth aspect the present invention relates to haptoglobin (Hp) for use in treating or preventing iron-mediated toxicity in the brain parenchyma of a subject suffering from a traumatic brain injury (TBI). Optionally the use according to the second, third, fourth, fifth, sixth, seventh, eighth and/or ninth aspect is characterized as defined in any of the embodiments of the first aspect of the invention described herein, that is these aspects are optionally characterized by any of the details described herein in the context of the first aspect of the present invention.

According to a tenth aspect the present invention relates to the use of haptoglobin (Hp) for the manufacture of a medicament i) for the neuroprotective treatment of a traumatic brain injury (TBI)

in a subject; or ii) for the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI); or iii) for the reduction of progressive brain damage in a subject suffering from a traumatic brain injury (TBI). Optionally the use according to the tenth aspect is characterized as defined in any of the embodiments of the first aspect of the invention described herein, that is these aspects are optionally characterized by any of the details described herein in the context of the first aspect of the present invention.

According to an eleventh aspect the present invention relates to a method of neuroprotective treatment of a traumatic brain injury (TBI) in a subject, comprising administering haptoglobin (Hp), and optionally hemopexin (Hx), as defined in any of the embodiment of the first aspect of the invention, to the subject. According to a twelfth aspect the present invention relates to a method of treating or preventing a secondary brain injury in a subject suffering from a traumatic brain injury (TBI), comprising administering haptoglobin (Hp), and optionally hemopexin (Hx), as defined in any of the embodiment of the first aspect of the invention, to the subject. According to a thirteenth aspect the present invention relates to a method for the reduction of progressive brain damage in a subject suffering from a traumatic brain injury (TBI), comprising administering haptoglobin (Hp), and optionally hemopexin (Hx), as defined in any of the embodiment of the first aspect of the invention, to the subject. Optionally the method of the eleventh, twelfth, and/or thirteenth aspect of the present invention may be further characterized by any of the details described herein in the context of the first aspect of the present invention; for example, the treatment or the subject may be characterized as described herein in the context of any of the embodiment of the first aspect.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the features, compositions, steps, and compounds referred to or indicated in this specification, individually or collectively and any and all combinations of any two or more of said features, compositions, steps, and compounds. Any feature that has been described above in relation to any aspect or embodiment of the invention is also disclosed hereby in relation to all other aspects and embodiments. Likewise, all combinations of two or more of the individual features or elements described above may be present in any aspect or embodiment. For brevity, all possible features and combinations have not been recited in relation to all aspects and embodiments, but they are expressly contemplated and hereby disclosed.

The amino acid sequences shown in the sequence listing are summarized in Table 1.

Table 1

SEQ ID NO:	Description
1	human haptoglobin isoform 1 preproprotein (NCBI Reference Sequence NP_005134.1; UniProt ID: P00738-1)
2	human haptoglobin isoform 2 preproprotein (alpha1-beta) (NCBI Reference Sequence NP_001119574.1; UniProt ID P00738-2)
3	alpha1F chain of human haptoglobin
4	alpha1S chain of human haptoglobin
5	alpha2 chain of human haptoglobin
6	beta chain of human haptoglobin
7	human hemopexin precursor (NCBI Reference Sequence NP_000604.1; UniProt ID: P02790)
8	mature human hemopexin (amino acids 24-462 of human hemopexin precursor [NCBI Reference Sequence NP_000604.1; UniProt ID: P02790])

Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the invention described hereinbefore.

EXAMPLES

Materials and Methods

Animals and proteins

Adult male mice of 12 weeks of age with average body weight of 23+3 g were used in all experiments. WT mice of C57BL/6J background were purchased from the Animal Resource Centre (ethics no: 181458.9). All experiments involving animal surgery were conducted in accordance with University of Melbourne Animal Ethics Committee. Human plasma-derived

haptoglobin (Hp) (predominantly phenotype Hp2-2 [54 % Hp2-2 and 46 % Hp2-1]) and hemopexin (Hx) were provided by CSL Behring (Kankakee, Illinois, USA).

Experimental groups

Mice were randomly grouped to receive either a SHAM (SHAM group) or mild form of controlled cortical impact (CCI) surgery (TBI group) (n=11-12 per group). 30 mins and 5 days following successful CCI, mice were given either 400mg/kg of Hp (TBI-Hp group), 400 mg/kg of Hx (TBI-Hx group), combination of 200mg/kg Hp and 200 mg/kg Hx (TBI-Hp+Hx group) or PBS (TBI-placebo/PBS group) intravenously. Throughout this report, these cohort of mice will be referred to their group identifier accordingly. MRI and DigiGait analysis performed measuring lesion size and behavioural outcome respectively, with biochemical analysis performed at 24 h, 48 h and 7 days after TBI (Figure 1). Throughout this study, the identity of each animal with respect to treatment was blinded to researchers who conducted the experiments and analysis.

Mouse Controlled cortical impact model (CCI)

All of the CCI procedures performed in this study were based on standard protocols as previously described and reported (Karve et al. 2016; Abdullah et al. 2018) . Mice were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg, Parnell)/xylazine (10 mg/kg, Parnell). A sagittal scalp incision was made to expose the underlying parietal skull. A 2-mm-diameter plate of bone (centered 1.5 mm posterior to bregma and 2.5 mm lateral to the midline) was then removed using a Dremel 10.8 V drill with a 0.8 mm tip (Dremel) to expose the underlying right parietal cortex. A 1.5 mm deep impact into the exposed cortex was made at 5 m/s using the computer-controlled impactor device (LinMot®-Talk 1100, LinMot). Following impact, the bone plate was replaced and held in place with a small section of parafilm to cover the injury site. The skin incision was then closed with sterile silk 5.0 metric sutures (Syneture Tyco Healthcare). Mice were administered intraperitoneal buprenorphine (0.6 mg/kg, Reckitt Benckiser Healthcare) and placed on a heat mat for postsurgical recovery. SHAM-operated controls underwent the same anesthesia, scalp incision, and bone plate removal, but were not injured by the impactor.

Western Blot

Protein concentration was measured using Braford assay with 50 µg of protein used for western blot analysis. Extracted proteins were incubated in 2x Novex® Tris-glycine SDS sample buffer (Invitrogen) for 10 mins at 100 °C and were resolved on 8 % or 12 % acrylamide SDS PAGE gels. Blots were then transferred to polyvinylidene fluoride (PVDF) membranes using a semi-dry transfer apparatus (Biorad). Membranes were blocked with 5 % w/v skim milk in TBS-T for 1 h and incubated with primary antibodies in 2 % w/v skim milk in TBS-T at 4 °C overnight. Membranes were washed 3 times for 10 minutes each with TBS-T prior to being incubated with HRP-conjugated secondary antibodies (diluted in 2 % skim milk in TBS-T) for 60 min at room temperature. Again, membranes were washed with TBS-T and signals were detected using an ECL prime® western blotting detection kit (Amersham) and visualized with the IQ350 imaging machine (GE Healthcare). Post-image densitometry was performed using Image J software (NIH), whereby signal intensity was calculated in arbitrary units. For densitometry calculations, phosphorylation intensity was measured in arbitrary units and normalized to the β-actin loading control. These values were then calculated as fold change compared to control.

Immunohistochemical analysis

Animals were sacrificed and perfused with ice-cold PBS followed by 4 % PFA before hemispheres were removed and fixed in 5 ml of chilled 4 % w/v paraformaldehyde, pH 7.4 (Sigma-Aldrich) overnight at 4 °C. They were then left at 4 °C overnight in 30 % w/v sucrose before being embedded in optimal cutting temperature and cryosectioned into 30 µm coronal sections. For immunohistochemistry, sections were permeabilized in 0.2% Triton X-100/PBS (PBS-T) for 20 minutes before being blocked for 1 hour in 10 % normal donkey serum/5 % BSA/PBS at RT°C. The following antibodies were diluted in 1 % BSA and incubated overnight at 4 °C; anti-rabbit IBA1 (1:200, WAKO) and anti-mouse GFAP (1:1000, Cell signaling). Sections were then washed 3 times in PBS before 2-hour incubation at room temperature with Alexa Fluor 594-conjugated donkey anti-mouse and Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibodies. Sections were again washed in three washes of PBS before being mounted in Vectashield plus DAPI (Vectashield). Slides were viewed using a Ziess Axio 123672641 microscope and images captured using an Axio Cam Mrm camera and Zen 2011 software (**Figure 1**).

Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) scans were performed for this study using a Bruker 4.7 Tesla small animal MRI scanner (Monash Biomedical Imaging) to quantify the progression of tissue damage, as described by Crack et al. (2014)(Wojciak et al. 2014). Mice were anesthetized with ~3 % isoflurane in a 1:1 mixture of medical-grade air and oxygen. Anesthesia was maintained throughout scanning with 0.25 to 1.5 % isoflurane through a nosecone placed over the animal's snout and respiration was continuously monitored throughout the experiment with a pressure-sensitive probe positioned under the animal's diaphragm. Anesthetized animals were laid supine on a purpose-built small-animal holder and their heads fixed into position with ear and bite bars. A surface receiver coil was placed over the animals' heads and the cradle was inserted into a transmitter coil fixed inside a BGA12S-HP gradient set for imaging. The MRI protocol consisted of a three-plane localizer sequence followed by multiecho T2 and diffusion-weighted sequences. The total scanning time was kept to <1 h per animal. Multiecho T2-weighted images were acquired using a rapid acquisition, relaxation enhanced (RARE) sequence with RARE factor = 2; repetition time = 2500 ms; effective echo time (TEeff) = 10, 30, 50, 70, 90, and 110 ms; field-of-view = 1.6 Å~ 1.6 cm^2; matrix = 192 Å ~192; and 16 slices with thickness = 0.5 mm. Volumetric analysis was carried out on T2-weighted images using ITK SNAP software (Yushkevich et al. 2006; Yushkevich et al. 2016).

DigiGait analysis

Behavioral testing assessing changes in gait parameters was performed using the DigiGait system (Quincy 2009). Digital images of paw placement were captured by a high-speed video camera positioned underneath a clear treadmill. This ventral plane videography of the animals captured automatically calculated numerous spatial and temporal gait indices including stance, swing, brake, propulsion, and stride duration of each limb by DigiGait proprietary software (Table 2). Baseline readings of all mice used in this study were recorded prior to CCI injury at a 15cm s-1 treadmill speed. Mice that were unable to walk on the treadmill at this speed were excluded from this study. All surgeries, behavioral and lesion size analyses for compound-treated mice were performed in a blinded fashion.

Table 2: The duration of each gait interval, which includes the duration of the stride, stance, swing, braking and propulsion phase (seconds) was used for gait analysis.

DigiGait indices	Definition	Unit
swing	time taken in swing phase (paw is off the treadmill)	seconds(s)
brake	time taken in brake phase (initial paw contact to maximum paw contact on the treadmill)	seconds(s)
propulsion	time taken in propulsion phase (maximum paw contact to end of stance phase)	seconds(s)
stance	time take in stance phase (total time taken in brake and propulsion phase)	seconds(s)
stride	time taken for a complete stride phase of one paw (total time taken in stance and swing phase)	seconds(s)
stride length	length of paw measured on a given stride	cm
stride frequency	number of complete strides per second	steps/second

Analysis of human CSF samples by label-based TMT-11 plex quantification method

Cerebrospinal fluid (CSF) is a colorless body fluid that circulates around the brain and spinal cord. This body fluid is an important source of potential biomarkers for brain-associated damage such as TBI. CSF screening to identify proteins that are altered in TBI is a highly clinically relevant approach. CSF was obtained from 16 TBI patients recruited to the Alfred Hospital, Melbourne, via extraventricular drainage on day 0, 1, 2, 4, 7 and 10 post-injury (between 8-16 samples for analysis per time point; total of 80 CSF samples). Control CSF samples were obtained from 11 individuals as part of an elective surgical procedure unrelated to TBI. The study was conducted in accordance with the National Statement on Ethical Conduct in Research Involving Humans of the National Health and Medical Research Council of Australia (NHMRC), following approval by the Alfred Hospital Human Ethics Committee. Included patients had a post-resuscitation Glasgow Coma Scale (GCS) score of ≤8, indicating severe TBI.

The expression of Hp, Hx and molecules involved in iron homeostasis were analyzed by mass spectrometry via the Monash Proteomics and Metabolomics Facility, Monash University, Clayton. In brief, CSF samples were lyzed then proteins were precipitated via chloroform/methanol prior to trypsin digestion. Samples were run on a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler. Samples were loaded via an Acclaim PepMap 100 trap column (100 µm × 2 cm, nanoViper, C18, 5 µm, 100 Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column, then peptides were analyzed with a QExactive Fusion mass spectrometer (Thermo Fisher Scientific). Raw data files were analyzed using MaxQuant v1.6.5.

Table 3: Proteins/genes of interests screened from human CSF screened in this study.

Gene Name	Protein ID	Protein name
CD163	Q86VB7	Scavenger receptor cysteine-rich type 1 protein M130; Soluble CD163
FTH1	P02794	Ferritin heavy chain; Ferritin heavy chain, N-terminally processed
FTL	P02792	Ferritin light chain
HEBP2	Q9Y5Z4	Heme-binding protein 2
HP	P00738	Haptoglobin; Haptoglobin alpha chain; Haptoglobin beta chain
HPR	P00739	Haptoglobin-related protein
HX	P02790	Hemopexin
TFRC1	P02786	Transferrin receptor protein 1; Transferrin receptor protein 1, serum form
HEBP1	Q9NRV9	Heme-binding protein 1

Statistical analysis

Data are expressed as mean ± SEM and analyzed using Graph Pad Prism 9.1 software. For MRI data, a one-way or two-way Analysis of Variance (ANOVA) was performed as appropriate followed by a Bonferroni's post-hoc analysis, with a value of $p<0.05$ considered statistically significant. For human CSF samples, statistical analysis was firstly performed using various statistical packages in R version 3.6.0 (2019) using an x86_64-pc-linux-gnu platform, and data was background corrected and normalized by variance stabilizing transformation (vsn) (https://bioconductor.org/packages/3.10/bioc/html/vsn.html). The limma package from R

Bioconductor was used to generate a list of differentially expressed proteins for each pairwise comparison, and the protein intensity data was converted to log2 scale. A cutoff of the adjusted p-value of 0.05 (Benjamini-Hochberg method) along with a |log2 fold change| of 1 was applied to determine significantly regulated proteins in each pairwise comparison.

Results

Summary

In the present examples 12-week-old male C57BL/6J wildtype (WT) mice were given 400 mg/kg of Hp or Hx alone or a combination of 200 mg/kg Hp and 200 mg/kg Hx via intravenous administration at 30 minutes and 5 days after controlled cortical impact (CCI). Western blot analysis was performed to confirm Hp and Hx protein expression in the mice brains. Lesion size was measured using MRI, and motor function was assessed using DigiGait analysis. Glial reactivity was evaluated through immunohistochemical staining assessing astrocytes and microglia reactivity. Proteomics analysis of human TBI cerebrospinal fluid (CSF) samples was conducted to examine the expression of Hp, Hx and iron-related proteins.

Western blot analysis confirmed Hp and Hx protein expression in the 24 hours TBI brains (i.e 24 h after CCI) which diminished 7 days after CCI. Importantly, a significant improvement in motor function was demonstrated that is associated with a smaller lesion size in mice given 400 mg/kg of Hp alone and a combination of Hp and Hx at 24 hours and 7 days after CCI as confirmed by MRI and DigiGait analysis, respectively, supporting a neuroprotective effect of Hp after TBI. Interestingly, mice treated with Hp alone and a combination of Hp and Hx showed reduced levels in astrocytes and microglia reactivity at 24 hours and 7 days after CCI as compared to the control group suggesting Hp neuroprotectivity in TBI is in part attributed to its role in mediating TBI-induced glial reactivity. Further, proteomics analysis of human TBI CSF samples demonstrated an upregulation in Hp levels that is associated with increased levels of its scavenging receptor protein CD163, suggesting a potential neuroprotective role of Hp at the early stage of TBI (Tentillier et al. 2016, Liu et al. 2017, Oyarce et al. 2022) . The increased Hp expression is also associated with high iron levels in the CSF indicated by a significant upregulation in ferritin level, supporting previous observations of iron dysregulation seen in TBI brains. These findings highlight a neuroprotective effect of Hp in improving neurological outcomes at early timepoints after TBI and identify Hp as a potential new therapeutic for reducing cellular damage in TBI.

Confirmation of delivery of administered Hp and Hx to brain in vivo after TBI

The delivery of Hp and Hx to the TBI brains were validated using western blot at 24 hours (h) and 7 days after TBI (that is, after CCI). As shown in **Figure 2**, Hp and Hx level were confirmed in both the ipsilateral cortex and the ipsilateral striatum of the Hp and Hx treated mice brains 24 h after CCI. Additionally, mice treated with a combination of Hp and Hx showed a reduced level in the brains 24 h after CCI. Hx expression were undetected 7 day after CCI while Hp showed multiple bands suggesting the breakdown of this compound to several monomers at 7 days after CCI.

DigiGait analysis detects gaits improvement in Hp-treated mice 24h after TBI mice

To determine the neuroprotective effects of Hp, Hx and its combination in TBI mice, alteration in gait impairment that reflect changes in motor behavior were assessed with DigiGait™ system. More importantly, to mimic a clinically relevant state, where treatment is only an option after injury, all compound treatments were administered 30 min post CCI. All animals were pretrained to run on a treadmill at a speed of 15 cms^{-1} prior to CCI injury and tested again at 24 h, 48 h and 7 days post compound treatment and CCI injury. Mice unable to walk steadily were excluded from this study. Previously it was shown that TBI mice displayed gaits deficits in temporal parameters including swing(s), stance (s), brake(s) and propulsion(s) duration (Neumann et al. 2008; Sashindranath et al. 2015).

Gait parameters known to be affected in TBI mice measured by DigiGait system were examined. Sham treated mice (SHAM mice) were used as a reference point to detect any changes between compound-treated mice and placebo-treated mice at 24 h, 48 h and 7 days after CCI. The DigiGait analysis revealed motor function deficits 24 h after CCI as compared to the SHAM mice in the parameters of swing(s) (TBI-SHAM=0.1139 + 0.002938 VS TBI-placebo=0.075287 + 0.005707; ****p<0.0001), propulsion(s) (TBI-SHAM=0.1460 + 0.004954 VS TBI-placebo=0.05684 + 0.005233; ****p<0.0001) and stride(s) (TBI-SHAM=0.3391 + 0.005768 VS TBI-placebo=0.2451 + 0.02640; ****p<0.0001) duration when assessing left forelimb function. These gait functions returned to sham levels at 7 days after CCI validating that the CCI model induces a mild form of TBI. However, DigiGait analysis revealed no effects of Hx treatment on gait parameters assessed between TBI-Hx and TBI-placebo group as compared to their sham counterparts at 24 h, 48 h and

7 days post CCI (**Figure 3**). It is noteworthy that in assessing left forelimbs brake parameter, it was found that TBI-Hx group had decreased brake duration as compared to their SHAM and placebo counterparts at 24 h after CCI **(Figure 3 D**). Additionally, DigiGait analysis identified significant improvement in motor behavior reflected by gaits parameters including propulsion(s) (TBI-SHAM=0.1460 + 0.004954 VS TBI-Hp=0.1581 + 0.01545), swing(s) (TBI-SHAM=0.1139 + 0.002983 VS TBI-Hp=0.1098 + 0.004073), and stride(s) (TBI-SHAM=0.3391 + 0.005768 VS TBI-Hp=0.3560 + 0.009536) duration, all of which showing SHAM levels as compared to their TBI-placebo group at 24 h after TBI (**Figure 4**). Interestingly, these TBI-Hp mice showed a significant decrease in brake duration at 48 h after CCI as compared to their SHAM and placebo group counterparts (**Figure 4 D**). Furthermore, the combination treatment of 200 mg/kg of Hx and 200 mg/kg of Hp at a final concentration of 400 mg/kg revealed a similar effect in behavioral improvement with TBI-Hp+Hx mice performing similarly as SHAM mice and contrary to their TBI-placebo group mice at 24 h after TBI (**Figure 5**). These data suggest that administration of 400 mg/kg of Hx and Hp 30 min post TBI either alone or in combination results in improved behavioral outcome following an acute timepoint after CCI injury.

Hp treatment reduces progressive brain damage after TBI in mice

To further confirm the neuroprotective effects of Hp, Hx and the combination of Hp/Hx in TBI mice, anatomical tissue damage captured by MRI was analyzed. Using a similar dosing regime and study design performed in the previous experiment, 400 mg/kg of Hx was administered intravenously and animals brains analyzed by MRI at 24h and 7day post TBI to measure lesion size (**Figure 6A**). No changes in lesion size between TBI-Hx mice (TBI-Hx= 7.71 + 0.882 VS TBI-placebo=6.22 + 0.2848) as compared to TBI-placebo group at these timepoints was. Interestingly, treatment of Hp alone showed a significant reduction in the lesion size as compared to the PBS-control group at 24 h and 7 days after TBI. Additionally, administration of 200 mg/kg of Hx and 200 mg/kg of Hp combined also showed a significant reduction in lesion size at 24 h (TBI-PBS=6.213 + 0.002938 VS TBI-Hp=3.687+ 0.3129; *$p<0.05$) and 7 days (VS TBI-Hp= 2.0794 + 0.1692; ****$p<0.0001$ after CCI as compared to 24 h TBI-PBS control group (**Figure 6B**). These results indicate a neuroprotective effect of Hp when administered alone or in combination with Hx, highlighting the therapeutic potential of Hp for treatment of TBI.

Hp contributes to astrocyte and microglia reactivity after TBI

To determine the effects of Hp and/or Hx treatment in TBI-induced astrogliosis, a hallmark neuroinflammatory response, mice brain sections were assessed by immunohistochemical analysis 24 h after CCI. Immunolabeling of microglia and astrocytes were performed using antibodies specific to IBA1 (cell surface marker on microglia/macrophages) and GFAP (intermediate filament on astrocytes), respectively, to investigate the changes in structural morphology and intensity of the glia cell types following compound treatment at 24 h after CCI. An increase in GFAP staining intensity was observed in TBI-PBS mice as compared to the SHAM group. This was supported by western blot analysis with increased GFAP protein expression detected in at 24 h after CCI (**Figure 7**) validating an astrogliosis response induced in the CCI model. More importantly, the combination treatment of Hp with Hx revealed a significant reduction in GFAP protein expression at 7 days after CCI as compared to 24 h TBI-PBS group suggesting a modulatory effect of these compounds in astrogliosis in TBI (**Figure 8**). Further, an activated form of microglia characterized by an amoeboid shape with a larger cell size was identified in the TBI-PBS mice as compared to the SHAM group 24 h after TBI. In contrast, SHAM brains at 24 h post CCI displayed ramified morphologies with branches and fine processes indicative of a less reactive microglial phenotype (**Figure 9**). This less reactive form of microglia was also detected in the compounds treated group. Whilst preliminary, these results strongly support a role for Hp and/or Hx in modulating the neuroinflammatory environment by driving glial reactivity in TBI.

Human TBI CSF analysis reveals a significant upregulation in Hp and iron-regulatory proteins

Analysis of the CSF components from human TBI samples is critical to better understand the pathological processes involved in this diseased brain. Label-based TMT-11 plex proteomics analysis was used to compare the CSF protein profile of severe TBI patients with uninjured controls. To identify proteins involved at early stage of TBI, 80 samples from a total of 16 patients across 6 time points post-TBI were analyzed in this study. Increased expression of CSF Hp (α and β chain subunits) was detected at day 1 after the initial trauma resulting in TBI with a significant upregulation in Hp-related proteins (HPR) identified at day 0 to day 7 post initial trauma as compared to the control group (**Figure 10**). This data implicates a role for Hp in the molecular process seen in human TBI. Interestingly, the peak upregulation of Hp levels is seen at day 1 and then subsides at day 2 to day 10 post initial trauma. This finding correlates with an upregulation

in scavenger receptor cysteine-rich type 1 (CD163) protein at day 4 to day 10 post initial trauma. CD163 acts as the endocytic receptor binding hemoglobin (Hb) in complex with Hp. The increase in CD163 levels suggests a neuroprotective mechanism maybe taking place, most likely by mediating the toxic Hb clearance at the early stage of TBI. Intriguingly, human TBI CSF Hx is detected at a low level as compared to the control group (**Figure 10 (F)**). This data correlated with a significant upregulation in heme-binding protein 2 (HEBP2) level at day 1 to day 10 post initial trauma (**Figure 10 (D)**). Interestingly, a reduced HEBP1 levels in the human CSF TBI samples as compared to the control group was identified (**Figure 10 (E)**). Collectively, these results suggest that increased expression of Hp most likely contribute to a neuroprotective role following TBI.

To better understand a relationship between Hp and/or Hx with iron-related neurotoxicity following TBI, it was screened for proteins involved in iron regulation and homeostasis in the human CSF TBI samples. As shown in **Figure 10 (G-I)**, the human TBI CSF samples showed a marked increase in the expression of iron-regulatory proteins. Both ferritin heavy chain (FTH1) and ferritin light chain (FTL) levels were significantly upregulated from day 4 to day 10 post initial trauma resulting in TBI as compared to the control group. This correlated with a significant down-regulation in transferrin receptor protein-1 (TFRC1) levels at similar time points suggesting a negative feedback mechanism to prevent accumulation of iron that is otherwise detrimental. Collectively, these results implicate the accumulation of iron confirming previous observations of iron burden in the human TBI CSF that is likely caused by the injured brains.

Discussion

To the knowledge of the present inventors the present results represent the first report revealing a neuroprotective effect of Hp with Hp-treated mice displaying a significant reduction in lesion size at 24 h and 7 days after TBI as compared to their placebo group in an acute neural injury model. This corroborates behavioral outcome that showed an improved locomotor function and a significant gaits improvement in mice treated with Hp at 24 h after TBI. Furthermore, combination of Hx and Hp treatment showed a similar effect in behavioral improvements as observed in Hp-treated mice alone at 24 h after TBI. Importantly, the present results show that the neuroprotective effects of Hp either given alone or in a combination with Hx in behavioral outcome is evident with a significant reduction in lesion size as compared to the control group confirmed by longitudinal MRI analysis in this study.

Assessing gait changes in experimental TBI provides useful information regarding motor function status that is highly relevant to clinal studies. The CCI model induces mild-form of TBI which lasted for 48 h after the surgery as validated by DigiGaitTM analysis. The experimental design of this study enables the investigation of CCI-injury induced behavioural changes in an acute post-injury up to 7 days. DigiGaitTM analysis revealed changes in gait parameters between TBI and SHAM group indicating that the injury led to gait instability, imbalance and affected the speed of mice when walking on the treadmill. More importantly, using this system it was possible to detect behavioral deficits in TBI mice which were ameliorated in mice treated with Hp and combination of Hp and Hx, demonstrating their neuroprotective effects.

Experimental TBI confirmed the involvement of gliosis, marked by increased in GFAP expression (astrogliosis) and IBA1 staining(microgliosis), in both mild and moderate TBI models (Mondello et al. 2011; Lafrenaye et al. 2015; Gorse and Lafrenaye 2018; Lafrenaye et al. 2020). The CCI model has confirmed an increase in microglial and astrocyte reactivity with the data showing a trend in reduced GFAP and IBA1 staining in the compound treated group as compared to the control group suggesting a role for Hp and/or Hx in driving this neuroinflammatory factor at 24 h after TBI.

Destruction of red blood cells termed hemolysis has been associated with poor outcome following TBI. Importantly the leakage of blood from the injured brain into the surrounding area is a responsible event exacerbating the secondary injury (Willmore and Ueda 2009; Agas et al. 2023). This liberates Hb and ultimately free heme and iron that is detrimental when in excess through induction of toxic radical species and the subsequent increased in oxidative stress. Iron accumulation in the injured brain can occur via hemorrhage (heme-bound iron) or independently as labile iron (non-heme bound) (Daglas and Adlard 2018; Tang et al. 2020). In the context of TBI, excess iron leading to a detrimental neuroinflammatory environment is a self-perpetuating process as increased in unbound iron levels caused by the brain injury will generate more toxic reactive species that can compromise cell viability contributing to worst outcome. Since these products are toxic and could drive a detrimental oxidative stress and neuroinflammatory processes that exacerbate TBI outcome, the effective clearance of these molecules mediated by Hp or Hx is of clinical important. Studies have shown that free heme released in diseased brains are cleared through high affinity binding with Hx and is rapidly scavenged by its CD91 receptor protein (Wagner et al. 2003; Aronowski and Zhao 2011; Ma et al. 2016; Tseng et al. 2023; Voltarelli et

al. 2023). This neutralization of redox activity generating toxic free radicals mediated by formation Hx-heme-CD91 complex making Hx a potential therapeutic TBI treatment. Similarly, Hp is known to neutralize toxic redox activity mediated by Hb. The high affinity binding of Hp to free Hb mediates Hb clearance via the CD163 membrane receptor. This study investigated the response of the human Hx-CD91 and Hp-CD163 scavenging system to TBI by proteomics analysis from human TBI CSF samples. The present inventors report that the increase in Hp and CD163 levels was evident at the early stage after TBI that is likely attributed to a high iron deposition in the injured brain. High iron levels in the human TBI CSF samples are reflected by increases in the levels of ferritin and decreased levels of the transferrin receptor proteins. Indeed, in the event of iron overload, ferritin will be rapidly synthesized to take up excess iron while transferrin receptor expression is halted preventing high influx of iron into the cells. The sustained and increased CSF CD163 levels at the early stage of TBI also suggests a saturation of Hp-Hb uptake supporting an active scavenging activity mediated by Hp. Collectively, the present results support a neuroprotective role of Hp potentially by limiting iron-mediated toxicity in the TBI brain through the Hp-Hb-CD163 scavenging system.

Intriguingly, the present results identified a sustained low level of CSF Hx with its CD91 receptor protein undetectable in the human TBI CSF as compared to the control group (results not shown). The presence of Hp and Hx in the human CSF could be of blood or brain-derived origin due to the compromised blood-brain barrier (BBB) integrity following TBI. The blood derived Hp and Hx can originate from the internal brain bleeding or increased transfer from the circulation via a more permeable BBB. Therefore, low levels CSF Hx observed can be attributed to the accumulation of this protein in the injured brain with active transfer of Hx-heme-CD91 complex to the peripheral circulation across the BBB. Additionally, the lack of CD91 expression can be attributed to the saturation in Hx-heme brain uptake at the early stage of TBI since the level of heme is also undetectable (results not shown) in the human TBI CSF. This differential levels of Hp and Hx detected in the human TBI CSF samples at the early stage of TBI is intriguing.

The discrepancies on the effects of Hp/Hx observed so far in the literature can be attributed to various factors such as animal models used, the location of injury, the timing after injury and cell and/or tissues type analyzed on the study.

Recent experimental evidence suggests that Hx is protective in stroke mouse model with intracerebroventricular injection of Hx reduced the infarct volumes and improved measurements of neurological function within 7 days after stroke injury (Yang et al. 2018; Dong et al. 2019). This study also revealed neuroprotective effects of Hx in behavioral outcome with Hx-treated animals showing improved learning and memory capacity. Their results further suggest that these neuroprotective effects of Hx can be attributed to its ability to maintain the blood-brain barrier integrity and improve neovascularization following stroke injury. In another model of brain injury looking at intracerebral hemorrhage (ICH), increased brain Hx levels was associated with improved outcomes. The present results show a discrepancy in the neuroprotective effects of Hx in an acute model of TBI with no changes in lesion size observed in Hx-treated mice as compared to the control group using MRI analysis. Hx is known to be synthesized in the brain with CD91 found to be expressed in neurons and glia (He et al. 2010). Formation of Hx-heme complexes can lead to intracellular deposition of heme iron moiety and this can be accumulated in surrounding neurons and glia expressing CD91 receptor. Hence, it is possible that when brain Hx level are too high, it becomes detrimental by binding heme, preventing its efflux from the brain leading to intracellular heme/iron overload that is toxic to delicate neurons and glia. A recent study using primary cultures of neurons and glia show an increased neurotoxicity of Hb mediated by Hx in the absence of Hp. This study suggests a combined therapy of Hx with Hp is preferable to Hx alone in the treatment CNS hemorrhage thus supporting the observations made by the present inventors.

On the other hand, a neuroprotective role of Hp in reducing the toxic effects of Hb breakdown and associated radical products following ICH has also been reported. While reports investigating the effects of Hp in brain injury are still limited, the present results demonstrate for the first time a significant reduction in lesion size and improved behavioral outcome following Hp treatment in a mouse model of TBI. More importantly, the neuroprotective effects of Hp is further supported by its increased expression identified in human TBI CSF samples that is associated with Hb-scavenging activity and impaired iron homeostasis in the injured brains early phase after an initial trauma, as it causes a rapid increase in the concentration of oxidants in the brain.

The present experiments thus strongly support a neuroprotective effect of 400 mg/kg of Hp administration alone or 200 mg/kg of Hp in combination with 200 mg/kg of Hx 30 min and 5 days post TBI with a significant reduction in lesion size and improved motor function identified in TBI-

Hp and TBI-Hp+Hx group as compared to the TBI-placebo group 24 h and 7 days after TBI. The neuroprotectivity of these compounds may be attributed in part to their roles in driving reactive astrogliosis as indicated in this study. Additionally, the present experiments also demonstrate an upregulation in Hp expression and its scavenging proteins that is associated with elevated iron levels in human TBI CSF samples implicating a role for Hp in the event of iron-induced neurotoxicity after TBI.

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Claims

1. Haptoglobin (Hp) for use in the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI).

2. The haptoglobin for use according to claim 1, wherein the secondary brain injury is caused by the TBI; and/or wherein the TBI is a primary brain injury preceding the secondary brain injury.

3. The haptoglobin for use according to claim 1 or 2, wherein the secondary brain injury occurs progressively after an initial trauma or primary brain injury as a direct consequence thereof.

4. The haptoglobin for use according to any one of claims 1 to 3, wherein the haptoglobin is administered intravenously.

5. The haptoglobin for use according to any one of claims 1 to 4, wherein the treatment comprises administering the haptoglobin within 7 days, preferably within 5 days, or 4 days, or 3 days, or 2 days, after the initial trauma of the traumatic brain injury.

6. The haptoglobin for use according to any of the preceding claims, wherein the treatment comprises administering the haptoglobin within less than 48 hours, or 36 hours, or 24 hours, or 12 hours, or 6 hours, or 4 hours, or 2 hours, after the initial trauma of the traumatic brain injury.

7. The haptoglobin for use according to any of the preceding claims, wherein the secondary brain injury involves excitotoxicity, mitochondrial dysfunction, oxidative stress, lipid peroxidation, brain iron accumulation, apoptosis, ferroptosis, necrosis, axonal degeneration, neuroinflammation, cerebral ischemia, cerebral hypoxia, cerebral hypotension, dysfunction of brain metabolism, cerebral hypo- or hyper-carbia, cerebral hypo- or hyper-glycemia, cerebral hypo- or hyper-thermia, cerebral edema, raised intracranial pressure, breakdown of blood-

brain barrier and/or seizures; preferably at least excitotoxicity, oxidative stress and/or neuroinflammation.

8. The haptoglobin for use according to any of the preceding claims, wherein the secondary brain injury comprises formation and/or progression of intracranial lesions, cerebral edema, cerebral edema-related damage, neuroinflammation-related damage, impaired motor function, impaired cognitive function, neurotoxicity, apoptosis, ferroptosis and/or necrosis of brain cells, nitric oxide depletion-related damage, oxidative tissue injury, iron-mediated damage, ischemia-related damage, blood–brain barrier impairment-related damage, and/or spreading depolarization.

9. The haptoglobin for use according to claim 8, wherein the secondary brain injury comprises intracranial lesions and/or neuroinflammation-related damage and/or impaired motor function.

10. The haptoglobin for use according to any of the preceding claims, wherein the traumatic brain injury (TBI) is moderate TBI or severe TBI.

11. The haptoglobin for use according to any of the preceding claims, wherein the haptoglobin is human haptoglobin; optionally wherein the haptoglobin is plasma derived Hp, and/or comprises Hp2-2 multimers and/or Hp1-2 multimers and optionally Hp1-1 tetramers.

12. The haptoglobin for use according to any of the preceding claims, wherein the treatment further comprises administration of hemopexin (Hx); optionally wherein the hemopexin is administered in the same manner as defined in claim 5 or 6 for haptoglobin.

13. The haptoglobin for use according to claim 12, wherein the hemopexin is human hemopexin; optionally wherein the hemopexin is plasma derived hemopexin.

14. The haptoglobin for use according to any of the preceding claims, wherein the secondary brain injury is an injury to the brain parenchyma and/or wherein the treatment comprises exposing the brain parenchyma of the subject to a therapeutically effective amount of the haptoglobin and optionally hemopexin.

15. The haptoglobin for use according to any of the preceding claims, wherein the use is in the neuroprotective treatment of the traumatic brain injury in the subject.

16. The haptoglobin for use according to any of the preceding claims, wherein the traumatic brain injury is accompanied by an intracerebral hemorrhage (ICH), an epidural hemorrhage, a subdural hemorrhage and/or a subarachnoid hemorrhage.

17. Pharmaceutical composition comprising haptoglobin (Hp), and optionally hemopexin (Hx), for use in the treatment or prevention of a secondary brain injury in a subject suffering from a traumatic brain injury (TBI); optionally wherein the pharmaceutical composition comprises one or more pharmaceutically acceptable additives, preferably selected from carriers, stabilizers, buffering agents, isotonifiers, surface active agents including non-ionic detergents, diluents, binders, thickeners, lubricants, preservatives, antioxidants, and combinations thereof; optionally wherein the use is characterized as defined in any of claims 1 to 16.