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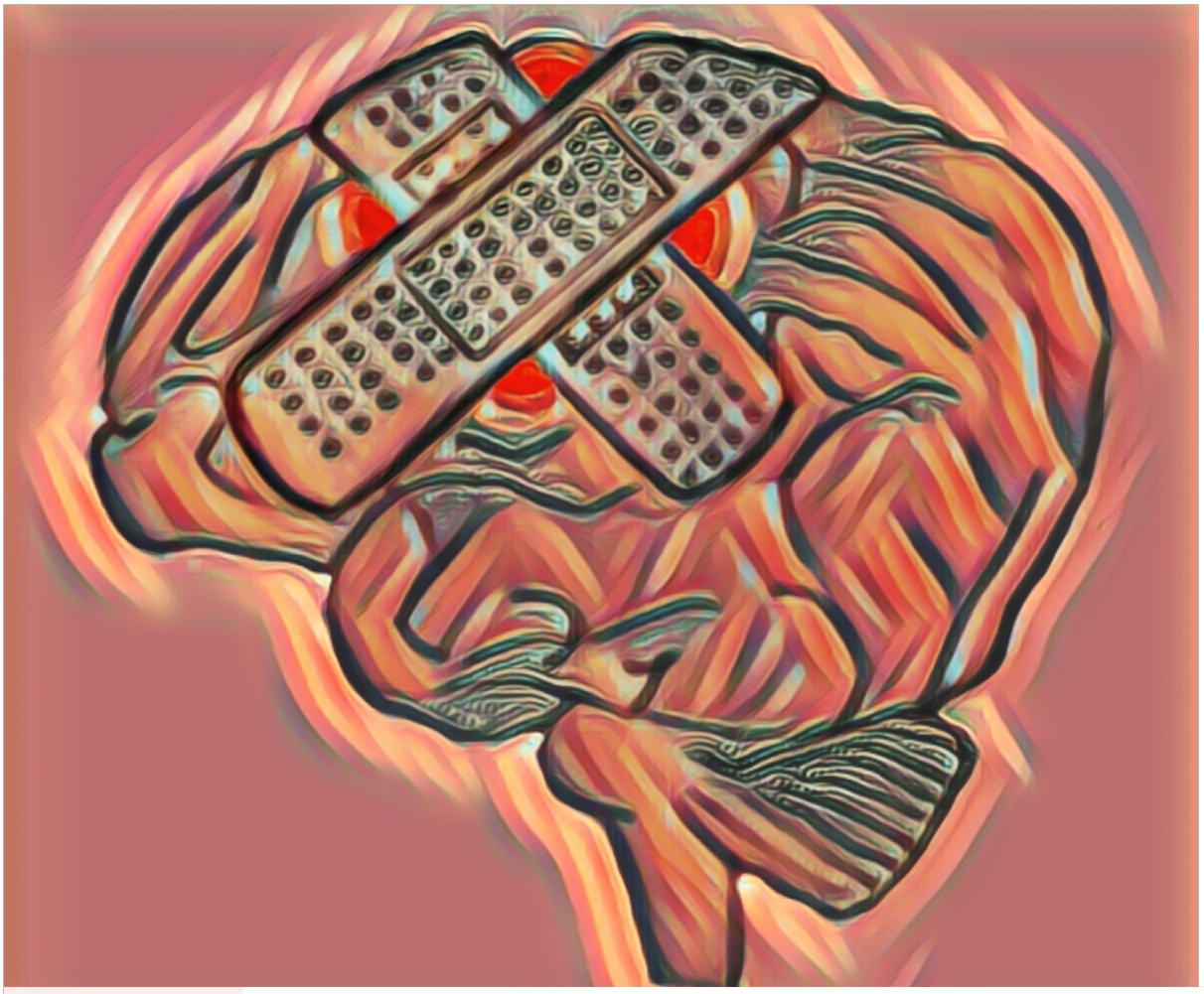

The inflammatory response to traumatic brain injury and a potential cure for it

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LUND UNIVERSITY

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Abstract:

The primary damage inflicted to brain tissue after mechanical trauma is always followed by secondary brain damage. The primary damage triggers the release of pro-inflammatory mediators that will lead to traumatic brain edema. The mechanisms behind traumatic brain edema are poorly understood, and currently no specific treatment against it exists.

Inflammatory mediators such as cytokines and chemokines will be secreted into the extracellular fluid of the brain after TBI.

Microdialysis with custom made high cut-off membranes were used to investigate the cytokine/chemokine response in an adult (7 patients) and a pediatric (7 patients) population with severe TBI.

In both adult and pediatric patients, the results outlined 3 groups of cytokines/chemokines based on the levels of secretion. The groups did not differ significantly between adults and pediatrics. Levels of the inflammatory mediators did not decline during the first week after trauma, which indicates a potent and prevailing inflammation in the CNS after severe TBI. The identified cytokines/chemokines may be used as outcome markers in future trials aimed at modulating the inflammation that ensues after TBI.

A food for special medicinal purposes (FSMP), Salovum® was first tested for feasibility as a concomitant treatment for severe TBI. The pilot trial (5 patients) showed that Salovum® can reduce ICP and that the substance was easy to deliver to the patients without any observed side-effects.

A research protocol for a prospective, randomized, placebo-controlled trial was developed.

The prospective trial (100 patients) was conducted in South Africa.

The trial showed that Salovum® significantly reduces 30-day mortality in adults with severe TBI.

The mortality was reduced from 39% in the control group to 20% in the Salovum® group.

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To Malin, Hugo, Nelson and Stella

Table of Contents

Populärvetenskaplig sammanfattning

Traumatisk skallskada (TBI), är ett resultat av trauma mot huvudet och spänner från mindre allvarliga, så kallade milda skallskador till de svåraste skadorna där den drabbade vanligtvis är långvarigt medvetslös efter traumat. De milda skallskadorna utgör den absoluta majoriteten av skador (89%) och många av de som drabbas, men långtifrån alla, återhämtar sig helt över tid. För svår TBI varierar dödligheten mellan 10 och 80% och bland de som överlever är det många som drabbas av neurologiska skador som innebär bestående handikapp. Skador vid TBI delas vanligtvis in i den primära och den sekundära skadan. Den primära skadan är de skador som uppstår till följd av att energi överförs från omgivningen till hjärnan i samband med skadeögonblicket. Den primära skadan kan således endast undvikas genom att förebygga trauma. Den sekundära skadan är de skador som utvecklas senare i förloppet, vanligtvis som en följd av en mängd processer som dras igång i den skadade hjärnan. Dessa skador skulle sannolikt till stor del kunna undvikas om det fanns läkemedel som kunde tämja de processer som äger rum. Det är känt sedan ganska många år att det vid TBI uppstår en inflammation i hjärnan. Det är också känt att denna inflammation kan kvarstå lång tid efter att skadan uppstått. Den första delen av mitt avhandlingsarbete syftade till att försöka mäta de signalsubstanser som uppstår vid inflammation och om möjligt koppla dessa till andra mätbara parametrar som sker under neurointensivvård. Mätningarna gjordes med så kallade mikrodialys-katetrar som in-opererades i hjärnan. Mikrodialys är en metod där en kateter vars spets har ett halvgenomsläppligt membran, opereras in i hjärnan. Genom katetern cirkulerar en vätska som är väldigt lik den vätska som finns i hjärnans extracellulär-utrymme och detta gör att utbyte av små molekyler kan ske över membranet. Vätskan som cirkulerat genom katetern kommer således att innehålla en fraktion av de ämnen som finns i hjärnans extracellulär-utrymme. Denna vätska kan sedan analyseras för att mäta hjärnans biokemi, men också för att mäta inflammatoriska signalsubstanser i hjärnan – s.k. cytokiner och kemokiner. I delarbete I gjordes mätningar på 7 vuxna patienter med svår TBI. I delarbete II gjordes samma mätningar på 7 barn med svår TBI. Vi kunde identifiera ett antal cytokiner/kemokiner som var konsekvent förhöjda och i koncentrationer som är jämförbara med de koncentrationer som uppmätts i blod hos patienter med svår COVID. Vi kunde se att det inflammatoriska uttrycket skiljde sig något mellan barn och vuxna. Slutligen kunde vi också konstatera att det inflammatoriska svaret kvarstod många dagar efter det primära traumat, något som tidigare studier inte med säkerhet visat. Att det finns en kvarstående inflammation långt efter en genomgången TBI stämmer väl med andra, nyare studier på milda skallskador, där man påvisat förhöjda cytokin/kemokin-värden i cerebrospinalvätska (ryggmärgsvätska), så långt som 6 månader efter genomgånget trauma.

I den andra delen av avhandlingen, som börjar med delarbete III, beskriver vi först en serie av patienter med svår TBI som erhållit ett kosttillskott (Salovum) med höga halter av proteinet antisekretorisk faktor. Hos flertalet patienter som erhöll Salovum, kunde det intrakraniella trycket (ICP) hållas under kontroll och ingen ytterligare neurokirurgi eller neurointensivvård behövdes. Mot bakgrund av att effekten av Salovum var så pass kraftig, valde vi att använda 30-dagars-dödlighet som primärt utfallsmått för en större studie då vi trodde att vi kunde påverka det verkliga utfallet för patienterna som ingick i den. Delarbete IV beskriver tankarna bakom forskningsprotokollet som låg till grund för mitt sista arbete – en prospektiv (framåtblickande), placebo-kontrollerad, dubbelblind, randomiserad studie där 100 vuxna patienter med svår TBI erhöll antingen Salovum eller placebo i tillägg till neurokirurgi och neurointensivvård. I studien såg vi att dödligheten hos patienterna som erhöll Salovum var 20% jämfört med 39% i placebo-gruppen. Resultatet var statistiskt signifikant och innebär att vi därmed bevisat att Salovum kan minska dödligheten i svår TBI om det ges i tillägg till övrig behandling. Resultaten presenteras i delarbete V.

List of Papers

This thesis is based on the following papers referred to in the text by their roman numerals. The papers are appended in the end of the thesis with permission from the publishers.

Paper I

Cederberg, D., Visse, E., Marklund, N. & Siesjö, P. Prolonged and intense neuroinflammation after severe traumatic brain injury assessed by cerebral microdialysis with 300 kDa membranes. J Neuroimmunol 578020 (2023) doi:10.1016/j.jneuroim.2023.578020.

Paper II

The inflammatory response in the pediatric brain after traumatic brain injury. David Cederberg MD, Edward Visse PhD, and Peter Siesjö PhD *Manuscript completed, currently not submitted.*

Paper III

Cederberg D, Hansson H-A 1939, Visse E, Siesjo P. Antisecretory Factor May Reduce ICP in Severe TBI-A Case Series. Frontiers in Neurology. 2020;11. doi:10.3389/fneur.2020.00095

Paper IV

Cederberg, D., Harrington, B. M., Vlok, A. J. & Siesjö, P. Effect of antisecretory factor, given as a food supplement to adult patients with severe traumatic brain injury (SASAT): protocol for an exploratory randomized double-blind placebocontrolled trial. Trials 23, 340 (2022).

Paper V

Antisecretory factor reduces mortality in severe TBI
David Cederberg, Bradley M. Harrington, Iain Walker, Ruan Grobler, Edward Visse, Adriaan Johannes Vlok, Peter Siesjö
Currently submitted to Lancet

Author's contribution to the papers

Paper I

David Cederberg played a major role in the design of the study, generated all results, participated in the analysis of data and wrote the first draft. He managed the submittal process and is first author of the article.

Paper II

David Cederberg played a major role in the design of the study, collected the results, participated in the analysis of data and wrote the first draft. He is first author of the article.

Paper III

David Cederberg participated in the design of the study and generated the more part of the results. He also alone wrote a first draft of the article and managed the submittal process. He is first author of the article.

Paper IV

David Cederberg participated in the design of the study and wrote the first draft. He managed the submittal process and is first author of the article

Paper V

David Cederberg played a major role in the design of the study, partially collected the results, participated in the analysis of data and wrote the first draft. He also presented the data at scientific meetings. He managed the submittal process and is first author of the article.

Aims of the thesis

The first aim of this thesis was to, in depth, investigate the inflammatory response in the extra-cellular space of the brain during brain trauma by using custom-made microdialysis (MD) catheters. The second aim was to use this information to define end-points in an interventional trial, using one or more anti-inflammatory drugs. During the time between when the first aims of the thesis were decided upon and now, the potentially anti-inflammatory substance Salovum, caught our attention and the second part of the thesis was re-formulated to test this substance in a clinical trial, using 30-day mortality as the primary end-point.

Paper I

To analyze the inflammatory response in the brain in adults with severe TBI, using double custom-made MD membranes with a pore-size of 300 kilodalton (kDa). The cytokines/chemokines response were analyzed in relation to other pseudo-markers for secondary injury.

Paper II

To analyze the inflammatory response in the brain in a pediatric population with severe TBI, using custom-made MD membranes with a pore-size of 300 kDa. The cytokines/chemokines response were analyzed in relation to other pseudo-markers for secondary injury and compared to the material collected in the adult population.

Paper III

To investigate if the medical food supplement, Salovum, was safe, feasible to administer and could show signs of efficacy in patients suffering from severe TBI.

Paper IV

To formulate a research protocol for a prospective, randomized, double-blinded, placebo-controlled trial with Salovum in severe TBI.

Paper V

To conduct a prospective, randomized, double-blinded, placebo-controlled trial with Salovum in adult patients with severe TBI.

Abbreviations

BBB	Blood brain barrier
AF	Antisecretory factor
CNS	Central nervous system
DAMP	Damage associated molecular patterns
DNA	Deoxyribonucleic acid
ECL	Electrochemiluminescence
ELISA	Enzyme-linked immunosorbent assays
EU	European Union
FasL	Fas ligand
FSMP	Food for special medical purposes
G-CSF	Granulocyte colony-stimulating factor
GCS	Glasgow coma scale

GFAP	Glial fibrillary acidic protein
GOSE	Glasgow outcome scale extended
ICP	Intracranial pressure
IFN	Interferon
IFP	Interstitial fluid pressure
IL Interleukin kDa Kilodalton	
LOS	Loss of consciousness
LPR	Lactate pyruvate ratio
MAC	Membrane attack complex
Mb	Morbus – disease in latin
MCP1	Monocyte chemoattractant protein-1
MD	Microdialysis
MIP-1β	Macrophage inflammatory protein-1beta
NETs	Neutrophil extracellular traps
NK cell	Natural killer cell
NOD-like	Nucleotide-binding oligomerization domain-like
PAMP	Pathogen associated molecular patterns
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
SPC	Specially processed cereals
TBI	Traumatic brain injury
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TIL	Treatment intensity level
US	United States

Introduction

During my training as a neurosurgeon in Lund, starting 2008, but also for 6 months in South Africa, I saw the deleterious effects of traumatic brain injury on peoples´ lives and the lives of their next of kin. Sometimes the initial trauma was serious, but survivable given that the patient had imminent neurosurgery. Then, during the days that followed patients deteriorated. The swelling that occurred, caused acute

herniation of the brain. The hemorrhagic transformation of cerebral contusions that initially were small, caused serious brain injury with anticipated significant neurological deficits. All these events occurred right in front of our eyes, and there was very little that we could do about it. In Sweden, where the number of head traumas is decreasing, numerous neurointensive care interventions were undertaken. Sometimes, these interventions resulted in actual benefits for the patients. Each neurointensive care unit in Sweden had their own local traditions and treatment algorithms, and why wouldn't they? The American Guidelines do not present much solid evidence for anything[1]. In South Africa, where brain trauma is an epidemic, patients were treated for their initial, primary injuries, however there was simply not enough beds to allow for weeks of neurointensive care, and the patients were often weaned off the ventilator on the 4th to 5th day.

Everybody that has experienced trauma to another part of their body, recognizes that swelling tends to occur. However, in no other parts of the body will the swelling have so dire consequences.

The notion that tissue edema can arise without an infectious agent, inflammation but not infection, has been recognized for a long time. It was my opinion that a very unfortunately designed trial with corticosteroids closed the door to a potentially efficient anti-inflammatory treatment against the sterile inflammation that supersedes TBI. In the study, corticosteroids were given in supra-normal doses due to a direct conversion from animal models, investigating lipid peroxidation in rats with spinal cord injury. The trial included patients that did not suffer from severe traumatic brain injury, and only 20% of the total patient population received any kind of neurosurgery, such as an intracranial pressure (ICP) monitor. But the results, rather than the methods, were widely spread, and henceforth steroids were banned in the already weak treatment arsenal of TBI[1].

I was convinced that much of the secondary injuries that occur after TBI were caused by inflammation and should be treated in such a manner. The initial goal of this thesis was to identify a pattern of inflammatory mediators that could be used as pseudo-markers for outcome in an interventional trial, using available and appropriately dosed anti-inflammatory drugs to treat the post-traumatic swelling of the brain that occurs after TBI. By chance however, we were introduced to the protein antisecretory factor (Salovum) and the pilot study that was conducted showed some remarkable results. We could conclude that Salovum sometimes reduces ICP in patients with severe TBI and may even spare the patient from further neurosurgery. The second half of my thesis was therefore dedicated to designing and conducting a trial to test the potency of Salovum and antisecretory factor in severe TBI. It has been a great pleasure and a whole lot of work, serious work, to complete this task, and I hereby proudly present my doctoral thesis.

Traumatic brain injury - TBI

Epidemiology

Traumatic brain injury (TBI) is a global pandemic. It is estimated that 50 – 60 million people experience a traumatic brain injury each year globally[2]. The morbidity after TBI is significant, ranging from severe life-long disability for the most severely affected individuals. Even for the least affected individuals, problems with persisting head aches and long term unemployment is a common problem[3].

The leading cause of TBI are falls, road traffic accidents, assault/violence, being struck by or struck against events and sporting or recreation accidents. In developed countries falls are the most common cause and in developing countries road traffic accidents predominate.

Men are generally more often affected by TBI. The male to female incidence varies between 1,2 to 4,8, depending on country.[4] Three peaks of incidence are noted: children, young adults and the elderly.[5]

Classification

TBI can be divided into subgroups depending on the clinical presentation of the patient. Most TBIs are mild, the exact proportion varies in different materials approximately ranging from 70-95%[6,7]. The higher number is likely the most accurate since many mild TBIs may not involve the health care system at all and are thus underrepresented in epidemiologic studies utilizing hospital records only. Mild TBI means that the patient may have a loss of consciousness (LOS) but limited to a maximum of 30 minutes, a Glasgow Coma Scale (GCS) score of 13-15 and a posttraumatic amnesia of less than 24 hours. 20 % of TBIs are considered moderate, which means LOS of 30 min – 24 hours, GCS 9-12 and a post-traumatic amnesia of 1-7 days. The last 10% is considered severe TBI, which means that the patient remains unconscious >24 hours, has a GCS of 3-8 and a post-traumatic amnesia of >7 days.[8]

The most severely injured patients, usually patients with severe TBI, often need neurocritical care and neurosurgery to survive. The mortality of severe TBI differs greatly in different materials, with death rates ranging from 10 – 80 percent[9-11], depending on geography, logistics, referral policy, availability of neurosurgery and neurocritical, or at least intensive care.

Although outcome, as measured by mortality, has greatly improved during the last decades, morbidity after moderate and severe TBI is still high[9]. The functional outcome after TBI depends on several factors besides the mechanism of trauma and

GCS at arrival. Comorbidity[12], geographic location in relation to a hospital with neurosurgery[13], ethnicity and socioeconomic status[14] all have an impact on the outcome.

Primary injury

The primary injury is what happens when the cranial cavity is subjected to trauma and impact energy is transmitted into the brain. Primary injury is often divided into focal injuries and diffuse injuries.

Focal injuries encompass epidural hematomas, subdural hematomas, traumatic subarachnoid hemorrhage and contusions. Diffuse injury involves tearing of mainly white matter nervous structures and are named diffuse axonal injuries[15].

The extent and character of the primary injury is under influence of multiple factors. The most important factor is the energy of the trauma, where high energy trauma will yield more serious primary injuries. The mode in which energy is transmitted into the brain at the time of trauma will also have an impact. Non penetrating trauma can be delivered through blast, acceleration, deceleration, rotation and combinations of these.

The age and medical history of the individual that is subjected to trauma, will also have a high impact on the types of primary injuries that will arise. The pediatric population will have different primary injuries depending on age, where infants with soft cranium and high water content of the brain will be prone to certain injuries, whereas small children with heads that are proportionally bigger than the rest of the body will be prone to other injuries. An elderly person with some degree of brain atrophy may develop an acute subdural hematoma, caused by a tear of a vein between the cortex and the dura, from a minor head trauma. A younger individual, with no brain atrophy, i.e. small distance between the cortex and the dura, is less prone to develop an acute subdural hematoma, but if he/she does, the trauma mechanism behind is usually much more severe. This means that the same diagnosis, an acute subdural hematoma, may have a better outcome for the elderly person because the initial trauma is much milder in this age group.

The primary injury can be averted through measures that address the root cause, such as road safety signs, lane separation, and the use of proper safety gear.

Secondary injury

The secondary injury is what follows as a result of the initial, primary injury. These events are potentially avoidable. The initial trauma gives rise to the primary injury, but the primary injury will, if left untreated, most likely lead to secondary injuries.

Neuroinflammation, excitotoxicity and oxidative stress are all components of the secondary injury that may further damage the brain[16].

A large hematoma from a primary injury may give rise to increased ICP. The increased ICP can make it difficult for the brain to receive enough blood through the circulation, which in turn may cause an ischemic injury to the whole brain, or parts of it. Early neurosurgical removal of the blood may normalize ICP and thus avoid ischemic injuries. But not all secondary injuries can be avoided by evacuating a hematoma or contused tissue. Both vasogenic and cytotoxic brain edema generated by disruption of the blood brain barrier (BBB) or swelling of astrocytes respectively, lead to an increase in ICP. The cerebral blood flow may also be impaired by micro-thrombosis, leukocyte/platelet aggregates and inflammatory changes in the cerebral endothelium.

When the brain is injured, cells will die and release factors normally not exposed to the extracellular space. An important fraction of these, damage associated molecular patterns (DAMPs), will trigger a sterile immune, or inflammatory response. The ensuing inflammation will lead to brain swelling that may increase the ICP even more or cause further direct or indirect cell damage. Areas of the brain that are close to or within injury sites may be hypo-perfused which can lead to hypoxia that can cause mitochondrial dysfunction, a state where cells are unable to properly manage their metabolism, which may cause more cell death. This is a cycle that begins with certain mechanisms and leads to worsening of the damage. Other mechanisms follow and the damage worsens even more. Secondary injury, if left un-attended, may behave like a snowball at the top of a steep hill.

The Inflammatory or Immune Response to TBI

It is believed that the immune system has evolved to be a highly efficient defense against microbial invasion, which in evolution has been the biggest threat against survival of the species.[17] The role of the neuro immune system has thus been designed to ward off any infectious disease that may affect the human in such a way that the individual cannot reproduce. Evolution over thousands of years has premiered individuals that have the capacity to fight infectious disease and survive. Thus, the immune system has been primed to fight external threats and the consequence of this has lead to morbidity and mortality invoked by the immune response itself.

Brain trauma has been shown to induce long standing immune-mediated inflammatory responses that can last for years post injury[18-20]. When trauma hits the brain, a series of immune events occur, these events can be divided into events that

are mediated by the innate immune system, and events that are mediated by the adaptive immune system.

The Innate Immune System

The innate immune system, a crucial component in the defense against pathogens and tumors, plays a multifaceted role extending beyond protection—it actively contributes to the restoration of tissue homeostasis following injury. It has been proposed that the mechanisms originally arose from tissue damage inflicted by pathogens. Evolution shaped this system to have a remarkable ability to rapidly respond in a non-specific manner, albeit with limited capacity to recall encountered antigens. Key cellular effectors of the innate immune system encompass monocytes, macrophages, microglia, dendritic cells, granulocytes, and natural killer cells.

DAMPs and PAMPs

PAMPs and DAMPs play crucial roles in the innate immune response[21]. PAMPs (Pathogen-associated molecular pattern) help the immune system identify and respond to infectious agents, while DAMPs signal the presence of tissue damage or stress, prompting an immune response to facilitate tissue repair and clearance of damaged cells. PAMP and DAMP can initiate an immune response via classical Pattern Recognition Receptors (PRRs), (discussed below) which include not only Toll-like receptors (TLRs), but also via multiple germ-line encoded receptors such as NOD-like receptors, other receptors and multiple intra-cellular DNA sensors. Since DAMP-initiated inflammatory responses are independent of pathogen infection, they can be considered a sterile inflammation. To understand the molecular patterns of the innate immune response is essential in understanding the immune system in relation to infections and injuries since these molecules appear in the presence of tissue damage or infection, instigating an immune response that is primarily directed towards tissue repair and clearance of damaged cells and foreign debris.

Pattern recognition and signaling

Central to the functioning of the innate immune system are Pattern Recognition Receptors (PRRs)[22]. PRRs are a class of proteins expressed by the cells of the innate immune system to recognize molecular patterns that are associated with pathogens or injurious processes. It was first thought that PPRs were only germ-line encoded and thus able to recognize conserved pathogen associated molecular patterns. However, this could only explain the innate immune response to pathogens and not injury free of microbes. Later it was discovered that injured or distressed cells

release endogenous molecules that initiate the innate immune response – DAMPs. In this way the cells of the immune system and other cells, constantly search the extracellular environment of the central nervous system (CNS) for exogenous pathogens or self-molecules that indicate cells in danger or distress.

Toll-like receptors (TLRs) are a specific subset of PRRs and consist of transmembrane proteins that are expressed on the surfaces of immune cells as well as some non-immune cells. Other PRRs subsets are germ-line encoded receptors such as NOD-like receptors (NLRs), scavenger receptors and multiple intra-cellular DNA sensors. All these play pivotal roles in identifying triggers that initiate innate immune responses. When a PRR identifies and binds to a protein that triggers it, a signaling cascade is initiated which activates the innate immune system.

Cells of the Innate Immune System

Monocytes/Macrophages**,** microglia, dendritic cells, granulocytes, and natural killer cells are central players in the innate immune system.

Microglia

The resident macrophage of the CNS monitor their surroundings and will become activated when they sense danger.

Microglia can be activated into a M1-like phenotype or a M2-like phenotype[23]. DAMP, free radicals and pro-inflammatory cytokines will induce M1-like cells, which are generally considered to be harmful. However, a well-timed response at the time of injury will likely be neuroprotective[24]. The M1 phenotype will most likely increase BBB permeability and result in migration of peripheral blood monocytes/macrophages and granulocytes into the site of injury. M2a and M2c-like phenotypes will initiate different sorts of anti-inflammatory and tissue-regenerating responses and thereby promote healing. Expression of the different phenotypes depend on a large range of variables such as genetic and epigenetic factors.

Astrocytes

Astrocytes may convert from the resting type to the reactive type in TBI. The conversion into the reactive type results in cell hypertrophy, heightened proliferation, secretion of inflammatory mediators and neurotrophic factors. It also leads to increased expression of intermediate filaments such as glial fibrillary acidic protein (GFAP) and vimentin which leads to gliosis, which is essential to start the healing process after the acute injury[25]. Some evidence suggest that reactive astrocytes are essential for the initial healing process in the acute phase of an injury, but may actually be counteracting the later stages of

recovery[26]. Astrocytes may also differentiate into a more pro-inflammatory type, or a more anti-inflammatory type and this may happen under the influence of microglia[27].

Dendritic cells

Dendritic cells are antigen-presenting cells critical in regulating the adaptive immune response. They can both present antigens and release appropriate stimulatory molecules to initiate an adaptive immune response. Dendritic cell counts decrease after TBI. In addition, the large amounts of DAMPs after TBI activate immature dendritic cells, which leads to a lower abundance of cells that can respond to secondary insults later. The dendritic cells may also induce tolerance and release anti-inflammatory signals[28].

NK cells

It is known that the number of NK cells decrease after TBI[29]. The low numbers persist for weeks and has been found to correlate with low GCS at admission. In patients with ICH, the predominant immune cell in the tissue surrounding the hematoma are NK cells. Two types have been identified, one that exert strong cytotoxicity and one that has a high chemokine production. NK cells may attract other neutrophils and contribute to edema, disruption of BBB and neurological deterioration[28]

Monocytes/Macrophages

Monocytes will follow chemokine gradients into the area of injury. Once in the brain they transform into macrophages within 24 hours after TBI.[30] The main mechanism of recruitment of monocytes is thought to be via monocyte chemoattractant protein-1 MCP1(CCL2).[31] The monocytes, turned into macrophages will execute phagocytosis and secrete inflammatory mediators.

Neutrophil granulocytes

Neutrophil granulocytes are the first cells to extravasate after TBI. They will appear in the subarachnoid space hours after trauma and will begin to infiltrate the extracellular space within the first 24 hours. Neutrophils are attracted to the site of injury and will be influenced by different factors such as the complement system[28]. They try to clear cell debris by phagocytosis but will increase inflammatory response by producing Neutrophil Extracellular Traps (NETs) and Reactive Oxygen Species (ROS). ROS are known to have a negative impact on outcome in TBI[32]. The impact of NETs has been suggested to contribute to
coagulopathy[33,34], but the underlying mechanism is largely unknown.

The Complement System

Another important effector of the innate immune system is the complement system. The complement system consists of over 50 glycoproteins that have diverse roles in the homeostasis and development of the uninjured CNS. The role of the complement system in the injured CNS has often been overlooked. It is thought that it may have several potentially deleterious effects in the traumatically injured CNS, making research about the complement system and TBI an important task. Complement activation ensues after tissue damage or microbial challenges and can be mediated in three separate pathways: classic, lectin and alternative pathway. All pathways result in the formation of the membrane attack complex (MAC). Regulatory proteins protect the host against over-activation. These proteins block different pathways for the formation of the MAC.

Cerebral biosynthesis of complement proteins is induced when tissue damage has occurred. It adds to the systemic pool that enters the CNS once the BBB is compromised. Complement opsonins (chemotactic molecules) facilitates clearance of debris at the injury site by microglia and macrophages bearing the CR3 receptor. C1q promotes transformation of microglia into pro-inflammatory phenotype M1. C1q and C3 also play an important role in microglia-mediated neurodegenerative mechanisms after trauma. The activated microglia induces pro-inflammatory phenotype astrocytes via secretion of C1q, Interleukin-1α (IL-1α) and Tumor necrosis factor α (TNFα). The pro-inflammatory astrocytes in turn secrete more complement components such as C3. Other components of the complement system, that will gather at the injury site, can act as chemo-attractants for granulocytes.

The complement system is also involved in the coagulation system and can cause coagulopathy with both increased bleeding and micro-thrombosis as a consequence.

The complement system has been less investigated than the rest of the immune system for its role in TBI.

The Adaptive Immune System

The adaptive immune system in TBI acts mainly through T-cells and B-cells. Many of the cells of the innate immune system have the ability to activate T- and B-cells by presenting antigens in different ways so that these cells become active. Cytokines can activate and differentiate T cells into CD4 or CD8 T cells and B cells into antibody secreting plasma B cells that undergo clonal expansion. NK cells may target damaged cells and promote different lymphocyte responses, depending on which cytokines they release. T helper cells may stimulate plasma B cells to produce antibodies that lead to opsonization of the injured cell or antigen that will flag the cell for phagocytosis. T helper cells may also activate complement pathways.

Cytotoxic T lymphocytes and B cells can be stimulated into causing cytotoxicity and FasL/Fas mediated cell death. Via blood and the lymph nodes, the systemic immune system will be activated once the adaptive immune system has been triggered.

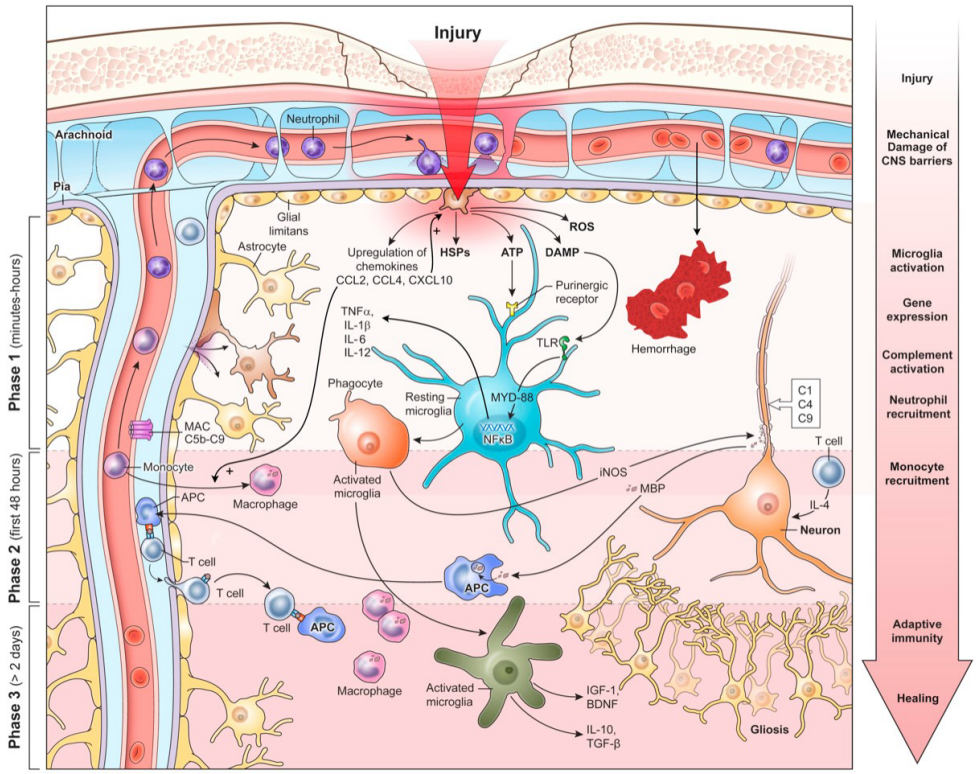

Figure 1 Inflammation in TBI

The relevant cells and molecules that will be activated in TBI. On the sides are time-axes depicting proposed times for when changes occur (left) and in which order and what occurs (right). The initial mechanical trauma will damage CNS barriers with ensuing hemorrhage and secondary events. Damage of neurons, astrocytes, pericytes and other resident CNS cells will release damage associated molecular patterns (DAMP) and other danger cues. Microglia and invading blood derived macrophages will sense tissue damage rapidly by stimulation of their TLR and other pattern recognition receptors by released DAMP molecules as HMBG1 and danger cues as reactive oxygen species (ROS), heat shock proteins (HSP) and free adenosine triphosphate (ATP). These cues will activate microglia and macrophages and transcription factors as nuclear factor kappa beta (NFkB), leading to release of pro-inflammatory cytokines as IL1beta, TNFα, IL-6 and IL-12, but also inducible nitric oxide synthetase (iNOS) and other inflammatory mediators. The complement system, which has a role in neuronal development and homeostasis, is activated by tissue damage and coagulation factors as thrombin leading to formation of the cell toxic membrane attack complex (MAC). Damaged cells will also release chemokines as CCL2, CCL4 and CXCL10 which will attract extravasating neutrophils and monocytes from blood. Adaptive immunity can induce cytotoxic T cells after presentation of components as myelin basic protein (MBP) from damaged neurons by antigen presenting cells (APC) that may further aggravate tissue damage. Release of proinflammatory cytokines and other damage inducing entities as MAC, CD8 and CD4 T cells, and neutrophil derived ROS can lead to tissue edema but also induction of cell death as pyroptosis. But tissue damage can also be reduced by the induction of regulatory T cells

and anti-inflammatory cytokines. In later stages of the CNS response to trauma signaling from both microglia, macrophages and T cells will induce tissue healing by release of growth factors as insulin like growth factor (IGF-1), brain derived neurotrophic factor (BDNF) and anti-inflammatory cytokines promoting healing as interleukin-10 (IL-10) and transforming growth factor -beta (TGF-β). Modified from Jassam, Y. N., Izzy, S., Whalen, M., McGavern, D. B. & Khoury, J. E. Neuroimmunology of Traumatic Brain Injury: Time for a Paradigm Shift. *Neuron* **95**, 1246–1265 (2017). with permission of Elsevier (license number 5724660710015).

Summary

Upon encountering trauma, hypoperfusion, metabolic distress, or environmental irritants, the innate immune system undergoes an "awakening." This event triggers a local production of cytokines and chemokines, setting the stage for subsequent immune reactivity at the site of tissue damage. The site of tissue damage will undergo secondary injury that includes dysfunction of the BBB, brain edema, and neuroinflammation. Secondary injury is mainly driven by astrocytes, microglia, and infiltrated immune cells from peripheral tissues, and causes continuous neuronal and vascular dysfunction[28]. Activation of the innate immune system will lead to activation of the adaptive immune system, the role of which, is much less understood in TBI. The activation of the adaptive immune system after TBI is most likely responsible for parts of the long-term negative effects such as progressive neurodegeneration and also specific neurodegenerative diseases such as Parkinson´s disease[35], a disease known to be far more common in boxers than non-boxers.

Cytokines/Chemokines

Cytokines

Cytokines are a category of small proteins involved in cell signaling. Although their definition is continuously altered, they include chemokines, interferons, interleukins, lymphokines, but generally not hormones or growth factors. They are soluble proteins and are crucial for orchestrating and regulating immune responses, ensuring a dynamic and coordinated reaction to various physiological and pathological stimuli. Cytokines are primarily produced by leukocytes, including macrophages, T cells, and B cells, as well as non-immune cells such as astrocytes, fibroblasts and endothelial cells. Cytokines primarily have an effector function in the immune and inflammatory system. They may regulate responsiveness of certain cell populations, as well as maturation and growth of others. Cytokines may be expressed by several different cell types, are almost always related to immune responses, and are often expressed in lower quantities than hormones, which are most often secreted in larger concentrations and only by one cell type. Unlike hormones that are secreted by fixed glands cytokines can thus be secreted

throughout the whole organisms and often execute their main effects at close range. When cytokines are secreted at high levels, they can also induce systemic effects e.g. multiorgan failure which can be seen in sepsis and other fulminant infections. These signaling molecules mediate intercellular communication and thus influence the behavior and function of target cells. Cytokines can be classified into different categories based on their functions, such as interleukins (IL), interferons (IFN), and tumor necrosis factors (TNF). They act in autocrine, paracrine, and endocrine manners, exerting pleiotropic effects on immune cells, hematopoiesis, and inflammation. The function of cytokines can be broadly divided into the following categories:

Immunoregulation

Cytokines regulate immune responses by modulating the activation, proliferation, and differentiation of immune cells. They can enhance or suppress the activity of various immune components based on the existing conditions of the microenvironment.

Inflammation

Many cytokines act as pro-inflammatory or anti-inflammatory mediators, influencing the initiation, progression, and resolution of inflammatory processes. Pro-inflammatory cytokines, such as IL-1β and TNF-α, IL-6 promote inflammation via production of other inflammatory mediators and promotion of migration of immune cells to sites of inflammation.

Hematopoiesis

Cytokines contribute to the regulation of hematopoiesis by influencing the development and differentiation of blood cells in the bone marrow. It has been shown that it is safe, and potentially beneficial for patients to receive granulocyte colony-stimulating factor (G-CSF)[36] after TBI.

Cellular communication

Cytokines facilitate communication between immune cells, enabling them to work in a coordinated manner. This communication is crucial for an effective immune response against pathogens and for maintaining immune homeostasis.

Chemokines

Chemokines are a subgroup of cytokines that specifically regulate the migration and positioning of immune cells within tissues. Some of the most common ones are IL8, Monocyte chemoattractant protein-1 (MCP1) and Macrophage inflammatory

protein-1beta (MIP-1β). They play a central role in directing leukocyte trafficking during immune surveillance, inflammation, and immune responses. The function of chemokines can broadly be divided into the following categories:

Cell Migration

Chemokines control the directional migration of immune cells by inducing chemotaxis. They guide leukocytes to specific locations within tissues, such as sites of infection or inflammation.

Inflammatory Responses

Chemokines contribute to the recruitment and activation of immune cells at inflammatory sites. They establish concentration gradients that guide immune cells towards the source of the chemokine, promoting an effective immune response.

Homeostasis

Chemokines are involved in the maintenance of immune cell homeostasis by regulating the trafficking of cells between lymphoid organs and peripheral tissues.

Summary

In summary, cytokines and chemokines constitute integral components of the immune system, regulating immune responses and maintaining homeostasis. Their intricate network of signaling pathways ensures a finely tuned and dynamic immune reaction to diverse challenges, playing crucial roles in health, immune defense, and resolution of inflammatory processes. A comprehensive understanding of these signaling molecules is essential for unraveling the complexities of immunology and holds promise for the development of targeted therapeutic interventions in various immune-related disorders.

Microdialysis for neuromonitoring

The method of microdialysis (MD) for monitoring biochemical processes in the brain has been around for over 30 years. MD measures the metabolism of the brain by continuously sampling and analyzing the molecules of the Kreb´s cycle: glucose, lactate and pyruvate[37-40]. The concentration of glucose and the ratio between lactate and pyruvate (LPR) have been shown to reflect the metabolic state of the tissue that is being monitored. LPR is considered the most clinically useful biomarker, since it

reflects the $NADH/NAD^+$ redox status, i.e. the balance between aerobic and anaerobic metabolism. A value of ≤25 in "healthy" brain tissue is usually considered favorable. Values above 25 are independent predictors of poor patient outcome[41].

MD depends on a small double lumen catheter with a semi-permeable membrane at the top. The tip of the catheter is placed into the brain and the catheter is connected to a fluid pump at one end and a collecting tube at the other side. A fluid that is isotonic with the extracellular fluid of the brain is slowly pumped through the tubing of the catheter. When the fluid passes over the membrane that is located in the extracellular space of the brain, molecules move down their osmotic gradient and pass into the fluid of the catheter that can later be collected at the end of the tube.

The fluid that is collected at the end of the MD catheter tubing contains a proportion of the same molecules that are existent in the extra cellular fluid of the brain.

The size of the pores of the semi-permeable membrane and the osmotic content of the perfusion fluid that is pumped through the catheter affects the size and recovery of the molecules that are being sampled. It is very difficult to predict how various molecules will react, i.e. if they will be captured by the MD catheter or not. Many factors will influence this, i.e. size of molecule, charge and hydrophilic properties. Also, the intracranial pressure may have a great impact on what is recovered[37,42,43]

The normal size of the pores of MD catheters is 20 kDa, the large size catheters are 100 kDa. The two pore-sizes were the only ones available in 2008. Today, the company HA sells MD catheters with a pore size of 500 kDa for animal use. In the present studies we used a custom-made catheter with a 300 kDa pore size. Our intention was to sample larger molecules than what would be possible using the standard 100 kDa membranes. It is well-known that higher pore size may experience problems such as imbalanced fluid recovery due to ultra-filtration, unstable catheter performance and low and unstable molecule extraction efficiency[44]. The pores of a custom-made membrane can be made exactly to the specified size, but by changing the pore-size, the hydrophilic properties and the propensity for a pore to let through particles of different charges may change the recovery rate. In addition, the properties of the perfusion fluid influences recovery, where addition of osmotic agents such as dextran or albumin has been reported to increase fluid and particle recovery[43].

A potential source of error that should be addressed, in particular when comparing different studies in the subject with each other is the issue of protein adsorption to the MD membranes. This will likely occur on all MD membranes[45], but may very well be different depending on MD location – peri-contusional vs. non-traumatized, membrane pore-size and perfusion fluid. Being consistent in method will at best ensure that you get the same potential errors every time. However, it does not

ascertain that different studies, using different methods, are comparable. A recent publication concluded that there is very little work done on cerebral MD in regards to adsorption[46]. This may, have a substantial negative effect on recovery and may very well explain why many MD trials in TBI have reported increases in cytokine/chemokine values after trauma[47,48], followed by a rather fast decrease just a few days later, contrary to what the few long term follow up studies investigating mild TBI have shown, where increased inflammatory mediators have been demonstrated in plasma and CSF, months after the initial trauma[16,49,50].

Microdialysis for Cytokine/Chemokine analysis

As part of trying to get a better understanding of the inflammatory processes that occur in the brain after traumatic brain injury, my supervisor and I began with writing a review article on brain trauma and inflammation[51].

The immunological response after TBI can be assessed by measuring the cytokine/chemokine expression that occurs following the trauma. It is known that the immune system is activated both locally and systemically[16]. There is some evidence that there is a gradient between the cytokine concentrations in the extracellular space as measured by MD, the cytokine concentrations in the CSF and the concentrations in plasma[48]. Although there are not a lot of studies, it makes sense to conclude that cytokines are higher in concentration at the site of the injury. However, even plasma concentrations in TBI patients are elevated and can remain elevated for months after the trauma itself[49,50]. When my research projects were being planned, none of the foundational scientific papers about intracerebral MD for cytokines had been written[47,52]. Only early reports with highly variable methodologies, yet some common denominators had been published on the topic[53][55][56]. Cytokines vary in size between 6 to 70 kDa , chemokines are generally smaller, 7 to 15 kDa[57]. There were reports suggesting that the actual molecular cut-off for 100 kDa MD membranes would be 20 kDa. The consequences would be that some of the larger cytokines may not be collected using 100 kDa. For that reason, custom made 300 kDa membranes were ordered from CMA MD. In the very first patient, 100 kDa MD catheters were implanted next to the 300 kDa catheters. Fluid recovery and biochemistry were found to be comparable. A pooled sample of the MD fluids was analyzed using proteomics to establish what the maximum size of the collected proteins were for the respective membranes. In the 100 kDa catheter, most of the proteins were less than or 25 kDa when using ordinary perfusion fluid. In the 300 kDa catheter, using the same perfusion fluid, there was a lot of proteins in the range of 70 kDa. We actually found an abundance of albumin in the samples from the 300 kDa catheter. Albumin was used as a plasma expander in TBI patients, according to the Lund Concept at the time.

Immunoassays for Cytokine/Chemokine analysis

When paper I and II of this thesis were being planned, the most commonly used technique to analyze cytokines were enzyme-linked immunosorbent assays (ELISA). ELISA is a widely used laboratory technique for detection of specific proteins such as cytokines/chemokines. The process of ELISA is as follows:

Coating

Microplates are coated with an antigen or antibody specific to the target protein which creates a solid phase which allows for any protein in the sample that can bind to this coated surface to be captured.

Blocking

Unoccupied sites on the microplate are blocked to prevent non-specific binding of other proteins. This is done by adding a blocking agent solution.

Sample addition

The sample to be analyzed is added to the microplate and allowed to interact with the coated antigen or antibody. If the target protein is present in the sample, it will bind to the coated molecules. To ensure accuracy, each sample is most often analyzed in duplicate or triplicate.

Washing

The microplate is washed to remove any unbound or non-specifically bound substances, which reduces the background noise and enhance the specificity of the assay.

Detection

A detection antibody, specific to a different epitope on the target protein, is added. This secondary antibody is conjugated with an enzyme

Substrate addition

A substrate specific to the enzyme is added. The enzyme catalyzes a reaction that produces a detectable signal, such as a color change, luminescence or fluorescence.

Measurement

The intensity of the signal is measured using a spectrophotometer or a plate reader. A validated standard is also added, which yields a standard curve. Each of the other samples´ signals are compared to this curve. The signal from each sample will then be directly proportional to the amount of target protein present in the sample.

New ways to utilize the technique of ELISA, making it possible to analyze numerous analytes simultaneously were introduced around year 2000. The leading companies at the time were Luminex® and MesoScale Discovery™ (MSD).

Both provided multiplex immunoassays capable of analyzing numerous analytes at the same time both utilizing ELISA but in different ways. While Luminex® worked with bead-based technology, in which microspheres (beads) are coated with specific capture molecules for different analytes and utilizes flow cytometry for detecting fluorescence signals, MSD uses electrochemiluminescence (ECL) which generates a light through a chemical reaction when an electric current is applied through the electrode surfaces of the plates. The process of ELISA is utilized by putting the capture antibodies on a plate with an electrode surface. After the sample has been added and relevant analytes have been captured by the antibodies, detection antibodies labeled with ECL tags are used to bind to the captured analytes. The instruments for analyzing are designed to apply an electric current to the plates which induces the ECL reaction. The light emission is then detected and quantified by the instrument.

There are many reports comparing different multiplex arrays, in 2008 there were fewer. One report stated a higher sensitivity in MSD and a higher precision in Luminex. We used MSD in paper I and II.

MSD has been validated many times and proven to have the best sensitivity in the low detection limit and the broadest dynamic range – meaning that it is capable of detecting and quantifying the analytes across a wide span of concentrations, from very low to very high levels[58]. The dynamic range is a strength since some analytes may be present in very low levels in one patient, while another patient may have levels that are significantly elevated due to the disease that is being studied.

It is likely that every available multiplex array have their own strengths and weaknesses. When tested, certain cytokines or chemokines may have a high detection level, such as IL-2 and IL-12 for MSD in one report[59], others for other multiplex assays. Of course, if the cytokine/chemokine that you are specifically interested in, is the one that is a certain multiplex arrays´ weakness, you should probably choose another. Otherwise, it is likely most important of all to use the same method in all your experiments, in order to avoid different method-related errors in different experiments.

For example, if we know that IL-2 and IL-12 in low concentrations may be even lower with MSD, first of all, these interleukins must not be the crucial to our study. Secondly, if we change multiplex arrays mid-study, or for our next project, and are not aware of this weakness – then all of a sudden we might have higher values of these two cytokines in the low-range and start giving the new “higher” values a significance.

Another source of consistent errors might well be the equipment that is used to bring the luminescence meaning – i.e. the optical readers, that may or may not have their own in-built faults. By using the same equipment, you get the same, potentially consistent errors which facilitates comparisons between different patient cohorts analyzed at different instances.

Interventional Trials in TBI

TBI incorporates a wide range of different diagnoses and severities. Thus, patients with TBI constitute a heterogenous group, where some of the outcome is predestined from the primary injury and cannot be influenced by interventional treatments. Other factors, such as time from trauma to neurosurgery, socio-economic status, geography, age, sex and other co-morbidities will also have a great impact on outcome. Any trial trying to improve outcome after TBI, must address these factors and stratify accordingly. In order for a trial to be practically feasible, it cannot take all these factors into account, but rather choose the ones that are expected to have the most effect on outcome.

The more factors a trial chooses to stratify according to, the more patients will be needed for the trial. If you have a large enough trial, you will need more than one center, and the more centers you need for a trial, the more the risk of confounding factors outside of the trial protocol. Multiple centers mean multiple local traditions. Traditions such as which patients will be admitted, which injuries are considered too severe, at which GCS is the patient beyond rescue? At what age will an injury be considered too severe? Each center will also have local treatment algorithms for neurointensive care, for when to perform neurosurgery, time it will take from when the decision to operate is made until the patient is in the operating theatre. Local traditions will dictate the threshold for performing decompressive craniectomy etc. Typically, in a low-income country with a high trauma-load there can be a substantial delay between the decision to operate and the actual surgery due to the fact that the anesthesiology staff may be occupied with other acute trauma. This can lead to long periods of un-controlled ICP for the patient, which will of course affect the outcome. Consequently, if operating time is difficult to obtain on demand, many centers in low-income countries with high trauma loads will perform prophylactic primary decompressive craniectomies, which we know will affect the outcome negatively[60].

There are several hundred clinical trials with the aim of improving outcome after TBI, that are ongoing and registered at www.clinicaltrials.gov according to a recent publication[61]. In these trials, a total of 125 different drugs are being tested.

A trial that has had great impact on steroid-use in TBI, the CRASH-1 trial included all patients with TBI that hade an affected GCS at admission. The total amount of patients that received neurosurgery in the two groups was 20,4 respectively 20,80% which is roughly half of the patients that were classified as severe TBI[62].

Does that mean that the rest of the patients, roughly 80%, did not need neurosurgical intervention? Perhaps the trial would have had other results if only patients that had at least an ICP-monitor and received neurointensive care would have been eligible for inclusion. Perhaps if the patients that did receive corticosteroids, would be given them in a dosage equivalent to what patients with a brain tumor are given and for slightly longer than 48 hours, the results would have been different. When trials are designed to include a large number of patients, they usually broaden their inclusion criteria, with the risk of including patients that possibly would never benefit from the intervention being tested, since they did not need the treatment in the first place. These patients will remain in the treatment group but since they were never in need for treatment, they will obscure the treatment group but with the potential adverse effects of the treatment that was tested.

A reason for the great difficulties with clinical interventional trials in TBI is the large variability of the primary injuries in TBI since this will have a substantial impact on the outcome of the individual patient. The GCS at admission is one of the strongest outcome predictors[63]. Current TBI classification using mild, moderate and severe TBI will cluster all patients that are unconscious in severe TBI for instance, but the reasons for the loss of consciousness may be very different within the groups. Some patients will be unconscious due to injuries to structures required for consciousness that may or may not be reversible with time. Other patients will be unconscious due to a critically high ICP with impaired blood flow to the entire brain as a consequence. If the ICP can be managed quickly, these patients may recover completely, whereas if the ICP crisis, with subsequent cerebral hypoperfusion, remains over hours, for that same type of injury, the entire brain may be affected and no neurointensive care may be able to change the outcome.

Antisecretory Factor - AF

Salovum® consists of freeze-dried egg yolk powder that contains large amounts of the antisecretory factor protein (500 times higher than in ordinary egg yolk) and is produced by feeding hens with specially processed cereals capable of inducing antisecretory proteins in the yolk. Salovum® has been commercially available in most pharmacies in the EU and US since the late 1990´s and has been used to treat

diarrhea in both adults and children[64,65]. Salovum® is classified as a food for special medical purposes (FSMP).

In the 1990´s, the use of antibiotics in Swedish piglet farming was strictly reduced, leading to a mass death of piglets being weaned off suckling, due to infant diarrhea. The Swedish farmers´ association (Lantmännen) contracted a research group with the task of allocating a functional food that could be given to the piglets to counteract the piglet diarrhea. Eventually, they developed the SPC flakes, consisting of malted oats, based on knowledge from home remedies for diarrhea. The process during which the oats are malted, leads to changes in the surface structure and this, in turn, initiates a reaction from the body upon digesting them.

Once the piglets were fed with SPC flakes, the death rates decreased to the same rates as before cessation of antibiotics.

A research group in Gothenburg set out to try to identify what happened to the piglets after ingestion of SPC flakes[66]. They found that within days, there was an increase in a particular protein with an unknown identity and a size of 43 kDa. The research group named the protein antisecretory factor[67]. The protein could be found in humans, as well as in all animals that were examined. Later studies have shown that the protein antisecretory factor plays an important role in the regulation of fluids between different spaces in the organism. During the 1990s´, research was focused on the effect antisecretory factor exhibited on cholera[68,69].

By feeding hen SPC flakes, the hens produced eggs with egg yolk containing high amounts of antisecretory factor. These egg yolks can then be collected and freezedried and ingested to elevate the levels of antisecretory factor in the body[70]. The freeze-dried egg yolk powder is commercially available in pharmacies across the EU and US and is called Salovum®.

Salovum® has been used in attempts to treat different medical conditions such as Mb Menières´ disesase, where some effect could be observed[71], and inflammatory bowel disease, where a lowering of inflammatory parameters and a histological recovery was demonstrated[72]. Salovum® has been most widely used in treating childhood diarrhea, where it seems to be an efficient treatment[65,73,74]. Recently, it has been shown that Salovum® increases blood perfusion in liver metastases by reducing interstitial fluid pressure (IFP)[75]. In patients with glioblastoma to the brain, it has been shown to do the same, hypothetically allowing for chemotherapy to reach the tumor cells more easily[76].

Figure 2 Salovum® and how it is prepared
The powder is weighed and given 1g/kg body weight/24 hours. The total dose will be divided, mixed with water and administered orally or via feeding-tube every 4 hours.

The active sequence of amino acids in antisecretory factor has been identified as an 8 amino acids N-terminal peptide, which has been attached to another 8 amino acids for stability. The resulting peptide is called AF-16 and is available for animal and in vitro experiments. AF-16 has been shown to decrease water content of the traumatized brain and decrease the intracranial pressure after trauma in animal models[77,78]. AF-16 has also been shown to reduce morbidity and mortality in encephalitis models in animals[78]

Recent experiments with AF-16 in combination with temozolamide have been shown to cure gliomas in mice[79].

The peptide AF-16 has undergone phase 1-trial for human intravenous use and is currently undergoing a phase 2a trial.

It is obvious that the protein antisecretory factor does have an important function in the bodies of most animals and humans. The fact that the protein exists in all animals that have been investigated for it, suggests that it is an important protein.

In our laboratory and others, attempts have been made to understand what the protein does[80,81]. In vitro, AF-16 is a potent regulator of chemokines and cytokines from both tumor cells and monocytes/macrophages.

However, so far, the exact working mechanisms of the protein antisecretory factor or its active peptides are still not fully understood.

One viable hypothesis is that antisecretory factor is part of the proteasomes and plays its´ important role by modulating inflammation this way.

One experiment has shown that a version of antisecretory factor called antisecretory factor 1 is a constituent of the 19S proteasome subunit. The subunit consists of 19 proteins and antisecretory factor 1 is one of them. Antisecretory factor 1 is also known as 26S Proteasome Regulatory Subunit (Rpn 10), which in humans is coded by the PSMD4 gene[82]. These regulatory subunits are important for the controlled and selective breakdown of cellular proteins, influencing various cellular processes and maintaining cellular homeostasis.

Bacterial enterotoxins and processed cereals were able to induce an altered form of the protein – antisecretory factor, which seemed to inhibit inflammation and fluid secretion in the gut. The experiments showed that antisecretory factor 1, as part of a proteasome complex in the blood, may bind to complement factor C3 and thus splitting it into its inactive form C3c.[83]

Proteasomes exist in all cells and become extra active in times of infection or inflammation. If antisecretory factor modulates the immune response by downregulating the activity of proteasomes, this may well explain the diversity of effects that have been observed and studied in the body.

It has been postulated, and studies have shown that non-selective attenuation of the inflammatory response in the early phase after TBI might be advantageous in some ways, and detrimental in others[84]. The main reason being that neuro-inflammation after trauma is a double-edged sword – it is both needed for restoration of tissue integrity and function, and un-wanted due to the secondary injuries that it may cause.

Salovum® in Clinical Use

In 2015, Salovum® was introduced in our department. Our aim was to investigate the effect of antisecretory factor, given as Salovum®, on different types of brain pathologies where cerebral edema was a major factor for morbidity. Salovum® is classified as a medical food, and as such is not under the laws that regulate drugs. Salovum® has been given to thousands of patients without any known side-effects and the only contraindication is egg-allergy.

The first patient to receive Salovum® was a young man with tuberculous meningitis. Despite full neurointensive care with the aim to control ICP, including barbiturate coma and ventricular drainage, ICP was 40 mm Hg. The patient was given Salovum® and there was an almost instant effect on ICP. The barbiturates could quickly be weaned off and the only ICP-instability that was noted was when the patient no longer received Salovum, on day 3. The patient eventually recovered completely.

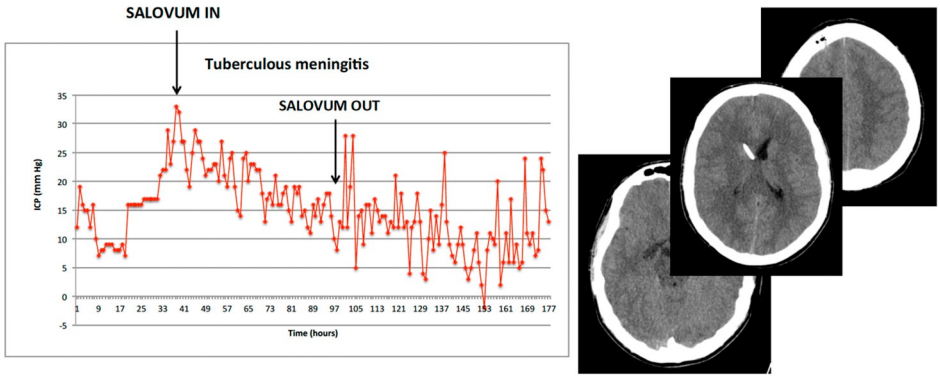

Figure 3 ICP and CT scans

Patient ICP data displayed as a red line. Black arrows mark when Salovum® administration (1g/kg body weight/24 hours), was commenced and concluded. Y-axis shows ICP in mm Hg, X-axis shows time in hours. CT scans from admission shows general edema, but no space occupying lesions.

The second patient who received Salovum® was a patient that was operated for a large lateral sphenoid wing meningioma. The patient had no deficits postoperatively, but 2 days post-op developed a complete aphasia. An MRI showed increasing swelling in the left frontal and temporal lobes, but no ischemia. The symptoms did not respond to increased doses of steroids. The patient was offered 16 g of Salovum® mixed in a glass of lemonade. 30 minutes after ingesting the drink, the patient had made a complete recovery of his symptoms.

A few more patients with various pathologies of the brain were given Salovum®. None of these cases have been published. Patients with steroid-resistant tumor edema usually responded well. Patients with meningitis usually responded well. Within a certain diagnosis, there were, at times, a large variation in how well the patients responded. The reasons for the variations may be interpersonal variation in uptake and different duration of symptoms and hence different edema. There might also be variations between patient's immunological response to the pathology and hence susceptibility to respond to treatment.

Salovum® in an Interventional Trial

We decided to conduct a prospective interventional trial with Salovum® in adult patients with severe TBI. We were not aware of all the difficulties that inevitably follows when planning an interventional trial for patients with TBI. Some of the things we had to learn the hard way along the way. We knew that Salovum® can be

very effective in patients with TBI and various other pathologies where alternative medical therapies failed. Many intracranial pathologies have a fairly well-defined treatment algorithm with which most patients can be treated successfully, which is not the case with TBI. The most efficient way of controlling ICP, decompressive craniectomy, has many side-effects and should only be considered when every other therapy has failed[85]. To ameliorate the effects of secondary injury would be very beneficial for the patients with TBI.

Since the 1970´s, the incidence of TBI has been decreasing in Sweden. The main reason is better automobile safety and improved road safety with rails separating the lanes on smaller roads. The age of TBI patients is also increasing in Sweden, adding yet another bad prognostic factor to a diagnosis whose prognosis was dire to begin with. Currently, it is not feasible to conduct an interventional trial in patients with severe TBI in a single center in Sweden, since there are not enough patients that would fit the inclusion criteria. An alternative would be to try to involve as many as possible of the seven neurosurgical sites that exist in Sweden. However, each center has their own local traditions, their own set of neuromonitoring, with a treatment algorithm for what to do with pathological values. Four centers use different variants of Lund Concept, three do not. In conclusion, there would be a lot of confounding factors in multi-center trial in Sweden.

We chose to start a collaboration with the Department of Neurosurgery at Tygerberg University Hospital, affiliated to Stellenbosch University and situated in Cape town, South Africa. The hospital is a tertiary trauma center which services approximately 3,6 million people with neurosurgery. The center receives more trauma per year than all the neurosurgical sites in Sweden combined. The trauma panorama consists in general of younger patients and the primary trauma mechanism is motor vehicle accidents, followed by violence and fall accidents.

The center has a neurointensive care ward with 9 beds and it uses ventricular drains, intra-parenchymal pressure monitors and LICOX as part of their standard of care. The neurointensive care is given according to a treatment algorithm that is based on the American guidelines[86]. The ethical regulations surrounding a medical trial are very strict in South Africa due to the large number of socially disadvantaged people in the country. Only trials that are strictly controlled, medically safe and ethically justifiable will be allowed and the process of attaining ethical permission for a medical trial in South Africa is currently more difficult than in Sweden.

The process of seeking ethical permission and constructing the scientific protocol for the Salovum® trial began in October 2016. The trial was granted ethical permission in September 2017. The first patient was recruited in September 2017, the last patient was recruited in October 2022. The results are presented in paper V.

Conclusions and Future Aspects

The inflammatory response in TBI is instant but also far-reaching and potentially chronic. The mechanisms behind this response are not fully elucidated. The reports from cytokine analysis in TBI are relatively few, and the methods for capturing the cytokines and for analyzing them vary, making comparison between studies difficult[48]. Up until recently, the volumes needed for analysis of cytokines from the extra-cerebral fluid has made time resolution another limiting factor, where typically MD vials equivalent to 6-12 hours of monitoring have been pooled in order to gain sufficient volumes for the multiplex assays, which typically required volumes around 200 μL[47,87]. It is reasonable to suspect that the cytokine/chemokine levels will change during this period of time, possibly as a result of important clinical events such as an ICP crisis or surgery. It is also possible that a rapid increase in cytokine/chemokine levels would precede an ICP crisis, but with the limited time resolution for analysis, this is something that has not yet been proven.[88] New methods, such as *Target 96 Inflammation* ®, a multiplex assay panel commercially available by Olink™ Proteomics, Uppsala, Sweden, requires substantially smaller volumes for analysis (1 μL)[89]. If these methods are used, they may change the temporal resolution and hence, potentially prove if there is a correlation between an increase in certain cytokines/chemokines and clinical events. Due to the dual nature of cytokines/chemokines, which can exhibit both beneficial and detrimental effects, and considering that these effects may vary over time and in different locations post-injury, our present comprehension of cytokines/chemokines in the central nervous system following traumatic brain injury is very limited.

To improve our understanding of the inflammatory reactions in TBI in the future, methods requiring smaller volumes of extra-cellular fluid for analysis should be used, adding a higher temporal resolution of the cytokines/chemokines that are being investigated.

Also, it may be problematic to only investigate the bio-markers that we think we understand, those that have been investigated before, and which already are mentioned in publications showing some sort of correlation. This itself will add to an investigational bias that likely will influence the way we interpret the data we acquire.

The medical data that is collected must be saved in high frequency and catalogued in a way so that all clinically relevant details can be analyzed. Most studies,

including paper I in this dissertation, list hours spent in theatre as “missing values” for MD sampling for example. This is of course correct, they are in fact missing, but with the risk of really missing the point – surgery itself could be what gives rise to a change in cytokine/chemokine expression. Also, the type of anesthesiology given may have substantial effects on cytokine levels, as has been shown in other types of surgery where patients were either anesthetized using sevoflurane or propofol[90]. In none of the papers investigating cytokine/chemokines after TBI, have either of the above issues been addressed.

Adding more advanced statistical methods for un-biased investigations may be of advantage when trying to make sense of the intricate balance between essentially hundreds of proteins that interact with each other in different ways. The most promising way forward likely involves employing different forms of pattern recognition methods, which involves the application of machine learning, data mining, and statistical methods to automatically recognize and interpret patterns, which can include trends, correlations, clusters, or outliers. One such method, principal component analysis, has been published and discussed in relation to cytokine/chemokine analysis and TBI[91].

In order to give future studies the best opportunities to gain new insight, machine learning should probably be incorporated at an early stage, linking physiologic data and events of importance, such as surgery, new anesthetic agents etc. to the cytokine/chemokine release that is being sampled.

Even though it may be quite some time before we fully understand the intricate relationships between all the inflammatory mediators of the brain and body, cytokine/chemokine levels may be used as pseudo markers for outcome in clinical interventional trials. They may also be used to monitor the impact on inflammation in trials encompassing interventions aimed at modulating inflammation In paper I and paper II, we identified a number of cytokines/chemokines that were consistently found in higher concentrations in both adults and children. These were MCP-1, MIP-1β, IL-8, IP-10 and IL-6. Of these, IL-6 is a proinflammatory cytokine with multiple downstream effects while the rest are chemokines. Among these MCP-1 and IL-8 have the best documented effects in recruitment of monocytes/macrophages and neutrophils respectively. However, recent reports also highlight the fact that chemokines have an array of non-chemoattractant features

including regulation of survival, cell adhesion, generation of ROS, degranulation, endocytosis, differentiation, chemo-repulsion, stimulation of immune synapse formation, and release of neutrophil extracellular traps (NETosis) and many more[92]. The chemokines/cytokines with high concentrations in Paper I and II are considered pro-inflammatory and these were further investigated in relation to clinical events, biochemistry measured by MD and catheter placement in relation to injury. There

was a difference in IL-6 between peri-contusional and non-traumatized areas in adults. No other correlations could be detected, likely due to all the confounding factors discussed above.

Using the data from paper I, we initiated a prospective double-blind, placebocontrolled trial on Salovum® in our department. The trial was started in parallel to the trial presented in papers IV and V. The main goal of this trial was to investigate if Salovum® could be proven to have an effect on ICP, brain oxygenation, biochemistry measured with MD or cytokines/chemokines in plasma and MD. The trial will also assess 30-day and 12 month mortality and Glasgow Outcome Scale Extended (GOSE) at 6 and 12 months. However, due to the low number of study participants, it would be highly unlikely to find any results of statistical significance in the morbidity/mortality outcome measures. The trial is planned for 10 + 10 patients. The study started recruitment in March 2020 and has currently recruited 17 out of 20 patients[93].

The discovery of antisecretory factor and Salovum® changed the course of this thesis. The effect that was observed in the few patients discussed in paper III were dramatic and having seen these effects, it did not seem proper to use pseudo-markers for outcome as the primary outcome measure. We did use a pseudo-marker as secondary outcome measure – ICP, and we expected ICP to differ between the groups since we had seen a remarkable effect on ICP in our pilot study (paper III). What we could not foresee, was how many things that are different in South Africa as compared to Sweden. In Sweden, a severe TBI patient would receive neurointensive care for longer periods of time than in South Africa. On the average, a patient in South Africa would have an ICP-monitor for 3.5-3.8 days, after that the patient is extubated and moved to the intermediary care in the NICU. Swelling after TBI is highly individual, but according to many reports, it may be at its peak at around day 3 – 5[94,95]. During this time, most of the patients in the trial were extubated and hade their ICP monitor removed, due to the high caseload at the clinic. To conclude, even though two tertiary trauma centers may look the same on paper, they seldom are. The extremely high case load of TBIs in South Africa significantly influences the duration a patient will be kept on a ventilator. The reason is clear and simple: if there is not a vacant bed, the next patient cannot be treated.

According to our colleague and friend who is the principal investigator of paper V, the South African Department of Health ordered a special inquiry about patients with cervical spinal cord injuries that were admitted into the public hospital system, i.e. patients without health care insurance. In South Africa, health care is free for everybody, but people with sufficient resources usually have a private health insurance and will be taken care of at a private hospital. The inquiry was aimed at investigating the outcome for patients with high cervical injuries that were admitted

and treated by the public health care system. The scope was to see how the patients would fare, but also if there were any socioeconomic consequences for the families at large. The results were very disheartening. Generally, a patient with a socioeconomically vulnerable background that sustained a high cervical injury with complete neurological deficits below the level of injury, lived for a maximum of 6 months. What made the report even worse was that the socioeconomic consequences for the rest of the family, if the patient had a family, were very discouraging. One person in the family, usually a young male that worked and provided an income was incapable of providing for the family when the accident struck. The patient would receive neurosurgical treatment that would stabilize the cervical injury and thereafter would receive neurorehabilitation until he or she could breathe on his/her own. After that, the patient would be discharged from the hospital, usually to a makeshift house in one of the townships. To care for the patient, one more person from the household would have to quit his/her job, which meant even less financial support for the family. The patient with the injury would eventually succumb to one of the many perils of tetraplegia, many of which potentially could be avoided if enough medical resources were present to aid in every-day life. At the time of death for the tetraplegic person, the rest of the family would be in shatters due to the double loss of income.

The results of the inquiry were clear, but could never be published, let alone be implemented. The bare notion of not treating someone for an injury that is treatable, however resource-demanding, when all the equipment and expertise are present, but the outcome actually might be predestined, is too much for any health care system to bear. To my knowledge, people from socioeconomically poor conditions with high cervical injuries and complete neurological deficits, still receive the best possible cervical stabilization and after-care, even though they will be discharged to a household that cannot cater for their future needs and that will suffer dire consequences for their disabilities.

The neurosurgeons that work in the public health care system are generally exceptionally experienced, since they are exposed to a large case-load of trauma. However, they will be forced to aim their efforts at the patients that have a fair chance of survival, and survival with only minor disabilities.

The major weakness in the Salovum® trial (paper V) is that we did not register morbidity in the surviving patients. The patient population are mostly individuals of lower socioeconomic status, many of which do not have an address or a registered telephone number. It was the investigators´ opinion that it would be very difficult to conduct long term follow-up due to these circumstances. There was no difference in how many days the surviving patients spent in the NICU, nor at the hospital in general. This indicates that the surviving patients in the Salovum® group did not survive at the cost of higher morbidity.

The inflammatory markers that we identified in paper I and II could not be used in the Salovum trial, since MD was not in clinical use at the trial center. However, blood was collected from the first 70 patients, before, during (day 3) and after Salovum administration was ended. The blood was centrifuged immediately and plasma was transferred to a -70° freezer for later analysis of cytokines and chemokines. These samples will be analyzed in order to investigate if Salovum® administration alters the systemic levels of cytokines/chemokines compared with the patients that were given placebo.

More trials on Salovum® are warranted. The next trial should be performed in one or more affluent countries. The information that will be gained from the ongoing Salovum® trial in Lund, can be used when planning such a trial.

To this date, nothing has ever been proven to be as efficient in reducing mortality in severe TBI as Salovum®. The results are clear and even though it is unlikely that they can be directly translated to advanced neurointensive care settings in developed countries, Salovum® will reduce mortality in those settings too. Once the paper is published, we expect Salovum® to become part of the treatment algorithm for TBI in many centers. There is no reason for why it shouldn´t.

Salovum® might just be the solution that ameliorates inflammation without stopping it, acting as a band-aid for the broken brain.

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Paper I

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Prolonged and intense neuroinflammation after severe traumatic brain injury assessed by cerebral microdialysis with 300 kDa membranes

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ABSTRACT

Background: A neuroinflammatory response that may lead to edema and secondary brain damage is elicited in Brain metabolism severe traumatic brain injury (TBI). Previous studies using microdialysis (MD) membranes with 100 k Dalton Brain injuries (kDa) cut-off found a transient intracerebral release of cytokines and chemokines without significant correlations Traumatic / pathology to clinical course, intracranial pressure (ICP) or metabolites. In this study, a (300 kDa) MD probe was used to measure the levels of cytokines and chemokines in relation to ICP and metabolites.

Methods: Seven patients with severe TBI received 2 MD catheters. In four patients sufficient dialysate could be retrieved for analysis from both catheters. MD samples were analyzed bedside, then frozen and analyzed for chemokines and cytokines using a multiplex assay (Mesoscale Discovery).

Results: MD sampling was performed from 9 to 350 h. In total, 17 chemokines and cytokines were detected. Of these, IL-6, IL-8, IP-10, MCP-1 and MIP-1β were consistently elevated, and investigated further in relation to metabolites, and ICP. Levels of chemokines and cytokines were higher than previously reported from TBI patients, and partially higher than those reported in patients with cytokine release syndrome.

There were no significant differences between the two catheters regarding cytokine/chemokine concentrations, except for IL-6 which was higher in the peri-contusional area. No correlation with metabolites and ICP was observed. No significant increase or decline of chemokine or cytokine secretion was observed during the study period.

Conclusion: Our data suggest that cytokine and chemokine levels reflect a perpetual, potent and pan-cerebebral inflammatory response that persists beyond 15 days following TBI.

1. Introduction

Traumatic brain injury (TBI) is a global epidemic with high rates of morbidity and mortality, despite advances in prevention and therapy in developed countries(Maas et al., 2017). TBI results in macroscopic tissue alterations as hemorrhage, lacerations and contusions, often appearing simultaneously. Microscopically, cellular damage, intra- and extracellular edema and various forms of cell death are observed. On the molecular level, hypoxic, damaged or dying cells release a plethora of mediators that will orchestrate an evolving secondary brain damage (Jha et al., 2019; Ladak et al., 2019).

Sterile inflammation, defined as an inflammatory cascade initiated by the release of disease associated molecular patterns (DAMPs), has been associated to both the events that lead to secondary brain damage but also in the evolving tissue regeneration and degeneration after the trauma (Huber-Lang et al., 2018).

Sterile inflammation is the result of a cascade initiated by release of DAMPs, such as HMGB1, mDNA, IL-16 and IL-33 from stressed or dying cells, followed by outflow of pro-inflammatory and anti-inflammatory cytokines from resident or recruited cells. Additionally, other inflammatory mediators such as complement factors, kinins, reactive oxygen and nitrogen species play a role in inflammatory secondary brain damage (Bains and Hall, 2012; Tisdall et al., 2013). In the brain the inflammatory cascade is purported not only by invading inflammatory cells but also by brain resident cells with inflammatory capacity as microglia, neurons and astrocytes (Wofford et al., 2019)However, Inflammation can also promote tissue healing and regeneration after TBI (Maas et al., 2017; Zwir et al., 2020).

Sterile inflammation has been implicated in the evolution of cerebral edema that in turn results in raised intracranial pressure with ensuing disturbances in perfusion and diffusion that lead to further tissue damage resulting in severe morbidity or death (Cederberg and Siesjo, 2010; Jha et al., 2019; Needham et al., 2019; Tucker et al., 2017). Thus, there is an increasing interest in attenuating inflammation in TBI, but yet no successful interventions have been performed (Corps et al., 2015; Huber-Lang et al., 2018).

Clinical data supporting the role of inflammation in TBI have come from measurement of inflammatory mediators, mostly during intensive care treatment, imaging and postmortem studies. The release of DAMP mediators and inflammatory cytokines/chemokines after TBI can be measured in blood, cerebrospinal fluid (CSF), brain tissue and from the brain

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extracellular fluid, the latter by microdialysis (MD) (Thelin et al., 2017). In the present literature there is evidence that a gradient for most inflammatory mediators from brain to periphery exists, supporting the notion of local CNS production after TBI (Giorgi-Coll et al., 2017). Furthermore, it has been reported that the inflammatory response to TBI is different to that of multi-trauma and that this affects outcome(Rowland et al., 2020).

Cerebral MD, originally devised for the analysis of brain metabolites, makes sampling of brain extracellular fluid possible for subsequent analysis of inflammatory mediators. Microdialysis data from experimental and clinical TBI have displayed heterogenous results. However, some mediators such as IL-6, IL-8, MIP-1β, MCP-1 and IP-10 are repeatedly detected, in addition to numerous others found at lower concentrations that include IL1-b, GM-CSF, IL-2, IL-7 and IFNα (Zeiler et al., 2017)Due to the use of various designs of MD catheters and multiple platforms for analysis, the absolute values of the specific inflammatory mediators diverge between reports (Giorgi-Coll et al., 2017; Hillman et al., 2007). Due to intrinsic features of MD catheters, only a fraction of the actual cytokine levels is recovered and analyzed adding to variability. As the size of many inflammatory mediators is higher than the operating cut-off of standard 20 k Dalton (kDa) catheters, catheters with larger cut-off such as 100 kDa, have mostly been used. Although larger pore size catheters would theoretically give a better recovery, they also introduce the risk of ultrafiltration (Snyder et al., 2001). Also inter-catheter variations due to fluctuations in cerebral perfusion pressure and temperature have been reported (Galea et al., 2014). No direct in vivo comparison of different catheter sizes in MD sampling for analysis of chemokines or cytokines in TBI patients has previously been reported, although the impact of using different perfusion fluids has been investigated (Giorgi-Coll et al., 2020).

With these aspects in mind, it is difficult to perceive which inflammatory mediators are decisive for the evolution of secondary inflammatory brain damage in TBI. Previous studies report quenching of cytokine/chemokine release between 4 and 6 days after trauma, a notion that partially contradicts results from post-mortem and blood analysis showing signs of a persistent, long-term inflammation after TBI (Chaban et al., 2020; Frugier et al., 2010; Vedantam et al., 2021). Therefore, the aim of the present study was to validate a large pore (300 kDa MW cut-off) MD membrane and to investigate if the cytokine levels differ based on location in the brain and if and how the patterns change in relation to secondary events such as periods of uncontrollable intracranial pressure (ICP) elevations. We also aimed at extending sampling time beyond 100 h after the trauma. Besides the necessity to pinpoint the qualitative and quantitative release of cytokines in TBI for more detailed unveiling of mechanisms, inflammatory mediators in TBI could also be used for monitoring in trials using anti-inflammatory drugs.

2. Materials and methods

2.1. Ethics

All research was conducted in accordance with the ethical standards given in the Helsinki Declaration of 1975, as revised in 2008. Ethical permission was granted by the regional ethical review board of Lund University, LU, Sweden (decision number LU-2017/469). As the use of microdialysis is clinical routine, the ethical permit covered the analysis of patients not included in clinical routine, eg cytokines and chemokines. Since the included TBI patients could not themselves consent to the study, a written informed consent was obtained from the patient's closest relative.

2.2. Clinical Aspects/patients

The study recruited 7 patients with isolated severe TBI, a Computed Tomography (CT) scan consistent with TBI, and a post-resuscitation Glasgow coma score (GCS) of ≤8 (Grande et al., 2002). All patients displayed extensive primary injuries that included traumatic intracranial hemorrhage. All patients but one had secondary surgery due to intractable ICP. The patient that only had surgery once underwent a primary decompressive craniectomy and simultaneous evacuation of two traumatic contusions. Monitoring was performed using an intra- parenchymal or intra-ventricular intracranial pressure (ICP) monitor and microdialysis was performed with 300 kDa membranes, inserted together with the ICP monitor, and also, in 4/7 patients, in a peri- contusional area at the time of craniotomy.

ICP and mean arterial blood pressure (MAP) was measured continuously and recorded automatically in the intensive care software IntelliSpace Critical Care and anesthesia (ICCA Philips Healthcare, Suresnes, France), Cerebral perfusion pressure (CPP) was calculated (MAP- ICP) and recorded.

2.3. Treatment according to TBI algorithm

Treatment according to modified principles of Lund Concept was given to all patients (Grande et al., 2002). Focal mass lesions (extracerebral hematomas or contusions) with a significant mass effect were evacuated, unless the traumatic hematomas were deep-seated. According to the protocol, blood pressure was controlled with clonidine and metoprolol. Sedation was maintained with midazolam and,occasionally, with thiopental.

Cerebrospinal (CSF) drainage was allowed when basic NICU treatment could not control ICP. If CSF drainage could not control ICP, thiopental was added.

Finally, if ICP could not be controlled, a decompressive hemicraniectomy was performed.

Fever was managed with paracetamol, and invasive cooling when needed to keep core temperature ≤ 38.5.

2.4. Microdialysis

300 kDa molecular weight cutoff MD catheters (CMA 320, Stockholm, Sweden) were used in all patients. 7 patients received 2 MD catheters, one placed in seemingly non-traumatized areas near the ICP monitor, and one placed in peri-contusional areas. All MD catheters were perfused with central nervous system perfusion fluid (CMA Microdialysis AB, Solna, Sweden) at 0.3 μL/min using CMA 106 micro infusion pumps. All microdialysis vials were kept at approximately the same height as the MD pumps in order to avoid hydrostatic forces that could affect the recovered volumes. Microdialysis catheters were categorized according to their position in relation to an injured area using the first postoperative CT-scan available. All catheters were clearly visualized on a CT-scan and termed "A" if placed in "seemingly normal" tissue, i.e. brain parenchyma without visible edema and blood. If the catheter was placed in close proximity to edema or blood, it was peri- contusional and named "B".

The locations of all monitoring devices were confirmed on CT scans.

MD vials were changed hourly in all catheters and analyzed on an ISCUS (CMA Microdialysis AB, Sweden) bedside analyzer according to clinical routine. Remaining contents of vials were stored on dry ice in a cooling bag until they were transported to a − 80 °C freezer daily.

2.5. Cytokine analysis

All samples were analyzed using a multiplex kit (MesoScale Discovery, USA) according to the manufacturers´ instructions. Microdialysates from a 6-h time period were pooled immediately before analysis, in order to yield sufficiently large volumes for analysis which was 200 μl. Cytokine measurements were plotted in the middle of the 6- h time period, to represent a mean value for the given period.

The microdialysates were analyzed with Proinflammatory cytokine- 1 V-plex kit and Chemokine V-plex kit (see Table 2). Due change of standards values for the proinflammatory cytokine-1 Vplex kit, concentrations were recalculated after linear regression computations with a constant of 0.7357. Both kits contain IL-8 but gave different values and the final IL-8 value was taken from the proinflammatory cytokine-1 V-plex kit. Samples were analyzed in duplicate. *2.6. Statistics*

ar regression. All co https://www.R-pr

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3. Results

3.1. Patient demographics

S 8 on admission ar
≤ months ranged frc

2/7 patients had an isolated TBI
3 out of 7 patients had a GCS < 6
GOSE 3 to 7. All patients survived (s

Table 1).

Duration of microdialysis monitoring

ers worked during t
es was initiated at a ven patients. The

of 51 h post trauma (range 9–350 is initiated at 196 h
pital and additional
present study, we

intended to monitor patients be
feasible in all patients. er trauma, which w

Name/Group	CCname	Receptor	Target	MW (kDa)
Top range MCP-1				
MIP1-β IL-8				
IP-10	CCL2			13-15
IL-6	CCL4			7.8
	CXCL8		Mcyte[a]	8.4
	CXCL10	CCR2,5	Mcyte	8,6
Mid range	–	CCR1,5	VPhil[b]	21–26
Eotaxin		CXCR1,2 CXCR3		
MCP-4 TARC		IL6R	Mcyte, Tcell[c] Misc.	
MDC				
	CCL11			8.4
	CCL13			8.6
Low range	CCL17	CCR2,3,5	Ephil[d]	10.3
IFNγ	CCL22	CCR1,2,3 CCR4	Ephil Tcell	8.1
GMCSF		CCR4	Tcell	
IL1-β				
TNFα IL-10				17
IL-2	–		Misc.[e] Misc.	14–35
IL-12	–	IFNGR CSF2R	Misc.	31
	–	IL1R	Misc.	17.3
	–	TNFR	Misc.	18
	–	Il-10R	Misc.	15.5
	–	IL-2RA	Misc.	75
	–	IL12RA	Misc.	

[a] Monocyte.
[b] Neutrophil granulocyte.
[c] T lymphocyte. [d] Eosinophil granulocyte. [e] Target cells undefined in TBI or miscellaneous

Table 1
Patient demographics.

Patient	A	B	C	D	E	F	G
Age (years) Sex (Male/Female)	46 M	22	42	32	61	44 M	42 M
GCS[1]	5	F 4	F 8	F 5	F 8	7	7
Pupils (+dilated/– normal)	– /–	– /+	– /–	+/– mv	– /–	– /–	– /+
Trauma mechanism	fall	mv	fall		fall	fall	fall
Neurosurgical Diagnosis EDH/SDH/ICH[2]	Y/Y/Y 12 h	N/N/Y 3 h	N/N/Y 16 h	Y/Y/Y 24 h	N/Y/Y 2 h	N/N/Y 6 h	N/Y/Y
Time trauma - 1:st surgery Time trauma - 2:nd surgery	35 h	45 h	51 h	216 h	37 h	73 h	6 h
DC[3]	N	Y	N	N	N	Y	N/A Y
Time – with MD in hours	(59–175)	(50–126)	(52–162)	(219–359)	(37–160)	(80–131)	(9–139)
Days in NICU[4]	17 3	7	9	9	7	11 6	16 5
GOSE[5]		3	5	7	3		

Whitney *U* test. Slope and *p* values for regression analysis of change over

Differences between catheters in seemingly non-traumatized and peri-contusional tissue were calculated with the non-parametric Mann

3.3. Definition of consistently elevated cytokines

Using the Mesoscale Multiplex assay, the following chemokines and cytokines were analyzed: MCP-1 (CCL2), MIP-1β (CCL4), eotaxin (CCL13), MCP-4 (CCL13), TARC (CCL17), MDC (CCL22), eotaxin-3 (CCL26), IL-8 (CXCL8), IP-10 (CXCL10), IL-1β, IL-2, IL-6, IL-10, IL- 12p70, GM-CSF, IFNγ and TNF-α (Table 2). Cytokines/chemokines that were found to be consistently elevated, defined as values >1000 pg/mL, at more than one time-point e.g. IL-6, IL-8, IP-10, MCP-1 and MIP-1β were tentatively considered to be most important in the development of inflammation following TBI.

These were investigated further in relation to glycerol, lactate- pyruvate ratio, ICP and CPP. Four other chemokines; Eotaxin, eotaxin- 3, TARC and MDC had concentrations between 100 and 1000 pg/mL with mean values over 100 pg/mL. A third group encompassing IL-1β, IL-10, GM-CSF, IFNγ and TNF-α displayed median values between 10 and 100 pg/mL while IL-2 and IL-12 had mean values below 10 pg/mL

Table 2
Summary of analyzed chemokines/cytokines with 300 kDa membranes.

[1] Glasgow Coma Scale.
[2] EDH – Epidural hematoma, SDH – Subdural hematoma, ICH - Traumatic intracranial hemorrhage.
[3] Decompressive craniectomy.
[4] NICU – Neurointensive Care Unit.
[5] GOSE – Glasgow Outcome Scale Extended.

(see Fig. 1).

Differences between catheters in peri-contusional and seemingly non-traumatized areas 4 of the 7 patients had consistently functioning catheters in damaged and non-damaged areas, i.e. peri-contusional or seemingly normal areas. In these patients, comparison of cytokine and chemokine values between peri-contusional tissue and non-traumatized tissue was possible. In the present study, only IL-6 levels differed significantly between the catheters, peri-contusional: 1467(22–2912) pg/mL versus non-traumatized: 429(314–544) pg/mL (median(IQR)), *p*- value = 0,000036. No significant differences between peri-contusional and non-traumatized areas of the chemokines IL-8, IP-10, MCP-1 and MIP-1β were observed. (Fig. 2).

No correlations between cytokine/chemokine secretion and ICP.

Using the modified Lund Concept algorithm, ICP could be controlled in all patients during monitoring time, and no secondary insults such as cerebral Ischemia or infarction were noted. As a result, we did not observe any major changes in ICP that could have mirrored changes in cytokine/chemokine levels.

3.4. Microdialysis metabolite pattern

The L/P ratio was defined as pathological when >25. A pathological L/P ratio was registered in patient B and C on the peri-contusional side, but also in patient D on the seemingly normal side. Glycerol was defined as pathological when >100 μmol/l (Belli et al., 2008). Glycerol was slightly elevated in patient A and pathological in patients C and D on the peri-contusional side, but also pathological in patient A on the non- traumatized side. Neither changes in glycerol nor L/P ratio covaried with the cytokine/chemokine secretion (Fig. 2).

3.5. Temporal pattern of cytokine/chemokine secretion

Slope and *p* values for each cytokine or chemokine were calculated for all 6 patients together. When all patients were included in the analysis no significant increases or decreases in chemokines/cytokines were observed. If the patient admitted at day 10 and monitored until day 14 was excluded from the analysis, IL-6, IP-10 and MCP-1 from the peri- contusional area were significantly decreased from 71 ± 5 h to 141 ± 14 h, whereas the slope for the non-traumatized area was not affected. (Fig. 3).

4. Discussion

The rationale for measuring cytokines from the cerebral extracellular space in TBI are multiple; firstly to verify that TBI induces tissue inflammation, secondly to classify the patterns of the inflammatory response and thirdly to assess whether the temporal or quantitative inflammatory profile influences, or is influenced by, alterations of other factors induced or observed in secondary injury. Finally, assessment of inflammation in TBI can be used to monitor interventions aimed at modulating outcome by targeting specific components of the inflammatory response. In the present study, we for the first time report

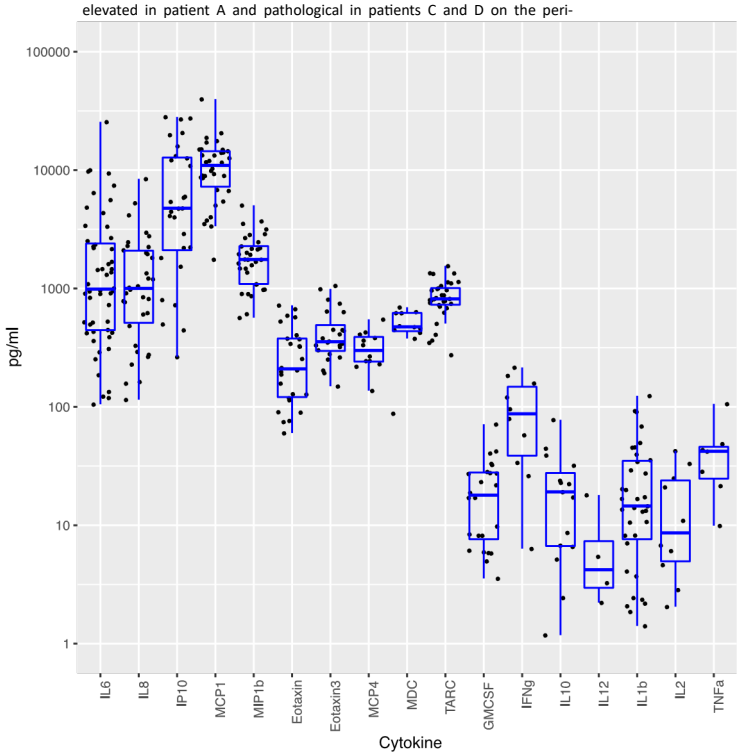

Fig. 1. Analyzed cytokines and chemokines for all patients depicted on a logarithmic scale.

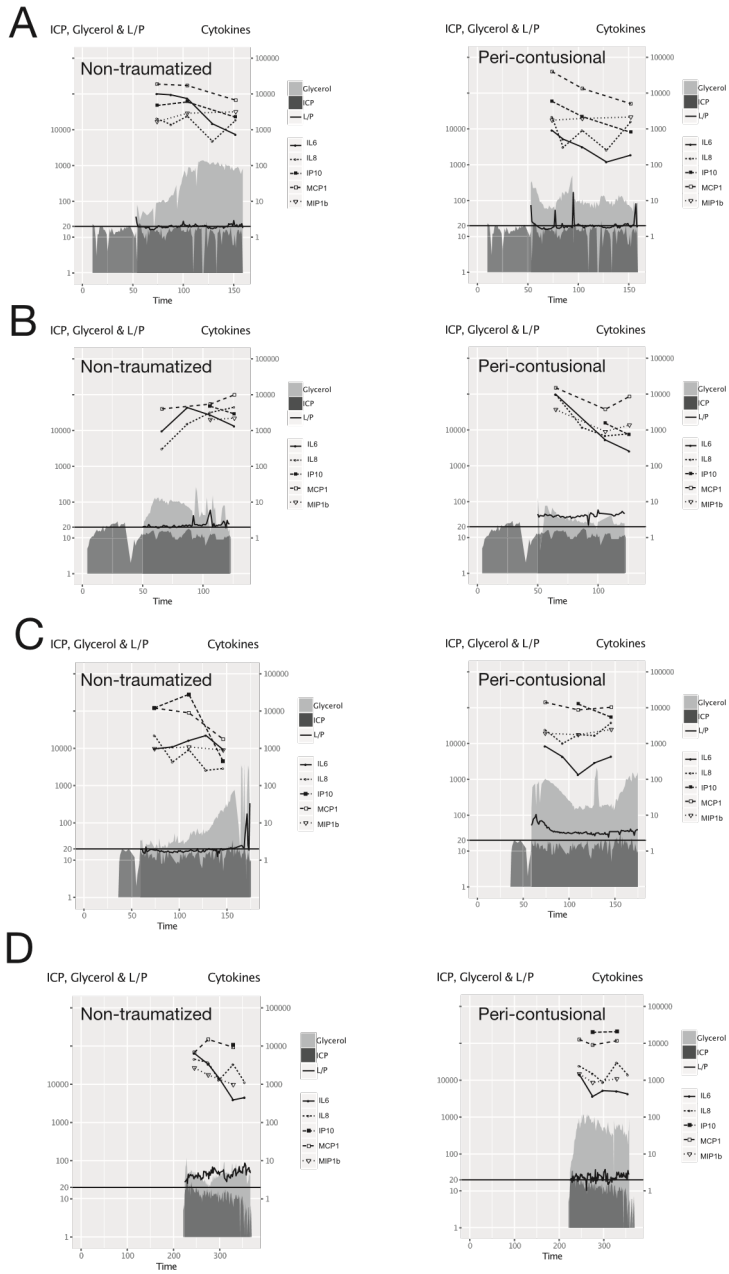

Fig. 2. Patient monitoring data is displayed as Glycerol (gray-filled line), ICP (black filled line), and MD Lactate/Pyruvate ratio (black line). Critical ICP threshold according to the TBI algorithm (20 mmHg) (A) patient 3 (B) patient 5, (C) patient 6 and (D) patient 7.

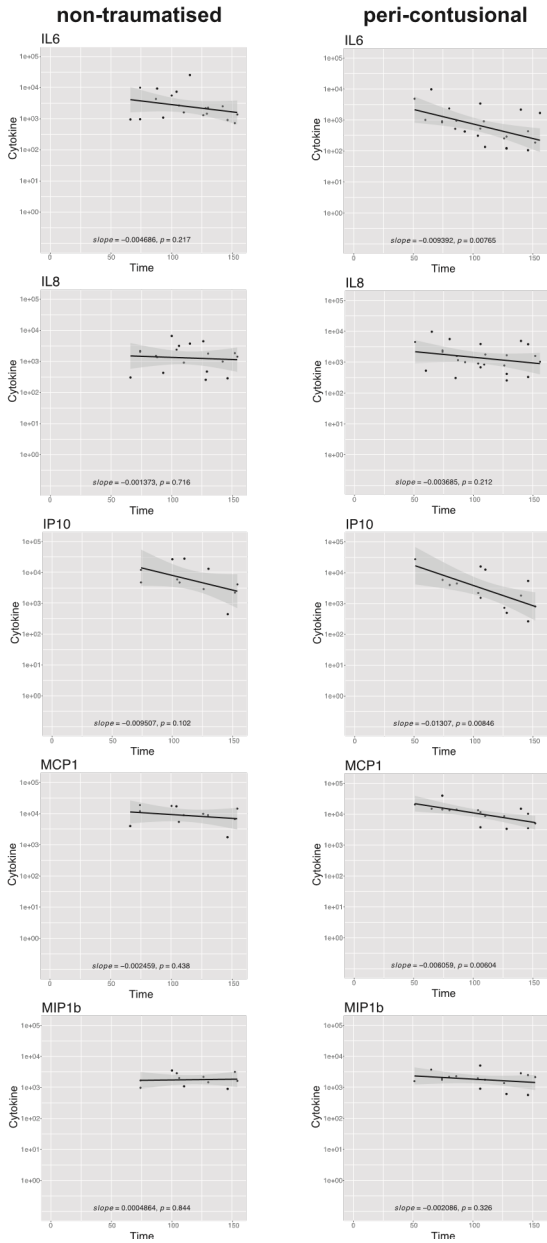

Fig. 3. Slope values denoting changes in pico-grams per hour. For non-traumatized areas, no significant increases or decreases chemokine/cytokine levels were observed. For peri-contusional areas, a significant decrease was observed for IL6, IP10 and MCP1.

measurement of cytokines and chemokines in TBI beyond 5 days after trauma and from seemingly non-traumatized and peri-contusional areas by cerebral MD using 300 kDa membranes,

4.1. Definition of consistently elevated inflammatory mediators

Due to the availability of multiplex kits, most studies have reported a heterogenous range of cytokines and chemokines, where the choice has depended more on kit content than on study hypothesis.

In the present case series, we could discern four categories of chemokines /cytokines based on quantitative levels defining a high range group (IL-6, IL-8, IP-10, MCP-1 and MIP-1β) a mid-range group (Eotaxin,

Eotaxin-3, MDC, MCP-4 and TARC), a low range group(IL-1β, IL-10, GM- CSF, IFNγ and TNF-α) and a very low range group (IL-2, IL-12). Both the high range and the mid-range group have been reported in previous MD studies using 100 kDa catheters in TBI (Bartek Jr. et al., 2019; Giorgi- Coll et al., 2017; Perez-Barcena et al., 2011). However, there is the possibility that certain mediators have a biological activity even at lower concentrations while others have their effects at higher concentrations, either directly or indirectly (Thelin et al., 2018). Using 300 kDa membranes, higher concentrations of all inflammatory mediators were observed compared to previous studies using 100 kDa membranes (see Table 2). Given that recovery of chemokines/cytokines is only partial, at best, the actual levels in the brain are likely even higher. This indicates that inflammation after TBI resembles a local cytokine storm of similar magnitude as those induced by CNS infections or cytokine release syndromes (Table 2), but confined to the CNS (Fajgenbaum and June, 2020; Gupta et al., 2017).

4.2. Patients

The subtype of TBI that induces the most vigorous tissue inflammation in humans is unknown but contusional injuries are most likely to do so (Woodcock and Morganti-Kossmann, 2013). Secondary events such as ischemia or hemorrhage, but plausibly the extent of surgical and intensive care treatments, could additionally influence the evolution of tissue inflammation. In the present study, traumatic contusions were found in all patients and in all patients cytokine and chemokine values over 1000 pg/mL could be detected at some time points thus indicating extensive tissue damage.

4.3. Temporal changes

Earlier reports have found significantly increased plasma levels of IL- 6, IL-8 and MCP-1 several months after severe and mild TBI and these partially correlate with outcome (Chaban et al., 2020; Rusiecki et al., 2020; Sun et al., 2019). In previous studies, cytokine/chemokine expression reach peak values within 24–48 h after MD-catheter implantation (Giorgi-Coll et al., 2017; Perez-Barcena et al., 2011; Winter et al., 2002). In our material, MD samples have been sampled from 9 to 359 h after the primary insult with only minor declines in cytokine/ chemokine expression, and thus in contradiction to previous results, as we did not observe any clear decline of the levels of inflammatory mediators during the sampling time, except for a partial decrease in the peri-contusional area for IL-6, MCP1 and IP10. The reasons for this discrepancy could be multiple; different injuries in our study population, different therapeutic interventions and smaller pores of the 100 kDa catheters in previous studies. The reported early peaks and fast decline of cytokines/chemokines may tentatively be due to loss of recovery from catheters, either by clogging or fibrosis in the vicinity of the membrane. We present our data with the zero-time point at the time of the trauma. Most patients were referred from other hospitals and therefore our zero- time point may differ from other studies where the zero-time point is presented as the time when the patient received the MD catheter, whereas this time point may well be 24 h after the trauma.Different methods to increase in vitro recovery in microdialysis studies have included antibody coated microspheres, nano-gold particles, dextran, albumin, plasma and hydroxyethyl starch (HES) but none have addressed the possibility of late partial or total blocking of catheters (Ao et al., 2004; Giorgi-Coll et al., 2017; Giorgi-Coll et al., 2020; Khan et al., 2015).

The levels of some chemokines/cytokines, mainly IL-6, IL-8 and IL-1β are extremely sensitive to tissue damage even as minute as that caused by inserting the MD catheter (Carson et al., 2015). Bouras et al. implanted 31 intracerebral MD catheters in 12 patients with epilepsy undergoing invasive EEG monitoring (Bouras et al., 2021). Surprisingly, this study found that the cytokines IL-1β, IL-6, IL-8, peaked within 24 h and thereafter declined to reach low steady state levels within 72–120 h in non-gliotic areas. In gliotic areas, levels of IL-6, IL-8 and TNF-α showed the same pattern but IL-1β was increased. This implies that IL-6, IL-8 and TNF-α were induced by the introduction of the MD catheter while IL-1β could represent true tissue inflammation. Taken together, these results do not exclude the possibility that cytokine levels in TBI MD studies initially may reflect catheter inflicted tissue damage. However, because Bouras et al. also used 100 kDa catheters, the early decline of cytokine levels reported could partially be caused by catheter clogging.

4.4. Differences between catheters in peri-lesional and seemingly non-traumatized areas

Despite that previous studies have reported difference for metabolites in peri-contusional and seemingly non-traumatized areas, no conclusive data exist for cytokine/chemokine expression in TBI. Helmy et al. (Helmy et al., 2011) used two MD catheters, both in non- traumatized areas for comparison of relative recovery of cytokines/ chemokines using two different perfusion fluids. Mellergard et al. (Mellergard et al., 2008). used two MD catheters placed in non- traumatized area and peri-lesional, but only used the catheter which yielded the highest concentrations of glycerol during the first 12–18 h after insertion for further analysis. In the present study only IL-6 levels differed between peri-lesional and non-traumatized areas. This could reflect that IL-6 is mainly produced by astrocytes and neurons while the chemokines may be produced by infiltrating cells. However, this does not exclude the possibility that there could be an initial difference before our first samplings at 50 h. Our findings could thus depend on either rapid spread of inflammatory mediators in brain parenchyma or that the inflammatory response is evoked simultaneously in multiple areas.

4.5. Covariation with ICP and metabolites

We could not observe any relation between cytokine/chemokine expression and potential secondary insults as measured by metabolites or minute ICP changes. This is consistent with the finding of a previous study that also failed to show any correlation between clinical events and cytokine/chemokine levels (Perez-Barcena et al., 2011). This may not be surprising since the raised levels of chemokines and cytokine were relatively unchanged during sampling time. There could still be a covariation at early time points prior to our first samples. It is also possible that though the inflammatory cascade induces cerebral edema over a longer time, other mechanisms as perfusion related events, regulate minute changes in ICP. *4.6. Influence of treatment algorithm*

Several drugs commonly used in different algorithms for severe TBI treatment including propofol, midazolam, clonidine, metoprolol and barbiturates may all influence the cytokine and chemokine expression. The Lund Concept algorithm used in the present study includes clonidine and metoprolol, which may have had an effect which cannot be identified in this study, since all patients were treated with clonidine and metoprolol. Which both can modulate the chemokine and cytokine levels (Schroeppel et al., 2010). Also, midazolam, pentothal and propofol have been shown to modulate cytokine/chemokine expression (Cruz et al., 2017; Kallioinen et al., 2019; Rossano et al., 1992).

4.7. Importance of pore size

Considering different sampling time and assays it is still obvious that the 300 kDa membrane gives a higher yield than those reported for 100 kDa membranes (see Tables 2 and 3). The recovered levels of the smaller size chemokines could be higher than for the larger size cytokines. Unfortunately, there are no relevant studies comparing MD with direct sampling from brain tissue for chemokines and cytokines.

4.8. Mechanisms of cytokine/chemokine influx

The sequence of cellular infiltration in experimental TBI encompasses early neutrophil influx followed by monocytes/macrophages and plausibly a later arrival of T- and B-cells (Alam et al., 2020; Needham et al., 2019). Simultaneously, CNS intrinsic cells as microglia, resident CNS macrophages, astroglia and neurons are activated in a complex and reciprocal network (Jacob Rodrigues et al., 2020). Chemokine receptors also have a skewed immune cell distribution where CCR2 is mainly located on monocytes/macrophages, CCR3 on eosinophils and basophils, CCR4 on microglia and CXCR2 on neutrophils and CXCR3 on T- cells(Gyoneva and Ransohoff, 2015). In the high and mid-range groups of detected chemokines/cytokines, all have a documented chemotactic effect on neutrophils or monocytes/macrophages, even the only non- chemokine IL-6 (Clahsen and Schaper, 2008). There is a certain redundancy of chemokines and their receptors at least at resting conditions where some share the same receptors as Eotaxin/MCP-4 (CCR3) Eotaxin-3/TARC/MDC (CCR4). This could mean that some of the measured molecules are mostly ¨noise¨in the local cytokine/chemokine storm. Although all chemokines have been shown to act as chemo- attractants during varying conditions, it is possible that some are more important, as has been proposed for MCP-1 for monocyte attraction and IL-8 for neutrophil attraction(Gyoneva and Ransohoff, 2015). These are both included in the group of high range chemokines and cytokines.

Despite the fact that certain chemokines and cytokines have been associated with worse outcome (MCP-1, IL-6), no persistent pattern has yet been put forward, emphasizing the notion that measurements of single mediators have no clear correlation to other parameters (Simon et al., 2017). The low range and very low range group consists of inflammatory cytokines that were not detected in all patients. It is possible that some of the cytokines were present, but at concentrations limited for detection by this assay (0,5–1,5 pg/mL). We do recognize that these cytokines may play an important role, even at very low concentrations. Some are classified as pro-inflammatory cytokines (IL-1β, TNF-α), others have been designated as anti-inflammatory (IL-10) while the remaining have an undecided role (GM-CSF, IL-12, IL-2). As they are not detected in all patients they might not only be the result of tissue damage but of events such as an ongoing infection, actual therapy or autoimmunity.

Besides functioning as chemo-attractants for infiltrating inflammatory cells, the high-range and mid-range chemokines could also have other effects in TBI. Both MCP-1, IP10, IL-8 and IL-6 have been implicated in neuronal damage and edema formation by signaling to CNS resident cells as microglia (Wang and Colonna, 2019) astrocytes (Sun et al., 2017) and neurons (Du et al., 2018).

As discussed, the levels of chemokines/cytokines after TBI might not mimic their impact in the evolution of secondary damage in TBI. In this respect both TNF-α and IL-1β have been proposed to initiate the release of other pro-inflammatory cytokines in an experimental in vitro model (Thelin et al., 2018).

5. Conclusions

The major limitation of this study, is that the results are based on analysis of 7 patients. However, the results show potent and persistent pan-cerebral cytokine and chemokine levels far beyond the first 5 days after the trauma with no signs of decline in patients with severe TBI.

A high range group, of mainly chemokines, was identified but only IL-6 from this group showed a gradient from traumatized to non- traumatized tissue when dual catheters were used. The results implicate that severe TBI induces a long standing cerebral neuro- inflammation. **Abbreviations**

Table 3
Comparison of chemokine/cytokine values in TBI and cytokine release syndrome.

Ch/Ckin	TBI-EC-Md100[a]	TBI-EC-Md300[b]	CRS-blood[c]	TBI-blood[d]
MCP-1	2500	11,000	60	<300
IP-10	2834	4000	2000	<200
MIP1-β	38	1500	60	<200
IL-6	570	1000	3200	<400
IL-8	339	1000	575	11
TNFα	1	40	52	<5
IFNγ	7	80	3722	NA
IL1-β	6	20	5	<1

[a] Cytokine/chemokine values from extracellular fluid /microdialysis with 100 kDa cut off membranes-ref 20,[30] (Helmy et al., 2011; Perez-Barcena et al., 2011).

[b] Cytokine/chemokine values from extracellular fluid /microdialysis with 300 kDa cut off membranes-present study.

[c] Cytokine/chemokine values from blood in patients with cytokine releases syndrome aka cytokine storm-ref [18] (Gupta et al., 2020).

[d] Cytokine values from blood in TBI patients-ref [8,13,36] (Chaban et al., 2020, Frugier et al., 2010, Smith et al., 2013).

Abbreviations

TBI	Traumatic Brain Injury
MD	Microdialysis
ICP	Intracranial pressure
kDa	Kilo Dalton
DAMPs	Disease associated molecular patterns
CSF	Cerebrospinal fluid
MW	Molecular weight

Data availability

Data will be made available on request.

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Paper II

The inflammatory response in the pediatric brain after traumatic brain injury.

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Introduction

Traumatic brain injury (TBI) in children constitutes a global epidemic with high rates of morbidity and mortality, despite advances in prevention and therapy in developed countries. As TBI in children affect a developing brain the consequences will be more far reaching and profound than in adult TBI with impact on cognitive, psychological and neurological development and function [1]. The unequal distribution of health resources globally also yields the paradox that where the incidence of pediatric TBI is the highest the resources are the scarcest. The primary brain damage is manifested by macroscopic tissue damage as hemorrhage, lacerations and contusions. Intrinsically, intra- and extracellular edema and various forms of cell death evolve. Hypoxic, damaged or dying neuronal, astrocytic, endothelial and inflammatory cells will subsequently release a plethora of mediators that will generate mechanisms leading to secondary brain damage [2, 3]

As in other parts of an injured organism a trauma induced inflammation, defined as a reaction initiated by the release of disease associated molecular patterns (DAMPs), has been associated to both the events that lead to secondary brain damage but also in the long-term tissue regeneration and degeneration after the trauma [4]

The inflammatory cascade following tissue damage, denoted sterile inflammation to distinguish it from pathogen induced inflammation, is kindled by release of DAMPs, such as HMGB1, mDNA, IL-16 and IL-33 from stressed or dying cells, followed by secretion of proinflammatory and anti-inflammatory cytokines from resident CNS cells or recruited immune cells . Also complement factors, kinins, reactive oxygen and nitrogen species play a role in inflammatory secondary brain damage [5, 6].

Trauma induced inflammation is correlated to cerebral edema that in turn results in raised intracranial pressure with perturbation of perfusion and diffusion that exacerbate tissue damage resulting in severe morbidity or death [2, 7-9].

Different interventions aimed at counteracting inflammation in TBI have been undertaken in clinical trials, but none have yet been successful [4, 10].

Proof of inflammation in TBI have come from sampling and analysis of inflammatory mediators, mostly during intensive care treatment, imaging and by postmortem studies. The release of DAMP mediators and inflammatory cytokines/chemokines after TBI can be measured in blood, cerebrospinal fluid (CSF), brain tissue and from the brain extracellular fluid, the latter by microdialysis (MD) [11]. Several reports have demonstrated that a gradient for most inflammatory mediators from brain to periphery is present, which strengthens the hypothesis of a primary CNS origin [12].

Cerebral MD (CMD), originally used for the analysis of brain metabolites and signaling substances, makes sampling of brain extracellular fluid possible for subsequent analysis of other molecules as inflammatory mediators. Microdialysis data from experimental and clinical TBI have displayed heterogenous results. However, some mediators such as IL-6, IL-8, MIP1β, MCP-1 and IP-10 are repeatedly detected, in addition to numerous others found at lower concentrations that include IL1-b, GM-CSF, IL-2, IL-7 and IFN type I and II [13]

Different MD catheters and methods for analysis, have been utilized which could explain why absolute values of the specific inflammatory mediators diverge between reports [12, 14]. Also actual cytokine levels vary due to the recovery of the catheters which is dependent on pore size, dialysis fluid, fluid velocity and characteristics of the analyte [15] [16]. Most inflammatory mediators are larger than the recovery limit of standard 20 kilo Dalton (kDa) catheters, consequently catheters with larger cut-off, such as 100 kDa, have mostly been used. Larger pore size catheters would theoretically give a better recovery but they also impose a

risk of ultrafiltration [17]. Also, inter-catheter variations due to fluctuations in cerebral perfusion pressure and temperature have been reported [18].

Despite the fact that post-mortem and blood analysis have indicated that TBI leads to a longlasting inflammation, previous reports of MD of inflammatory mediators have shown a gradual decline after day 4-6, implicating a time limited, brief period of inflammation.[19-21]. Contrary to these results we have shown that there is a prolonged and intense neuroinflammation beyond day 4 to 6 in adult patients with severe TBI when evaluated using 300 kDa MD.[22] Although it has been assumed that inflammation is pertinent also in the evolution of secondary brain damage of pediatric TBI few reports have actually addressedthis. Therefore the aim with this study was to investigate how intracerebral release of chemo- and cytokines , as evaluated with 300 kD MD catheters , evolves in a pediatric patients treated for severe TBI.

Methods

Clinical material and methods

The study was conducted at Red Cross War Memorial Children's Hospital (RCWMCH), a university-affiliated hospital and referral center for severe pediatric TBI in the Western Cape Province of South Africa. Approval for the study was obtained from the scientific and human research ethics review boards of the University of Cape Town (HREC 741/2010).

Patient selection

Data were prospectively collected from patients with severe TBI who underwent monitoring with intracranial pressure (ICP), brain oxygenation (PbtO2), and MD catheters between January 10:th 2011 and March 22:nd 2011. All patients were less than 13 years of age and had a post-resuscitation Glasgow Coma Score (GCS) $\leq$ 8 or with deterioration to this level during

their admission. The protocol used standard monitoring of ICP and PbtO2 for severe TBI unless patients had an improving clinical status where extubation was planned within 24–48 h or there was diagnosed or imminent brain death as evidenced by GCS 2, fixed and dilated pupils, and “black brain” on computed tomography scan (CT).

Patient management

Patients were resuscitated according to the Pediatric Advanced Life Support guidelines [23], and surgically drainable lesions were evacuated in theatre. All study patients were monitored for ICP (Codman ICP Express; Codman), PbtO2 (Licox; Integra Neurosciences), and CMD (CMA 320 catheter, MDialysis, Stockholm, Sweden). The length of the MD catheter was 30mm with a membrane pore size of 300kDa. The position of the catheter was at the surgeon’s clinical discretion: as a general principle, for focal injuries, the catheter was placed in the proximity of the lesion. For diffuse axonal injuries, the catheter was placed in right frontal white matter. Patients were then admitted to the pediatric intensive care unit (PICU) where they were ventilated and managed according the institutional protocol [24]. Broadly, initial therapy aimed to maintain ICP < 20 mmHg, cerebral perfusion pressure (CPP) >40 mmHg (age < 4 years) or, > 50 mmHg (age>4 years), and PbtO2> 20 mmHg by applying various therapies which include sedation, analgesia, neuromuscular paralysis, ventricular drainage, osmotic agents, carbon dioxide control, surgical decompression, and barbiturate coma[25] [24]. After baselines were established, therapy was individualized based on patient characteristics and data from multimodal monitoring. A repeat head CT scan was standard to evaluate evolving injury and to check the catheter position.

Microdialysis

300 kDa molecular weight cutoff MD catheters (CMA 320) were used in all patients . 2/7 patients received 2 MD catheters, one placed in non-traumatized areas near the ICP monitor, and one placed in peri-contusional areas. All MD catheters were perfused with central nervous system perfusion fluid (CMA Microdialysis AB, Solna, Sweden) at 0.3 μL/min using CMA 106 micro infusion pumps. All MD vials were kept at approximately the same height as the MD pumps in order to avoid hydrostatic forces that could affect the recovered volumes. MD catheters were categorized according to their position in relation to an injured area using the first postoperative CT-scan available. All catheters were clearly visualized on a CT-scan and termed “A” if placed in non-traumatized tissue, i.e. brain parenchyma without visible edema and blood. If the catheter was placed in close proximity to edema or blood, it was called peri-contusional and named “B”.

The locations of all monitoring devices were confirmed on CT scans.

Chemokine and Cytokine Analysis

All samples were analyzed using a multiplex kit (MesoScale Discovery, USA) according to the manufacturers´ instructions on MultiPark's MSD MESO QuickPlex SQ120 at the Biomedical Center, Lund University, Sweden. Microdialysis samples from a 6-hour time period were pooled immediately before analysis, in order to yield sufficiently large volumes for analysis which was 200 μl. Cytokine measurements were plotted in the middle of the 6hour time period, to represent a mean value for the given time period.

The microdialysates were analyzed with Pro-inflammatory cytokine-1 V-plex kit and Chemokine V-plex kit (see table 2).

Due to change of standard values for the proinflammatory cytokine-1 Vplex kit, the concentrations were recalculated after linear regression computations with a constant of

0.7357. Both kits contain IL-8 but gave different values and the final IL-8 value was taken from the proinflammatory cytokine-1 V-plex kit. Samples were analyzed in duplicate and the same multiplex-kits as had been used in a similar project for adult patients with severe TBI were used, to enable comparison between the two materials[22].

Statistics

Differences between catheters in seemingly non-traumatized and peri-contusional tissue were calculated with the non-parametric Mann Whitney U test. Slope and p values for regression analysis of change over time were calculated with linear regression. All computations and graphics were performed with the free software R (https://www.R-project.org/)

Results

Patient demographics

All patients were under the age of 13 at the time of trauma. 5/7 patients had an isolated TBI. All patients were ≤ GCS 8 on admission. Trauma mechanism were motor-vehicle accidents. 1 patient did not survive. (see Table 1).

Patient no	1	2	3	4	5	6	7
GCS[1]	7	6	6	7	6	8	7
Age	10	6	6	9	10	10	12
Pupils (+dilated/-normal)	-/-	-/-	-/-	-/-	-/+	-/-	-/-
Trauma mechanism	MVA	MVA	MVA	MVA	MVA	MVA	MVA
Neurosurgical Diagnosis							
EDH/SDH/ICH[2]	N/N/N	N/N/N	N/N/Y	N/Y/N	N/N/Y	N/N/Y	N/N/N
Time trauma - 1:st surgery[3]	10 h	6 h	4 h	5 h	6 h	6 h	12 h
Time trauma - 2:nd surgery	32 h	N/A	N/A h	N/A	N/A	115 h	N/A
DC[3]	Y	N	N	N	N	Y	N
Time – with MD in hours	(10-78)	(6-110)	(4-87)	(5-71)	(6-107)	(6-115)	(12-75)
Days in NICU[4]	3	8	5	4	5	7	10

Table 1. Patient demographics

[1]Glasgow Coma Scale

[2]EDH – Epidural hematoma, SDH – Subdural hematoma, ICH - Traumatic intracranial hemorrhage

[3]Decompressive craniectomy

[4]NICU – Neurointensive Care Unit

Duration of microdialysis monitoring

2 patients had 2 MD catheters which worked during the entire monitoring time. MD monitoring for metabolites was initiated at a mean time of 7 hours post trauma (range 4-12 hours). The mean monitoring time was 118 hours (range 80-200).

Definition of consistently elevated cytokines

Using the Mesoscale Multiplex assay, the following chemokines and cytokines were detected: MCP-1 (CCL2), MIP-1β (CCL4), eotaxin (CCL13), MCP-4 (CCL13), TARC (CCL17), MDC (CCL22), eotaxin-3 (CCL26), IL-8 (CXCL8), IP-10 (CXCL10), IL-1β,IL-2.

IL6, IL8, IP10, MCP-1 and MIP-1β were found in relatively high concentrations and were named top range. These were tentatively considered to be most important in the development of inflammation following TBI and were investigated further in relation to glycerol, lactatepyruvate ratio, ICP and CPP. Four other chemokines; Eotaxin, MCP-4, TARC and MDC had concentrations between 200 and 600 pg/mL with mean values at 200 pg/mL or higher. GMCSF displayed median values of 62 pg/mL, whereas a third group encompassing IFNγ, IL-1β,

TNF-α, IL-10 IL-2 and IL-12 displayed median values between below 5 pg/mL. (Figure 1)

Differences between catheters in peri-contusional and seemingly non-traumatized areas

2 of the 7 patients had consistently functioning catheters in damaged and non-damaged areas, i.e. peri-contusional or non traumatized tissue In these patients, comparison of cytokine and chemokine values between peri-contusional tissue and non-traumatized tissue was possible.

No correlations between cytokine/chemokine secretion and ICP.

ICP was controlled in all patients during monitoring time and as a result, we could not discern any changes in cytokine/chemokine levels that could be attributed to changes in ICP.

Microdialysis metabolite pattern

The L/P ratio was defined as pathological when >25. Glycerol was defined as pathological when >100 μmol/l [26]

A pathological L/P ratio was registered in patient 1, patient 3 on the peri-contusional side, patient 5, 6 and 7. Glycerol was highly elevated in patient 1 and in patient 6, both catheters. No changes in glycerol or L/P ratio covaried with the cytokine/chemokine secretion (figure 2).

Top range			
MCP-1	2112	1961	4460
MIP1-b	648	586	2148
IL-8	939	545	4788
IP-10	1447	911	5949
IL-6	1306	841	5753
Mid range			

Eotaxin	257	204	524
MCP-4	317	338	547
TARC	256	221	472
MDC	294	254	654
Low range			
IFNg	28,7	0,5	277
GM-CSF	59,6	62,6	156
IL1-b	8,8	4,5	87
TNFa	28,6	1,5	169
IL-10	23,8	2,1	120
IL-2	13	0,5	95,8
IL-12	8,9	4,5	87

Table 2. Summary of analyzed chemokines/cytokines with 300 kDa membranes. Concentrations in pg/mL

Temporal pattern of cytokine/chemokine secretion

The slope and p-values for each cytokine or chemokine were calculated for all 7 patients together. For IL-6, IL-8 and IP-10, there was a slight decrease over time, for MCP-1 and MIP1β, there was a slight increase. In the 2 patients that had 2 MD catheters, the peri-contusional catheters were analyzed separately as well. In these 2 catheters, there was an increase for MCP-1, MIP-1β and IP-10, however, the values were few and the variation was high. No differences were significant.

(Figure 3).

Discussion

In the present study intracerebral secretion of inflammatory mediators e.g. chemo-and cytokines were investigated in 7 pediatric patients with severe TBI, using 300 kDa MD. The CNS immune and inflammatory systems are not fully mature in children which is reflected in that CNS infections are more common in children than in adults [15]. Furthermore autoimmune CNS disease differ both quantitatively and qualitatively between adults and children [27].The tentative differences between pediatric and adult inflammatory reactivity in the CNS would thus predict differences in the secretion of inflammatory mediators in cases of pediatric TBI.

The results in the present investigation show that the cyto- and chemokine levels in a pediatric population are significantly lower for most analyzed mediators than in adults, previously sampled using the same 300 kD MD catheters and the same method of analysis. Still the qualitative pattern of release mimics that of adult TBI patients with one group of mediators with very high intracerebral levels (IL-6, IL-8, MCP-1, IP-10, MIP-1a), one group with intermediate levels (Eotaxin, MCP-4, TARC and MDC) and one group with lower levels (IFNγ, IL-1β, TNF-α, IL-10 IL-2 and IL-12). The absolute values in children are significantly lower but still dramatically increased and partially even higher than those reported for CSF in inflammatory complications of infectious disease as COVID-19 [28] Furthermore, the immune system develops continuously and thus the responses to tissue damage will also differ depending of the age of the child afflicted [29]. In the present report the patients ages ranged from 6 to 12, thus the present results might be different in younger and older children Reports on trauma induced CNS inflammation in pediatrics are few and no comparisons to adult TBI victims have been published To our knowledge there are no previous publications reporting

analysis of inflammatory mediators by CMD in pediatric TBI alone or in comparison with adult TBI.

Previously release of inflammatory chemokines and cytokines in pediatric TBI patients have been reported by analysis of CSF and blood identifying the major inflammatory cyto- and chemokines discussed here[30-32].

In the present pediatric TBI patients, we could discern the same categories of chemokines /cytokines as in adult patients, based on quantitative levels defining a high range group (IL-6, IL-8, IP-10, MCP-1 and MIP-1β) a mid-range group (Eotaxin, MDC, MCP-4 and TARC) , a low range group (IFNγ, GM-CSF IL-1β, TNF-α ,IL-10, IL-2 and IL-12). Both the high range and the mid-range group have been reported in previous MD studies using 100 kDa catheters in adult TBI [12, 33, 34]. However, there is the possibility that certain mediators have a biological activity even at lower concentrations while others have their effects at higher concentrations, either directly or indirectly [35] The measured values, even by using a 300 kD pore size in the MD membrane, constitute only a fraction of the actual levels in the brain tissue thus implying that the true levels are higher.

The subtype of TBI that induces the most vigorous tissue inflammation in humans is unknown but contusional injuries are most likely to do so [36]. Secondary events such as ischemia or hemorrhage, but plausibly the extent of surgical and intensive care treatments, could additionally influence the evolution of tissue inflammation. In the present study, traumatic contusions were found in all patients and in all patients cytokine and chemokine values over 1000 pg/mL could be detected at some time points thus indicating extensive tissue inflammation.

Earlier reports have found significantly increased plasma levels of IL-6, IL-8, IP-10 and

MCP-1 several months after severe and mild TBI , somewhat correlating with outcome [19, 37, 38]. In previous adult TBI studies, cytokine/chemokine expression reach peak values within 24-48 hours after MD-catheter implantation but we could show that this is not the case when using 300 kD catheters in adults with severe TBI[12, 22, 34, 39] In the present series, MD samples have been sampled 10-120 hours with no significant decline except for IP10 in the peri-contusional area. As discussed previously, the decline in cyto- and chemokine values in studies using 100 kD catheters could depend on clogging of catheters, different patient populations and the actual therapy delivered [22].

Experimentally, it has been shown that inflammatory cytokines as IL-6, IL-8 and IL-1beta can increase due to the tissue damage inflicted by the MD cathether [40]. Bouras et al could show that IL-6, IL-8 and TNF-α but not IL-1beta increased transiently in patients with epilepsy after CMD catheter insertion into gliotic brain tissue using 100 kDa catheters [41]. Our results thus strengthen the notion that TBI produces a potent and long-lasting inflammation in pericontusional but also in adjacent seemingly non-traumatized tissue.

Only two patients had patent catheters both in peri-contusional and non-traumatized tissue. In these, no significant differences of cyto- and chemokine levels could be observed, compared to adult TBI were only IL-6 differed in peri-contusional areas [22]

As in our previous study in adults, we could not see any relation between cytokine/chemokine expression and potential secondary insults as measured by metabolites or minute ICP changes, which also has been reported in another previous study in adult TBI using 100 kD catheters[34]. This notion implies that other mechanisms regulate the minute changes in physiological parameters as ICP and CPP

In the discussed report using 300 kD catheters in adult TBI patients, clonidine and metoprolol, which both can modulate the chemokine and cytokine levels, were used [42]. Also, midazolam, pentothal and propofol, used in both the adult and pediatric patient cohort, have

been shown to modulate cytokine/chemokine expression [43-45]. The use of clonidine and metoprolol, which mostly have been reported to decrease cyto- and chemokine secretion, in the adult group could mean that the difference between adults and children are actually larger than reported here. In the high level group of chemo- and cytokines in both adult and pediatric cohorts 4 of 5 are chemokines e.g. chemo-attractants where MCP-1 mainly orchestrate monocyte influx and IL-8 mainly neutrophil influx [46]. As for the two others chemokines in this group the picture is not so clear cut. IP-10 is believed to be released by IFNγ and recruit T -lymphocytes but it also a sensor in general tissue damage, infection and cancer [47]. MIP1-a recruits both monocytes, neutrophils and lymphocytes and is also found elevated in blood long time after TBI [19].

Several studies have addressed the task of using levels of inflammatory mediators in TBI to predict outcome in various aspects [48]. In both children and adults IL-8 levels has been shown to predict out come after severe [54], [55]. These and other reports on the predictive potential of inflammatory mediators in TBI stem from retrospective correlational studies but recently a prospective intervention with prehospital plasma administration in trauma patients showed that the intervention significantly lowered both levels of inflammatory mediators and mortality in the more severely injured encompassing > 50% of patients with GCS <8.[53]

Besides functioning as chemo-attractants for infiltrating inflammatory cells, the high-range and mid-range chemokines could also have other effects in TBI. Both MCP-1, IP10, IL-8 and IL-6 have been implicated in neuronal damage and edema formation by signaling to CNS resident cells as microglia[49] astrocytes [50] and neurons [51].
It might also be that case that some mediators in the low range group consisting mainly of cytokines, are more potent at lower concentrations, are secreted briefly, are metabolized more

rapidly or orchestrate their action through secondary mediators. To this end, IFNγ induces high levels of IP-10 and IL-1beta induces high levels of tentatively protective IL-RA [47, 52] [56],.

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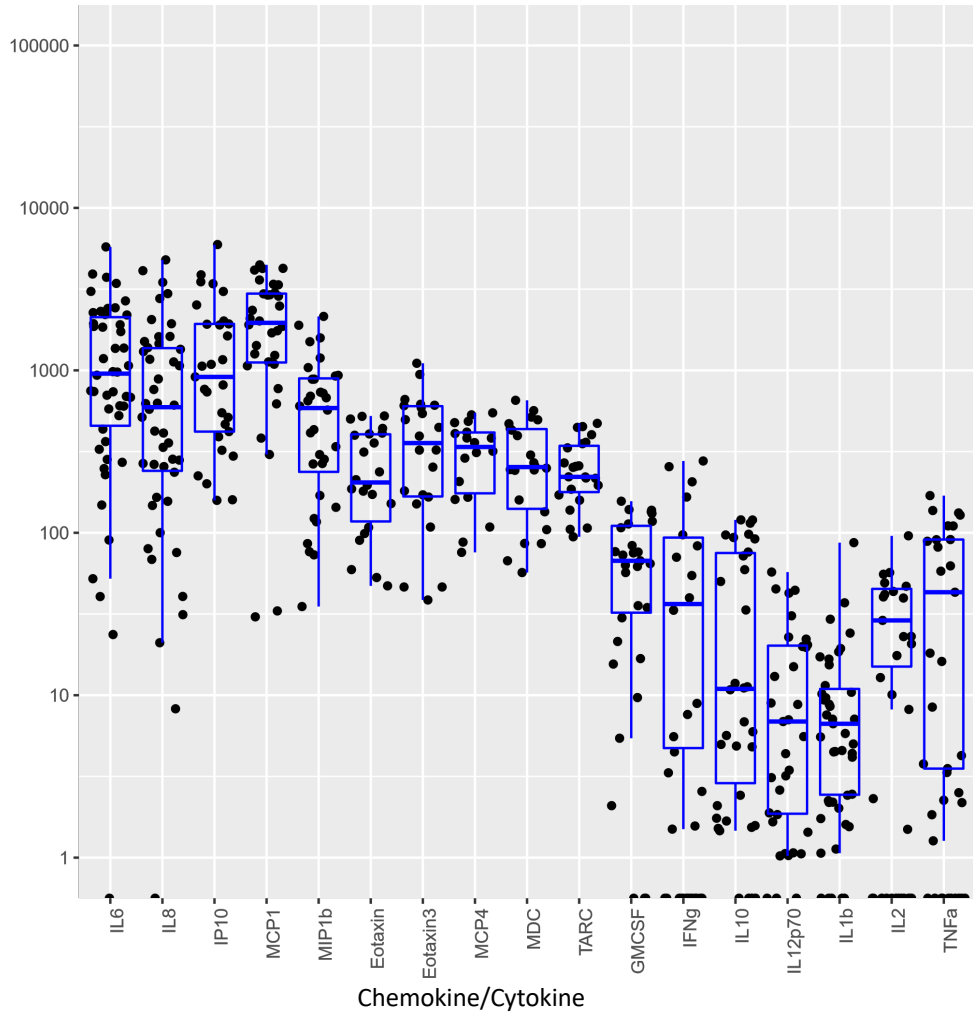
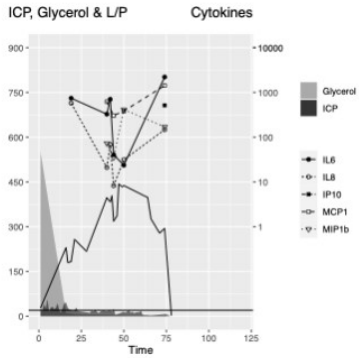
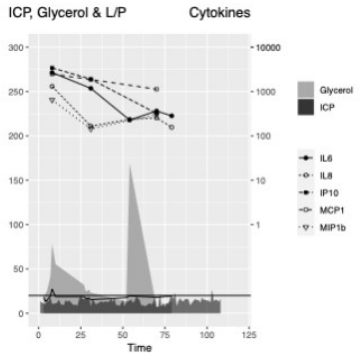


Figure 1. Total Chemokine/cytokine concentrations in pg/m

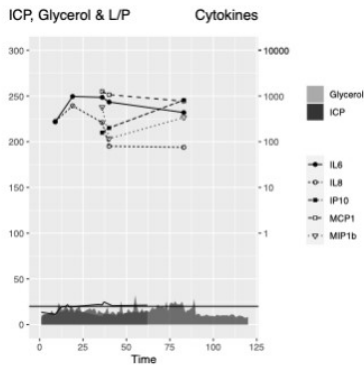
Patient 1.



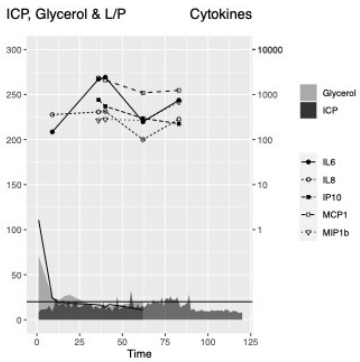
Patient 2.



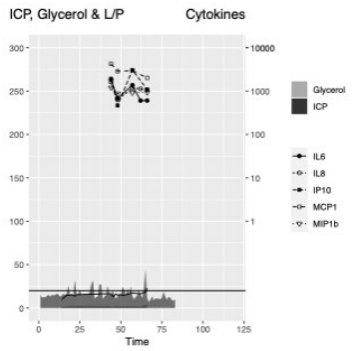
Patient 3 - A catheter



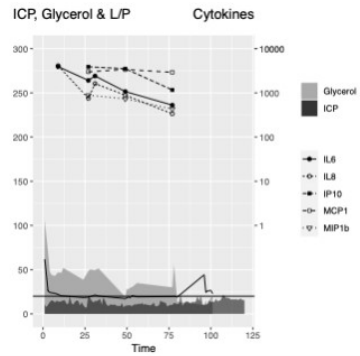
Patient 3 - B catheterP

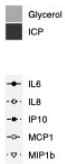


Patient 4



Patient 5





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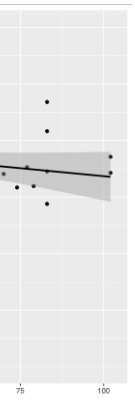
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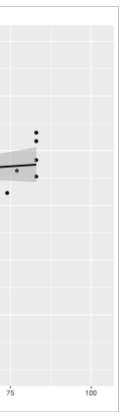
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Paper III

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Antisecretory Factor May Reduce ICP in Severe TBI—A Case Series

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Traumatic brain injury (TBI) constitutes a global epidemic. Overall outcome is poor, with mortality ranging from 10 to 70% and significant long-term morbidity. Several experimental reports have claimed effect on traumatic edema, but all clinical trials have failed. Antisecretory factor, an endogenous protein, is commercially available as Salovum®, which is classified as a medical food by the European Union and has been proven effective in experimental trauma models. It has, however, previously not been tested in humans with severe TBI. We hereby report a case series of five adult patients with severe TBI, treated with Salovum. The objective of the intervention was to evaluate safety and, if possible, its effect on intracranial pressure and outcome. Patients received 1 g Salovum per kilo of body weight divided into six doses per 24 h. Each dose was administered through the nasogastric tube. Patients were scheduled for 5 days of treatment with Salovum. Intracranial pressure was controlled in all patients. In three of five patients, intracranial pressure could be controlled with Salovum and deep sedation (no barbiturates), except during periods of gastroparesis. Five of five patients had a favorable short-term outcome, and four of five patients had a favorable long-term outcome. No toxicity was observed. We conclude that at least three of the five treated patients experienced an effect of Salovum with signs of reduction of intracranial pressure and signs of clinical benefit. In order to validate the potential of antisecretory factor in TBI, a prospective, randomized, double-blind, placebo-controlled trial with Salovum has been initiated. Primary outcome for the trial is 30-day mortality; secondary outcomes are treatment intensity level, intracranial pressure, and number of days at the neurointensive care unit.

Keywords: traumatic brain injury, intracranial hypertension, therapeutic agents per oral treatment, ICP reduction, anti-inflammatory therapy, novel treatments against traumatic brain edema

INTRODUCTION

Traumatic brain injury (TBI) constitutes a global burden despite the fact that mortality and morbidity have been reduced in several countries during the last decades (1, 2). Advances in neurointensive care, cerebral monitoring, and neuroradiology have improved outcome for patients with severe TBI, but the results globally are still poor with a mortality ranging from 10 to 70% and significant long-term morbidity (3).

Traumatic brain injury encompasses several pathogenic mechanisms as primary mechanical injury and hemorrhage followed by secondary events such as vasospasm, inflammation, excitotoxic cell damage, and energy deprivation but also long-term progressive brain tissue degeneration. One common denominator in TBI is cerebral edema, which may cause raised intracranial pressure (ICP) and is a major factor responsible for mortality and morbidity in TBI (4). The pathophysiologic mechanisms of cerebral edema are, however, only partially known (5).

Although several experimental reports have claimed effect on traumatic cerebral edema, all clinical trials have failed (6).

Antisecretory factor (AF) is a 41-kDa endogenous protein proposed to possess both antisecretory and anti-inflammatory effects (7). The exact mechanism of AF is unknown, but it has been proposed to act by modulation of proteasomes, complement, and myeloid cells (8–10). A recent report shows that AF inhibits the NKCC1 ion pump; the latter also has been implicated in the evolution of edema in TBI (11, 12).

Salovum ® is an egg yolk powder enriched for AF and classified as food for specific medical purposes in the EU. Salovum has been used in clinical trials for gastroenteritis and Ménière inflammatory bowel disease, and no toxicity has been reported [Lantmännen Functional Foods AB Besöksadress: S:t Göransgatan 160, Stockholm, Sweden, (13)].

The functional part of AF has been synthesized within a 16amino-acid peptide, AF16. AF16 and AF have shown effects against cerebral edema and increased ICP in models of herpes encephalitis and TBI (14, 15).

We hereby report the first five patients with severe TBI, treated with the AF-enriched dietary supplement Salovum with the aim to assess ICP control and clinical outcome.

PATIENTS AND METHODS

Patients

Patients with severe TBI (Glasgow Coma Scale score <9) were admitted to the neurointensive care unit (NICU), Skåne University Hospital. The treated patients were recruited during 2015 and 2017. Follow-up data were collected in December 2018.

ICP Monitoring

All patients were monitored for ICP, using Spiegelberg™ tunneled parenchymal probes placed in the right frontal lobe through a separate burr hole.

Treatment Algorithm

Patients were treated according to the local treatment algorithm for TBI, also known as the Lund Concept (LC) (16, 17). Five patients where first-tier treatment with LC did not control ICP were treated with Salovum after consent from next of kin. See **Appendix** (S1).

Treatment Intensity Level

Treatment intensity level (TIL) was used to display the measures taken to control ICP during treatment (18).

Ethical Permission

Ethical permission was granted by the regional ethical review board of Lund University, no. 2013/144.

Administration of Salovum

The patients received 1 g Salovum per kilo of body weight divided into six doses per 24 h. Each dose was mixed with 100 mL of water and administered through the indwelling nasogastric tube. Patients were scheduled for 5 days of treatment with Salovum.

The dosage of Salovum and the dosage interval were chosen from the lower ranges of dosages as reported from prior human trials with Salovum (19).

Control of Gastroparesis

There is no simple way to measure how much AF is delivered to the patient. Because of these circumstances, we can merely conclude when we almost certainly know when no AF was delivered.

Salovum was given every 4 h via the nasogastric tube. Gastroparesis was suspected prior to the administration, if the volume acquired by aspiration via the tube was more than 150 mL.

RESULTS

General patient characteristics are summarized in **Table 1**. During Salovum treatment, ICP >25 mm Hg occurs in 0–11% of hourly measurements. After 24 h with Salovum, ICP >20 mm Hg occurs in 3–33% of hourly measurements. Gastroparesis (gastric fluid volume >150 mL) is suspected in 0–28% of measurements.

Patient 1

A 13-year-old boy was involved in a bicycle accident. No mass lesion was seen on the initial computed tomography (CT). Because of uncontrollable ICP with first-tier treatment according to LC, barbiturates and Salovum were administered (**Figure 1A**). Intracranial pressure decreases to <20 mm Hg within 12 h, but during periods of gastroparesis, ICP increases to >20 mm Hg. Barbiturates could be discontinued, and ICP remains low. Treatment intensity level score was between 6 and 9. Salovum was administered during 4 days (107 h). Intracranial pressure >25 mm Hg occurs in 10 of 107 hourly measurements (11%) during Salovum treatment. After 24 h of treatment, ICP >20 mm Hg occurs in 23 of 84 of hourly measurements (27%). Gastroparesis is suspected in 2 of 26 measurements. At follow-up, the patient is Glasgow Outcome Scale–Extended (GOSE) (2).

Patient 2

A 35-year-old man was involved in a bicycle accident, with small frontal contusions and traumatic subarachnoid hemorrhage on initial CT. Because of uncontrollable ICP, using first-tier treatment of LC, Salovum is administered (**Figure 1B**). Twentyfour hours after admission, one of the patient's frontal contusions has greatly expanded and is surgically evacuated despite ICP of <20 mm Hg. On day 3 (71 h) after admission, CT shows progress of numerous contusions. One contusion is surgically evacuated, and the patient receives a bilateral hemicraniectomy. After surgery, ICP is controlled until day 5 (141 h), when gastroparesis recurs. Salovum is discontinued, and barbiturate treatment is initiated whereby ICP can be controlled during the rest of the NICU period. Treatment intensity level score was between 9 and 14. Salovum is given during 5 days (137 h). Intracranial pressure >25 mm Hg occurs in 8 of 137 hourly measurements (6%) during Salovum treatment. After 24 h of treatment, ICP >20 mm Hg occurs in 22 of 103 hourly measurements (21%). Gastroparesis is suspected in 4 of 14 measurements. The patient wakes up and

TABLE 1 | Summary of patient characteristics and interventions.

Patient no	1	2	3	4	5
Age/sex	13/male	35/male	16/male	56/male	63/male
Injury type	No mass lesion	Contusion SAH	Contusion SAH	ASDH contusion	ASDH contusion
[a]GCS	3	7	6	7	5
[b]Pupils	–/–	–/–	–/–	–/–	+/–
Trauma	[c]mv	mv	mv	mv	fall
Barbiturates	Y	Y	N	N	N
Non [d]DC surgery	N	Y	N	Y	N
DC	N	Y	N	N	N
[e]GOSE	7	[f]1	7	6	6
[g]ICP>25 (%)	11	6	0	6	4
[h]ICP>20 (%)	27	22	3	33	31
[i]Gastroparesis (%)	4	28	0	8	10

[a] *Glasgow Coma Scale. [b]On admission. [c]Motorvehicle accident. [d]Decompressive craniectomy. [e]Glasgow outcome scale extended.*
[f]Death after hemorrhage under scalp after gaining consciousness. [g]Percent of ICP measurements >25 mm Hg during Salovum treatment. [h]Percent of ICP measurements >20 mm Hg after 24 h during Salovum treatment. [i]Percent of gastric retention measurements >150 ml during Salovum treatment.

has GCS of 14 but later goes ad mortem due to an unexpected extracerebral bleeding under the scalp flap.

Patient 3

A male patient younger than 18 years was involved in a motor vehicle accident. Initial CT shows traumatic subarachnoid hemorrhage, obliterated basal cisterns, and small hemorrhagic contusions. Because of uncontrollable ICP using the first-tier treatment of LC (>30 mm Hg between the sampling times), a new CT scan was obtained, which shows no progression of the contusions, but slightly increased edema. Salovum is administered 13 h after admission (**Figure 1C**). Almost immediately, a decrease in ICP can be observed, and ICP is thereafter controlled. No other ICP-lowering measures were taken. Treatment intensity level score was between 1 and 3. Salovum was given during 3 days (55 h). Intracranial pressure >25 mm Hg occurs in none of 55 hourly measurements during Salovum treatment. After 24 h of treatment, ICP >20 mm Hg occurs in 1 of 31 of hourly measurements (3%). Gastroparesis is suspected in none of 14 measurements. At follow-up, the patient is GOSE 7.

Patient 4

A 56-year-old man was involved in a moped accident, where initial CT shows an acute subdural hematoma and numerous small contusions. The subdural hematoma is evacuated. Postoperatively, ICP is uncontrollable using first-tier treatment of LC. Salovum is administered 13 h after admission (**Figure 1D**). No other ICP-lowering measures were taken. Except for a brief period of gastroparesis, ICP is under control. Treatment intensity level score was between 2 and 3. Salovum is given during 5 days (103 h). Intracranial pressure >25 mm Hg occurs in 6 of 103 hourly measurements (6%) during Salovum treatment. After 24 h of treatment, ICP >20 mm Hg occurs in 26 of 79 hourly measurements (33%). Gastroparesis is suspected in 1 of 13 measurements. At follow-up, the patient is GOSE 6.

Patient 5

A 63-year-old man was involved in a falling accident. Initial CT shows an acute subdural hematoma and numerous middlesized contusions. The subdural hematoma is evacuated. Salovum is administered 15 h after admission (**Figure 1E**). Because of high ICP, several new CT scans are performed, revealing no progression of the contusions. Intracranial pressure is kept at <25 mm Hg at all times; no further surgery is needed. On day 5, gastroparesis is diagnosed with an ensuing slight elevation of ICP. When gastroparesis is attenuated, ICP returns to normal. Treatment intensity level score was between 2 and 3. Salovum is given during 3 days (81 h). Intracranial pressure >25 mm Hg occurs in 3 of 81 hourly measurements (4%) during Salovum treatment. After 24 h of treatment, ICP

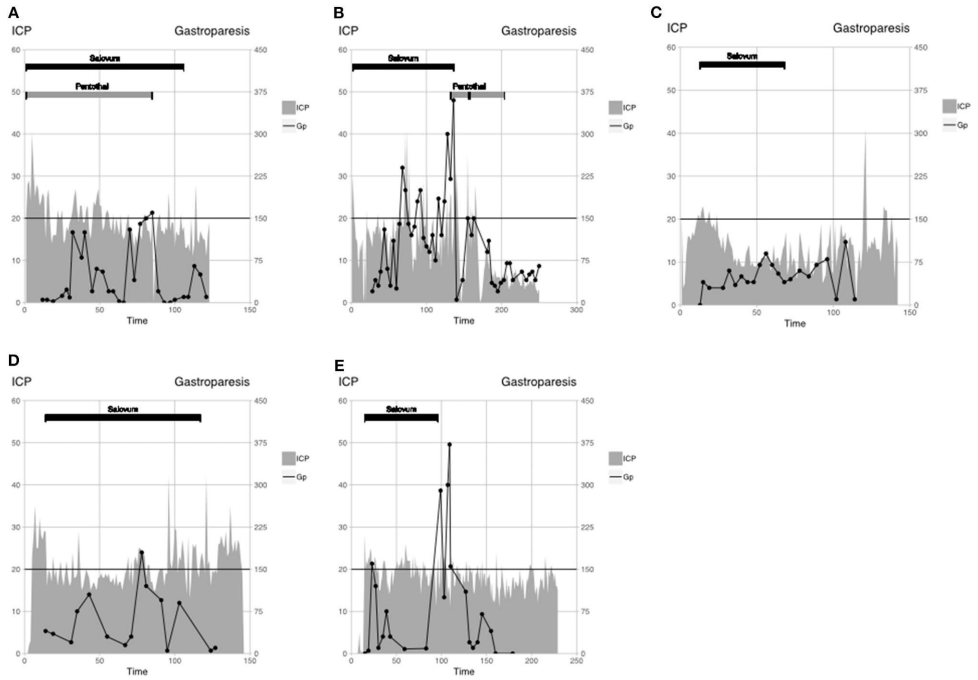

FIGURE 1 | Patient monitoring data are displayed as ICP (gray-filled line), gastroparesis (black line), and interventions: Salovum (black bar), Pentothal (gray bar). Critical ICP threshold according to the TBI algorithm (20 mm Hg) and threshold for gastroparesis are shown by horizontal black line. (A) patient 1, (B) patient 2, (C) patient 3, (D) patient 4, and (E) patient 5.

>20 mm Hg occurs in 18 of 57 hourly measurements (31%). Gastroparesis is suspected in 1 of 10 measurements. At follow-up, the patient is GOSE 6.

DISCUSSION

The present case study is the first publication on treatment of severe TBI with AF. We conclude that at least three of five treated patients may have experienced a beneficial effect of Salovum with reduction of ICP and very low TIL scores, since few other ICP-lowering measures were made.

The LC treatment algorithm is very restrictive in terms of which interventions are readily utilized. Lund Concept uses a physiology-oriented approach in which the patients' cerebral perfusion pressure is kept at low levels compared to traditional guideline recommendations. By doing so, it is hypothesized that the development of brain edema is kept at a minimum. Development of secondary injury is thought to be avoided partly because of less edema, partly by optimizing circulation physiologically and avoiding vasopressors that may compromise microcirculation. See **Appendix**.

The TIL score for LC generates low values, even when secondtier therapies are initiated (maximum 12/38). See **Appendix**.

Salovum is an egg powder enriched for AF but with no specified quantity or concentration of the protein. The delivered amount of AF could thus vary among the treated patients.

The uptake of AF after oral administration is, as for most oral drugs, dependent on functioning gastrointestinal passage. Gastroparesis is common in patients treated according to the LC, because of high doses of fentanyl. Gastroventricular fluid volume is not the gold standard for diagnosing gastroparesis, but a gastric fluid volume of 100–150 mL indicates decreased gastric emptying and therefore decreased uptake of oral drugs. In three of the patients, ICP was reduced during administration of Salovum as the only ICP-lowering measure taken. We could see that the effect was attenuated at periods with suspected gastroparesis.

All patients had good outcomes as assessed by GOSE at 6 months, with the exception of one patient who died of a very unusual complication, which was most probably related to the preceding decompressive craniotomy. This patient did, however, wake up to a GCS score of 14, indicating a good prognosis.

Treatment of patients with severe TBI by the LC has reported a reduction of mortality and morbidity in selected patients, but overall mortality and morbidity for all patients admitted with severe TBI are still considerable (unpublished data). The patients included in this report had an uncontrollable ICP despite application of the treatment algorithm's first-tier treatments. The first two patients were administered pentothal and Salovum because of the uncertainty of the effect of

the latter, whereas the three ensuing patients were given only Salovum and did not need any other ICP-lowering treatment. As in previously reported clinical trials, we could not see any obvious toxicity in the current case series.

Although the present results do not prove that AF can reduce ICP in TBI, and thus improve outcome, there are strong implications for this. The patients had all been treated according to the first tier of the LC TBI algorithm, currently a modification of the original LC, but despite this, ICP could not be controlled until Salovum treatment was initiated. The LC treatment algorithm allows for relatively few ICP-lowering measures according to the TIL score. Second-tier treatment options were utilized in only two of these patients, which means that numerous other actions could have been taken and proven equally effective. However, it appears that Salovum might be an effective agent in controlling ICP and with no observed toxicity. In order to validate the potential of antisecretory factor in TBI, a prospective, randomized, double-blind, placebo-controlled trial with Salovum in severe TBI has been initiated (NCT03339505). Primary outcome for the trial is 30-day mortality; secondary outcomes are TIL, ICP, and number of days at the NICU.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional ethical review board of Lund University, LU, nr 2013/144. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur. 2020.00095/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper IV

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STUDYPROTOCOL Open Access

Effect of antisecretory factor, given as a food supplement to adult patients with severe traumatic brain injury (SASAT): protocol for an exploratory randomized double blind placebo-controlled trial

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Abstract

Background: Traumatic brain injury (TBI) constitutes a global epidemic. Overall outcome is poor, with mortality ranging from 10 to 70% and significant long-term morbidity. Several experimental reports have claimed effect on traumatic edema, but no clinical trials have shown effect on edema or outcome. Antisecretory factor, an endogenous protein, is commercially available as Salovum®, which is classified as a medical food by the European Union and has shown effect in experimental trauma models and feasibility with signs of effect in 2 pilot case series. The aim of this study is to assess the effect of antisecretory factor in adult patients with severe traumatic brain injury as measured by 30-day mortality, treatment intensity level (TIL), and intracranial pressure (ICP).

Methods/design: This is a single-center, double-blind, randomized, placebo-controlled clinical phase 2 trial, investigating the clinical superiority of Salovum® given as a food supplement to adults with severe TBI (GCS < 9), presenting to the trauma unit at Tygerberg University Hospital, Cape Town, South Africa, that are planned for invasive ICP monitoring and neurointensive care, will be screened for eligibility, and assigned to either treatment group (n = 50) or placebo group (n = 50). In both groups, the primary outcome will be 30-day mortality, recorded via hospital charts, follow-up phone calls, and the population registry. Secondary outcomes will be treatment intensity level (TIL), scored from hospital charts, and ICP registered from hospital data monitoring.

Trial registration: ClinicalTrials.gov NCT03339505. Registered on September 17, 2017.
Protocol version 3.0 from November 13, 2020

Introduction

Background and rationale

Traumatic brain injury (TBI) constitutes a global burden despite the fact that mortality and morbidity have been reduced in several countries during the last decades [11, 13]. Advances in neurointensive care, cerebral monitoring, and neuroradiology have improved outcome for patients with severe TBI, but the results globally are still poor, with a mortality ranging from 10 to 70% and significant long-term morbidity [18].

Traumatic brain injury encompasses several pathogenic mechanisms such as primary mechanical injury and hemorrhage followed by secondary events such as vasospasm, inflammation, excitotoxic cell damage, and energy deprivation, but also long-term progressive brain tissue degeneration. One common denominator in TBI is cerebral edema, which may cause raised intracranial pressure (ICP) and is a major factor responsible for mortality and morbidity in TBI [21]. The pathophysiologic mechanisms of cerebral edema are, however, only partially known [20].

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Although several experimental reports have claimed effect on traumatic cerebral edema, all clinical trials have failed [2].

Antisecretory factor (AF) is a 41-kDa endogenous protein proposed to possess both antisecretory and antiinflammatory effects [16]. The exact mechanism of AF is unknown, but it has been proposed to act by modulation of proteasomes, complement, and myeloid cells [5, 14, 22]. A recent report shows that AF inhibits the NKCC1 ion pump which also has been implicated in the evolution of edema in TBI [10, 23].

Salovum® is an egg yolk powder enriched for AF and classified as food for specific medical purposes in the EU. Salovum has been used in clinical trials for gastroenteritis, Meniere disease, and inflammatory bowel disease with no toxicity reported [15]. Salovum is currently registered in the Republic of South Africa.

An active part of AF has been synthesized within a 16amino-acid peptide, AF-16. AF-16 and AF have shown effects against cerebral edema and increased ICP in models of herpes encephalitis and TBI [8, 12]. Currently, two case-series with reported beneficial effect from Salovum® on ICP in adults with severe TBI have been published [4, 6].

To this date, no medical interventional trials in TBI have succeeded in demonstrating a significant difference in mortality.

In patients were ICP can be controlled, morbidity and mortality is likely to be reduced compared to patients where ICP cannot be controlled [3, 7]. However, the strategies implemented to control ICP could have an impact on morbidity and mortality itself. One of the most efficient ways to control ICP after TBI is by performing a decompressive craniectomy, a procedure that also can influence morbidity and mortality by itself [9]. Due to the fact that Salovum® appears to have no side effects, severe TBI is a diagnosis with high mortality and morbidity without a cure, and promising results from two case-series have been reported, this trial is clinically motivated.

Objectives

The present trial intends to assess whether Salovum®, an egg yolk powder enriched for AF given to patients with severe traumatic brain injury, will improve outcome as defined by 30-day mortality, ICP, and TIL compared to a control group given placebo egg yolk powder.

Methods/design

Trial design

This is a single-center investigator sponsored, phase 2, double-blind, randomized, placebo-controlled, and parallel-arm trial to assess the superiority of AF given as Salovum®, in adult patients with severe traumatic brain injury. Allocation ratio is 1:1. Recruitment commenced in September 2017.

Trial population and eligibility

A total of 100 adult patients with severe TBI will be enrolled at a single study site, Tygerberg University Hospital, Tygerberg, Cape Town South Africa. Patients with GCS < 9 and indication for invasive ICP monitoring will be screened for inclusion.

Inclusion criteria

The inclusion criteria are as follows: patients with severe TBI, i.e., Glasgow Coma Score (GCS) < 9 on admission or within 48 h after injury, aged between 18 and 65 years with non-penetrating, isolated head trauma; admission to study hospital within 24 h of injury (for patients with GCS < 9 on admission) and within 24 h of deterioration for patients deteriorating to GCS < 9 within 48 h of injury; no known history of allergy to egg protein; planned for intracranial pressure monitoring and neurointensive care; absence of bilaterally dilated pupils; and CT scan with traumatic pathology that is more than an isolated epidural hematoma.

Exclusion criteria

The exclusion criteria are as follows: systolic blood pressure below 90 mmHg post resuscitation, epidural hematoma with no other signs of intracranial injury, penetrating injury, and non-fulfillment of inclusion criteria after screening and inclusion.

Management of traumatic brain injury

The study site will treat all the study patients according to hospital standard of care that may include assisted ventilation, use of invasive ICP monitors, head elevation, hyperventilation, barbiturate coma, mannitol, hypertonic saline, and surgical measures to lower ICP, including decompressive craniectomy.

Study participants

Study participants will be composed of 100 patients with severe TBI (GCS < 9) that are planned for neurointensive care and an invasive ICP monitor.

Ethics and protocol

Ethical approval has been granted by the Health Research Ethics Committee (HRECs), Stellenbosch University, Stellenbosch, South Africa (M16/10/040). The study will comply with the ethical principles as set down in the Declaration of Helsinki and will be conducted in accordance with good clinical practice as defined by the International Conference on Harmonization (ICH). The trial is registered with ClinicalTrials.gov, NCT03339505; any amendments to the protocol will be published on ClinicalTrials.gov.

On 14 December 2018, an amendment to the former ethical application was approved by HRECs, stating that recruitment of patients for the trial could be performed with delayed consent for next of kin. The reason for this was that investigators found that relatives of people with no current

address were difficult to find within the time frame for inclusion into the trial, thereby creating a potential selection bias.

Randomization

Patients in the trial are allocated to treatment with Salovum® or placebo egg yolk powder at a ratio of 1:1. Permuted block randomization, with blocks of 4–6, was used and was compiled with Math lab®. Each patient is assigned an envelope, and inside the envelope, the patient study number is written. The patient study number corresponds to a box with the same number, containing the study substance, either active (Salovum®) or placebo (normal egg yolk powder). Study substance and placebo look identical in regard to packaging and the powder itself. Both investigators and patients are blinded during the entire trial. If a single patient needs unblinding, a box with 100 envelopes marked with the patient study number can be accessed. If the envelope with study number is opened, the content will reveal if the patient has received substance or placebo.

Trial interventions

Active therapy

The active therapy is Salovum®, an egg yolk powder enriched for AF, which is manufactured from freeze dried egg yolk (Lantmännen Functional Foods AB, Stockholm, Sweden).

Placebo therapy

A placebo powder, containing low amounts of antisecretory factor, made from freeze dried egg yolk and identical in taste, texture, smell, and color to Salovum®, will be used.

Dosage

Patients are assigned to 1 out of 3 groups, according to their weight: group 1, < 60 kg, 11 g dose × 6; group 2, 60–80 kg, 14 g dose × 6; and group 3, > 80 kg, 17 g dose × 6. The dosages correspond to doses dispensed in previous studies [4, 6]. The study site is equipped with digital scales for weighing the trial substance before administration.

Dispensing

Both the Salovum® and placebo substance are packaged in identical bags. After opening of a bag, each containing 20 g, a weighed aliquot depending on patient weight will be mixed with 50–100 ml tap water in a glass container. Electrical milk frothers are used for mixing. The mixture will then be aspired into a syringe and administered to the patient via the nasogastric tubing, used for enteral nutrition.

Protocol adherence

Protocol adherence will be encouraged via regular site visits, monitoring, and continuous sponsor-study site communications.

Study endpoints

The primary endpoint is the effect of AF, given as a dietary supplement in the form of Salovum®, compared with placebo on mortality at 30 days in adult patients with severe TBI. Comparisons will be made using chi-square/ Fisher exact test between active and placebo groups.

Secondary endpoints are ICP and TIL. For ICP, mean values and time over 20 mmHg will be analyzed. Both ICP and TIL will be compared not only between treatment and placebo groups but also between deceased and alive participants within the study groups using nonparametric tests. For comparisons within the groups before and after intervention, the Wilcoxon signed rank test will be used, and for comparisons between the groups, Mann-Whitney U test will be used. ICP and TIL will be presented as mean and median values in the respective groups (Table 1).

Data collection of outcome parameters and predefined covariates

All data will be compiled in a paper based CRF and transferred to an electronic CRF continuously. After screening, basic parameters are noted: age, gender, trauma mechanism (motor vehicle accident, falls or blunt trauma), and type of injury (EDH, SDH, contusion, no-mass lesion, SAH).

ICP and mean arterial blood pressure (MAP) will be noted hourly in the patient charts, and TIL will be scored every 24 h. For comparisons between groups, only ICP and TIL during the intervention will be used. For comparisons within groups also, ICP and TIL before intervention can be used.

Once the patient is included in the trial, blood will be drawn and sent for centrifugation of plasma and storage in a − 80 °C freezer. Additionally, a blood sample will be drawn 2–3 days into treatment and after last dosage of

Table 1 Trial schedule

	Study period							
	Enrolment	Allocation	Post-allocation					Close-out
Time point	Admission		Admission to NICU	Day 2	Day 3	Day 4	Day 5*	
Enrolment:								
Eligibility screen	X							
Informed consent	X							
Allocation	X							
Interventions:								
Intervention group			X	X	X	X	X	
Control group			X	X	X	X	X	
Assessments:								
Basic demographic data	X							
Type of injury	X							
Blood sample			X				X	
MAP/ICP/TIL/			X	X	X	X	X	
30-day mortality								X

Abbreviations: NICU meurointensive care unit, MAP mean arterial pressure, ICP intracranial pressure, TIL treatment intensity level
*Or earlier if substance administration is discontinued before 5 days

trial substance. The plasma will be analyzed for AF levels, markers of brain damage, and cytokines/chemokines for exploratory ad hoc studies.

The total number of days in the neurointensive care unit and days in hospital are noted at the end of the trial for exploratory purposes. The patient or next of kin will be contacted via telephone after 30 days for registration of 30-day mortality. If it is not possible to contact the participants or their next of kin, the population registry will be used. Age, gender, and GCS at inclusion may be used as covariates in exploratory regression analysis.

Statistical power of proposed endpoints

The power of the proposed end points was calculated with R statistical software using the Fisher test (1) and power t-test (2 and 3) acknowledging the fact that there are no adequate power tests for skewed data.

1. Mortality. Reduction of rate of mortality from 40% (20/50 patients) to 16% (8/50 patients) after intervention will give a p = 0.01, odds ratio = 0.29, 95% and confidence interval = 0.10–0.80.
2. Treatment intensity level. Proposed reduction by 5 grades (delta) after intervention with n = 50 in intervention and control group gives power 0.80 with sd = 7.19.
3. Intracranial pressure (ICP). Reduction of ICP after intervention. Proposed reduction by 5 mmHg (delta) or 5 h over 20 mmHg after intervention with n = 50 in intervention and control group each gives a power of 0.80 with standard deviation (sd) = 7.19

Handling of missing data

With the exception of 30-day mortality, the data collected in this trial is limited to data that is normally measured and registered during standard care at the NICU. Therefore, the amount of missing data is expected to be low. Data missing at random will be handled using last observation carried forward (LOCF). Data not missing at random will be analyzed using mean substitution.

Strategies to achieve adequate participant enrolment The Tygerberg Hospital is tertiary unit with full neurosurgical capacity that serves approximately 3.6 million people and

treats approximately 200 patients with TBI and 60 patients with severe TBI each year.

Handling of protocol deviations and protocol violations

We defined 4 types of possible protocol deviations/violations in this trial: faulty enrolment of patients, faulty randomization, faulty intervention, and faulty data collection.

The trial is monitored by an external body with intention to disclose any protocol deviations/violations at the end of the trial.

Adverse events

As Salovum® is commercially available in Swedish pharmacies and has been available for human use for many years without any reported toxicity, it is not expected to cause any adverse events. Allergy to egg yolk protein (Gal 5; alpha-livetin) has an estimated low incidence in adults (< 0.1%), and anaphylactic reactions are very rare among these. However, special care will be taken to ensure that no vital parameters are changed for the worse in conjunction with the start-up of administering the drug/placebo and at the time for each dose administration, i.e., every 4 h.

The physician responsible for the patient will assess if any adverse or serious adverse events have occurred during the course of each day. The daily patient chart states: "Do you consider that there is a reasonable possibility that an adverse event has been caused by the study compound administered?" The question must be answered daily.

Adverse events: Skin rash and hives

Serious adverse events Serious anaphylactic reaction with hypotension and bronchospasm requiring intervention with corticosteroids and/or vasopressors

Data and trial monitoring and interim analysis

An external body, Novotech (see Monitor) monitors the quality of the trail. Three site visits are planned. AE and SAE events related to the trial substance or placebo except a potential egg yolk allergy are not anticipated as no previous side effects have been reported after use of Salovum® in non TBI and TBI patients.

An independent Data and Safety Monitoring Committee (DMC) will perform an unblinded interim analysis when 95 patients have been included. The interim analysis will be conducted by an unblinded statistician and reviewed by the DMC, based on clean data on the primary and secondary outcome variables. The outcome of this interim analysis will result in one of three possible recommendations of the DMC to the sponsor to do one of the following:

Stop the study because of futility

Continue and finalize the study as planned

Continue the study as planned but increase the sample size to a specified number of patients

Discussion

Endpoints

Antisecretory factor given as a food supplement, Salovum®, preliminarily appears to have an effect on the, often deleterious, secondary events following severe head trauma [4, 6]. The common view on TBI is that the pathogenic mechanisms are heterogenous and that trials aiming to improve outcome should enroll a large number of participants [17] and use prognostic tools as the IMPACT [19]. This approach could also be based on the fact that no medical intervention has yet changed the outcome of TBI.

Thirty-day mortality, although a crude measure, is robust. The 30-day mortality rate in the placebo group will also give an estimation of the outcome of therapy at the study site for comparison with other sites and settings, as this has not previously been reported. The demographics of the catchment area of the Tygerberg University Hospital makes it difficult to record GOSE at 6 months as part of the outcome. No subgroup or adjusted analyses will be performed. Possibly, attempts to record GOSE or Rankin scores will be made for later ad hoc analysis.

In order to compensate for an increased number of clinical interventions in the arms of the study, the full (summary) treatment intensity level scale (TIL) as opposed to the basic TIL will be used. As the full TIL scale is composed of 8 categories of interventions, it has the capacity to describe virtually all clinical interventions that occur. However, measures, especially decompressive craniectomy (DC), initiated before or just at the beginning of inclusion might obscure the outcome as points for DC will be added for each new 24-h TIL score. Additionally, some patients might not receive the prescribed trial substance for 5 days. Therefore, TIL scoring will be computed for each 24-h period after inclusion. Scores for 24, 48, 72, 96, and 120 h and mean scores divided by time will be used for computations. TIL scoring will begin at the time when the trial substance has been administered for comparisons between the intervention groups, but TIL scores before intervention can be used for comparisons within the intervention groups.

Despite the fact that ICP has been significantly correlated with outcome of TBI in numerous studies, there is no consensus of what level of ICP should be regarded as pathological [3]. With this in mind, analysis of ICP values will be performed addressing both mean changes over the intervention time period between the arms but also within the active arm before and after intervention, when possible. Additional analysis will be conducted comparing the time above proposed normal ICP levels (20 mmHg) for each arm and within the active arm. Further computations of TIL and ICP may be used in exploratory ad hoc studies.

Dosage

The optimal dosage of Salovum® is not known, nor the optimal interval between doses. However, the substance has

been given to a large number of patients with equivalent doses, without any side effects. The dosage used in this trial is also similar to the ones used in reported cases series using Salovum in TBI [4, 6], a pilot trial for treatment of cholera [1] and trials for pediatric diarrhea [24, 25], the latter with a dose up to 16 g to infants 6–24 months old. The rationale behind the dosage and intervals is that previous studies have shown zero toxicity and that the protein is endogenous, which justifies administration of high doses of protein in order to ensure that enough AF-16 is available in the blood to make a clinical difference. AF-16 cannot be accurately measured in blood currently, which makes a dose titration trial futile.

Trial status

The protocol is version 3, dated November 13, 2020.

This trial is active and has been recruiting since September 22, 2017. Due to the current COVID-19 situation, recruitment has been paused from March to August 2020. Recruitment is estimated to be finalized during 2021.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13063-022-06275-z.

Additional file 1. Full research protocol.

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Marit Bäckström is the administrator for the trial. Late Peter Höglund, MD, Professor, performed the power analysis and randomization algorithms for the trial protocol.

External monitoring body
Novotech (novotech-cro.com, Ground Floor, Building 4, Quadrum Office Park,
50 Constantia Boulevard, Constantia Kloof Johannesburg Gauteng 1709 South Africa).

Communicating the trial results
The results of this trial will be presented in an appropriate scientific journal after finishing the trial.

Authors' contributions
DC contributed to the design of the trial and the statistical analysis plan, drafted the manuscript, and is sponsor/investigator. BH is investigator at the trial site and reviewed the manuscript. AV is the principal investigator at the trial site and reviewed the manuscript. PS is the principal sponsor /investigator, who contributed to the trial design, reviewed the manuscript, and drafted the statistical analysis plan. All authors read and approved the final manuscript.

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Availability of data and materials
The full protocol is available as Additional file 1. The full anonymized data set will be available to the public upon reasonable request. Declarations

Ethics approval and consent to participate See ethics part

Consent for publication
When the patient/next of kin is informed of the trial, information is given about the intent of publishing the results, and if consent is given for participation in the trial, consent for publications is given as well.

Competing interests
The authors declare that they have no competing interests

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Publisher's Note

Paper V

Abstract (248)

Background: Severe traumatic brain injury (TBI) is associated with high mortality and long-term morbidity. Despite promising results from experimental therapies, no clinical trials have demonstrated efficacy. Antisecretory factor (AF) is an endogenous protein with immunomodulatory and antiedema capacity. AF is enriched in Salovum®, a food for special medical purposes. Salovum® has demonstrated efficacy in experimental trauma models and safety/feasibility in 2 pilot case series of TBI. This trial aimed to assess the effect of Salovum® in severe TBI.

Methods: A prospective, double-blinded, placebo-controlled phase 2 trial was conducted at a single center, including 100 adult participants with severe TBI (GCS 6-8). Participants were randomized (1:1) to receive Salovum® or placebo. The primary outcome was 30-day mortality. Secondary outcomes were treatment intensity level (TIL) and mean time of intracranial pressure (ICP) over 20mmHg.

Results:100 participants were included in the trial, with 49 and 51participants in the treatment and control group respectively. There were no significant differences between the groups for age, gender, trauma type, time to intervention, treatment time, hospital stay, or neurointensive care unit (NICU) stay. The 30-day mortality rate was 20% in the treatment group and 39% in the control group, yielding a risk ratio of 0.52 (relative risk) and a pvalue of <0.05 (chi-square test). There were no significant differences between the groups in ICP or TIL.

Conclusions: Administration of Salovum® resulted in a significant reduction in mortality in participants with severe TBI. This study was funded by Region Skane and others and registered under NCT05669820.

Antisecretory factor reduces mortality in severe TBI

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3038 words

Introduction

Traumatic brain injury (TBI) constitutes a global pandemic with a significant impact on society in terms of human suffering and health care costs. Globally more than 70 million people will experience a TBI and every year over 2 million die due to TBI [1]. The advances in neurointensive care, cerebral monitoring and neuroradiology have improved outcome for patients with severe TBI, but global results are still poor, with reported mortality ranging from 20 to 70% and significant long term morbidity.[2-4] TBI encompasses several pathogenic mechanisms as primary mechanical injury and hemorrhage, followed by secondary events as edema, vasospasm, hypo-perfusion, spreading depolarization, mitochondrial dysfunction, inflammation and excitotoxicity, but also long-term progressive tissue degeneration. One common denominator in TBI is cerebral edema, which may cause raised intracranial pressure (ICP) and is considered a major cause of mortality and morbidity in TBI.[5] The definition of severe TBI encompasses patients that have suffered trauma to the brain which lead to loss of consciousness for at least 30 minutes and are classified as GCS < 8 at admission [6]. Patients presenting with these features at admission, have the highest mortality with a reduction in life expectancy of 13 years and also the highest morbidity rates [7] [8]

Although uncontrollable ICP has been regarded as a causative agent for mortality in severe TBI it does not explain all cases and the proportion has diminished as algorithms aimed at controlling ICP have been implemented [9] [10]

Different ways of presenting mortality and morbidity data in severe TBI have been used as pre-hospital mortality, in hospital mortality, 28- or 3- day mortality, 6 month mortality/morbidity or 12 month and longer time morbidity. Although there is no consensus on which is most appropriate, 28- or 30-day mortality are regarded as direct measures of immediate hospital intervention in severe and moderate TBI while later follow up aims to evaluate long term consequences of TBI.[11] [12]

The pathophysiological mechanisms of traumatic cerebral edema are only partially known and although several experimental reports have claimed beneficial effects, clinical trials have failed.[13,14]

Antisecretory factor (AF) is a 41-kDa endogenous proteasomal protein proposed to possess both antisecretory and anti-inflammatory effects. The exact mechanism of AF is unknown, but several mechanisms have been suggested such as direct effects on ion pumps and myeloid cells[15,16]
Salovum® is an egg yolk powder with high concentrations of AF and is classified as a food for specific medical purposes (FSMP) in the EU and US. Salovum® has been used in clinical trials for gastroenteritis[17], Menieres disease[18] and inflammatory bowel disease[19] with no toxicity reported. An active part of AF has been identified and synthesized into a 16 amino acid peptide; AF16. AF16 has shown promising effects against cerebral edema and increased ICP in animal models of herpes encephalitis and TBI.[20,21] . Probably due the short half-life of AF16 and other active peptides, no reliable method to measure AF or AF derived peptides is currently available. Currently, two case-series, one from our department, have been published, demonstrating possible beneficial effects of Salovum® administration on ICP in adults with severe TBI.[22,23]
Based on our initial findings, a trial was designed to evaluate the effect of Salovum® in adults with severe TBI. The primary outcome of this trial was 30-day mortality and the secondary outcomes were treatment intensity level (TIL) and ICP.[24]

Methods

Trial design

The full research protocol have been published previously.[24] The trial is a single-center, investigator initiated, prospective, double-blinded, placebo-controlled trial conducted at the Division of Neurosurgery, Tygerberg Academic Hospital, Cape Town, South Africa. The trial was designed by the sponsors, conducted by the investigators and monitored by an external monitoring body (see *supplementary appendix*). The trial was supported by unrestricted grants from Lantmännen AB and regular funding from Lund University and Region Skane. The funders had no influence on the design or conduct of the trial, and were not involved in

datacollection, analysis, the writing of the manuscript nor the decision to submit it for publication. The trial protocol (available at supplementary material) was approved by the regional ethics committee, Human research ethics committee (HREC M16/10/040) Stellenbosch University, Stellenbosch, South Africa, and conducted in accordance with the Declaration of Helsinki. Due to a misunderstanding, the first study participant was recruited on September 18, 2017, which was two days before the final submission release of the research protocol on www.clinicaltrials.gov (NCT03339505). A total of 1 participants were recruited before the final release of the protocol. In order to avoid bias when recruiting from multiple centers with different treatment algorithms and referral patterns [3,25] we chose to conduct the trial in a single tertiary TBI center with basic standard of care e.g.

logistics for mechanical ventilation and ICP monitoring.

Participants

Adults with severe traumatic brain injury and a GCS of 6-8 after resuscitation, admitted to the neurointensive care unit at Tygerberg Academic Hospital and planned for invasive ICP monitoring, were recruited between September 2017 and February 2022. In December 2018 an amendment to the ethical application was approved, stating that recruitment of participants for the trial could be performed with delayed consent from next of kin. The reason for this was that the investigators found that relatives of people with no current address were difficult to find within the time frame for inclusion into the trial, thereby creating a potential selection bias. A complete list of inclusion and exclusion criteria is provided in the *supplementary appendix.*

Standard treatment

The NICU at Tygerberg Academic Hospital use a standardized treatment protocol which is in accordance with the Brain Trauma Foundation guidelines.[26] The treatment is aimed at controlling ICP, using one or all of the following; 30° head elevation, sedation-including barbiturates, hyperosmolar therapy with Mannitol or NaCl and CSF-drainage. Surgical hematomas are evacuated and decompressive hemicraniectomy may be performed in conjunction with the primary surgery or when medical treatment has failed at controlling ICP.

Trial procedures

Participants were randomly assigned in a 1:1 ratio to either the Salovum® group or the control group. Block randomization with block sizes ranging from 4-6 was used.

The participants were assigned either Salovum® or placebo egg yolk powder. Participants were given the substance during 5 days, or until the ICP monitor was removed, if this occurred earlier. Based on previous clinical trials and case series in TBI, substance was administered as 10 g/dosage (participants <60kg), 14 g/dosage (participants 60-80 kg) and 17 g/dosage (participants >80kg),via a naso-gastric feeding tube every four hours. During the trial, ICP and mean arterial blood pressure (MAP) were recorded hourly, and Treatment Intensity Level (TIL) every 24 hours.

Outcomes

The primary outcome was 30-day mortality. The secondary outcomes were ICP in mm Hg and TIL. All safety outcomes were based on adverse-event reporting. The trial was monitored by an external monitoring body (Novotech, Gauteng, SA). An independent safety and monitoring committee (DMC) performed an unblinded interims analysis at 93 included participants and a decision to finalize the trial at the prescribed number of participants was made. Plasma samples were collected from participants were consent was given from next of kin. The samples were intended for exploratory endpoints and are thus not addressed in the present trial.

Statistical analysis

Baseline comparisons between the groups were computed with Mann Whitney U test or Fisher test for continuous or binary data respectively. Non-baseline characteristics of the trial groups as hospital stay, NICU stay, substance dose, time to intervention, decompressive craniectomy, treatment days and reason for ending ICP monitoring were analyzed as for baseline characteristics. Based on previous case series, a formal sample size estimate of 100 participants was made using the chi-squared power test (pwr.chisq.test in R). An estimated sample size of 100 participants would allow us to detect a reduction of mortality of 25% percent points, a reduction of ICP of 5 mmHg and a reduction of 5 points on the TIL scale. As no differences were

noted for ICP comparisons as prespecified, we extended the analysis to time over ICP 22, 25 and 30 mmHg. In the original SAP, also analysis of difference in ICP and TIL between alive and dead participants and within trial groups were specified but these showed no significance. Also comparisons within groups before and after study intervention were pre-specified but had to be omitted because very few data were available before study intervention. 30 day mortality was calculated with the prespecified chi square test, but calculation of relative risk was also added as this method is recommended for binary outcomes of e.g. mortality. Both ICP and TIL were handled as nonparametric due to non-Gaussian distribution. ICP and TIL comparisons were calculated with the non-parametric Mann-Whitney U test. All computations were performed with the free R software [27]. See also SAP for the trial in the *supplementary appendix*.

Results

Participant demographics

From September 22, 2017 a total of 100 participants with isolated TBI were enrolled (Figure 1). A total of 49 participants were randomly assigned to the Salovum® group and 51 participants to the control group.

Male gender was predominant and the mean age was 33 years. Trauma was distributed as no mass lesion (2%).

subdural hematomas (23%), isolated contusions (41 %) and mixed trauma types (34%). There were no statistically significant differences for baseline characteristics as age, gender, trauma type nor for time to intervention, treatment time or hospital or neurointensive care unit (NICU) stay (Table 1 and Table 2).

Outcome

30-day mortality

30-day mortality was 10/49 participants in the Salovum® group and 20/51 participants in the control group. This difference was statistically significant (p=0.040) using Chi-Squared test and the relative risk ratio was 0,52 (CI:

0.271-0.997).

ICP and TIL

ICP in the Salovum® group and control group measured as mean ICP and/or total time with ICP above thresholds of 20, 22, 25 and 30 respectively were not significantly different. However, mean ICP during the whole intervention period was below 20 mmHg in both groups.

There was no significant difference between the Salovum® and control group for total TIL during the trial period or for daily TIL during treatment (Table 2). However, in alive participants, the median for TIL on day 3 was 4 in the Salovum® and 8 in the control group, this was statistically significant (p=0.041) using Man Whitney U.

DC and treatment days

There was no significant difference between the number of decompressive craniectomies performed in the groups (Salovum®; 16/49, control 20/51). 34/36 decompressive craniectomies performed were primary.

There was no significant difference in number of treatment days. Number of days with substance was 3,8 in the Salovum® group and 3,5 in the control group due to that treatment was stopped prematurely if the ICP monitor was removed before 5 days.

Safety

Adverse events and serious adverse events were noted in the CRF daily, by the physician responsible for the participant. The daily chart states: “Do you consider that there is a reasonable possibility that an adverse event has been caused by the study compound? This question was answered daily.

Adverse events were defined as skin rash and hives, Serious adverse events were defined as a serious anaphylactic reaction with hypotension and bronchospasm, requiring medical intervention with corticosteroids and/or vasopressors. No other adverse events were reported. Allergic reactions to egg yolk are, according to literature, extremely rare. [28]

One participant was unblinded because the trial substance was accidentally locked up over the weekend, This participant was excluded from the trial.

Discussion

In this randomized, controlled and double blinded single center trial, oral administration of Salovum®, an egg yolk powder containing increased amounts of the endogenous protein antisecretory factor, significantly reduced mortality in participants with severe traumatic brain injury.

Multiple randomized controlled trials on patients with severe TBI have previously have failed to significantly reduce mortality, morbidity or improve long time outcome using interventions as hypertonic saline, , erythropoietin, progesterone, tranexamic acid and hypothermia.[29] For decompressive craniectomy, one trial has succeeded in showing a significant decrease in mortality at 6 months[30] Most recently, the CRASH III trial did show an effect on 30-day mortality in participants with moderate traumatic brain injury[11], tentatively due to reduction of secondary hemorrhagic events in this participant group. However, the administration of tranexamic acid had no effect on mortality in participants with severe traumatic brain injury with a mortality of 39,6 % in the intervention group versus 40.1 % in the control group.

RCTs in traumatic brain injury are hampered by several factors such as heterogeneity in respect to both type of trauma, recruitment policy, catchment area and prevailing treatment algorithms. In tertiary NICUs, off label use of pharmacological and non-pharmacological interventions are frequent outside the treatment algorithms[31] In order to reduce these potential biases, we conducted the trial in a single, tertiary TBI center with a high caseload of severe TBI.

AF is an endogenous proteasomal protein found in humans and many other species. When administered to humans and rodents as whole protein and peptides it has several effects. These effects could originate from either proteasomal modulation or by mechanisms outside the proteasome. However, unlike other proteasome modulators, no side effects of Salovum® have been noticed in various reports including two case series of severe TBI. In the present trial 49 participants were administered Salovum® for a median of 3.8 days without signs of adverse effects which is in line with previous reports[22,23]. In a phase 1-2 trial of participants with primary glioblastoma, Salovum® was given at an equivalent dose (1g/kg) for more than 50 days without signs of side effects or toxicity further strengthening the case for safety.[32]

Hospital and post-treatment mortality rates (hospital case fatality) of severe TBI patients vary substantially between 10-70% in reports from retrospective studies, RCTs and the very few population-based studies.[33] This notion makes comparisons and reference data ambiguous depending on inclusion and exclusion criteria. In RCTs, there can also exist additional causes of non-representativeness as exclusion after prior

inclusion, standard or logistics-based exclusion criteria from centers involved or that eligible participants are not admitted at the trial center due to lack of transportation. In the present trial, mortality at 30 days was 20% in the Salovum® group and 39% in the control group. The mortality in the control group corresponds well with the numbers from large, randomized trials from low-income countries such as the CRASH trial and available data from South Africa.[34,35,] There was a significant difference in treatment intensity on day 3 for participants that survived, with a median of 4 in the Salovum® group versus 8 in the control group. Since the number of days where patients were monitored for ICP and treated with neurointensive care is low in this trial, this difference likely reflects the patients that needed neurointensive care the most. Day 3 after TBI may be the day where swelling of the brain is at its worst. This indicates that Salovum® does not only prevent deaths, but diminishes the need for neurointensive care interventions as well.

There was no significant change in ICP neither as mean nor as time over 20, 22, 25 and 30 mmHg between the groups. Several trials have put forward that using ICP monitoring does not influence the outcome in moderate or severe TBI.[36,37] In the present trial, several explanations can be proposed; firstly, that the hourly measurements of ICP do not reflect the true variation of ICP . Secondly, that the sample size is too small to detect significant changes in ICP as there were numerically longer periods of increased ICP in the control group. Thirdly, that the standard of care actually controlled ICP in both groups, and fourthly that the mode of action after exogenous administration of antisecretory factor in TBI patients indeed does not only involve control of ICP. The high number of primary decompressive craniectomies in both Salovum® and control groups could also have blunted ICP levels.

The causation of death in severe TBI has traditionally been associated with uncontrollable ICP, specifically during the first 48 days after the initial trauma[9,38]. However, the rationale for this statement is mostly supported by circumstantial evidence without no real causative proof as ICP monitoring may be coupled to other measures to manage severe TBI. Randomized trials have both shown that ICP monitoring reduces mortality and morbidity or that it does not influence outcome [37,39]. This may indicate that ICP management does not per se reduce mortality or morbidity but, depending on the specific trial design and recruitment policy, may or may not correlate to outcome.

The trial protocol did not specify a minimal time of treatment, but due to the requirement for ICP monitoring during trial intervention, the treatment length was dictated by the clinical indications for ICP monitoring. In

both arms intervention time was slightly less than 4 days. This implies that even a shorter intervention time than that applied in the previous case series of Salovum® administration to patients with severe TBI significantly reduces mortality.

Other factors that could be dependent on trial intervention such as days in NICU and days in hospital were likewise similar between the treatment arms. This indicates that these parameters are neither correlated to nor causative of the reduced mortality. A high proportion of the population served by Tygerberg Academic Hospital is from a low socioeconomic background. Resource limitations dictate that only participants unable to be discharged are referred to a rehabilitation facility which restricts the short and long term follow-up. There was no difference in number of days spent in hospital between the groups, even though 10 more participants died in the control group, which may suggest that days in hospital in the control group would actually be higher than in the Salovum® group.

Due to resource limitations at the trial site, almost only patients with a post-resuscitation GCS of 6-8 qualify for admission to NICU. To minimize the risk of an uneven distribution, we chose to only include these participants. We contend that while the present trial may be generalizable to the population experiencing severe TBI in many countries, it may not necessarily reflect the situation in countries with advanced neurointensive care. Such settings may admit patients with lower Glasgow Coma Scale (GCS) scores and may utilize a more varied care, thus introducing a greater degree of heterogeneity to the participant population.[40]

The study's main advantage lies in its use of the 30-day mortality rate as an objective indicator of treatment efficacy, which is a widely accepted and utilized approach. The trial's limitations include the relatively small sample size of 100 participants, which may restrict the generalizability of the findings in relation to TBI interventions. Additionally, the study did not collect data on outcomes beyond the predetermined 30-day period, and thus, neurological recovery could not be assessed beyond this time point.

Conclusion

Administration of Salovum in conjunction with conventional treatment to adults with severe traumatic brain injury (TBI) resulted in a statistically significant decrease in mortality rates as compared to the control group. While the exact mechanisms of action of Salovum and antisecretory factor remain unclear, the findings of this study indicate a substantial and noteworthy impact on TBI outcome.

	Active	*Placebo*	*Total*	*Stat*
Participants	49	51	100	NA
Age (Mean/SD)	32±10	34±11	33±10	NS
Gender (F/M)	1/48	3/47	4/96	NS
Trauma mechanism				
Contusion				
SDH				
NML	17/49 (35)	24/51(47)	41/100 (41)	NS
Mixed	12/49 (24)	11/51 (24)	23/100(23)	NS
	1/49 (2)	1/51 (2)	2/100	NS
	19/49(39)	15/51(29)	34/100(34)	NS

Table 1. Participant demographics

SD = Standard Deviation, F/M = Female/Male, SDH = Subdural hematoma, NML = No mass lesion

	Active	*Placebo*	*Total*	*Stat*
Participants	49	51	100	NA
NICU stay (Mean(SD))	10.2±4.5	10.5±6.7	10.4±5.7	NS
Hospital stay (Mean(SD))	28.1±20.4	28.3±32.3	28.2±26.9	NS
Substance dose	14.6±1.2	14.8±1.4		NS
Time to intervention (Mean(SD))	32.9±11.6	32.9±8.7	32.9±10.7	NS
TIL d1 (median/(QR))	6(3.5-8.5)	7(7-9)	6.7(5.2-9.2)	NS
TIL d2 (median/(QR))	6(3.5-8.5)	6(3.5-8.5)	6(3-9)	NS
TIL d3 (median/(QR))	6(3.5-8.5)	8(5.3-10.8)	6(3-9)	NS
TIL d3 -alive (median/(QR))	4	8	-	P=0.041
TIL d4 (median/(QR))	5(1.9-8.1)	7(5.5-8.5)	6(3.3-8.8)	NS
TIL d5 (median/(QR))	5.5(2.8-8.3)	7(6-8)	6.5(4.9-8.1)	NS
TIL total DECRA[1]	6(3.4-8.6)	6.5(4-9)	6(5.8-7.2)	
ICP > 20 (mean/SD hours)	17	19	34	NS
ICP > 22 (mean/SD hours)	30.7±29.2	30.8±29.7	30.8±29.3	NS
ICP > 25 (mean/SD hours)	21.9±25.8	24.4±26.7	23.0±26.2	NS
ICP > 30 (mean /SD hours)	12.8±18.1	14.5±19.1	13.7±18.5	NS
ICP total	4.8±8.6	5.6±12.9	5.2±11.0	NS
CPP (mean/SD)	19.4±6.5	18.7±6.3	19.0±6.4	NS
Treatment days (mean/SD)	72.0±9.7	72.0±10	72.1±9.8	NS
ICP monitoring ended[2]	3.8±1.1	3.5±1.3	3.6±1.2	NS
Mortality	29/4/16	31/2/18	60/6/34	NS
	10/49	20/51	30/100	
	RRisk			0.52(0.271-0.997)
	ChiSquare			P=0.040

Table 2. Primary and secondary outcome

[1]Decra = Decompressive Craniectomy, [2]Controlled ICP/Uncontrolled ICP/Other

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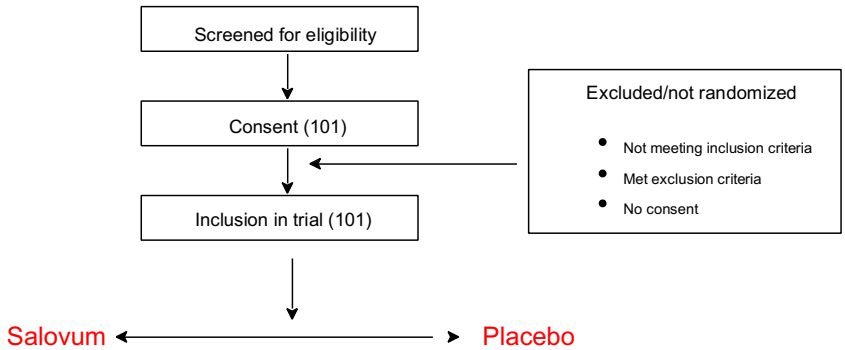

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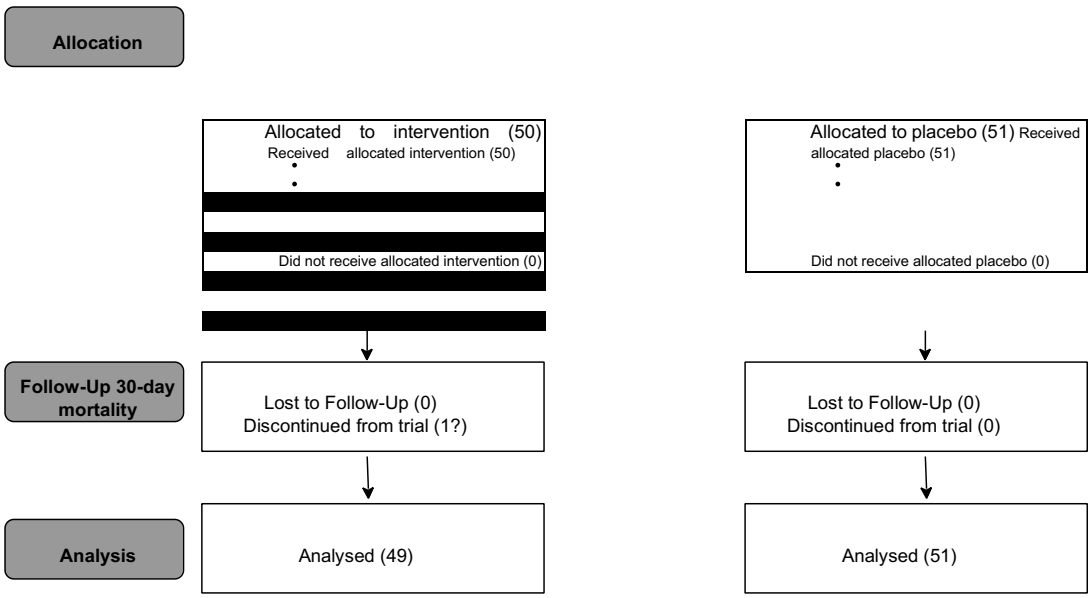
Enrollment


Allocation
Allocated to intervention (50)
Received allocated intervention (50)
Did not receive allocated intervention (0)
Allocated to placebo (51) Received allocated placebo (51)
Did not receive allocated placebo (0)
Follow-Up 30-day mortality
Lost to Follow-Up (0)
Discontinued from trial (1?)
Lost to Follow-Up (0)
Discontinued from trial (0)
Analysis
Analysed (49)
Analysed (51)

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