SINGLE-CENTER TBI TRIAL CLUJ

FOUNDATION OF THE SOCIETY FOR THE STUDY OF NEUROPROTECTION AND NEUROPLASTICITY

UMF IULIU HAȚIEGANU UNIVERSITY OF MEDICINE AND PHARMACY CLUJ-NAPOCA

RoNEURO INSTITUTE FOR NEUROLOGICAL RESEARCH AND DIAGNOSTIC

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Protocol Title

Cerebrolysin Trial in Neuroprotection and Neurorecovery after Traumatic Brain Injury (CAPTAIN – RO)

Sponsor: Foundation for the Study for Nanoneuroscience and Neuroregeneration

Investigator Initiated Study

Confidential Information

Coordinating Investigator

Prof. Dr. Muresanu Fior Dafin

Trial Site

Clinical Center: Emergency Clinical County Hospital Cluj-Napoca, Neurology Clinic and Neurosurgery Clinic

Signature Pages

I confirm that I have read this protocol and agree that it contains all of the necessary information required to conduct the study and I agree to conduct it as described. I will work according to this protocol and adhere to the ethical principles stated in the latest version of the Declaration of Helsinki, the applicable guidelines for the International Conference on Harmonisation - Good Clinical Practices and local regulations.

Clinical Study Coordinator

Prof. Dr. Muresanu Fior Dafin

Sub-investigators

Prof. Dr. Florian Ioan Stefan

Dr. Stan Horatiu

Dr. Stan Adina

Dr. Birle Codruta

Dr. Slavoaca Dana

Dr. Lacan Sorin

Dr. Rosu Olivia

Dr. Jemnea Nicoleta

Dr. Blesneag Alina

Dr. Balea Maria

Psyhologist Muresanu Ioana

Psychologist Costin Cezara

Biometrician

Johannes C. Vester

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1. Background information

In addition to mechanical damage, traumatic brain injury (TBI) induces the impairment of cerebrovascular autoregulation, metabolic dysfunction, inflammation and neuroplasticity as well as neuronal and astroglial death. All these process are interconnected and complicate the TBI neurodegenerative process with a significant risk for cognitive decline later in life. For example, inflammation and its primary detrimental effects cause increases in tissue regeneration and plasticity (Morqanti-Kosmmann, 2002). Several mechanisms are considered responsible for the brain's post-injury reparative process, and these mechanisms function at both the microscopic and macroscopic levels. The most important described mechanisms are neurogenesis, synaptic plasticity, compensation, resolution of diaschisis, reorganization within functional networks and cross-modal plasticity as well as dendritic and axonal sprouting (Nudo, 2001; Duffau, 2006; Thompson, 2006). However, neuroplasticity is a complex and balanced process, and this process also involves pathological plasticity, which consists of the abnormal reorganization of damaged networks, the alteration of neurotransmission and alterations in molecular signals (Giza and Prinz, 2006). All these processes interact with other factors, such as the maturation grade of the brain (Giza and Prinz, 2006), gene expression (Diaz-Arrastia, 2006; Jordan, 2007), environmental factors (Whiteneck, 2004), exercise (Griesbach, 2009; Hoffman, 2010) and self-directed neuroplasticity (Schwartz, 2005). Neurotrophic factors play a key role in neuroprotection and plasticity by modulating neuron survival (Barrett, 1994; Kurokaua, 1999; Kim, 2005) and by enhancing neurogenesis and neurotrophicity (Mahmood, 2002; Kim, 2010).

1.1 Treatment with neurotrophic factors

After an acute brain lesion, there is always an endogenous continuous brain defense response consisting of two main sequences: an immediate one that aims to reduce brain damage, known as *neuroprotection*, and a later one that aims to repair the brain damage, known as *neurorecovery*, which is based on *neurotrophicity*, *neuroplasticity* and *neurogenesis*.

Neurotrophic factors are the most important endogenous molecules involved in brain protection and recovery. They are modulating molecules with immediate pleiotropic neuroprotective activity and long-term multimodal effects. Due to this unique therapeutic effect, the principle oftreatment with neurotrophic factors is based on, in addition to acute administration, repetitive periods of treatment. This principle is applied to stimulatelong-term endogenous capacity for neurorecoverythat is induced by neurotrophicity, neuroplasticity and neurogenesis.

Cerebrolysin has a neurotrophic factor-like activity based on the four important endogenous neurobiological processes: neurotrophicity, neuroprotection, neuroplasticity and neurogenesis. Additionally, this activity may have similar effects as the real sequence of endogenous post-lesional regulation.

Experimental studies (Sharma, 2010) in rat models have demonstrated the beneficial effects of Cerebrolysin on blood-brain barrier permeability, neuron loss and brain edema formation. Cerebrolysin-treated rats also showed improved sensory-motor function.

Cerebrolysin has previously beenshown to exert a beneficial effect as part of the initial therapy (Wong, 2005) in severe and moderate acute traumatic brain injury in terms of neurological functions and cognitive performance. When neuro-motor rehabilitation is taken into consideration, administering this modern multimodal molecule can speed recovery (Onose, 2009). A recent study on patients with mild TBI tested the efficacy of Cerebrolysin treatment on cognitive outcome. There were included 92 patients,

from which 32 completed the trial. 17 patients received Cerebrolysin and 15 patients reiceved placebo. The scales used for the measurement of cognition function were Mini- Mental Status Examination (MMSE) and Cognitive Abilities Screening Instrument (CASI). The results of the study indicated that Cerebrolysin treatment improves CASI scores at 3rd month after the injury, especially for lon-term memory and drawing function (Chen, 2013).

1.2. Treatment with repetitive trancranial magnetic stimulation

Transcranial magnetic stimulation (TMS) operates on Faraday's principle of electromagnetic induction.

There are several studies regarding a beneficial role of repetitive TMS (rTMS) in neurorehabilitation, including in TBI patients such as: motor recovery including spasticity, depression treatment, and speech rehabilitation (Fitzgerald, 2011; Bonni, 2013; Castel-Lacanal, 2013, Krewer, 2014).

According with the paradigm that rTMS effects are based on the induction of potential actions, most of the studies regarding the effect of rTMS on cognition are based on the idea that stimulating with different frequencies on a certain area, will activate/deactivate specific regions or even networks and so will enhance/inhibit specific functions. However, the experimental studies, showed that rTMS influence also the molecular and cellular level, influence that can be independent from the induction of action potentials (Rodger and Sherrard, 2015). One of the key targets by which rTMS improves cognitive function appears to be BDNF, magnetic stimulation having a stimulating effect on its genetic expression (Wang, 2015).

Cognitive treatment with rTMS was tested in Alhemer's diseases and the combination between rTMS combined with cognitive treatment seems to have a beneficial effect upon cognition (Bentwich, 2011; Rabey, 2013). Regarding rTMS as an add-on to pharmacological treatment in cognitive rehabilitation, there are too few data to establish its efficacy. (Haffen, 2012). There are several studies on rTMS as add-on

treatment in depression, with good results when the magnetic stimulation was performed with high frequencies (Mogg, 2008; Chen, 2013). In TBI, this study is the first one in order to test the efficacy of the combining treatment rTMS + pharmacological intervention (CRB) in cognitive rehabilitation.

Even that rTMS has extensively been tested in patients with Alzheimer's disease (Liao, 2015), in TBI there is a little date about rTMS in cognitive rehabilitation.

A recently published article described the effects of rTMS in various post-concussive symptoms, including cognititve dysfunction. There were administrated 20 seesions of RTMS (20 x 5-sec trains; 10-Hz at 110% threshold), with clinical and functional magnetic resonance imaging (fMRI) assessments before and after intervention, and clinical assessment at 3-month follow-up, in 15 patients with mild TBI. The results showed cognitive improvement and a decrease of post-concussive symptoms (Koski, 2014). The use of high frequency stimulation on DLPFC is concordant with data existing in literature in TBI, dementia, stroke and depression (Miniussi, 2011; Ahmed, 2012; Kedzior, 2012; Luber, 2014; Nardone, 2014; Koski, 2014, Nadeau, 2014). There is a single case presented in literature with rTMS treatment for depression in TBI which represent a common complication in this pathology and is independently associated with decreased cognitive functions (Fitzgerald, 2011).

The main area of concern regarding the use of TMS in stroke or TBI patients has been the triggering of kindling activity, which can induce seizures Seizure induction, however, has rarely been reported following rTMS, and animal studies have shown that there is no clear evidence that rTMS leads to increased seizure susceptibility (Miniussi and Rossini, 2011).

1.3. Biomarkers

Taking in account the dynamic and complex state of the brain after a traumatic injury, the estimation of outcome represents a real challenge; this estimation is possible

only by understanding the extent of pathological mechanisms including inflammation and astro-neuronal damage and by determining the genetic background of each patient.

Biomarkers represent a potential evaluation tool for TBI patients that can serve as an internal indicator of cerebral damage with the added advantage that these markers can provide dynamic information about cellular, biochemical and molecular changes.

Despite the fact that, until now, the study of biomarkers in TBI has not identified clinically significant biomarkers (The *Clinical Practice Guideline for the Management of Concussion/Mild Traumatic Brain Injury, 2009*), recently published articles have emphasized the idea that a realistic approach can be made only by combining multiple types of biomarkers with other investigative tools (imaging, outcome scales, genetic polymorphisms) (Kövesdi, 2010; Dash, 2010; Li, 2010; Mondello, 2011). Additionally, there is an increasing interest in using biomarkers as treatment monitoring tools (Mondello, 2011).

S100 Calcium-Binding Protein (S100). The members of the S100 protein family are multifunctional proteins with a regulatory role in a variety of cellular processes. They exert their actions usually through calcium binding, although Zn^{2+} and Cu^{2+} have also been shown to regulate their biological activity. Through their interaction with several effector proteins within cells they are involved in the regulation of a variety of cellular processes such as contraction, motility, cell growth and differentiation, cell cycle progression, transcription, structural organization of membranes, dynamics of cytoskeleton constituents, protection from oxidative cell damage, protein phosphorylation and secretion (Sedaghat and Natopoulos, 2008). *S100B* is a Ca^{2+}-binding protein expressed by astrocytes, oligodendrocytes and Schwann cells and therefore can be considered a marker of astroglial damage. In addition to the nervous system, S100B can also be found in chondrocytes and adipocytes and is a marker of malignant melanoma (Harpio , 2004; Yardan , 2011) and ischemic heart disease (Mazzini, 2009, Snyder-Ramos, 2009). While there are extraneuronal sources of this protein, recent studies have shown that these sources do not influence the

serum levels of S100B in TBI (Pham, 2010). In terms of TBI, S100B is characterized by low specificity, high sensibility and a pooled negative predictive value (Unden, 2010, Leidel, 2011). Until now, a direct correlation between the levels of S100B and the evolution of TBI patients had not been demonstrated (Biberthaler, 2001; Bazarian, 2006; Raynei, 2009; Ballender, 2010). However, the predictive value of S100B was found to be significantly better when it was correlated with other data such as other biomarkers, CT, MRI, GCS, clinical data, disability scales, and cognitive scales (Kövesdi, 2009; Kleindinest, 2010; Vos , 2010; Wiesmann, 2010; Mondello, 2011). There are also studies on S100, that corellate that biomarker with cognitive impairement in TBI (de Boussard, 2005) and unfavourable outcome (Yan, 2014).

Neuron-specific enolase(NSE) is a glycolytic isoenzyme that is expressed in all neuronal cell including neuroendocrine cells.The levels of NSE in the serum and cerebrospinal fluid have been used as markers for injury and neuronal cell death. Regarding screening in TBI patients, the use of NSE as a biomarkerhas several serious limitations, especially in those patients with polytrauma because extracranial trauma, bleeding, liver damage, or kidney damage may also increase the levels of NSE. In patients with isolated head injuries, NSE levels were negatively correlated with the Glasgow Coma Scale scores, and higher levels were correlated with an increased rate of mortality (Guzel, 2008). Additionally, increased serum concentrations of NSE and S100B within the first 3 days in patients with diffuse axonal injury (DAI) were associated with poor outcomes despite mild CT findings (Chabock, 2012). Cerebral hypoxia may be predicted by elevated serum levels of biomarkers, including NSE, S100B and glial fibrillary acidic protein (GFAP) (Stein, 2012).

1.4. Genetic polymorphism

Several genes have been found to a play a role in the outcome of TBI patients, e.g., apolipoprotein E (APOE) promoter, catechol-o-methyltransferase, dopamine D2

receptor, p53, and CACNA1A as well as inflammatory genes. Studies suggest that inflammatory genes (such as IL-1, TNFα and TGFβ) may be associated with apoptosis-related genes (Sojo, 2010). APOE is the most studied gene in TBI, and several clinical studies have demonstrated a correlation between the APOE ε4 allele and poor outcome (Friedman, 1999; Samatovicz, 2000; Alexander, 2007). Additionally, APOE is considered a genetic risk factor for the development of Alzheimer Disease later in life. Significantly, patients with this allele who die from a head injury are four times as likely to present evidence of cortical Aβ deposition as those without APOE (Nicoll, 1995; Gentleman, 2004). In a recent study, patients with the APOE ε4 allele were more predisposed to brain cellular damage measured by S100B and NSE levels (Olivecrona, 2012), but more studies are necessary to identify a genetic polymorphism that can help in the prognosis of TBI.

1.5. Eye tracking

Eye tracking implies looking at targets on a computer screen while a special system records eye movements and changes in pupil diameter in response to the movements of the targets. Patterns of eye movements offer information about what a person is processing at a particular moment and the time course of processing visual information. Because the saccades and fixations recruit the same neuroanatomical circuitries as attention, involving the dorsolateral prefrontal cortex (DLPFC), and also because eye movements can be influenced by emotions, by it has been suggest that eye-tracking can be used as a biomarker for cognitive dysfunction (Munoz, 2002; Seligman and Giovannetti, 2015).

1.6. Quantitative electroencephalogram (qEEG)

qEEG technique, by its highly accurate temporal resolution, provides a unique window to assess brain dynamics underlying cognitive functions. qEEG has been use for the evidentiation of Alzheimer disease (Chen, 2013), vascular dementia (Neto,

2015), Parkinson's disease associated dementia (Caviness, 2015). It also can reveal abnormalities in preclinical stages of cognitive impairment (When, 2015). Typical EEG findings in patients with Alzheimer's disease (AD) are increased slow wave and decreased fast wave activities. Also in TBI patients, qEEG was used as a treatment monitoring tool in several studies on CRB (Alvarez, 2000; Alvarez, 2003; Alvarez, 2008).

2. **Methodology:** This pilot study is the first to compare the cognitive outcome in TBI after the two types of treatment: CRB and CRB + rTMS.

- CRB treatment: single center, prospective, randomized, double-blind, placebo-controlled clinical trial with 4 visits (screening baseline, day 10, day 30, day 90). Eligible 140 patients will be consecutively enrolled in the study and will be randomly devided in two groups (4:3) using computer-generated random numbers. The first group will receive pharmacological treatment with CRB, while the second group will receive a placebo of identical appearance.
- CRB + rTMS treatment: single center, prospective, randomized, open-label clinical trial with 4 visits (screening baseline, day 10, day 30, day 90). Eligible 20 patients will be consecutively enrolled in the study, after the complete enrollment of the patients which will receive only CRB/placebo.

2.1 Treatment Duration: Three months

2.2 Study Population:

TBI patients with Glasgow Coma Scale (GCS) scores between 7 and 12.

2.3. Number of Subjects:

- CRB treatment group: 140 patients will be enrolled, divided into 2 arms: 80 patients in the active treatment group (CRB) and 60 patients in the control group.

- CRB + rTMS treatment group: 20 patients that will receive Cerebrolysin plus rTMS.

All the 160 patients will be assessed by biomarkers, genetic polymorphism and neuropsychological tests. The last 70 patients will perform also eye-tracking and qEEG.

3. Study Objective(s):

3.3. Primary objectives

- The primary objective is to assess the effects of Cerebrolysin on general and neurocognitive outcomes after traumatic brain injury. Three ensembles of appropriate single efficacy criteria for Day90, Day 30 and Day 10 shall be tested according to the principle of *a priori* ordered hypotheses by a multivariate, directional test approach, reflecting the "global status of patients in TBI

3.4. Secondary Objectives:

- To assess the efficacy of the combining rTMS and Cerebrolysin treatment upon neurocognitive outcomes (measured using clinical scales: Processing Speed Index, Stroop Color-Word Test – Victoria Version, Color Trails Test, Digit Span, Finger Tapping Test, Mini-Mental State Examination, Hospital Anxiety and Depression Scale) at 90 days after TBI.
- To investigate whether the levels of serum biomarkers (NSE and S-100) can independently predict the clinical outcome (measured using clinical scales: Processing Speed Index, Stroop Color-Word Test – Victoria Version, Color Trails Test, Digit Span, Early Rehabilitation Barthel Index, Finger Tapping Test, Mini-Mental State Examination,Hospital Anxiety and Depression Scale, Extended Glasgow Outcome Scale) at 90 days after TBI.
- To test whether the levels of serum biomarkers (NSE and S-100) can

independently predict the clinical outcome (measured using clinical scales Mini-Mental State Examination, Processing Speed Index, Stroop Color-Word Test – Victoria Version, Early Rehabilitation Barthel Index, Glasgow Outcome Scale Extended) at 10 days after TBI.

- To investigate whether genetic polymorphisms (APOE ε2-4) can independently predict clinical outcomes (measured using clinical scales: Processing Speed Index, Stroop Color-Word Test – Victoria Version, Color Trails Test, Digit Span, Early Rehabilitation Barthel Index, Finger Tapping Test, Mini-Mental State Examination,Hospital Anxiety and Depression Scale, Extended Glasgow Outcome Scale) at 90 days after TBI.
- To investigate whether genetic polymorphisms (APOE ε2-4) can independently predict clinical outcomes (measured using clinical scales Mini-Mental State Examination, Processing Speed Index, Stroop Color-Word Test – Victoria Version, Early Rehabilitation Barthel Index, Glasgow Outcome Scale Extended) at 10 days after TBI.
- To investigate whether BPRS can independently predict clinical outcomes (measured using clinical scales Mini-Mental State Examination, Processing Speed Index, Stroop Color-Word Test – Victoria Version, Early Rehabilitation Barthel Index, Glasgow Outcome Scale Extended) at 10 days after TBI.
- To investigate whether genetic polymorphisms associated with the levels of serumbiomarkers (NSE and S-100) and BPRS can predict outcomes (measured using clinical scales: Processing Speed Index, Stroop Color-Word Test – Victoria Version, Color Trails Test, Digit Span, Early Rehabilitation Barthel Index, Finger Tapping Test, Mini-Mental State Examination,Hospital Anxiety and Depression Scale, Extended Glasgow Outcome Scale) at 90 days after TBI.
- To investigate whether genetic polymorphisms associated with the levels of serum biomarkers (NSE and S-100) and BPRS can predict outcomes (measured using clinical scales Mini-Mental State Examination, Processing Speed Index,

Stroop Color-Word Test – Victoria Version, Early Rehabilitation Barthel Index, Glasgow Outcome Scale Extended) at 10 days after TBI.

- Comparison between CRB treatment versus CRB + rTMS treatment upon cognitive neurocognitive outcomes (measured using clinical scales: Processing Speed Index, Stroop Color-Word Test – Victoria Version, Color Trails Test, Digit Span, Finger Tapping Test, Mini-Mental State Examination, Hospital Anxiety and Depression Scale) at 90 days after TBI.
- To investigate Eye-tracking as a prognostic factor of cognitive dysfunction and as a treatment monitoring tool in TBI patients.
- To investigate the changes in qEEG activity frequency bands related to treatment with CRB and rTMS.
- To investigate the qEEG data analyses in order to study the neurophysiological patterns of cognitive dysfunction.
- Mortality of any cause
- rTMS adverse events

4. **Study Settings:**

- Emergency Clinical County Hospital Cluj-Napoca, Neurology Clinic and Neurosurgery Clinic.
- RoNeuro Institute for Neurological Research and Diagnosis

5. **Treatment Description.**

5.1. Cerebrolysin treatment

- IV Cerebrolysin (CRB), 50 ml daily for first 10 days (days 1-10); 20 days off, followed by CRB 10 ml daily for 10 days: days 31-40 and 61-70 diluted in

0.9% saline solution up to 250 ml.

- Placebo: 0.9% saline solution, 250 ml.

5.2. Cerebrolysin + rTMS treatment

- IV Cerebrolysin (CRB), 50 ml daily for first 10 days (days 1-10); 20 days off; followed by CRB 10 ml daily for 10 days: days 31-40 and 61-70 diluted in 0.9% saline solution up to 250 ml.

- The rTMS treatment will be performed 10 consecutive working days: from days 31-40 (+/-4 days) and 61-70 (=/- 4 days).

  A device MagPro X100 (MagVenture, Denmark) will be used for repetitive stimulation with a figure-8 coil (C-B60). The coil will be hold tangential to the scalp with the handle pointing upwards. For localizing the left dorsolateral prefrontal cortex (DLPFC) stimulation, we will use the 10-20 EEG system by placing the coil at F3. The stimulation parameters for DLPFC will be set up at 10 Hz and 1,200 stimuli/day, with an intensity of 120% of resting motor treshold. The resting motor threshold is determined at the beginning of the first treatment session and is defined as the minimal intensity at which at least five of 10 motor evoked potentials are 50 μV in amplitude in the policis abductor brevis.

6. Inclusion and screening criteria

6.1. Glasgow Coma Scale

The Glasgow Coma Scale (GCS) was first introduced in the 1970s to provide a simple and reliable method for recording the level of consciousness of patients and monitoring change. It consists of three items: Eye (E), Verbal (V) and Motor (M). The sum score of the all three ranges between 3 and 15, 3 being the worst score (deepest coma)

6.2. Karnofsky Index

The Karnofsky index (KI) allows patients to be classified according to their performance status, where 100 is perfect and 0 indicates death.

6.3. Abbreviated Injury Scale

The Abbreviated Injury Scale (AIS) is an anatomical severity scoring system that classifies each injury in every body region according to its relative importance on a six-point ordinal scale.

6.4. Baseline Prognostic Risk Factor

The Baseline Prognostic Risk Factor (BPSR) is the only validated and weighted prognostic risk score existing for TBI (Hukkelhoven, 2005). BPRS items include age, motor score (taken from GCS), pupillary reactivity, hypoxia, hypotension, CT classification (Marshall Criteria) and the presence or absence of traumatic subarachnoid hemorrhage.

7. Outcome Evaluation:

7.3. Biomarkers

- S100 Calcium-Binding Protein (S100)
- Neuron Specific Enolase (NSE)

Despite the non-specificity of S100, the decision to choose it as a biomarker for outcome evaluation was driven by the fact that analyses of S100B is not performed in Romania. Also in literature there are studies on S100, that corellate this biomarker with cognitive impairement (de Boussard, 2005) and unfavourable outcome (Yan, 2014) in TBI.

7.4. Genetic polymorphism:

- Apolipoprotein E (ApoE) ε2-4 allele

7.3. Evaluation Scales:

7.3.1. Early Rehabilitation Barthel Index

The Barthel Index (BI) has been shown to be a good measure of reduced activities of daily living; this index can be applied in routine clinical practice in a valid and reliable manner. However, patients with severe brain damage cannot be differentiated appropriately as floor effects manifest as the severity of neurological impairment increases, e.g., in comatose and near- or post-comatose patients during early rehabilitation. Aspects of functional deficits relevant for early rehabilitation patients have been introduced to the Barthel Index in a separate section, the Early Rehabilitation Barthel Index (ERBI). These aspects are as follows: a state requiring temporary intensive medical monitoring, tracheostoma requiring special treatment (suctioning), intermittent artificial respiration, a confused state requiring special care, behavioral disturbances requiring special care, swallowing disorders requiring special care, and severe communication deficits.

7.3.2. Extended Glasgow Outcome Scale

The Extended Glasgow Outcome Scale (GOS-E) was developed to address the limitations of the original GOS, including the use of broad categories that are insensitive to change and difficulties with reliability due to the lack of a structured interview format. The GOS-E extends the original 5 GOS categories to 8. The 8 categories are as follows: Dead, Vegetative State, Lower Severe Disability, Upper Severe Disability, Lower Moderate Disability, Upper Moderate Disability, Lower Good Recovery, and Upper Good Recovery. A structured interview has been provided to improve the reliability of the rating. Good inter-rater reliability and content validity have been demonstrated for the GOS-E (see Properties for further details). Compared to the GOS, the GOS-E has been shown to be more sensitive to change in mild to moderate TBI.

7.3.3. Mini Mental State Examination

The mini-mental state examination (MMSE) is a brief 30-point questionnaire test that is used to screen for cognitive impairment. It is commonly used in medicine to screen for dementia. It is also used to estimate the severity of cognitive impairment at a given point in time and to follow the course of cognitive changes in an individual over time, thus making it an effective method to document an individual's response to treatment.

7.3.4. Stroop Color-Word Test

The Stroop Color-Word Test is based on the observation that individuals can read words much faster than they can identify and name colors. The cognitive dimension tapped by the *Stroop* is associated with cognitive flexibility, resistance to interference from outside stimuli, creativity, and psychopathology – all of which influence the individual's ability to cope with cognitive stress and process complex input. Whether the test is used as a screener or as part of a general battery, its quick and easy administration, validity, and reliability make it an especially attractive instrument. Furthermore, it is not culturally biased (Cohen, 2002). Thus, this unique test is an ideal way to screen for neuropsychological deficits.

7.3.5. Processing Speed Index, Wechsler Adult Intelligence Scale, Third Edition

The Processing Speed Index (PSI) assesses skills such as focusing attention and quickly scanning as well as discriminating between and sequentially ordering visual information. It requires persistence and planning ability, but it is sensitive to motivation, difficulty working under time pressure, and motor coordination, too. It is also related to reading, mathematical, and memory skills. Cultural fac-

tors seem to have little impact on processing speed. Processing Speed (PS) refers to the speed at which cognitive processes can be performed.

7.3.6. Finger Tapping Test

The Finger Tapping Test (FTT) was initially part of the Halstead-Reitan neuropsychological tests. It is a simple and quick test, widely used to assess the motor functioning, especially the motor speed and coordination. The testing procedure is designed to obtain a reliable measure of the patient's maximal performance on each hand.

7.3.7. Digit Span, Wechsler Adult Intelligence Scale, Third Edition

The Digit Span task exercises a patient's verbal working memory. Attention and comprehension also contribute to performance. The digit span task is a common component of many IQ tests, including the widely used WAIS (Wechsler Adult Intelligence Scales). Performance on the digit span task is also closely linked to language learning abilities. The procedures for this assessment of working memory are considered standard. A list of numbers is read out loud at a rate of one number per second, and the participant is then asked to recall the numbers in order. The first list will consist of three numbers and increase until the person begins to make errors. Lists with recognizable patterns (e.g., 1,3,5,7, and 9) should be avoided, as people may remember these numbers more easily. At the end of each sequence, the participant is asked to the recall items in order. The average adult can remember a sequence of seven numbers, plus or minus two. This test can be distributed both backwards and forwards. Scores are thought to correlate with age and not intelligence.

7.3.8. Hospital Anxiety and Depression Scale

The Hospital Anxiety and Depression Scale (HADS) is commonly used to determine a patient's levels of anxiety and depression. It uses a 10-point scale such

that, if a patient scores the lowest possible value of 1, he/she is considered to possibly need clinical psychiatric treatment. If the patient scores the maximum points (10), he/she will be considered clinically stable. These points are determined by a series of questions asked by a clinician. Certain questions are geared towards anxiety, while others are geared toward depression. The patient would be asked to answer with his/her feelings during the past week. While answering the questions, the patient should answer with immediate reactions, thus giving a more accurate representation of his/her feelings.

7.3.9. Color Trails Test

The Color Trails Test (CTT) was developed to meet the need for a test with the sensitivity and specificity of the standard Trail Making Test (TMT) but that was as free as possible from the influences of language and cultural bias. The CTT retains the psychometric proprieties of the standard TMT, but the CTT substitutes the use of color for the use of English alphabet letters, making it more suitable in cross-cultural and other special needs contexts.

7.5. Eye tracking

Eye movements will be recorded by a human infrared eye tracking system (Tobii TX300) with 300 Hz temporal rezolution. The target stimulus, which will be created in Toby Studio program, will be presented on a computer screen 40 cm from the subject, in a darkened room. Before testing, an eyechart will be used to verify that all subjects will have a normal or corrected-to-normal vision. Calibration based on 9 points, including center and peripheral, will be performed before each session, which also will ensured that all subjects will have a full range of oculomotor movement. There will tested 2 paradigms. The first one consists in a succession of 20 paired images, in which the patient must identify the item that was changes in the second from the first image. When the patient identifies

the modified item, he has to perform mouse click. There will be calculated the following parameters: time to first fixation on the modified item, time from first fixation to next mouse click and fixations before. The second paradigm will consists in a presentation of 24 slides, each one of them with 4 emotional expressions. The patient will be asked to identify a certain emotional expression before each slide. There will be calculated fixation duration regarding each emotion image. One eye-traking session will last 15 minutes.

7.6. qEEG technique with 19 channels

Continous EEG recordings will be performed in both resting conditions and cognitive tasks, during the following sequences: 1) eyes open – 5 minutes; 2) eye close – 5 minutes; 3) labirint test – 3 minutes; 4) Krapelin test -3 minutes; 5) Benton test – 3 minutes; 6) eyes open -5 minutes; 7) eyes closed - 5 minutes. Will be used scalp electrodes fixed in an elastic cap, located according to the international 10–20 system and electrode impedances will be keep below 5 kΩ. EEG data will be recorded using 19 channels and analysis will be conducted with Nicolet One Topographic Brain Mapping Analysis software. The following frequency bands will be studied: delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz) and beta (13–30 Hz). Signals will be visually inspected offline in order eliminate artifacts and we will analyse all artefact-free epches with a duration of 5 seconds from all sequences. The reference parameters will be relative power (%) and coherence.

8. Study Design:

- Eligible TBI patients with a GCS between 7 and 12 will be randomized by a 3:4 ratio into 2 groups:
  - Treatment group “Control”:
    - Days 1-10: Placebo IV

- Days 31-40: Placebo IV
- Days 61-70: Placebo IV

Placebo: 250 ml 0.9% NaCl

- Treatment group "Acute" + "Rehabilitation":
  - Days 1-10: 50 ml CRB IV
  - Days 31-40:10 ml CRB IV
  - Days 61-70: 10 ml CRB IV

  CRB is diluted in 0.9% NaCl to a total volume of 250 ml.
- CRB group + rTMS:
  - Days 1-10 (± 4): 50 ml CRB IV
  - Days 31-40 (± 4) :10 ml CRB IV + rTMS
  - Days 61-70 (± 4): 10 ml CRB IV + rTMS

- **Screening and baseline** should occur within 4 hours post-TBI event onset and includes the following:
  - Neurological exam
  - CT scan
  - ECG
  - Hematology, blood chemistry, urinalysis
  - Biomarkers: S100, NSE
  - Evaluation Scales: GCS, KI, AIS
  - Baseline Prognostic Risk Score (BPRS)
    - Age
    - Motor score
    - CT Scan (Marshall criteria)
    - Pupillary reactivity
    - Hypoxia

- Hypotension
- Subarachnoid hemorrhage

- Patients will be treated according to the standard protocols for TBI.
- Blood samples for biomarker assessment and genotyping will be obtained according to the study's protocol.
- Patients' treatment with CRB/placebo will start according to the study's protocol.
- **At 72 hours**: Biomarkers S100, NSE, Genetic polymorphisms APOE ε2-4

- **Visit 1**: 10th day:
  - Vital signs
  - ECG
  - Hematology, blood chemistry, urinalysis
  - Adverse reactions
  - Concomitant medication
  - Neurological and general exam
  - Scales:
    - Mini-Mental State Examination
    - Processing Speed Index
    - Stroop Color-Word Test – Victoria Version
    - Early Rehabilitation Barthel Index
    - Glasgow Outcome Scale Extended

A subgroup of 70 patients will do also eye tracking and qEEG.

- **Visit 2**: 30th day
  - Vital signs
  - ECG
  - Hematology, blood chemistry, urinalysis

- Adverse reaction
- Concomitant medication
- Neurological and general exam
- Scales:
    - Processing Speed Index
    - Stroop Color-Word Test
    - Color Trails Test
    - Digit Span (Wechsler adult intelligence scale – third edition)
    - Early Rehabilitation Barthel Index
    - Finger Tapping Test
    - Mini-Mental State Examination
    - Hospital Anxiety and Depression Scale
    - Glasgow Outcome Scale Extended

A subgroup of 70 patients will do also eye tracking and qEEG.

- **Visit 3**: 90th day:
    - Vital signs
    - Hematology, blood chemistry, urinalysis
    - Adverse reaction
    - Concomitant medication
    - Neurological and general exam
    - Scales:
        - Processing Speed Index
        - Stroop Color-Word Test
        - Color Trails Test
        - Digit Span (Wechsler adult intelligence scale – third edition)
        - Early Rehabilitation Barthel Index
        - Finger Tapping Test
        - Mini-Mental State Examination

- Hospital Anxiety and Depression Scale

- Glasgow Outcome Scale Extended

A subgroup of 70 patients will do also eye tracking and qEEG.

9. Inclusion Criteria:

- Diagnosis of TBI and a GCS score of 7-12 at the time of hospital admission. Pre-hospital intubation/sedation/paralysis is accepted if the GCS score has been assessed before intubation/sedation/paralyses by trained personnel
- Pre-treatment GCS score of 7-12. If intubation/sedation/paralysis occurs after hospital admission, the pre-treatment GCS score has been assessed before intubation/sedation/paralyses
- Only isolated TBI (AIS score in other body regions of ≤ 2)
- CT (Marshal classification)
- Pre-Trauma Karnofsky Index = 100. If no corresponding information is available before the start of treatment (e.g., patient is unconscious or not able to communicate) and no information is retrieved within 24 hours after the start of treatment, the patient stays in the study. If no information is available before the start of treatment and a violation of the Karnofsky-Index is detected within 24 hours after the start of treatment, the patient is withdrawn from the study, and the treatment medication is stopped.
- Age 18-80
- Able to provide written informed consent to enrollment
- Willing and able to comply with the protocol requirements for the duration of the study
- Time to needle for study medication should be within 4 hours
- Patients were able to speak, read and write before the accident. If no corresponding information is available before the start of treatment (e.g., patient

is unconscious or not able to communicate) and if no information is retrieved within 24 hours after the start of treatment, the patient should remain in the study. If no information is available before the start of treatment and if a violation of this inclusion criterion is detected within 24 hours after the start of treatment, the patient should be withdrawn from the study, and the treatment medication should be stopped.

- Reasonable expectation, in the investigator's judgment, of completion of outcome measures at follow-up.

10. Exclusion Criteria:

- Patients with polytrauma (AIS score in other body regions of >3)
- Patients with spinal cord injury
- History of intracranial interventions as well as ischemic or hemorrhagic stroke
- Evidence of pre-existing major health problems (e.g., cancer, hematological, renal, hepatic, or coronary disease, psychiatric disorder, diabetes, myocardial infarction or other known heart diseases, disabling or musculoskeletal problems as rheumatoid arthritis, epilepsy, evidence of degenerative or inflammatory diseases affecting nervous system [e.g., Alzheimer, Parkinson]). Patients with well controlled diabetes and hypertension can be included if there is no evidence of secondary damage to major organs.
- Any neurological or non-neurological condition independent from TBI that might influence the functional outcome or other efficacy outcome measures.
- Injury of writing hand influencing cognitive or other outcome measures, in the investigator's judgment.
- Clear clinical signs of intoxication influencing the evaluation, in the investigator's judgment.
- Major drug dependency including alcohol, in the investigator's judgment.
- Chronic treatment with steroids, Ca2+-channel blockers or major anticoagulants (e.g., warfarin and other coumarin derivates), monoamine oxidase inhibitors,

antipsychotic drugs or nootropic molecules.

- Patient with penetrating brain injury.
- Patients with spinal cord injury.
- Females who are pregnant or lactating.
- Females who are of child bearing potential and not taking adequate contraceptive precautions are excluded from the trial. Females of child bearing potential taking acceptable contraceptive precautions can be included. A highly effective method of birth control and one which is acceptable for this study, is defined as those which result in a low failure rate (i.e. less than 1% per year) when used consistently and correctly such as implants, injectables, combined oral contraceptives, some IUDs, sexual abstinence or vasectomised partner.

11. Blinding

This study will be performed under double-blind conditions to keep investigators, other study personnel and patients blinded to treatment allocation. Cerebrolysin is an amber-colored solution; therefore, colored infusion lines will be used for drug administration.

A set of envelopes for each patient enrolled should be distributed to the study nurse preparing the ready-to-use-infusion solution. These nurses are only responsible for the preparation and administration of infusion solutions, and they should not be involved in any further study-related procedures. This person should not be allowed to disclose any information about treatment allocation. A treatment envelope should not be opened until the patient's first ready-to-use-infusion has been prepared.

12. Route and Dosage Form:

Cerebrolysin or placebo is given as an add-on therapy to the standard treatment protocol for TBI. The study nurse prepares the ready-to-use infusion solution freshly prior to administration according to the randomization number provided.

An infusion is administered as an intravenous infusion in a peripheral vein over a time period of 30 min. The first infusion is administered within four hours after traumatic brain injury. The subsequent infusions take place at approximately the same time every day in the morning.

In the CRB treatment group, patients will receive 50 ml Cerebrolysin once daily during the first treatment course and 10 ml once daily during treatment courses 2-3 (days 31-40, 61-70). Cerebrolysin is diluted with physiological saline (0.9% NaCl) to a total volume of 250 ml. An identical amount of physiological saline (250 ml) is used as a placebo in the control group.

13. Subject withdrawal:

The subjects will be informed that they have the right to withdraw from the study at any time, without prejudice to their medical care and without needing to state reason.

Reasons for discontinuation are classified according to the following scheme:

- Lack of efficacy
- Adverse event
- Consent withdrawn
- Lost-to-follow-up
- Administrative reasons

14. Concomitant medication

The concomitant use of medications specified in the exclusion criteria should not be allowed during the course of the study. Nootropic medications, cholinesterase

inhibitors, NMDA antagonists, the chronic administration of corticosteroids and the prophylactic use of antiepileptic drugs are not allowed.

15. Safety and Tolerability Measures:

- Adverse Events (AE)
- Serious Adverse Events (SAEs)
- Vital signs (blood pressure, heart rate, respiration rate, body temperature and weight)
- Changes in neurological exam
- Rates of drug discontinuation
- Concomitant diseases
- ECG findings
- Laboratory tests:
  - hematology: red blood cell count, hematocrit, hemoglobin levels, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, white blood cell and platelet count
  - blood chemistry: glucose, total cholesterol, triglycerides, uric acid, creatinine, urea, total bilirubin, total protein, aspartate transaminase, alanine transaminase, gamma-glutamyl transferase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, sodium, potassium
  - urinalysis: glucose, bilirubin, ketones, density, blood, pH, protein, nitrites and leukocytes

16. Adverse events

The severity of AEs should be graded as follows:

- **Mild:** the patient is aware of the event, but the event is easily tolerated by the patient;

- **Moderate:** the patient experiences sufficient discomfort to interfere with normal daily activities;
- **Severe:** the patient experiences significant impairment of functioning; the subject is unable to perform usual activities or the subject's life is at risk due to the event.

Causality to study medication is assessed as follows:

- **Definite**: Clinical or laboratory test abnormality, with a plausible time relationship to drug intake/rTMS, which cannot be explained by disease or other treatment;
- **Probable**: Clinical or laboratory test abnormality, with a reasonable time relationship to drug intake/rTMS and a reasonable response to withdrawal;
- **Possible**: Clinical or laboratory test abnormality, with a reasonable time relationship to drug intake/rTMS;
- **Unlikely**: Clinical or laboratory test abnormality, with a time relationship to drug intake/rTMS that makes a relationship improbable;
- **Not related**: A causal relationship can be definitively excluded, and another documented cause of the AE is most plausible.

Possible action taken to study drug due to an AE:

- dose not changed
- stop medication
- reduced dose medication
- stop rTMS

17. Biomarkers – handling, procedures and schedule

Venous blood samples will be taken immediately prior to study-drug administration (within 4 hours post-injury) and again at 72 hours post-trauma. For each patient, 3-4 ml of serum and 3 ml of plasma should be collected for the analysis of biomarker levels. For the collection of serum and plasma, the collection of 16 ml of blood will be necessary at each time point. The use of a 10 ml dry tube and a 6 ml EDTA tube is recommended.

18. Statistical Methods

Primary Efficacy Criteria

Justification for Primary Efficacy Criteria

In the last 30 years, no TBI trial with a traditional design on neuroprotective agents showed a significant treatment effect in moderate to severe TBI (Maas, Roozenbeek, 2010). Thus, the challenge to demonstrate benefit of a novel agent in TBI is great, but the rewards are regarded as correspondingly high (Maas, Roozenbeek, 2010).

Almost all failed studies used a single measure approach. Nevertheless, outcome after TBI is by definition multidimensional, including neurophysical disabilities, disturbances in mental functioning (*e.g.,* cognitive and executive functioning), and consequential problems in social reintegration (Maas, Steyerberg, 2010).

It is assumed, that no single measure can capture the multidimensional nature of TBI outcome. Therefore, for future trials, the use of multiple measures to address the breadth of potential deficits and recovery following TBI is recommended by an increasing number of authors (Zafonte, 2009; Marguiles, 2009; Temkin, 2007; Bagiella, 2010).

A major problem in the past was the statistical solution of the multidimensional approach. Today, several statistical methods are available to compare two groups with respect to more than one outcome (see *e.g.,*Bagiella, 2009; Temkin, 2007; Bagiella, 2010; Lachin, 1992; Dimitrenko, 2010; O'Brien, 1984; Lu, 2001; Huang, 2008).

Thus, in agreement with current recommendations, a multidimensional approach for outcome assessment and classification will be chosen for the present study.

Three ensembles of appropriate single efficacy criteria shall be tested separately by a multivariate, directional test approach, reflecting the "global status of patients in TBI" (Bagiella, 2010) at three different points in time (overall recovery, early recovery, neuroprotection):

- Outcome Ensemble at Day 90 (overall recovery phase, 5 scales reflecting the recommendations of the TBI Clinical Trials Network Group (Bagiella, 2010) supplemented by motor function, mental state examination, basic activity of daily living, and depression as recommended by the Trial Advisory Board; total ensemble: 9 scales)

- Outcome Ensemble at Day 30 (early recovery phase, 5 scales reflecting the recommendations of the TBI Clinical Trials Network Group (Bagiella, 2010) supplemented by motor function, mental state examination, basic activity of daily living, and depression as recommended by the Trial Advisory Board; total ensemble: 9 scales)

- Outcome Ensemble at Day 10 (neuroprotection phase, 5 scales as recommended

by the Trial Advisory Board for acute phase efficacy assessment)

Multiplicity due to multiple points in time (Day 90, 30, 10) will be controlled by the principle of *a priori* ordered hypotheses (see below).

Efficacy Criteria Recovery Phase

The following ensembles of appropriate single efficacy criteria shall be tested separately by a multivariate, directional test approach, reflecting the global status of patients in TBI at different points in time:

1. PSI (Processing Speed Index, Wechsler adult intelligence scale – third edition)
2. Stroop Color-Word Test – Victoria Version (VST) (Marcus, 1976)
3. Color Trails Test (Posch, 2005)
4. Digit Span (Wechsler adult intelligence scale – third edition(Wechsler, 1997)
5. Early Rehabilitation Barthel Index (Schoenle, 1995)
6. Finger Tapping Test (Huang, 2008)
7. Mini-Mental State Examination (MMSE) (Folstein, 1975)
8. Hospital Anxiety and Depression Scale (Zigmond, 1983)
9. Glasgow Outcome Scale Extended (GOS-E) (Wilson,1998)

Efficacy endpoints are Day 90 (overall recovery) and Day 30 (early recovery) after brain injury.

Efficacy Criteria Acute Phase (Neuroprotection)

The following ensemble of appropriate single efficacy criteria shall be tested separately by a multivariate, directional test approach, reflecting the global status of patients in TBI:

1. Mini-Mental State Examination (MMSE) (Folstein, 1975)
2. PSI (Processing Speed Index, Wechsler adult intelligence scale – third edition)
3. (Wechsler, 1997)Stroop Color-Word Test – Victoria Version (VST)(Marcus, 1976)
4. Early Rehabilitation Barthel Index (Schoenle, 1995)
5. Glasgow Outcome Scale Extended (GOS-E) (Wilson, 1998)

Efficacy endpoint is Day 10 after brain injury (neuroprotection phase).

Secondary Efficacy Criteria

- **Secondary Efficacy Criteria Day 90, 30, 10**
  - Mortality from any cause

Level of Significance

The multiple level alpha of the study (multiple level of significance) is defined as alpha = 0.025 (one-sided).

Multiplicity

General Considerations

Three ensembles of appropriate single efficacy criteria (outcome ensemble at Day 90, at Day 30, and at Day 10) shall be tested in an *a priori* defined order by a multivariate, directional test approach. Further details of this procedure are described in section 18. ("Confirmatory Analyses").

Sample Size Calculation

Design Specifications

The power for this study is determined based on the following design specifications:

(a) One-sided type I error defined as alpha = 0.025 (multiple level alpha)

(b) Day 90/30: 90% power (1 – beta), Day 10: 85% power (1 – beta)

(c) Design alternative effect size: Mann-Whitney statistic (MW) = 0.64 (medium-sized difference according to Cohen (Colditz, 1988); assuming a normal distribution the effect size MW may easily be re-expressed as the well-known Co-

hen effect size (Cohen, 1988) of a standardized difference (Cohen's d): MW = 0.64 means Cohen's d = 0.5)

(d) Estimated correlations among the single outcome scales included in the global statistics (based on results of the Traumatic Brain Injury Trials Group (Bagiella, 2010), further assumptions for additional scales introduced by the Trial Advisory Board)

Nonparametric sample size calculations within the framework of a multiple outcome approach (Wei-Lachin procedure- Wei and Lachin, 1984; Lachin 1992) was performed applying the validated software Nnpar 1.0 from idv Data Analysis and Study Planning, Krailling/Munich (see also Tang, 1989; Lachin, 1981). A good example for sample size calculation according to the Lachin approach (Wei and Lachin, 1984; Lachin 1992; Lachin, 1981) in multidimensional trials with neuroprotective agents is also given by Huang (2008) .

Please note: further details of the effect size (Mann-Whitney statistic) are described in section "Confirmatory Analyses", further details of sample size assessment are described in the separate document "Sample Size Assessment Based on a Multidimensional Efficacy Approach ".

Sample Size Assumptions Day 30/90

- One-sided, multivariate, directional Wilcoxon test of nine outcome measures
- Estimated average correlation rho of nine outcome measures = 0.5
- Reduction due to multidimensional directional approach for 9 single outcome criteria: 44% (sample size 0.56 as compared to sample size required

for one single criterion)

- Effect Size Mann-Whitney = 0.64 („medium-sized“ difference, see above)
- Testpower 90% (1 – beta)
- Unbalanced sample size approach 1:0.75 (active treatment: 1, placebo 0.75)

The total required sample size for Day 30/90 results in 72 patients for the active treatment and in 55 patients for placebo (including 15% enhancement for usual „ambiguities“, e.g., dropouts). With this total sample size of 127 patients a „medium-sized“ group difference (MW = 0.64) with regard to the multivariate outcome ensemble at Day 30/90 can be detected with a power of 90%.

Sample Size Assumptions Day 10

- One-sided, multivariate, directional Wilcoxon test of five outcome measures
- Estimated average correlation rho of five outcome measures = 0.6
- Reduction due to multidimensional directional approach for 5 single outcome criteria: 32.0% (sample size 0.68 as compared to sample size required for one single criterion)
- Effect Size Mann-Whitney = 0.64 („small“ difference, see above)
- Testpower 85% (1 – beta)
- Unbalanced sample size approach 1:0.75 (active treatment: 1, placebo 0.75)

For Day 10 the total required sample size results in 80 patients for the active treatment and in 60 patients for placebo (including 5% enhancement for usual „ambiguities", e.g., early dropouts). With this total sample size of 140 patients a „medium-sized" group difference (MW = 0.64) with regard to the multivariate outcome ensemble at Day 30/90 can be detected with a power of 85%.

Total Sample Size

While a total of 127 patients is required under the above design assumptions for Day 90/30, a total of 140 patients is required for Day 10. Thus, the largest required sample size is 140 patients.

The total sample size of this study will be based on the largest required sample size, *i.e.,* on 140 patients. This way, at least 85% power for all multivariate tests at all points in time is guaranteed.

Confirmatory Analyses

Minimizing the required assumptions is a recommended approach for confirmatory statements on efficacy (LaVange, 2005). This applies especially in scales with skewed distributions including floor and ceiling effects as is known from many scales used in TBI. Furthermore, data types can be of different nature (binary, ordinal, continuous). Thus, a non-parametric assessment of treatment effects independent of data type and distribution should be chosen as the primary analysis method.

The analysis will be performed using the Wei-Lachin procedure, a multivariate generalization of the Wilcoxon-Mann-Whitney test, which takes account of the correlation

among univariate Mann-Whitney tests for each outcome to produce an overall average estimate of benefit and test for treatment differences. The summarizing test used is, however, not the undirectional or omnibus test of the classical procedure, but instead a directional test which is most efficient in the case of known direction for superiority.

The procedure is described by Wei and Lachin (1984) and Lachin (1992). Practical examples are given in modern textbooks on multiple testing problems(see *e.g.,* Dimitrenko, 2010).Incidentally it should be noted that the nonparametric Wei-Lachin procedure is similar to the frequently used parametric procedure of O'Brien (O'Brien, 1984). We prefer, however, the Wei-Lachin procedure as it is more robust for practical data sets (minimization of required assumptions (LaVange 2005)) and because the O'Brien procedure has been shown to give too liberal results (Frick 1997).

It is important to note, that the multivariate, directional test procedure chosen for this study can cope simultaneously with binary, ordinal and continuous data. Thus, there is no technical need for the widely used dichotomization of original scales which is associated with substantial loss of information and reflects a major disadvantage of previous TBI studies.

The multiple level alpha of the study (global level of significance for the whole study) is defined as alpha = 0.025, one-sided test for superiority.

The three points in time (Day 90, Day 30, Day 10) can then be tested individually in a confirmatory manner according to the principle of *a priori* ordered hypotheses.The procedure of *a priori* ordered hypotheses is most powerful with full control of alpha (for control of alpha using stepwise testing see Maurer, 1995).

According to the ICH Guideline E9 (ICH Topic E9, Statistical Principles for Clinical Trials, Step 4, Consensus guideline, 5 February 1998, CPMP/ICH/363/96) the results

will be given as P-values as well as effect size measures with their confidence intervals (Mann-Whitney statistic as corresponding effects size measure of the Wilcoxon-Mann-Whitney test), so that the direction and quantity of the treatment effects are determined with their precision.The Mann-Whitney statistic is the most valuable effects size measure for the Wilcoxon-Mann-Whitney test because it is appropriate where the Hodges-Lehmann shift parameter is no longer valid. Furthermore, the Mann-Whitney effects size measure is appropriate for continuous, ordinal and binary data at the same time and represents an ideal effects size measure for multiple outcomes. Incidentally, the 25th Anniversary of the journal Statistics in Medicine dedicated a whole issue to papers about the Mann-Whitney statistic (D'Agostino, 2006).

The Mann-Whitney effects size measure (MW) gives the probability that a randomly chosen patient of the test group is better off than a randomly chosen patient of the comparison group, defined in statistical shortcut: P (X<Y) + 0.5 P (X = Y).

Applying the Mann-Whitney effects size measure, the null and alternative hypothesis for the comparisons of the test treatment to control treatment (superiority) can be formulated as follows:

H_0: $MW_{TC} \leq 0.50$

H_A: $MW_{TC} > 0.50$

H_0: Null-hypothesis; H_A: Alternative Hypothesis; T: Test Treatment; C: Control

The traditional benchmarks for the Mann-Whitney effects size measure (MW) are as follows (Colditz,1988):

0.29 large inferiority
0.36 medium inferiority
0.44 small inferiority

0.50 equality

0.56 small superiority

0.64 medium superiority

0.71 large superiority

The sequence and nature of the *a priori* ordered hypotheses of the study is as follows:

Outcome Ensemble at Day 90 (overall recovery phase):

1. Multivariate Global Test (combining the 9 single endpoints of hypotheses no. 2 - 10)
2. Glasgow Outcome Scale Extended (GOS-E) (Wilson, 1998)
3. PSI (Processing Speed Index, Wechsler adult intelligence scale – third edition) (Wechsler, 1997)
4. Stroop Color-Word Test – Victoria Version (VST) (Lee, 2000)
5. Finger Tapping Test (Reitan, 1993)
6. Digit Span (Wechsler adult intelligence scale – third edition) (Wechsler, 1997)
7. Color Trails Test (Dugbartey, 2000)
8. Mini-Mental State Examination (MMSE) (Folstein, 1975)
9. Early Rehabilitation Barthel Index (Schoenle, 1995)
10. Hospital Anxiety and Depression Scale (Zigmond, 1983)

Outcome Ensemble at Day 30 (early recovery phase):

1. Multivariate Global Test (combining the 9 single endpoints of hypotheses no. 2 - 10)
2. Glasgow Outcome Scale Extended (GOS-E) (Wilson, 1998)
3. PSI (Processing Speed Index, Wechsler adult intelligence scale – third edition)

   (Wechsler, 1997)
4. Stroop Color-Word Test – Victoria Version (VST) (Lee, 2000)
5. Finger Tapping Test (Reitan, 1993)
6. Digit Span (Wechsler adult intelligence scale – third edition) (Wechsler, 1997)
7. Color Trails Test (Dugbartey, 2000)
8. Mini-Mental State Examination (MMSE) (Folstein, 1975)
9. Early Rehabilitation Barthel Index (Schoenle, 1995)
10. Hospital Anxiety and Depression Scale (Zigmond, 1983)

Outcome Ensemble at Day 10 (neuroprotection phase):

1. Multivariate Global Test (combining the 5 single endpoints of hypotheses no. 2 - 6)
2. Glasgow Outcome Scale Extended (GOS-E) (Wilson, 1998)
3. Early Rehabilitation Barthel Index (Schoenle, 1995)
4. Mini-Mental State Examination (MMSE) (Folstein, 1975)

5. PSI (Processing Speed Index, Wechsler adult intelligence scale – third edition)

   (Wechsler, 1997)
6. Stroop Color-Word Test – Victoria Version (VST) (Lee, 2000)

In order to control heterogeneity of the study population, all multidimensional comparisons (Wei-Lachin procedure) will be performed within predefined baseline strata of prognostic risk (for basic procedure see Lachin, 2000). The validated BPRS scale (Baseline Prognostic Risk Score) will be used for stratification (Hukkelhoven, 2005) including seven predictors of outcome obtained before randomization - age, motor score, CT classification, pupillary reactivity, hypoxia and hypotension, and traumatic subarachnoid hemorrhage. It is a weighted and validated prognostic scale and includes the criteria recommended by IMPACT (Maas, Steyerberg, 2010). The patient population will be ordered on the basis of the BPRS and split into octiles in order to obtain eight strata of comparable size (with respect to the use of percentiles for stratification of a TBI patient population using BPRS see also Maas 2006). The predefined strata will then be combined to a summarized, baseline-adjusted overall result applying the formal meta-analytic approach (Hedges, 1985). This procedure is robust, leads to valid P-values, effect sizes and confidence intervals also in the presence of multidimensional comparisons, and confirms to the recommended approach of minimizing required assumptions for confirmatory statements on efficacy (LaVange, 2005; Lachin, 2000). The aim of the described stratification with subsequent meta-analytic pooling is not the analysis of subgroups but the control of heterogeneity of the study population.

The confirmatory analyses are performed with the ITT population according to the ICH Guideline E9 (full analysis set).

Exploratory Analyses

All primary and secondary efficacy criteria will be analyze with descriptive group statistics.

In addition, nonparametric effect sizes and confidence intervals (Mann-Whitney effects size measure) will be provided for all primary and secondary efficacy criteria at all points in time.

Biomarker levels will be analyze in order to address the question of whether biomarkers improve the diagnosis and prognosis of traumatic brain injury. Details of these exploratory analyses will be specified in the final statistical analysis plan.

Accounting for missing data

Missing Data Problems – General Considerations for a TBI Study

Missing data are a problem in every data analysis. Of course, there are always missing data of the type ´missing completely at random´ (MCAR), which in principle will not bias the results; the analysis procedure should be able to cope with partially missing data of such a type. In many studies this type of data is treated by LOCF replacement (Last Observation Carried Forward) as far as there exist follow-up measurements at previous visits.

In a study like the one planned there might also be informatively missing data (missing not at random, MNAR): participants of the study died or are unable to complete the tests because of brain-related impairment. Neglecting these missing data might introduce bias.

A reasonable policy for minimizing bias in the case of informatively missing data (MNAR) is the replacement of these missing data by assigning the worst possible

score, or a score worse than those observed. It should be noted that this strategy is only reasonable if rank-based robust procedures are used for the data analysis.

The worst rank imputation procedure was recommended by Lachin in his seminal paper about the missing data problem for data missing because of mortality when performing an exercise test (Lachin, 1999). This procedure was also used when analyzing non-fatal outcomes in studies where mortality was a problem (Lusben, 2002; McMahon, 2001). Recently a similar procedure has been proposed by the ´Traumatic Brain Injury (TBI) Clinical Trials Network´ when designing the COBRIT study (Bagiella, 2010).

In order to identify each type of missing data the eCRF should assign each test an identification code (see also Bagiella, 2010) :

1 = valid (complete task)
2 = unable to complete (TBI-related neurological reason) [describe reason]
3 = not completed (different reasons, not TBI related) [describe reason]

Temkin (2007) included deaths with the worst rank for the significance tests of neuropsychological scales, but excluded deaths for the calculation of descriptive statistics (*e.g.,* mean, SE estimates). Thus, significance tests reflect all patients with estimation of missing data while the descriptive statistics reflect only the actually observed assessments. We prefer not excluding deaths from descriptive statistics, since the study treatment with more deaths would artificially have better neuropsychological scores while a study treatment preventing deaths would be burdened by rather severe scores of survived patients. The use of robust descriptive statistics in this study allows the inclusion of worst rank scores for deaths also in descriptive analysis. This way, confirmatory analyses and descriptive analysis can be based on the same analysis data and contradictory results are avoided.

Handling of Missing Data

Outcome scales will be coded for every patient and visit according to the following scheme:

(1) valid (complete task)

(2) unable to complete due to death or TBI-related neurological reason [describe

reason]

(3) not completed (different reasons, not TBI related) [describe reason]

Worst Rank Imputation

For outcome scales with code "2" a worst rank imputation will be introduced for the corresponding patients since these data are informatively missing (missing not at random, MNAR). These missing data are replaced by the worst possible score of the corresponding outcome scale.

LPCF Imputation

For outcome scales with code "3" a LPCF replacement will be introduced (Last Percentile Carried Forward) as far as previous follow-up evaluations exist. This method carries forward the actual status information of the patient population, using the percentile value with back transformation to raw scale, instead of last value carried forward. This approach was recently developed and recommended by O´Brien, Zhang and Bailey (2005) for the analysis of data from chronic, progressive diseases as de-

mentia. According to their simulation study the calculated estimators should be negligibly biased by missing data. If no general change of patients over time occurs the method is more or less identical with LOCF (Last Value Carried Forward), if change occurs bias is minimized.

If no previous follow-up measurement exists, the outcome scale remains missing. It is important to note that the chosen multivariate test procedure (Wei-Lachin procedure) can handle partially missing single scales of type MCAR (missing completely at random).

Definition of study population

General Issues

Before the study is unblinded, a blind review will be performed. In this process, possible protocol violations will be classified as “severe”, “major”, “minor“, or “none”. Patients will be allocated to the individual data sets with regard to the classification of possible protocol violations. The analysis populations (Safety, ITT, and PP) will be listed individually in the final statistical analysis plan.

Safety Population

Safety population includes all patients who have had at least one dose of study medication and one contact with the Investigator afterwards. It will be used for safety analysis.

ITT Population (Full Analysis Set)

ITT population is defined as all patients who have no “severe” violation of entry crite-

ria, had at least one dose of medication and at least one post-baseline observation of at least one primary efficacy criterion ("modified" ITT). This way ITT is defined in the sense of the "full analysis set" according to ICH E9 § 5.2. ("Analysis Sets") . ITT population will be used for all efficacy analyses.

Per Protocol Population (PP)

A sensitivity analysis will be performed for a per protocol (PP) data set as an exploratory approach. The PP population includes all patients who are eligible for ITT evaluation and who additionally do not show major protocol deviations.

Homogeneity Analyses (Exploratory Interpretation)

Homogeneity analyses for baseline shall be performed based on the ITT population.

In addition to descriptive analyses robust nonparametric Mann-Whitney effects size measures and their two-sided 95% confidence intervals shall present an overview on demographic-anamnestic variables and on the primary efficacy criteria at baseline. This allows comparison of baseline variables across different scales and data types.

As benchmark for relevant baseline differences, a Mann-Whitney effects size measure of 0.36 and 0.64 respectively will be applied (referring to a standardized difference of 0.5 according to Cohen, which is regarded as a medium-sized difference).

In the case of heterogeneities, stratified analyses will be performed as second line analyses.

Compliance

Patients with compliance for the entire study below 80% for the treatments will be

considered protocol violators and will not be included in the per protocol analysis.

Additional Specifications:

- The compliance calculation will be performed as follows:

$$\% \text{ Compliance} = \frac{\text{Total number of infusions administered}}{\text{Total infusions that should have been consumed during actual time in study}} \times 100$$

- Patients with calculated compliance ≥ 80% are considered as "compliant". All other patients are considered as "not compliant" (**major** protocol deviation). This definition is an "active" compliance definition since patients with missing compliance data (no information about infusions administered) are considered as "not compliant" ("no proven compliance").

Blind Review / Final Statistical Analysis Plan / Confidentiality of Stage

I Results

A blind review of the data shall be performed within the framework of the requirements of the ICH Guideline E9.The statistical analysis plan will be finalized by the statistician before the decoding takes place. The analysis populations (Safety, ITT, and

PP) will be listed individually in the final statistical analysis plan.

Software Applied

Nonparametric sample size calculation was performed applying the validated software Nnpar 1.0 from idv Data Analysis and Study Planning, Krailling/Munich.

The data analysis will be performed in a validated working environment according to the requirements of the ICH-Guidelines E3 (1995). The software to be used for data evaluation will be described in the final statistical analysis plan.

Fig.1. Flowchart of Assessments

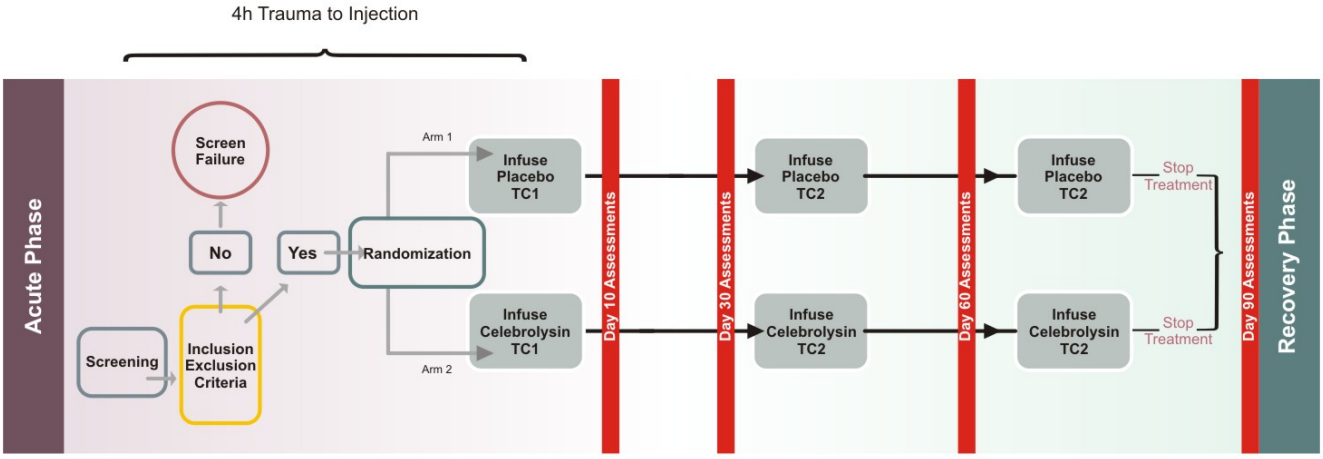

Fig. 2: Schedule of Assessments (patients with CRB without rTMS)

	Treatment Day	*IMP	Procedure
Injury			
Admission	1		GCS
Baseline	1		Informed Consent Exclusion/Inclusion: • Karnofsky Index • AIS • GCS Baseline Prognostic Risk Score • CT scan • Pupillary reactivity • Age • Motor score • Hypoxia • Hypotension • Subarachnoid hemorrhage Demographics Safety • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Medical history • Negative pregnancy test Blood samples – biomarkers (prior to study drug administration); GCS assessment shortly before administration of IMP
	1(or day 2)	Infusion (*TC 1-1)	The first infusion is administered within four hours after TBI, TC1 is 1x50 ml Cerebrolysin/0.9% NaCl per day for 10 days

	2 (24 hr +/- 4 hr after injury)	Infusion (TC1-2)	
	3	Infusion (TC1-3)	Serum biomarkers at 72 hours after TBI Genetic polymorphism APOE
	4	Infusion (TC1-4)	
	5	Infusion (TC1-5)	
	6	Infusion (TC1-6)	
	7	Infusion (TC1-7)	
	8	Infusion (TC1-8)	
	9	Infusion (TC1-9)	
Visit 1	10 (or day 11)	Infusion (TC1-10)	Primary Efficacy Criteria Day 10 • Mini Mental State Examination • Processing Speed Index • Stroop Color-Word Test – Victoria Version • Early Rehabilitation Barthel index • Glasgow Outcome Scale Extended Eye tracking qEEG Safety: • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Adverse Events • Negative pregnancy test
Visit 2	30 (+/-2)		Primary Efficacy Criteria Day 30 • Processing Speed Index • Stroop Color-Word Test – Victoria Version • Color Trails Test

			• Digit Span (Wechsler adult intelligence scale- third edition • Early Rehabilitation Barthel Index • Finger Tapping Test • Mini Mental State Examination • Hospital Anxiety and Depression Scale • Glasgow Outcome Scale Extended Eye tracking qEEG Safety • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Adverse events • Negative pregnancy test
	31 (+4)	Injection (TC2-1)	1x10 ml CRB/0.9%NaCl once daily for 10 days
	32	Injection (TC2-2)	
	33	Injection (TC2-3)	
	34	Injection (TC2-4)	
	35	Injection (TC2-5)	
	36	Injection (TC2-6)	
	37	Injection (TC2-7)	
	38	Injection (TC2-8)	
	39	Injection (TC2-9)	
	40	Injection (TC2-10)	
Visit 3	61 (+/-2)	Injection (TC3-1)	1x10 ml CRB/0.9%NaCl once daily for 10 day

	62	Injection (TC3-2)	
	63	Injection (TC3-3)	
	64	Injection (TC3-4)	
	65	Injection (TC3-5)	
	66	Injection (TC3-6)	
	67	Injection (TC3-7)	
	68	Injection (TC3-8)	
	69	Injection (TC3-9)	
	70	Injection (TC3-10)	
Visit 4	90 (+/-4)		Primary Efficacy Criteria Day 90 • Processing Speed Index • Stroop Color-Word Test – Victoria Version • Color Trails Test • Digit Span (Wechsler adult intelligence scale- third edition) • Early Rehabilitation Barthel Index • Finger Tapping Test • Mini Mental State Examination • Hospital Anxiety and Depression Scale • Glasgow Outcome Scale Extended Eye tracking qEEG Safety • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Adverse events

*IMP – Investigation Medicinal Product

*TC – Treatment Cycle

Fig. 3: Schedule of Assessments (patients with CRB + rTMS)

	Treatment Day	*IMP	Procedure
Injury			
Admission	1		GCS
Baseline	1		Informed Consent Exclusion/Inclusion: • Karnofsky Index • AIS • GCS Baseline Prognostic Risk Score • CT scan • Pupillary reactivity • Age • Motor score • Hypoxia • Hypotension • Subarachnoid hemorrhage Demographics Safety • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Medical history • Negative pregnancy test Blood samples – biomarkers (prior to study drug administration); GCS assessment shortly before administration of IMP
	1(or day 2)	Infusion (*TC 1-1)	The first infusion is administered within four hours after TBI, TC1 is 1x50 ml Cerebrolysin/0.9% NaCl per day for 10 days

	2 (24 hr +/- 4 hr after injury)	Infusion (TC1-2)	
	3	Infusion (TC1-3)	Serum biomarkers at 72 hours after TBI Genetic polymorphism APOE
	4	Infusion (TC1-4)	
	5	Infusion (TC1-5)	
	6	Infusion (TC1-6)	
	7	Infusion (TC1-7)	
	8	Infusion (TC1-8)	
	9	Infusion (TC1-9)	
Visit 1	10 (or day 11)	Infusion (TC1-10)	Primary Efficacy Criteria Day 10 • Mini Mental State Examination • Processing Speed Index • Stroop Color-Word Test – Victoria Version • Early Rehabilitation Barthel index • Glasgow Outcome Scale Extended Eye tracking qEEG Safety: • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Adverse Events • Negative pregnancy test
Visit 2	30 (+/-2)		Primary Efficacy Criteria Day 30 • Processing Speed Index • Stroop Color-Word Test – Victoria Version • Color Trails Test • Digit Span (Wechsler adult intelligence scale- third

			edition • Early Rehabilitation Barthel Index • Finger Tapping Test • Mini Mental State Examination • Hospital Anxiety and Depression Scale • Glasgow Outcome Scale Extended Eye tracking qEEG Safety • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Adverse events • Negative pregnancy test
	31 (+4)	Injection (TC2-1) + rTMS	1x10 ml CRB/0.9%NaCl once daily for 10 days
	32	Injection (TC2-2) + rTMS	
	33	Injection (TC2-3) + rtMS	
	34	Injection (TC2-4) + rTMS	
	35	Injection (TC2-5) + rTMS	
	36	Injection (TC2-6) + rTMS	
	37	Injection (TC2-7) + rTMS	

	38	Injection (TC2-8) + rTMS	
	39	Injection (TC2-9) + rTMS	
	40	Injection (TC2-10) +rTMS	
Visit 3	61 (+/-2)	Injection (TC3-1) + rTMS	1x10 ml CRB/0.9%NaCl once daily for 10 day
	62	Injection (TC3-2) + rTMS	
	63	Injection (TC3-3) + rTMS	
	64	Injection (TC3-4) + rTMS	
	65	Injection (TC3-5) + rTMS	
	66	Injection (TC3-6) + rTMS	
	67	Injection (TC3-7) + rTMS	
	68	Injection (TC3-8) + rTMS	
	69	Injection (TC3-9) + rTMS	
	70	Injection (TC3-10) + rTMS	
Visit 4	90 (+/-4)		Primary Efficacy Criteria Day 90 • Processing Speed Index • Stroop Color-Word Test – Victoria Version • Color Trails Test • Digit Span (Wechsler adult intelligence scale- third

			edition) • Early Rehabilitation Barthel Index • Finger Tapping Test • Mini Mental State Examination • Hospital Anxiety and Depression Scale • Glasgow Outcome Scale Extended Eye tracking qEEG Safety • Vital signs • ECG • Laboratory test • Neurological evaluation • Physical evaluation • Concomitant medication • Adverse events

*IMP – Investigation Medicinal Product

*TC – Treatment Cycle

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