

Arta Bārzdiņa

Evaluation of the role of biomarkers in diagnostics and prognostication of head injuries

Dissertation summary

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Scientific supervisors:

Dr. habil. med., professor **Māra Pilmane** AAI, RSU

Dr. habil. med., professor **Aigars Pētersons**

Department of Paediatric surgery, RSU

Official reviewers:

Dr. med. associated professor **Ilze Štrumfa** RSU

Dr. med. vet. associated professor **Ilze Matīse-Van Houtana** VMF,
LLU

MD PhD DEAA EDIC **Māris Dubņiks** University of Lund, Sweden

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Secretary of Promotion Council:

Dr. habil. med. professor **Līga Aberberga-Augškalne**

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LIST OF ABBREVIATIONS

Abbreviation	English	Latvian
<i>BBB</i>	<i>Blood brain barrier</i>	Hematoencefālā barjera
<i>BKUS</i>	<i>University Children's hospital</i>	Bērnu klīniskā universitātes slimnīca
<i>CNS</i>	<i>Central nervous system</i>	Centrālā nervu sistēma
<i>CSF</i>	<i>Cerebrospinal fluid</i>	Cerebrospinālais šķidrums
<i>CSN</i>	<i>Road accident</i>	Ceļu satiksmes negadījums
<i>CT</i>	<i>Computer tomography</i>	Datortomogrāfija
<i>DAI</i>	<i>Diffuse axonal injury</i>	Difūzi aksonāls bojājums
<i>EGF</i>	<i>Epidermal Growth Factor</i>	Epidermālais augšanas faktors
<i>GFAP</i>	<i>Glial fibrillary acidic protein</i>	Glijas fibrilārais skābais proteīns
<i>GCS</i>	<i>Glasgow Coma Scale</i>	Glāzgovas komas skala
<i>GOS</i>	<i>Glasgow Outcome Scale</i>	Glāzgovas iznākuma skala
<i>HT</i>	<i>Head trauma</i>	Galvas trauma
<i>i/c</i>	<i>Intra cranial</i>	Intrakraniāli
<i>ICP</i>	<i>Intra cranial pressure</i>	Intrakraniālais spiediens
<i>ICU</i>	<i>Intensive Care Unit</i>	Intensīvās terapijas nodaļa
<i>IF</i>	<i>Intermediate filaments</i>	Starpdiedziņi
<i>IL-α</i>	<i>Interleukin-1 alfa</i>	Interleikīns-1 alfa
<i>IL-1β</i>	<i>Interleukin-1 beta</i>	Interleikīns-1 beta
<i>IL-1RA</i>	<i>Interleukin-1 receptor antagonist</i>	Interleikīna-1 receptoru antagonists
<i>IL-4</i>	<i>Interleukin-4</i>	Interleikīns-4
<i>IL-6</i>	<i>Interleukin-6</i>	Interleikīns-6
<i>IL-8</i>	<i>Interleukin-8</i>	Interleikīns-8
<i>IL-10</i>	<i>Interleukin-10</i>	Interleikīns-10
<i>IL-12</i>	<i>Interleukin-12</i>	Interleikīns-12
<i>IL-17</i>	<i>Interleukin-17</i>	Interleikīns-17
<i>INF-α</i>	<i>Interferon-alfa</i>	alfa Interferons

<i>INF- γ</i>	<i>Interferon-gamma</i>	gamma Interferons
<i>IQR</i>	<i>Interquartile range</i>	Starpkvartīru izkliede
<i>K</i>	<i>Control patient</i>	Kontroles pacients
<i>M</i>	<i>Girl</i>	Meitene
<i>MCC</i>	<i>Median cell count</i>	Vidējais šūnu skaits
<i>MCP-1</i>	<i>Monocyte chemotactic protein-1</i>	Monocītu hemotakses proteīns-1
<i>mL</i>	<i>Milliliters</i>	Mililitri
μ m	<i>Micrometers</i>	Mikrometri
<i>MMPs</i>	<i>Matrix Metalloproteinases</i>	Matrices metaloproteināzes
<i>mRNA</i>	<i>Messenger Ribonucleic Acid</i>	Ziņotāja ribonukleīnskābe
<i>MSC</i>	<i>Mesenchymal stem cell</i>	Mezenhimālās cilmes šūna
<i>NF</i>	<i>Neurofilaments</i>	Neirofilamenti
<i>nm</i>	<i>Nanometers</i>	Nanometri
<i>NSE</i>	<i>Neuron-specific enolase</i>	Neironspecifiskā enolāze
<i>P</i>	<i>Patient</i>	Pacients
<i>pNF-H</i>	<i>Phosphorylated neurofilament-H</i>	Fosforilētais neirofilaments-H
<i>RNA</i>	<i>Ribonucleic Acid</i>	Ribonukleīnskābe
<i>RSU</i>	<i>Riga Stradins University</i>	Rīgas Stradiņa Universitāte
<i>S</i>	<i>Woman</i>	Sieviete
<i>S-AMPA</i>	<i>Selective-amino glutamate agonist</i>	Selektīvais amino glutamāta agonists
<i>S100B</i>	<i>S100 calcium binding protein B</i>	S100 kalciju saistošais proteīns B
<i>T</i>	<i>T cells</i>	T šūnas (T limfocīti)
<i>Th</i>	<i>T helpers</i>	T palīgšūna
<i>Th0; Th1; Th2</i>	<i>Different types of T helpers</i>	Tpalīgšūnu dažādas diferenciacijas
<i>TNF-α</i>	<i>Tumor Necrotic Factor alfa</i>	Tumora nekrozes faktors alfa
<i>UCH-L1</i>	<i>Ubiquitin C-Terminal Hydrolase-L1</i>	Ubikvitīna C-termināla hidrolāze-L1
<i>V</i>	<i>Man</i>	Vīrietis
<i>Z</i>	<i>Boy</i>	Zēns

INTRODUCTION

Head injuries are one of the most common causes of mortality and irreversible disability in children's population throughout the world. It causes serious socio-economic problems (Ghajar, 2000). In Europe and in the USA the frequency of head injuries varies from 100 to 1115 per 100 000 children, but in the age group up to 2 years it is on average 1150 to 1400 per 100 000 children (Falk *et al.*, 2008). In the last years the number of studies has increased, which emphasise the anatomical differences between adult brain and children brain still in development stages. It explains why head traumas of similar severity in infants and pre-school children have higher mortality rates than older children and adults (Giza *et al.*, 2007). For determination of severity of head injury the Glasgow coma scale is used (*GCS*), but for infants and small children – adapted modification of *GCS* (Simpson, 1982; Raimondi, 1984). Radiological methods provide limited information about severity and prognostics of head trauma. Computed tomography does not provide precise information about diffuse axonal brain damage, which is the most common in children; magnetic resonance imaging has its limitation when patient's condition is unstable. Light and medium severe head trauma form around 90% from all head injuries, and for this group of patients' precise acute diagnostics and the end stage prognostication are crucial. Studies from the last years emphasise that the least prognosticated, and with that the most dangerous, are the light head injuries, which are evaluated 13- 15 points by *GCS*; later after trauma patients can suffer from intracranial bleeding and diffuse axonal damage. In the future it can cause disability, disturbances in cognitive and psycho-social functions (Millis *et al.*, 2001).

With all this special attention is focused on biomarkers as indicators of prognosis of head injuries. Recently published studies identifies several

potentially valuable biomarkers, which are characteristic for brain tissue damage; inflammatory markers and/or markers of other biochemical and physiological processes, e.g., regeneration and apoptosis markers. A significant process of brain damage is the damage of cytoskeleton, one of its main markers is glial fibrillary acidic protein (*GFAP*). Less information in the literature is available about neurofilaments (*NF*), which are abundantly found in neuronal bodies. In case of neuronal destruction levels of *NF* in cerebrospinal fluid and serum could be in high concentration (*Shaw et al., 2005*). There are still no clear data in literature about expression of these biomarkers in brain tissue in spots of trauma and counterstroke in different time points after trauma. Detection of biomarkers in the spots of trauma and counterstroke has a significant role mostly because mechanics of head injuries in children is mostly linear acceleration, slow-down and rotational force interactions, which results in local damage in the spot of impact, and more extensive diffuse axonal damage in the spot of counterstroke. That is why it is important to understand the tissue response reactions in both, spot of impact and spot of counterstroke regions (*Drew and Drew, 2004*). Another pathological process that promotes secondary brain damage is inflammation. Studies turn special attention to complex, immune and inflammatory response reactions of brain tissue. Literature clearly indicates several inflammatory mediators that can be detected after traumatic brain injuries in animals, but there are no clear data about the expression of these biomarkers in human brain tissue in spots of direct impact and counterstroke in several time points after the injury. That is the reason of our study to focus on investigation of biomarkers, characteristic for secondary brain damage, and their expression in brain tissue and peripheral blood in several time points after the injury.

STUDY HYPOTHESIS

1. In cases of head injuries of similar severity expression of biomarkers (cytokines, chemokines, cytoskeleton markers) is more manifest in children of early age than ontogenetically older children and adults.
2. Expression of several biomarkers (cytokines, chemokines) specific for head injury is dependant of time period after the trauma.

AIM OF THE STUDY

Studies of biomarkers characteristic for secondary brain damage, and examination of their expression in brain tissue histological material and in peripheral blood samples in different time points after the injury.

OBJECTIVES

1. Studies the expression of *GFAP* and *NF* in brain tissue in the spot of impact and counterstroke in children and adults, which had died on the spot of the accident, and in those, which had died in delayed periods of time.
2. Studies the expression of *IL-6* in brain cortex and in the white substance, expression of *IL-10* in the white substance in spots of impact and counterstroke in children and adults, which had died on the spot of the accident, and in those, which had died in delayed periods of time.

3. Detection of amount and distribution of apoptotic cells in the spots of direct impact and counterstroke in patients after fatal head injury.
4. Detection of the possible correlations of the morphological data.
5. Detection of concentrations of inflammatory biomarkers *IL-1 β* , *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL-17*, *MCP-1*, *EGF* and *INF- α* in serum in children up to 7 years of age in four defined time points (24, 48, 72 and 96 hours after the trauma) in cases of severe, medium severe and light head trauma,, and also in a control group – healthy children up to 7 years of age.
6. Statistical analysis of the acquired data for determination of possible correlations of biomarkers in peripheral blood samples.

NOVELTY OF THE STUDY

Detection of biomarker differences in adults and children in the traumatised brain tissue, detection of biomarkers in peripheral blood samples in children.

MATERIAL AND METHODS

For the morphology studies brain tissue material from patients with fatal head injuries and control group, both from the archives of Institute of Anatomy and Anthropology of Riga Stradins University was used. Before being stored in this archive, the material was harvested in the Latvia State Centre for Forensic Medical Examination from the spots of direct impact and counterstroke, most often from frontal, occipital and right and left temporal regions, in 12 – 24 hours after patients' death. If the spot of the trauma was parietal regions, then counterstroke spots were lower regions of temporal regions. From the spots of direct impact mechanically traumatized tissue, and the adjacent tissue, consistent with the *penumbra* zone were sampled. Material was processed in the laboratory of Institute of Anatomy and Anthropology of Riga Stradins University with the routine histological and immunohistochemistry methods (permission of the RSU Committee of Ethics Nr. E-9(2) – 17.12.2009).

Total number of morphological material units were 28, which were divided into 4 subgroups and one control group: 1st group – 7 children up to 18 years of age that died in the spot of accident; 2nd group – 5 children up to 18 years of age that died in delayed time period after the trauma; 3rd group – 13 adults that died on the spot of the accident; 4th group – 3 adults that died in delayed time period after the trauma; 5th group – control group consisting of 5 adults.

5 µm thick slides were produced from the brain tissue material, haematoxylin and eosin staining was performed in each sample, and they were prepared for detection of glial fibrillary acidic protein (*GFAP*), neurofilaments (*NF*), interleukin-6 (*IL-6*) and interleukin-10 (*IL-10*) biotin – streptavidin

immunohistochemistry method. The antibodies used for this study are shown in Table 1.

Table 1.

Immunohistochemistry antibodies

No	Factor	Code	Extraction	Dilution	Manufacturer
1.	<i>GFAP</i>	M 0761	Mouse	1:100	<i>DakoCytomation, Denmark</i>
2.	<i>NF</i>	M 0762	Mouse	1:100	<i>DakoCytomation, Denmark</i>
3.	<i>IL-6</i>	sc-73319	Mouse	1:100	<i>Santa Cruz Biotechnology, USA</i>
4.	<i>IL-10</i>	ab-34843	Rabbit	1:400	<i>Abcam, Great Britain</i>

IL-6 – interleukin-6; *IL-10* – interleukin-10, *GFAP* – glial fibrillary acidic protein; *NF* – neurofilaments

For detection of apoptosis with *TUNEL* method an apoptosis kit *In Situ Cell Deth Detection, POD* catalogue No1684817 *Roche Diagnostics DNase I* was used.

Slides were viewed, using light microscope (*Leica*), and analysed with *Image Pro Plus 60* software. Histological pictures were captured using *Leica Microsystem AG* (Germany) digital camera.

GFAP and *NF* positive structures, detected by immunohistochemistry were counted using semi-quantitate method. The amount of these structures

was analysed in one slide's three random views. The descriptive of the semi-quantitate method are shown in Table 2.

Table 2.

Legends of the semi-quantitate method

Legends	Descriptions
–	No positive structures in the field of view
0/+	Occasional positive structures in the field of view
+	Few positive structures in the field of view
+ / ++	Few to moderate number of positive structures in the field of view
++ / +++	Moderate to numerous positive structures in the field of view
+++	Numerous positive structures in the field of view
+++ / ++++	Numerous to abundant positive structures in the field of view
++++	Abundance of positive structures in the field of view

IL-6 and *IL-10* positive cells were counted in one slide's three random fields of view, and the average cell count was calculated.

For data analysis of *TUNEL* method, an apoptotic index from the random fields of view of every slide, where the number of apoptotic cells out of 100 cells was counted, and the average number was calculated and then divided by 100.

For the clinical study blood samples were collected in the time frame from 2010 to 2012 in the Intensive care unit and Clinics of neurosurgery and neurology. These samples were analysed in the Biochemistry laboratory of Riga Stradins University (permission of the *BKUS* Committee of Ethics 30.08.2010).

In the clinical study venous blood samples from 18 children with severe, medium severe and light head trauma from age of 1 month to 7 years were analysed. The severity of head injury was determined upon admission by *GCS* (light HT – 13 to 15 points, medium severe 9 – 12 points, and severe – 3 to 8 points) (see appendix for further information on *GCS*), and computed tomography (*CT*) results. Patients with politrauma and/or other acute or chronic illnesses of different organ systems were excluded. All patients were divided into groups as follows: Group 1 – children aged 1 month to 2 years with head injuries of different severities (n=8), group 2 – children aged 2 years to 7 years with head injuries of different severities (n=10); group 3 – control group (n=16).

The venous blood samples were taken in the morning, from fasting patients in 2 vacu-tainers without anti-coagulant. Serum was harvested through spinning, and then stored frozen in -70° C. Serum samples were delivered to the RSU Biochemistry laboratory. Concentrations of cytokines were determined by *Milliplex kit Luminex xMAP* system.

Depending from the data structure routine medical research statistics methods were used. For data interpretation we used non-parametrical statistical methods. Central tendency variables with mean values and standart deviation and dispersion rates were used for comparison of study groups. For determining the mutual relations of two variables *Spearman* or *Pearson* correlations or linear regression analysis were used. *Mann – Whitney U* and *Wilcoxon Signed*

Ranks tests were used for comparing two independent groups. *P* value of less than 0.05 were considered statistically significant. Correlation coefficient *r* as the quantitate measurement of mutual correlation between two or more variables were calculated by ordinal scales - *Spearman* correlation coefficient. If *r* was more than 0.7 then correlation was determined as strong, if *r* was more than 0.5 the correlation was moderate, but if *r* was less than 0.3 correlations is weak. Statistical analysis was performed with *SPSS (Statistical package for social sciences for Windows 18.0 USA)*.

RESULTS

Morphological studies of brain cells' cytoskeleton, cytokines and apoptosis

In the brain tissue samples of the control group 6 layers of brain cortex were full blooded capillaries and some macrophages were seen. Also, the white matter of the control group samples revealed almost intact histological view with minimal glial cell oedema, nerve fibres and some plethoric capillaries.

All patients' samples after fatal head injuries revealed changes in the brain tissue. Both, direct impact and counterstroke spots had large pia mater damage with its fragmentation and wide areas of haemorrhaging among intact pia mater and the molecular layer of grey substance of brain.

All patients that died in the spot of the accident in the grey matter at the spot of direct impact had three of six layers of grey substance with oedema and focal necrosis; the white substance showed glial cell, basal substance oedema and plethoric capillaries. In the spot of counterstroke in the brain grey substance six layers of cortex were noted, the most clearly visible being three of them with remarkable cell oedema. The white substance had several stages of glial cell and blood vessels' wall oedema and plethora.

All patients that remotely after the trauma had six layers of grey substance with oedematous tissue and some destruction foci in the spot of direct impact. The white substance showed nervous fibre and glial oedema. In the spot of counterstroke these patients had all six layers of cortex visible, with remarkable neuronal structures' oedema and possible destruction foci. The white matter revealed nervous fibre, glial cell oedema of different stages and glial cell proliferation.

1.1 Results of control group brain tissue analysis.

All five control group patients had glial fibrillary acidic protein (*GFAP*) in the white substance of the brain in astrocytes and neuronal structures, also neurofilament (*NF*) expression in neuronal fibres and individual cells' nuclei. Interleukin *IL-6* positive pyramidal neurons were not detected in any of control group patients, just individual *IL-6* positive glial cells, but all patient had positive *IL-10* (mean 36.93 ± 1.89) and some *IL-6* positive glial cells (16.67 ± 2.87) in the white substance. All control group patients had apoptotic glial cells in the white matter (mean 58.13 ± 2.00 and apoptotic index (AI) (0.61 ± 0.05)).

1.2. Brain tissue analysis in children with head injuries, died in the spot of accident

In the spot of direct impact only three patients had *GFAP* positive nervous fibres and glial cells but *NF* positive neuronal structures in the white substance were noted in only four out of seven patients that died in the spot of accident. The white substance in the spot of counterstroke showed *GFAP* positive nervous fibres, immune-reactive astrocytes and factor-positive microglial cells and *NF* positive neuronal structures were seen in all seven fatalities. *GFAP* and *NF* positive neuronal structure mean relative amount in the white substance in the spot of direct impact was little (+), in the spot of counterstroke *GFAP* immune-reactive structure mean relative amount was from average to large (++)/+++), *NF* positive neuronal structure mean relative amount was large (+++). All seven patients that died in the spot of accident had *IL-6*

positive cortical pyramidal neurons, *IL-6* and *IL-10* positive glial cells in the white substance, and their number in the spot of direct impact was less than in the spot of counterstroke (Table 3). Apoptotic cells in the white substance were noted in all seven children in both, spots of direct impact and counterstroke. It must be emphasized that a one-year old child had three times more apoptotic cells in the spot of direct impact (62.33 ± 8.62) than in all other six patients of this group. The average number of apoptotic cells is displayed in Table 4. All children, which died in the spot of accident, except the one-year old, had only slightly larger number of apoptotic cells in the spots of counterstroke than in spots of direct impact.

1.3. Analysis of brain tissue in children, which died remotely after the trauma.

The white substance in the spots of direct impact and counterstroke showed *GFAP* positive nervous fibres, astrocytes, glial cells and macrophages, and *NF* positive nervous fibres, glial cells and macrophages in all five children, which died in a remote period after the injury. These children had average to large (++) average number of *GFAP* positive neuronal structures, and small (+) average number of *NF* positive neuronal structures in the spot of direct impact. In the spot of counterstroke *GFAP* and *NF* positive neuronal structure average relative number was large (+++). All patients, that died remotely after the head injury had *IL-6* positive cortical pyramidal neurons, *IL-6* and *IL-10* positive glial cells in the white substance, and its count in the spot of direct impact was smaller than in the spot of counterstroke. *IL-6* and *IL-10* positive glial cell number in the white substance in both, spots of trauma and counterstroke was smaller than in patients that died immediately after the accident (Table 3). Apoptotic cells were detected in all 5 children that died

remotely after the trauma. Notable, that in one-year and ten-months old patient apoptotic cell number in the spot of direct impact was larger (68.67 ± 3.48) than in the spot of counterstroke (59.33 ± 6.18) and larger than all the average numbers in this group, where all the adolescents had different counts of apoptotic cells in spots of trauma and counterstroke. The average number of apoptotic cells can be seen in Table 4.

Table 3

Average number on cytokine positive brain cells in spots of direct impact and counterstroke

	Group	<i>IL-6</i> PVPN, PV (avg.±SD)	<i>IL-6</i> PVPN, TV (avg.±SD)	<i>IL-6</i> BV, PV (avg.±SD)	<i>IL-6</i> BV, TV (avg.±SD)	<i>IL-10</i> BV, PV (avg.±SD)	<i>IL-10</i> BV, TV (avg.±SD)
Average	Group 1	43.81± 2.59	35.81± 2.76	93.52± 4.11	78.33± 3.05	37.33± 2.29	21.33± 1.39
Average	Group 2	39.13± 2.60	29.87± 2.67	52.40± 2.73	32.27± 1.97	17.40± 1.25	6.93± 0.52
Average	Group 3	43.82± 2.25	31.02± 2.53	90.33± 2.62	77.13± 3.40	38.13± 2.21	23.67± 1.57
Average	Group 4	44.89± 2.82	36.44± 2.00	61.89± 2.58	37.57± 2.52	6.11± 0.94	1.45± 0.79

BV – white matter; *IL-6* – interleukin-6; *IL-10* – interleukin-10;; *PV* – counterstroke; *PVPN* – pyramidal neurons of grey matter; *TV* – direct impact; SD – standard deviation; avg - average

Average number of apoptotic cell count in the white substance in spots of direct impact and counterstroke

	Group	Mean number of apoptotic cells in 3 fields of view, PV (avg. \pm SD); AI	Mean number of apoptotic cells in 3 fields of view, TV (avg. \pm SD); AI
Average	Group 1	37.33 \pm 14.11 (p=0.017) AI 0.48 \pm 0.04	40.47 \pm 26.31 (p=0.006); AI 0.40 \pm 0.14
Average	Group 2	50.00 \pm 8.00 (p=0.940) AI 0.48 \pm 0.04	47.83 \pm 3.66 (p=0.199); AI 0.40 \pm 0.14
Average	Group 3	55.33 \pm 24.51 (p=0.014); AI 0.65 \pm 0.05	60.44 \pm 10.86 (p=0.013); AI 0.63 \pm 0.04
Average	Group 4	50.67 \pm 19.92 (p=0.930); AI 0.64 \pm 0.11	59.00 \pm 19.73 (p=0.133); AI 0.77 \pm 0.05

AI – apoptotic index; *PV* – counterstroke; *SD* – standard deviation; avg - average
TV- direct impact

1.4. Brain tissue analysis in adults with head injuries, died in the spot of accident

None of the 13 patients, which died in the spot of accident, had *GFAP* and *NF* positive neuronal structures. All 13 patients had *GFAP* positive neuronal fibres, astrocytes and glial cells, also individual macrophages in the spots of counterstroke. *NF* positive nervous fibres, glial cells and individual macrophages were noted in 12 patients. There were no *GFAP* and *NF* positive cytoskeleton structures in the spots of direct impact; therefore their relative amount was not calculated.

In the spot of counterstroke *GFAP* positive neuronal structure average number changed from average to large (++)/+++), *NF* positive structures were seen in large numbers (++)). All 13 patients that died in the spot of accident had *IL-6*

positive cortical pyramidal neurons, IL-6 and IL-10 positive glial cells in the white substance; their number in the spot of direct impact was smaller than in the spot of counterstroke (Table 3). Apoptotic cells were also noted in all 13 patients, both in spots of direct impact and counterstroke. The average number of apoptotic cells in spots of direct impact and counterstroke in Group 3 is shown in Table 4.

1.5. Brain tissue analysis in adults with head injuries, died remotely after the trauma

All patients that died remotely after the trauma had *GFAP* positive nervous fibres, astrocytes, glial cells and *NF* positive nervous fibres and glial cells in the white substance both, spots of direct impact and counterstroke. Patients of this group had average number (++) of *GFAP* positive cells in the white substance in the spot of direct impact, but large (+++) in the spot of counterstroke. The average number of *NF* positive structures in the spot of direct impact was large (+++), in the spot of counterstroke – from large to abundant (+++/++++). All three adults, which died remotely after the injury had *IL-6* positive pyramidal cortical neurons, *IL-6* and *IL-10* positive glial cells in the white matter, and their amount in the spot of direct impact was smaller than in the spot of counterstroke (Table 3). Apoptotic cells in the white matter were noted in all three of these patients, both, in spots of direct impact and counterstroke (Table 4).

Results of clinical study

All patients of the clinical study, both from head injury group and control group, had 10 biomarkers detected in serum; but *IL-1β*; *IL-4*; *IL-* and in control group *IL-1β*; *IL-4*; *IL-12* and *IL-17* concentrations were lower than 3.2 pg/mL,

which is lower than the manufacturer’s recommendations, thus they were excluded from further studies.

2.1. Data of control group patients

Control group patients were divided in 2 groups – from 1 month to 2 years of age (5 children), and from 2 years to 7 years of age (11 children). The data showed that control group patients up to 2 years of age had 2 times higher medians of cytokine levels than patients older than 2 years (Table 5) .

Table 5

Levels of median cytokine concentrations in both study groups

	Group	<i>IL-6</i> pg/mL	<i>IL-8</i> pg/mL	<i>IL-10</i> pg/mL	<i>EGF</i> pg/mL	<i>MCP-1</i> pg/mL	<i>INF-α</i> pg/mL
Median	0-2 y	<3.2	13.20	6.02	122.10	637.89	19.45
IQR		0	33.19	38.14	225.82	332.71	11.33
Median	2-7 y	<3.2	8.27	3.87	41.37	335.78	14.34
IQR		0	4.10	3.31	48.54	139.93	5.19

EGF – epidermal growth factor; y - years; *IL-6* – interleukin-6; *IL-8* – interleukin -8; *IL-10* – interleukin -10; *INF-α* – alfa interferon; *IQR* – interquartile range; *MCP-1* – monocyte cheomotaxe protein-1; pg/mL – pictograms/millilitre

2.2. Data from children aged one month to two years with head injuries of different severity

This group consisted of 8 children with head injuries of different severity: 5 children with light, 2 children with medium severe and one with severe head trauma (*HT*). In this group higher concentrations of *IL-6* in serum were detected in patients with medium severe (30.81 and 34.56 pg/mL) and severe *HT* (36.97 pg/mL); also elevation of concentration was seen in 4 days after

trauma. **IL-8** concentration levels in patients from the first group were various: light *HT* 5.35 – 65.94 pg/mL, medium severe and severe *HT* 30.42 – 71.17 pg/mL in four days after trauma. Patients from the first group with light *HT* did not reveal significant changes in serum concentrations of **IL-10** in first four days after the trauma (<3.2 – 22.94 pg/mL). The highest level of *IL-10* serum concentration was seen in a patient with medium severe *HT* on the first day after the injury (71.17 pg/mL). Four patients of this group with light *HT* had similar serum levels of **EGF** in four days after the trauma – from the first to the third day it decreased from 196.26_{min}/ 437.69_{max} pg/mL to 79.35_{min}/318.92_{max} pg/mL, but on the fourth day it increased up to 290.85_{min}/516.64_{max} pg/mL. In patients with medium severe and severe *HT* the levels of **EGF** did not change in linear pattern, they varied between 9.34 and 132.96 pg/mL, without common tendency. 4 patients with light head trauma had steady levels of serum **MCP-1** concentration, except one patient whose serum **MCP-1** concentration was 2 times higher and decreased in four days from 1170.53 to 828.67 pg/mL. In patients with medium severe *HT* levels of serum **MCP-1** concentrations were between 266.82 and 1419.18 pg/mL. The patient with severe *HT* did not have significant differences in **MCP-1** serum concentrations in four days after the trauma. All 5 patients with light *HT* did not show significant changes in **INF- α** levels in four days after trauma. Serum concentrations of **INF- α** varied between 13.27 pg/mL and 31.73 pg/mL. Similar concentrations in four days were detected in one patient with medium severe and one patient with severe *HT*. The highest **INF- α** concentration was seen in an one month old patient with medium severe *HT* from 30.19 pg/mL to 42.11 pg/mL.

2.3. Data from children aged two to seven years with head injuries of different severity

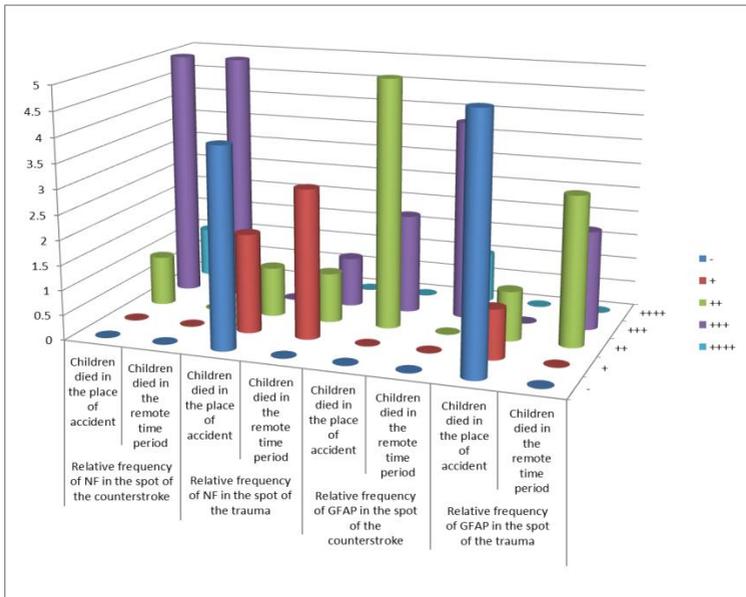
This group consisted of 10 children aged two to seven years with head injuries of different severity – 3 children with light, 4 children with medium severe and 3 children with severe head trauma (*HT*). Elevated serum concentration of IL-6 was detected in 7 children of this group; one of them with light head trauma had it up to 34.56 pg/mL, all 4 patients with medium severe *HT*; two of these patients had IL-6 levels up to 450.96 and 533.40 pg/mL, and both patients with severe *HT*. All 3 patients with light *HT* had serum concentrations of ***IL-8*** from 4.48 pg/mL to 32.80 pg/mL. All 4 patients with medium severe *HT* had elevated serum concentrations of *IL-8*, and they varied in the four days after the injury from 6.89 to 754.96 pg/mL. All 3 patients with severe *HT* had elevated serum concentrations of *IL-8* but the four day time period did not show abrupt variations, the levels were from 3,23 to 76.13 pg/mL. From this group only 2 children with severe head trauma had elevated serum concentrations of ***IL-10*** from 12.39 to 39.67 pg/mL. All other 8 patients, independently from severity of injury did not show significant changes in *IL-10* serum levels in four days after the trauma. All 3 patients with light *HT* had elevated serum concentrations of ***EGF*** in the four following days after the trauma from 50.62 to 365.00 pg/mL. All 4 children with medium severe and 3 children with severe *HT* had waveform changes of serum levels of ***EGF***. Patients with medium severe *HT* had its levels from 50.52 to 226.21 pg/mL, patients with severe *HT* showed *EGF* levels from 34.28 to 146.88 pg/mL. All patients from this group had elevated concentrations of ***MCP-1***. 2 patients from this group (one with light, and one with medium severe *HT*) had widely variable changes of serum concentrations of *MCP-1* in the four following days after the trauma from 343.29 to 1605.96 pg/mL. In all other 8 patients' serum

concentration of *MCP-1* in the four time period after the injury varied within 200 ± 50 pg/mL. All patients with light HT from this group did not show significant changes in serum concentrations of *INF- α* . Patients with light *HT* had it from 10.75 to 28.45 pg/mL, patients with medium severe *HT* - from 6.98 to 50.63 pg/mL, and patients with severe *HT* had slightly higher serum levels of *INF- α* concentrations than other children in this group – from 14.27 to 44.23 pg/mL.

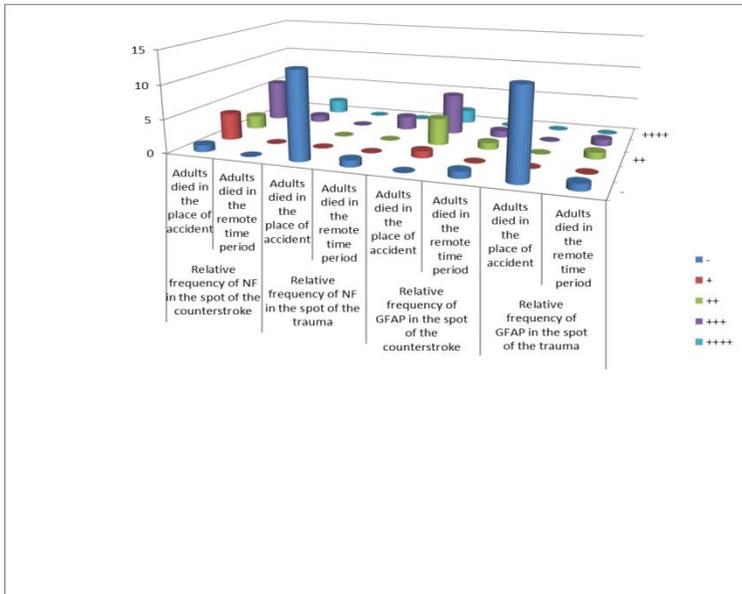
DATA STATISTICAL ANALYSIS

In data statistical analysis we used non-parametrical statistical methods Mann Whitney U test for comparing 2 independent samples (patients that died in the spot of accident and patients that died remotely after the injury) and Wilcoxon Signed Ranks test for comparison of 2 dependent samples (spots of direct impact and counterstroke).

Patients were divided in 2 groups – children and adults. Each of these groups were divided in 2 sub-groups: 1) children, which died in the spot of the accident, and those, who died remotely after the trauma (Picture 1), and 2) adults, which died in the spot of the accident, and those, who died remotely after the trauma (Picture 2).



Picture 1. Relative GFAP and NF amount in the brain's white matter in children, which died in the spot of accident and in those, who died remotely after the trauma in the spots of direct impact and counterstroke.



Picture 2. Relative GFAP and NF amount in the brain’s white matter in adults, which died in the spot of accident and in those, who died remotely after the trauma in the spots of direct impact and counterstroke.

Statistically significant differences were found in positive *GFAP* structures in the white matter of brain between both sub-groups in children in the spots of counterstroke (*Mann-Whitney U* test, $p=0.015$ and *Wilcoxon Signed Ranks* test, $p=0.030$) and positive *NF* structures in the spots of counterstroke only in both adult sub-groups (*Mann-Whitney U* test, $p=0.019$ and *Wilcoxon Signed Ranks* tests $p=0.025$).

All patients, either those died in the spot of accident (children and adults), and those died remotely (children and adults), had smaller numbers of **interleukin-6 (*IL-6*)** positive pyramidal neurons in the spot of direct impact than in the spots of counterstroke. Further information about average *IL-6*

positive pyramidal neurons and interquartile range (*IQR*) is shown in Table 6. In comparison of both independent groups – patients that died in the spots of accident and those who died remotely after the accident, no statistically significant differences between average numbers of *IL-6* positive cortical pyramidal neurons were found in the spots of counterstroke (*Mann-Whitney U* test, $p = 0.890$) and both groups *IL-6* positive pyramidal neurons in the spots of direct impact (*Mann-Whitney U* test, $p = 0.827$). Using *Wilcoxon Signed Ranks* test for 2 dependant groups, statistically significant differences between spots of counterstroke and direct impact in *IL-6* positive pyramidal neurons were found in patients that died remotely after trauma (*Wilcoxon Signed Ranks* test, $p < 0.001$), and patients that died in the spot of accident (*Wilcoxon Signed Ranks Test*, $p = 0.011$) (Table 6).

All patients, both the ones, which died in the spot of accident and those, which died remotely after head trauma (*HT*), -- **interleukin-6 (*IL-6*)** and **interleukin-10 (*IL-10*)** positive glial cell amount in the white matter of the brain was smaller in the spot of direct impact than in the spot of counterstroke. Further data about average *IL-6* and *IL-10* positive glial cell count and interquartile range(*IQR*) can be seen in Table 6. There were statistically significant differences between patients that died in the spot of accident and patients that died remotely in *IL-6* and *IL-10* positive glial cell count in the white matter in spots of direct impact (*Mann-Whitney U* test $p_{IL-6} < 0.001$; $p_{IL-10} < 0.001$) and in white matter in spots of counterstroke (*Mann-Whitney U* test, $p_{IL-6} < 0.001$; $p_{IL-10} < 0.001$), which shows that in patients that died in the spot of accident had statistically higher number of *IL-6* and *IL-10* positive glial cells than in patients that died remotely after the trauma. Using *Wilcoxon Signed Ranks* test for 2 independent samples statistically significant differences between *IL-6* and *IL-10* positive glial cells in spots of direct impact and counterstroke in patients that died immediately after the accident (*Wilcoxon*

Signed Ranks test, $p_{IL-6}=0.038$; $p_{IL-10}=0.050$), and those, which died remotely after the accident (*Wilcoxon Signed Ranks* test, $p_{IL-6}<0.001$; $p_{IL-10}<0.001$), (Table 6).

Apoptotic cell count was compared in the spots of direct impact and counterstroke in the white matter in children and adult groups. In children the average apoptotic cell count in the spot of direct impact was 42.57 ± 22.35 , in adults – 59.96 ± 14.05 . The average apoptotic cell count in the spot of counterstroke was 40.95 ± 13.78 in children, and 53.78 ± 22.80 in adults.

Comparing all four groups, the results were as follows: in the children's group that died immediately after the accident there were positive correlation between children's age and increase of apoptotic cell count in the spots of direct impact and counterstroke ($p_{\text{direct impact}}=0.006$; $p_{\text{counterstroke}}=0.017$). With increased age of children, also apoptotic cell count increase was seen in both, spots of direct impact and counterstroke. In adults, which died in the spot of accident, statistically significant differences between apoptotic cell count in the spot of direct impact ($p=0.013$) and counterstroke ($p=0.014$) were seen; in children and adults, died remotely after the trauma no statistically significant differences in apoptotic cell count in the white matter in spots between spots of direct impact and counterstroke were seen.

Table 6

IL-6* positive pyramidal neuron, glial cells and positive *IL-10* glial cell average values, interquartile ranges and statistical significance

	Patients died remotely after the trauma				Patients died in the spot of accident				
	Min	Max	Median	IQR	Min	Max	Median	IQR	p^1
PVPN counterstroke <i>IL-6</i>	13	67	41	27.8	34	53	44	10.5	0.890
PVPN direct impact <i>IL-6</i>	6	49	34.5	17.8	18	42	33	14.5	0.827
p^2	<0.001*				0.011*				
BV counterstroke. <i>IL-6</i>	21	75	61	23.5	76	113	101	25	<0.001*
BV direct impact <i>IL-6</i>	8	52	36	9.3	67	91	76	15	<0.001*
p^2	<0.001*				0.038*				
BV counterstroke. <i>IL-10</i>	4	33	12	9	22	51	37	18.5	<0.001*
BV direct impact <i>IL-10</i>	0	18	4	5	12	31	25	13	<0.001*
p^2	<0.001*				0.050*				

BV –white matter; PVPN – grey matter pyramidal neurons

p^1 –Mann– Whitney for 2 independent samples (patients, died remotely and immediately after the trauma);

p^2 – Wilcoxon test for 2 dependent samples (spots of direct impact and counterstroke)

Statistical data analysis from the clinical study

In the first group statistically significant changes in all four days after the trauma in comparison with control group were seen only in serum ***IL-6*** (*Mann Whitney U* test $p_{IL-61}=0.004$; $p_{IL-62}=0.009$; $p_{IL-63}=0.014$; $p_{IL-64}=0.009$). In the second group statistically significant differences were seen between serum ***IL-6***_{1,2,4} concentrations on the first, second and fourth day after the trauma (*Mann Whitney U* test $p_{IL-61}=0.003$; $p_{IL-62}=0.016$; $p_{IL-64}=0.003$); serum ***EGF***_{2,3,4} concentrations on the second, third and fourth day after the trauma (*Mann Whitney U* test $p_{EGF2}=0.024$; $p_{EGF3}=0.011$; $p_{EGF4}=0.014$) and serum ***INF- α_2*** concentrations on the second day after the trauma, in comparison with the control group of the same age (*Mann Whitney U* test $p_{INF-\alpha_2}=0.049$).

Calculation of *Spearman* correlation coefficient in the first and second group patients for 6 biomarker concentration in four days after the trauma compared one biomarker concentration correlation in four days, and the biomarker mutual correlations. In the first group statistically significant correlations among one biomarker's levels in four days were seen in all 6 biomarkers, but it did not apply in all four days. In the second group statistically significant correlations of one biomarker levels in four days were seen in five biomarkers – ***IL-6***; ***IL-8***; ***IL-10***; ***EGF*** and ***MCP-1***, but mutually not in all four days in a row. Significant correlations were assigned those, where the correlation coefficient r was >0.7 at significance level $p < 0.05$.

Serum concentration of biomarkers of patients from the first group showed that 4 patients with light *HT* serum ***EGF*** concentration changes were similar in 4 following days after the trauma. The data analysis with non-parametrical statistical method *Wilcoxon Signed Ranks* test, showed statistically significant

EGF concentrations in the first and second day after the trauma ($p=0.048$) and between the third and fourth day after the trauma ($p=0.048$).

These significant correlations, especially in one biomarker in four days after the trauma, could show relevance of this biomarker in diagnostics and prognostication of head injuries.

In patients of the first group mutual correlations of biomarkers were seen in one and several days after the trauma. Mutually statistically significant correlations were detected between ***IL-8*** and ***EGF***, ***IL-6*** and ***EGF***; ***IL-6*** and ***IL-8*** ($r>0.7$; $p<0.05$). In patients of the second group more biomarker concentrations showed correlations in one and several days after the trauma than in the first group. Mutual statistically significant correlations were seen between ***IL-8*** and ***MCP-1***, ***IL-8*** and ***INF- α*** , ***IL-8*** and ***IL-6***, ***INF- α*** and ***IL-6***, ***IL-10*** and ***EGF***, ***MCP-1*** and ***INF- α*** ($r>0.7$; $p<0.05$).

These mutual and statistically significant correlations of some biomarkers in the four days after the trauma leads to following interconnections: if correlation coefficient r is positive, then by increase in one biomarker's concentration, the other biomarker's concentration increases, if the correlation coefficient r is negative, then increase of one biomarker's concentration means decrease of another's biomarkers concentration. It could mean, that in cases when it is not possible to measure all desirable biomarkers for diagnostics of HT, changes of one biomarker could hypothetically predict increase or decrease of another biomarker, depending on the acquired value of the correlation coefficient.

DISCUSSION

First of all it should be mentioned that studies about the brain tissue damage in cases of head injuries have been mostly performed on experimental animal in controlled laboratory settings by modelling these injuries. This is why discussion is mostly based on the available data about experimental animals.

Our result show that patients in both groups – both, those, who died in the spot of the accident and those, which died remotely after the trauma, had less *GFAP* and *NF* in the spot of direct impact than in the spot of the counterstroke in the white matter. Most children that died in the spot of the accident and all adults did not show *GFAP* and *NF* positive neuronal structures in the white matter of the brain. Fatal head injury, which results in immediate death in the spot of the accident is a result of very strong, combined mechanical forces, which leaves the traumatized region as a mixture of damaged brain structures and massive hematomas. This morphological picture is being seen in the time of death, which does not allow for the primary damage to turn into secondary damage, i.e., the damaged brain tissue had not yet engaged in inflammatory processes which result in secretion of inflammation stimulant, neuro-chemically active substances and biomarkers that are significant for brain structure damage. As these patients died immediately after the trauma, in the period that lasts only minutes these reactions does not have time to happen.

Separately the group of children, which died in the spot of accident was analysed, and its results were different than in adults. This difference could be explained by the dynamic development theory in ages younger than 2 years, when all the biochemical processes, connected with brain development are more rapid than in adults. That is why there was no surprise that some children, which died immediately after the injury, had positive neuronal

structures. Results from children and adult groups show that in children, which died immediately after the trauma, *GFAP* and *NF* immune-reactive structure average count in the spot of direct impact was higher than in adults, who died immediately, but it was similar in spots of counterstroke. Special attention was focused on both children patients younger than 2 years – one year old child, who died in the spot of accident, and 1 year 10 month old baby, who died 2 days after the trauma. Both children had average number (++) of *GFAP* and *NF* positive neuronal structures in the white matter, but 1 year 10 month old child had more *GFAP* positive neuronal structures than the other child, who died in the spot of accident. The number of *NF* positive structures was similar in both children. This reaction could be explained by the specifics of brain development at this age. I. e., children up to 2 years of age has active cell differentiation, which is a background for last neuroblastic mitosis process (*Ross and Pawlina, 2006*). If in this period below 2 years of age *CNS* is subjected to mechanical forces, the necrotic and apoptotic cell death is more dynamic than in older children and adults (*Lenroot and Giedd, 2006*). So with great probability we can say that dynamic interfilament production mechanism in children's brain white matter right after the trauma signifies plasticity and self-defence ability of children's brain. In a short period of time it provides formation of glial scar. This way the primary damage gets restricted, and also the destructive impact of inflammatory cytokines to penumbra zone and further, healthy brain tissue lessens (*Faulkner et al., 2004*).

In studies with experimental animals – rats and mice – a scientific group revealed that in rats with controlled light *HT GFAP* immune-reactive changes in astrocytes progress from the 24th hour, reaching the maximum on the third day after the *HT*, but it remains intact in the white matter (*Ekmark-Lewén et al., 2010*). Another group of neuroscientists (*Bolouri et al., 2012*), in experiments with rats, imitating the light or medium severe *HT* n football

players, did not detect reactive astrogliosis in the first day after several controlled *HT*. It was fixed only after 7 – 10 days. It contradicts the experiments of *Li* and *Graham*, where in cats and dogs the controlled *HT* resulted in positive *GFAP* immune-reactive subcortical structures already in the first 24 hours (*Li et al.*, 1998; *Graham et al.*, 2000). In a study from 2012, reactive astrocytes were seen only in the spots of trauma after repeated blows on rats' skulls, but were not detected in spots of counterstroke in the white matter (*Bolouri et al.*). In our study, just like *Bolouri et al.* study from 2012, especially in the adult group, the greatest amount of *GFAP* and *NF* positive structures were found in adults that lived longer, from 7 to 15 days. But also differences were noted - *GFAP* and *NF* positive neuronal structures were seen in the highest number especially in spots of counterstroke not in direct impact. It is possible, that spots of direct impact, where the mechanical forces cause total tissue destruction, the damaged tissue inflammation reactions happen slower than in tissue that have been concussed in the spots of counterstroke. It corresponds with the study of *Verhkratsky et al.*, from 2012, where in cases of traumatic brain injury astroglial reaction was dependant from the mechanical force impact and the degree of tissue damage. That is why more active astroglial reaction can be seen in average blow force not massive blow, in which astroglial activity can be restricted or even suppressed.

Comparing the time of survival of children and adults and the mean number of *GFAP* we concluded that children, which mostly lived 48 hours after the trauma had similar amount of *GFAP* positive neuronal structures as adults, which lived 7 to 15 days. These data correspond with American study from experiments with mice (*Sandhir et al.*, 2004). This study revealed that older mice after controlled head trauma had gradually increasing concentrations of *GFAP* and reactive astrocytes in different time points in the white matter of

hippocampus. Younger mice had momentarily *GFAP* expression and large number of reactive astrocytes.

Analysing patients of both groups and time, which they lived after the trauma, it was seen that adults, lived longer than children (7 to 15 days), had significantly higher relative amount of intrafilamental *NF* positive neuronal structures in the spots of direct impact of the white matter than in children, which lived from 36 hours to 7 days after the trauma. Also the spot of counterstroke showed more *NF* positive structures in adults than in children's group. It is consistent with the experiment in mice and *NF* changes after the trauma. In this study in cases of light trauma the *NF* positive neuronal structure reaction was seen up to 1 month following trauma, when most likely *NF* dephosphorylation promoted survival of neurons, preventing their damage or dysfunction (Huh *et al.*, 2002). In the study with severe experimental *HT* in rats, loss of *NF* positive structure in the brain cortex ipsilaterally was seen already in 3 hours after the injury (Posmantur *et al.*, 1994). This data corresponds with our findings in patients, which died in the spot of accident 0 only few of these children had *NF* positive structures, but they were not found in any of the adults.

During last years special attention is focused on brain tissue cytoskeleton biomarkers - *GFAP* and amino acid heavy chain phosphorylated *NF* (*pNF-H*) studies, their serum level correlation with severity and result of head injuries. Also the role of *GFAP* in prognosticating and treatment of head injuries in children has become more important, by detecting its concentrations in serum. Missler *et al.* in 1999 studied *GFAP* serum concentrations in adults. A factor concentration, which is higher than 0.033 $\mu\text{g/L}$, confirms brain pathology but concentrations higher than 15 $\mu\text{g/L}$ confirm lethal outcome. In mammals and humans normal levels of *pNF-H* have not been confirmed. Right

now even more sensitive methods for detection of *pNF-H* have been developed. In this context the data about our local findings of *NF* in patients, which died immediately after the accident, and in patients, who lived more than 24 hours after *HT*, can be viewed as valuable original data.

The pathological processes and amount of head injuries are determined by hypoxia/hypotension with following cerebral ischemia, which is one of the inflammation promoters (*Morganti-Kossmann et al.*, 2001). From all the known secondary damage biochemical processes scientists have turned their attention to necrotic death of brain cells. This secondary brain damage is connected with wide endogenous inflammatory molecule productions, and their participation in different biochemical reactions in different periods after the trauma, providing both, inflammatory promoting and antagonistic inflammatory processes in brain tissue in penumbra zone, and in the adjacent healthy brain tissue (*Leker and Shohami*, 2002). In patients from both groups, the ones that died in the spot of accident and the ones that died remotely, ***IL-6*** positive pyramidal neuron count, ***IL-6*** and ***IL-10*** positive glial cell number in the white matter in the spot of trauma was smaller than in the spot of counterstroke. Larger number of *IL-6* and *IL-10* positive glial cells was seen in patients that died immediately after the trauma in both, spots of direct impact and counterstroke in comparison of patients that died remotely after the injury. It corresponds with Australian study (*Frugier et al.*, 2010) about inflammatory mediators in brain tissue of 21 people after fatal head injury, which concluded that brain tissue inflammatory reaction begins already after several minutes after the trauma. It is much earlier than believed. This observation could be explained with active astrocyte participation in the inflammatory process – formation of glial scar (*Schmidt et al.*, 2005). Glial scar is formed rapidly after the head injury, when in the spot of secondary injury, right after the primary damage, active cytokine production is started. In an experiment with rats it was

shown that right after *HT* neutrophils, monocytes and lymphocytes release inflammatory mediators, which concentrations remotely after the trauma decreased (*Das et al.*, 2011). It lets us presume, that healthy glial cells of white matter and penumbra zone has the most active inflammatory reaction right after the primary damage. Clinical studies have proven that *IL-6*, which is released in intracranial brain tissue after the trauma, diffuses through damaged chematoencephalic barrier, moves to peripheral circulation and promotes acute phase response reactions outside the central nervous system. During that *IL-6* is acting as an inflammatory promoting cytokine in the central nervous system, but in regeneration phase – as neuroprotective cytokine (*Schmidt et al.*, 2005). Analysing data from our study, statistically significant changes in *IL-6* concentrations in patients from both groups were seen only in the first and second day after the trauma. In some aspects it consists with the data from Italian – American science group data from children after *HT* of different severity, were biomarker concentrations on cerebrospinal fluid (*CSF*) were detected in the 2nd and 48th hour after the trauma. It was concluded that the highest concentration of *IL-6* in *CSF* was in the 2nd hour after *HT* – it was 10 to 23 times higher than in control group. The concentration of *IL-6* in *CSF* was lower in the 48th hour after the trauma (*Chiaretti et al.*, 2008). Several patients with medium severe an severe *HT*, and some patients from the first group with light *HT* had elevates serum *IL-6* concentrations in the second, third and fourth day after the trauma. One explanation for this could be based on studies with experimental animals. After brain tissue damage cytokines activate glial cells, which produce cytokines as a response to a trigger (*Barone and Kilgore*, 2008) and in animal brain tissue neurotoxic or neuroprotective microglial reactions were seen in correlation with the severity of head injury. In cases of light head trauma, i.e., light neuronal damage, microglial cells produced inflammation promoting chemicals, but neurotropic substances that promote neuronal regeneration were produced in all three severity grades of neuronal damage.

Another explanation could be based on studies about stem cell role in the pathological processes of *HT*. In rats mesenchymal stem cell (*MSC*) significance in inflammatory response reactions is proven. In rats, which had *MSC* administered, *IL-6* concentration decreased already after first 24 hours, but in those which did not receive *MSC*, *IL-6* levels initially decreased but later increased and reached a plateau 30 days after the trauma. It allowed concluding that *MSC* had immune response reaction modulating properties – in the first hours after *HT* when *IL-6* worked as inflammatory promoting mediator, *MSC* decreases its activity; after several days when *IL-6* activity is connected with re-vascularization, inhibition of *TNF- α* and stimulating nervous growth factors, *MSC* promotes *IL-6* activity. In our study *IL-6* concentration increase in the previous mentioned patients up to the fourth day after *HT* is, possibly, also a result of *MSC* activity.

In studies with experimental rats, in which controlled *HT* were modulated, no correlation between expression of *IL-10* in serum and its levels in brain tissue were found (*Kamm et al.*, 2006). In rat pups (*Gonzalez et al.*, 2009) and piglets (*Lyng et al.*, 2005) *IL-10* expression in immature brain tissue were different in comparison with mature animals and their brain tissue's inflammatory reactions. In adult rats systemic *IL-10* administration promotes neurological improvement after *HT* and parallel decreases inflammatory cytokine production (*Knoblach and Faden*, 1998), in animal pups (rats and pigs), systemic *IL-10* administration did not improve the neurological outcome, and in several cases it was even worse. In clinical studies with patients with severe head injuries biomarker expression was studied in *CSF* and blood serum. *IL-1* and *IL-6* in serum and in *CSF* were detected right after *HT*, *IL-10* in serum and in *CSF* was detected even after longer period of time, i.e., 24 hours (*Venetsanou et al.*, 2007). In studies with children it has been seen that *IL-10* reduces central local inflammation in brain tissue after *HT*, but in patients with

politrauma it can cause suppression of peripheral immune reactions, thus worsening the total outcome (*Morganti-Kossmann et al.*, 2007). In our study some patients with medium severe HT and one with severe HT had the highest level of *IL-10* in serum in the first day, but with every next day concentration of *IL-10* gradually decreased. This data corresponds with studies from patients with severe HT, in which the highest levels of *IL-10* were seen in the 24th hour after the injury, and then gradually decreased in 4 days (*Dziurdzik et al.*, 2004; *Hayakata et al.*, 2004).

In both groups patients with medium severe and severe *HT* showed 4 to 20 times higher concentration of *IL-8* on the first day after the trauma than patients with light *HT*. It corresponds with Swiss and Austrian scientists' study, where it was proven that higher concentration of *IL-8* in serum and in *CSF* depicts more severe trauma and more severe brain tissue damage. (*Kossmann et al.*, 1997). American scientists (*Stein et al.*, 2011) in a clinical study with patients with severe *HT* concluded that *IL-8* is an important factor for prognosticating severe *HT* and determining the amount of damage, as the concentration of *IL-8* in serum and *CSF* increases even before appearance of clinical symptoms (raise in intracranial pressure and progression of cerebral hypoxia). Our study patients from both groups showed the highest correlation activity of *IL-8* with other biomarkers. The correlations between *IL-8* and *IL-6*; *IL-10*, *MCP-1*, *EGF* and *INF- α* in serum in different days after the trauma were significant, and they could prove the role of chemokine *IL-8* in understanding pathological processes and prognosticating the outcome of *HT*.

When analysing each individual patient's concentration of *MCP-1* and its changes, we had to conclude that there is no connection between the age groups and severity of head injuries. For example, children up to 2 years of age with light HT on the first day can show very high serum concentrations of

MCP-1 but a child with severe *HT* from other age group had half of the concentration of *MCP-1* in serum. It matches the data from a British clinical study conclusion that in adults with severe brain contusion the changes of chemokine *MCP-1* concentrations could mean worse outcome of brain contusion, but *MCP-1* concentration in serum does not change if patient condition deteriorates and the amount of damage increases (*Rhodes et al.*, 2009). Published data gives a lot of information about interferon gamma (*INF- γ*) expression in brain tissue in experimental animals, and the positive significance of *INF- γ* in neuronal differentiation and growth stimulation is emphasised (*Wong et al.*, 2004). Whereas studies about significance of *INF- α* prognosticating the amount and outcome of head injuries are not published. Also, our study results show that *INF- α* is not significant in brain degeneration or regeneration processes after *HT*.

In our study *EGF* did not show statistically significant differences between patients of the first group and matched control group. Similar to our results, a clinical study conducted in Pittsburgh with infants with light *HT*, did not show statistically significant changes in concentration of *EGF* between patients and a control group of matched age (*Berger et al.*, 2009). But in our study high levels of serum *EGF* were seen in patients from the first group (children up to 1 year of age) with light *HT*. Thus, common tendency of serum *EGF* changes were seen in four following days after the injury. Also in infants of a control group, in comparison with other control group patients, the serum concentrations of *EGF* were three times higher. Data about changes of serum *EGF* concentration in four days after the trauma in 4 patients below 1 year of age from the first group with light *HT* showed statistically significant mutual correlation between the first and the second, and the third and the fourth day after the injury. Whereas patients with severe *HT* had 2 to 3 times lower concentrations of *EGF* than in the control group. This data is difficult to

interpret, as there are no many published reports about changes of *EGF* serum concentrations in different time points after head trauma. Groups of independent scientists have proved that *EGF* is a mediator for nervous cell proliferation and migration in mice and rats (*Teramoto et al., 2003; Sun et al., 2009*), showing that with the highest probability human brain has congenital potential to replace the population of damaged neurons as a part of endogenous neurogenesis. We can only hypothesise about the highest concentration results of *EGF* in our study in children up to 2 years of age with light head trauma. It is possible, that children below 2 years of age, when especially active brain development takes place due to neuroblastic differentiation, in cases of *HT* concentrations of *EGF* react to light *HT* in extremely sensitive way. In cases of severe and medium severe *HT* serum concentrations of *EGF* changed minimally. It is possible, that in cases of severe *HT* in these age groups several parallel physiological processes take place, and suppress the mediator function of *EGF*. In our study children older than 2 years had not had remarkable *EGF* expression, but it is possible that proliferation and migration of neuronal cells in cases of medium severe and severe *HT* is active. Thus, *EGF* could be one of potential prognosticating biomarkers in children below 2 years of age with light head injury.

Apoptosis

In human brain tissue after severe *HT* apoptotic cells are more seen in the white matter than in the grey matter of the brain (*Smith, 1997*). That is the reason, why we studied glial cells only in the white matter in spots of direct impact and counterstroke. Analysing the patients, included in our study, only few of them, which died remotely after the trauma, revealed large number of apoptotic cells. Special attention must be paid to the group of children, died in the spot of accident, which showed correlation between children's age and

increase of apoptotic cell number in spots of trauma and counterstroke. Exception was a one-year-old, who had remarkable differences in the average apoptotic cell count in the spots of trauma and counterstroke – it was remarkably higher in the spot of direct impact. It shows that children below 2 years of age have different process of apoptosis, more active than in older children. Studies about brain development have proven that if central nervous system gets subjected to mechanical forces, both necrotic and apoptotic cell death is more dynamic in children older than 2 years and in adults (*Paus et al.*, 2001; *Lenroot and Giedd*, 2006). Children below 2 years of age have very active necrotic processes, releasing high amounts of lysosomal enzymes in intracellular space, which can cause death of adjacent cells (*Clausen*, 2004) and/or starting the mechanism of programmed cell death. Studies with humans, which died remotely after severe *HT*, showed high numbers of apoptotic glial cells in the white matter of brain in comparison of grey matter, where the apoptotic cell count decreases after the 10th day of *HT* (*Smith et al.*, 2000). This allows presuming, that glial cell apoptosis in the white matter in prolonged period of time is a sign of severe *HT* in comparison of apoptotic cell death of neurons in the grey matter in cases of degenerative and other *CNS* diseases.

As the process of apoptosis takes from few hours to several days, it is very important to realise this time frame, in which it is possible to save the healthy nerve cells from the impact of damaged cells in penumbra zone. When decreasing the production of inflammatory promotion substances, also the apoptosis cascade decreases in the penumbra zone, which usually progresses after the lysosomal enzymes, released during brain cell necrosis process. It is especially relevant in children with severe *HT*, as the demyelination caused by *HT* is connected with loss of oligodendroglia via apoptosis (*Bell and Natale*, 2006).

CONCLUSIONS

1. Cytoskeletal proteins GFAP and filamental NF statistically significant increase in the white matter of the brain in adults and children, which lived more than 24 hours after the trauma, signifies momentane and remarkable adaptation of cytoskeletal for the damage in the white matter in the spot of counterstroke.
2. Dynamic polymorph (proliferation, expression, cell cytoskeleton changes) reaction of glial cells, which changes in cases of trauma in children below 2 years of age, signifies greater plasticity of brain and adaptation to traumatic changes in the brain tissue in this age group.
3. Pacients, that died in the spot of accident after a fatal head injury, had statistically significant increase in number of IL-6 and IL-10 positive glial cells in the spot of counterstroke versus spot of direct impact; this signifies spontaneous expression of these cytokines after a powerful trigger and rapid increase of tissue adaptation/anti-inflammatory reactions, which decrease in longer time periods if patient survives the trauma moment.
4. In most cases apoptotic cell count in the spot of direct impact was less in children than in adults, with exception of children up to 2 years of age. Thus, active brain tissue reaction (plasticity, regeneration) in children in early childhood can be argueded. In older children, who died immediately after the injury, correlation between growing ontogenetic age and increase of apoptotic cell count in spots of trauma and counterstroke, shows slowing of regeneration processes in growing brain.
5. In total, short term survival after the trauma and received treatment does not influence apoptotic rates in spots of trauma and counterstroke. It presents that

apoptosis rate show the real level of programmed cell-death in time of trauma/death.

6. Decrease in EGF serum concentration from the first to the third day after head injuries, and it increase from the third to fourth day after the trauma has a common tendency in children up to 1 year of age, which suggest that EGF could be informative diagnostic biomarker in children below 1 year of age with light head injuries.

7. Increase of IL-6 serum concentration statistically significantly correlated in all patients between the first and second day after the trauma; thus its shows that IL-6 is a potentially informative marker for diagnostics and regeneration starting from the 12th hour after the trauma.

8. Variable serum concentrations of IL-6 in both groups in four following days after the head injury shows the polymorph functions of IL-6 in brain tissue. IL-6 serum concentration decrease in the first 24 hours signifies blocking of regeneration processes in brain tissue; but abrupt increase in the third to fourth day signifies active role of IL-6 in regeneration processes.

9. In patients with head trauma chemokines IL-8 and MCP-1, cytokines IL-10 and INF- α did not show statistically significant changes in comparison with control group data. It points out, that by themselves these biomarkers do not provide information about severity of head trauma, its amount and outcome prognosis.

10. Chemokine's IL-8 tight and statistically significant correlation varriations with EGF and IL-6 in children up to two years of age, and with IL-6, MCP-1 and INF- α in older patients should be viewed as individual changes, but in

perspective – potentially informative in individual patients in diagnostics and prognosticating head injuries.

PUBLICATIONS AND PRESENTATIONS ABOUT THE STUDY

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