UDC 616.36-74/-78:577.112]-091.8
DOI: 10.14739/2310-1210.2022.5.261327

Key astroglial markers in human liver cirrhosis of different degree: immunohistochemical study

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

The aim of the study – determining the immunohistochemical levels of the GFAP, GS and AQP4 in different regions of the human brain in the conditions of liver cirrhosis of different degree.

Materials and methods. The study was performed on sectional material of 90 patients who suffered during lifetime from liver non-alcoholic cirrhosis of classes A (n = 30, group "A"), B (n = 30, group "B") and C (n = 30, group "C") according to Child–Pugh classification, including 59 (65.55 %) cases with clinical symptoms of I–IV grade hepatic encephalopathy. Cortex, white matter, hippocampus, thalamus, striopallidum, cerebellum, were examined using immunohistochemical method for evaluation of GFAP, GS and AQP4 levels.

Results. GFAP expression gradually decreased from classes A to C of cirrhosis. The most expressed GFAP decline was found in class C in the cortex and thalamus (6.74- and 6.23-fold decrease). Contrary to GFAP, GS expression gradually increased along with aggravation of cirrhosis. The most prominent augmentation of GS was related in the cortex and thalamus in "C" group, respectively 4.34- and 4.26-fold increase. AQP4 levels also showed growing mode correlated with cirrhosis aggravation. The highest increase was found in the cortex and thalamus in "C" group (4.25- and 4.34-fold increase, respectively). Starting from class B, altered GFAP, GS, and AQP4 levels showed region-dependent relationships. GS and AQP4 were positively correlated in all 6 studied regions, while the inverse relationships were found between GFAP vs. GS and GFAP vs. AQP4 proteins.

Conclusions. As early as in class A of cirrhosis, dynamic molecular alterations are occurred in the brain astrocytes, indicating the progressive development of astroglial remodeling with a violation of its cytoskeleton and redistribution of molecular domains within cells. This phenomenon is region- and time-specific; its signs get stronger with time from class to class, becoming most pronounced in class C. Among studied brain regions, cortex and thalamus are characterized by the most pronounced protein changes. Starting from class B, the remarkable relationship is seen between molecular changes of both direct and inverse type. Simultaneously emerging links might indicate synergistic involvement of these molecules in astroglial remodeling in chronic hepatic encephalopathy. Alterations in the mentioned astroglial molecular complex can serve both as a diagnostic marker of reactive astrogliosis during liver cirrhosis and represent a target for novel therapeutic approaches regarding encephalopathy in cirrhotic patients.

Key words: liver cirrhosis, astroglia, reactivity, GFAP, GS, AQP4, hepatic encephalopathy, immunohistochemistry.

Zaporozhye medical journal 2022; 24 (5), 529-537

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Ключові маркери астроглії при цирозі печінки різних ступенів тяжкості у людини: імуногістохімічне дослідження

Т. В. Шулятнікова, В. О. Туманський

Мета роботи – визначення імуногістохімічних рівнів GFAP, GS та AQP4 у різних ділянках головного мозку людини за умов цирозу печінки різних ступенів тяжкості.

Матеріали та методи. Дослідження здійснили на секційному матеріалі 90 пацієнтів, які протягом життя хворіли на неалкогольний цироз печінки класів A (n = 30, група «A»), B (n = 30, група «B») та C (n = 30, група «C») за класифікацією Чайлд–П'ю, у тому числі 59 (65,55 %) випадків із клінічною симптоматикою печінкової енцефалопатії I–IV ступенів. За допомогою імуногістохімічного методу досліджували кору, білу речовину, гіпокамп, таламус, стріо-палідум і мозочок для оцінювання рівнів GFAP, GS та AQP4.

Результати. Експресія GFAP поступово знижувалася, починаючи з цирозу класу A, набула найменших значень у корі та таламусі хворих класу C (зменшення у 6,74 і 6,23 раза). На відміну від GFAP, експресія GS поступово зростала разом з обтяженням цирозу. Найвиразніше підвищення GS виявили в корі, таламусі в групі «C» (у 4,34 і 4,26 раза відповідно). Рівні AQP4, що зростали, також корелювали з підвищенням класу цирозу. Найбільший приріст виявили в корі, таламусі в групі «C» (підвищення в 4,25 і 4,34 раза). Починаючи з класу B, у змінах експресії GFAP, GS і AQP4 встановили регіон-залежні кореляційні зв'язки. GS і AQP4 позитивно корелюють між собою в усіх 6 регіонах, що досліджували, а між білками GFAP/GS, GFAP/AQP4 виявили зворотний зв'язок.

Висновки. Починаючи з цирозу класу A, в астроцитах головного мозку відбуваються динамічні молекулярні зміни, що свідчать про поступовий розвиток астрогліального ремоделювання з пошкодженням цитоскелета та перерозподілом молекулярних доменів у клітинах. Це явище має залежність від регіону мозку та періоду хвороби; його ознаки посилюються з кожним наступним класом цирозу, набуваючи найбільшої виразності в класі C. З-поміж відділів головного мозку, що вивчали, кора й таламус характеризуються найсуттєвішими змінами експресії білків. Починаючи з класу B, визначали очевидні кореляційні зв'язки між молекулярними змінами і прямого, і зворотного типу. Одночасне виникнення такої кореляції вказує на синергетичну участь цих молекул в астроцитарному ремоделюванні при хронічній печінковій енцефалопатії. Зміни цього астрогліального молекулярного комплексу можна використовувати як діагностичний маркер реактивного астрогліозу при цирозі печінки, а також вони можуть бути мішенню для нових терапевтичних підходів щодо енцефалопатії в таких пацієнтів.

Ключові слова: цироз печінки, астрогліальна реактивність, GFAP, GS, AQP4, печінкова енцефалопатія, імуногістохімія.

Запорізький медичний журнал. 2022. Т. 24, № 5(134). С. 529-537

According the WHO data, liver cirrhosis is among leading causes of non-infectious morbidity and one of the twenty most common causes of death worldwide (http://www.who.int/) [1]. Hepatic encephalopathy (HE) is a severe consequence of liver cirrhosis with incidence about 30–45 % [2], manifesting as a complex neuropsychiatric disorder and leading to lethal outcome in about 90 % of patients with acute-on-chronic liver failure [3]. The American Association for the Study of Liver Disease (AASLD)/European Association for the Study of the Liver (EASL) practice guidelines for HE defines HE as "Brain dysfunction caused by liver failure and/or portal-systemic shunting, ..." [4].

HE is classified in type "A", linked to acute liver failure; type "B" related to porto-systemic bypass; and type "C" reasoned primarily by liver cirrhosis [5]. Regarding the severity and according the West–Haven criteria, HE is categorized as: (I) Grade 0 – "minimal" or latent HE (subclinical) when diagnostics of neuro-cognitive deficit is complicated without special psychometric tests; (II) Grade I – mild neuro-psychiatric abnormalities, "sleep-wake" cycle inversion; (III) Grade II – significant neurocognitive disorders in form of asterixis, seizures, hyperreflexia, time disorientation, prominent behavioural alterations, lethargy; (IV) Grade III – drowsiness, profound neurocognitive dysfunction, amnesia, disorientation in place and time, stuporous state with response to stimuli; (V) Grade IV – coma (precoma-1, precoma-2, coma-1, coma-2 – corresponded to the Glasgow Coma Scale) [5,6]. The first two grades (0 and I) refer to "covert" forms, while II–IV grades – to "overt" HE [6]. Along with ascites and varicose bleeding, overt HE reflects disease decompensation, hereby serves as a clinical marker of high risk for lethal outcome [5].

Current hypotheses of HE is linked to abnormal metabolism of ammonia, glutamine, amino acids (Tyrosine, Phenylalanine, Valine, Isoleucine, Leucine), action of inflammatory cytokines IL-1, IL-6, TNFα, manganese, neuroinflammation, all resulted in dysregulated neurotransmission, neurocognitive and functional disability of patients with HE [3,4,7]. In the brain hyperammoniemic conditions, the most vulnerable appear astrocytes being the only source of glutamine synthetase to metabolize ammonia [8]. Hyperammonemia leads to glutamine accumulation in astrocytes, which induces development of cerebral edema-swelling ending with coma and lethal outcome in case of decompensated acute-on-chronic live failure [9]. Recent studies evidenced a special role of astrocyte senescence in HE pathophysiology, which was suggested to be linked to abnormal growth factor signaling, altered glutamate metabolism and synaptic dysfunction [10,11]. It was proven experimentally and on human postmortem material, that in hyperammonemia, astrocytes gain specific reactive signs characterized by acquiring Alzheimer Type II phenotype with profound molecular and functional remodeling [12–14]. This state of astroglia in HE, C. Escartin et al. recommended to define as "diseased astrocytes" [15]. The central homeostatic neuroglia, losing its principal functions, initiate critical violations of fundamental physiological processes in the nervous tissue reflected in synaptic dysfunction, neurotransmitter imbalance, abnormal water homeostasis, disturbance of glymphatics and accumulation of toxic products, reduced microcirculation, blood-brain barrier (BBB) leakage, etc. [14,16].

Among central specific astrocytic molecules glial fibrillary acidic protein (GFAP) is one of the most critical. Known functions of the intermediate filament GFAP are focused mainly on regulation of cell volume, motility and structural stability [7]. It was evidenced in *in vitro* and *in vivo* studies that GFAP appeared regionally downregulated in HE, which was related to hyperammonemia-induced decline of ATP levels, oxidative/nitrosative stress, nitration of tyrosine residues, oxidation of RNA and astrocyte swelling [7]. Despite a long history of research on the revealing the precise role of GFAP in the mechanisms of edema development during HE, this issue is still uncovered. Blood-borne and liquor-borne ammonia crossing the BBB or brain-cerebrospinal barrier, once in the brain, is metabolized solely by glutamine synthetase (GS) into glutamine at the astrocytic perivascular end-feet [17]. Glutamine, a precursor of glutamate and gamma-aminobutyric acid, being released by astrocytes, then taken up by neurons, where it can be converted into the aforementioned neurotransmitters [18].

It was reported in earlier animal, *in vitro* and instrumental human studies that GS appeared to be upregulated in different brain regions in the conditions of acutely developed hyperammonemia [9,19], but not altered, downregulated or elevated in chronic forms of HE or liver disease without HE [7,9]. Given the mixed and conflicting earlier findings regarding the response of GS to acute/chronic hyperammonemia, this question still needs to be addressed. Aquaporin-4 (AQP4) is another critical protein of astrocytes, predominant water channel in the brain, which undergo alteration in response to hyperammonemia. Numerous studies have evidenced that this transmembrane protein enriched in astrocytic perivascular end-feet and responsible for the brain water homeostasis can be upregulated as well as reduced in acute and chronic liver diseases accompanied by hyperammonemia [7,20]. Despite the controversial findings, it was supposed that AQP4 alteration may play a principal role in cytotoxic and/or vasogenic edema formation occurring in HE brains. This statement needs further arguments to uncover the mechanisms that control edematous changes during liver cirrhosis in humans.

In sum, all above central proteins of the astrocytes are substantially altered and undergo changes of their regulation, expression and activity in HE. However, despite numerous studies with unambiguous results, the roles of each protein in compensated and decompensated HE is obscure, determining the relevance of the present research.

Aim

Immunohistochemical determination of the GFAP, GS and AQP4 levels in different regions of the human brain in the conditions of liver cirrhosis of different degree.

Materials and methods

The study was performed on sectional material of 90 patients who suffered during lifetime from liver non-alcoholic cirrhosis of classes A (30 cases, group "A"), B (30 cases, group "B") and C (30 cases, group "C") according to the Child–Pugh classification [21]. Etiologically, cirrhosis was classified as viral 64 (71.12 %), secondary biliary 13 (14.44 %), congestive 9 (10.00 %), drug-induced 2 (2.22 %), and cryptogenic

2 (2.22 %). Cases with concomitant diseases or conditions that could be accompanied by intoxication effect, such as renal diseases, cancer, necrotizing pathologies, severe inflammatory processes in the organism, etc., were excluded from the study cohorts, as well as the alcoholic, cancerous nature of liver failure and exogenic intoxications. Among the patients, 56 (62.22 %) were male aged 39–86 years and 34 (37.77 %) female aged 43–72 years. The mean age of all patients was 65 ± 3 years.

In groups "A" and "B", liver cirrhosis was diagnosed as an underlying or concomitant disease. Morphologically, class A cirrhosis was represented by inactive incomplete septal cirrhosis in 27 (90.00 %) cases and it was clinically accompanied by HE symptoms of Grade I in 8 (26.66 %) cases, which was documented in the medical history and case diaries. In group "B", cirrhosis was found in inactive in 20 (66.66 %) cases and in 10 (33.33 %) cases – in active forms; represented by macronodular 13 (43.33 %), micronodular 4 (13.33 %), mixed 8 (26.66 %), and incomplete septal 5 (16.68 %) variants. HE was characteristic of 21 (70.00 %) cases of class B cirrhosis being presented by Grade I or Grade II symptoms and figured either in case histories in a list of clinical symptoms and/or was reflected in the clinical diagnosis. Causes of death in groups "A" and "B" were complications of the main disease manifested by acute cardiovascular and/or respiratory failure.

In group "C", liver cirrhosis represented the main disease with the disease history of 11–35 years and lethal complications in form of varicose bleeding from the esophagus and the gastric cardia, acute cardiac failure, pneumonia, acute hepatorenal failure, HE and their combinations. Morphologically, class C was ranged: 23 (76.66 %) micronodular, 2 (6.68 %) macronodular and 5 (16.66 %) mixed variants. In 100 % of cases of class C, HE was indicated either in the clinical diagnosis or in the diaries of the case histories, being presented by HE symptoms of Grade II–III at a time of death in 23 (76.66 %), including Grade IV, hepatic coma, in 7 (23.33 %) cases, it was assessed on the Glasgow Coma Scale [22].

The control group was represented by 30 cases of death from acute cardiovascular failure with conditionally intact liver and the absence of any other intoxication factors; mean age 59.0 ± 2.5 years.

In each case, the clinical and laboratory data of the case histories were analyzed in detail. During the autopsy, sectional material of the organs was taken in the amount provided for making a pathoanatomical diagnosis. For histopathological analysis, brain and liver specimens were fixed in 10 % buffered formalin, subjected to a standard processing steps, embedded in paraffin blocks; histological examination was performed in sections stained with hematoxylin and eosin. Immunohistochemical (IHC) study of astroglial reactivity was carried out using material from sensorimotor cortex, subcortical white matter, hippocampus, thalamus, striopallidum and cerebellum (molecular layer, granular layer and white matter) of the brain. IHC study was carried out according to the standard protocol provided by the antibody manufacturer using primary antibodies: mouse monoclonal anti-GFAP (clone ASTRO6), rabbit polyclonal anti-GS, rabbit polyclonal anti-AQP4 (all from Thermo Scientific, USA) and Ultra VisionQuanto Detection imaging system with diaminobenzidine (Thermo Scientific Inc., USA). In all cases, IHC reactions were assessed in 5 standardized fields of view (SFV) in each listed brain region at a magnification of ×200 with a microscope Scope. A1 "Carl Zeiss" (Germany), Jenoptik Progres Gryphax 60N-C1"1,0x426114 (Germany) camera and the program Videotest-Morphology 5.2.0.158 (VideoTest LLC). Expression of GFAP, GS and AQP4 was assessed as a percentage of the relative area (S rel, %) of immune-positive labeling to the total area of SFV.

Data were analyzed using the package of Statistica® for Windows 13.0 (StatSoft Inc., license No. JPZ804I382130ARCN10-J). Compliance of quantitative indicators with the law of normal distribution was determined using the Kolmogorov–Smirnov test. The results were expressed as median (Me) with range (Q1; Q3) or as mean ± SD. The Student's t-test or Mann–Whitney U-test were used for comparison between two groups and Kruskal–Wallis test for comparing more than two. Correlation analysis was calculated using Spearman Rank Order Correlation coefficient (r). The results were considered statistically significant at 95 % ($P < 0.05$).

Results

The IHC study of GFAP, GS and AQP4 expression revealed unequal levels among the control and cirrhotic groups as well as diverse distribution within different regions of the brain.

In the control group, GFAP cytoplasmic expression appeared to be the highest in the white matter and the lowest in the cortex region, 18.20 (17.11; 18.43) %; 4.52 (4.23; 5.57) %, respectively (*Table 1*). Morphologically, control astrocytes were characterized by region-dependent heterogeneity. Cortical immune-positive astroglia displayed typical morphology of protoplasmic, glia limitans, interlaminar and varicose projection astrocytes, while the white matter, hippocampal, thalamic, sttriopallidar and cerebellar regions shared diverse astroglial morphology with the prevalence of the fibrous phenotype. In all studied brain regions astroglia showed non-overlapping territorial domain pattern (*Fig. 1*).

In cirrhotic groups, GFAP expression gradually decreased (compared to the control, $P < 0.05$) with rising the class of cirrhosis. In all 3 groups, decreased expression was conditioned by shortening and thinning of GFAP+ processes, as well as by decrease in the number of GFAP+ astrocytes (*Fig. 2*). Thus, class A of cirrhosis was characterized by decline of GFAP in all studied brain regions with the most prominent in thalamus – 2.19-fold, less pronounced in the cortex and hippocampus – 1.75-fold and the least in the striopallidum – 1.44-fold reduce (*Table 1*). In class B, decrease of GFAP level worsened to 5.13-fold in the cortical region, 3.78-fold in the thalamus and the least pronounced drop in the cerebellum – 2.20-fold compared to the control (*Table 1*). The most expressed decline of GFAP levels was found in class C of cirrhotic patients. The cortical region showed the lowest scores of GFAP expression, which were equal to 6.74-fold decrease compared to the control (*Fig. 2*). The second most altered region was the thalamus, where reduction in the expression was equal to 6.23-fold. The least valuable decrease was related to the cerebellum – 3.10-fold (*Table 1*). In the cortex, hippocampus, striopallidum and cerebellum, alteration of GFAP expression differed significantly among groups

Table 1. Brain GFAP, GS and AQP4 levels in liver cirrhosis of classes A, B and C expressed in the percent of positive labels in standardized field of view of the microscope

Brain region	Class "A"	Class "B"	Class "C"	Control
GFAP				
Cortex	2.57 (2.23; 3.21)*†	0.88 (0.81; 1.52)*†	0.67 (0.23; 0.78)*†	4.52 (4.23; 5.57)
Subcortical white matter	11.26 (10.70; 12.23)*†	7.31 (5.41; 9.83)*#	5.25 (4.81; 5.76)*#	18.20 (17.11; 18.43)
Hippocampus	4.05 (3.95; 4.52)*†	2.95 (2.83; 3.50)*†	2.26 (1.90; 2.73)*†	7.10 (6.58; 7.89)
Thalamus	2.90 (2.57; 3.25)*†	1.68 (1.22; 1.95)*#	1.02 (0.95; 1.37)*#	6.36 (5.91; 6.79)
Striopallidum	4.30 (3.71; 4.52)*†	2.21 (1.72; 3.62)*†	1.34 (1.12; 1.67)*†	6.23 (5.70; 7.84)
Cerebellum	3.84 (3.10; 4.10)*†	2.54 (2.45; 2.93)*†	1.80 (1.63; 2.11)*†	5.59 (5.18; 5.83)
GS				
Cortex	10.14 (7.11; 11.21)*†	15.54 (14.71; 17.12)*#	18.66 (16.14; 19.03)*#	4.29 (2.26; 5.63)
Subcortical white matter	0.74 (0.65; 0.88)*†	1.32 (1.27; 1.45)*†	1.62 (1.52; 1.88)*†	0.53 (0.34; 0.60)
Hippocampus	3.52 (3.28; 5.43)*†	6.12 (5.58; 8.15)*#	7.88 (7.64; 8.45)*#	2.25 (0.53; 1.90)
Thalamus	4.22 (4.15; 5.11)*†	7.43 (6.25; 8.74)*#	9.18 (8.23; 10.40)*#	2.15 (1.73; 3.45)
Striopallidum	3.43 (2.72; 5.02)*§	3.84 (3.07; 5.11)*§	5.43 (5.27; 6.14)*†	1.84 (1.33; 2.12)
Cerebellum	5.32 (5.27; 6.72)*§	6.22 (5.47; 6.86)*§	7.28 (7.14; 8.67)*†	2.43 (0.63; 1.84)
AQP4				
Cortex	6.32 (5.48; 8.23)*†	11.46 (10.18; 12.23)*#	14.45 (11.85; 14.74)*#	3.40 (3.22; 4.25)
Subcortical white matter	2.23 (2.12; 2.75)*†	3.23 (2.89; 4.15)*†	4.40 (4.25; 7.61)*†	1.25 (0.75; 1.34)
Hippocampus	8.15 (6.25; 9.14)*†	10.25 (9.33; 11.44)*#	13.84 (11.25; 14.23)*#	4.26 (4.17; 5.25)
Thalamus	3.14 (2.39; 4.27) *†	5.34 (4.59; 5.65)*†	6.21 (5.73; 7.45)*†	1.43 (0.43; 1.68)
Striopallidum	5.12 (4.75; 5.22)*†	6.23 (5.75; 6.72)*†	7.25 (6.85; 8.17)*†	1.95 (1.65; 2.43)
Cerebellum	5.25 (4.25; 7.34)*†	8.27 (7.67; 8.34)*†	9.22 (8.93; 10.43)*†	3.16 (2.47; 3.75)

Data are presented as median (Me) with lower and upper quartiles (Q1; Q3); ***:** significant difference in the same brain region compared to the control (P < 0.05); **†:** significant difference in protein expression compared to two other classes of cirrhosis in the same brain region (P < 0.05); **#:** significant difference compared to "A" class of cirrhosis in the same brain region (P < 0.05); **§:** significant difference compared to "C" class of cirrhosis in the same brain region (P < 0.05).

depending on the gradual worsening of cirrhotic classes, P < 0.05. Herewith, GFAP scores in the white matter and thalamus showed no difference between classes B vs. C of cirrhosis, P > 0.05 (*Table 1*). Representative dynamics of the cortical GFAP alteration among classes of liver cirrhosis can be seen in *Fig. 7*.

GS expression in the control group was found to be the highest in the cortex and the lowest in the white matter, 4.29 (2.26; 5.63) %; 0.53 (0.34; 0.60) %, respectively (*Table 1*). In all studied brain regions, GS cytoplasmic labeling was mostly related to perivascular astroglial end-feet and less often – to parenchymal astrocytic processes and cell bodies (*Fig. 3*).

Contrary to GFAP alteration, GS expression in cirrhotic groups gradually increased (compared to the control, P < 0.05) along with aggravation of liver cirrhosis. Increased expression was caused by wide acquisition of immunolabeling by cytoplasm of cell bodies and astroglial parenchymal processes in all studied regions (*Fig. 4*). Class A of cirrhosis was characterized by elevation of GS in all studied regions with the highest values in the cortex – 2.36-fold and the least in the white matter – 1.39-fold rise (*Table 1*). In class B, GS elevation gained maximal values to 3.62-fold in cortical region, 3.45-fold in thalamus and the least increase in striopallidum – 2.08-fold compared to control (*Table 1*). The most prominent augmentation of brain GS expression was identified in class C of cirrhosis. Cortical (*Fig. 4*) and thalamic regions presented the highest indicators of GS compared to the control, 18.66 (16.14; 19.03) %, (4.34-fold increase); 9.18 (8.23; 10.40) %, (4.26-fold increase), respectively. The least elevation of GS scores was found in the striopallidum and cerebellum, 2.95-fold and 2.99-fold increase, respectively, compared to the control (*Table 1*). GS expression differed significantly in all pairs of subsequent cirrhotic classes only in the subcortical white matter, P < 0.05. In the cortex, hippocampus and thalamus, GS expression differed significantly between A vs. B and A vs. C (P < 0.05), but not between B vs. C (P > 0.05) classes (*Table 1*). Wherein, in the striopallidum and cerebellum, GS expression differed significantly between A vs. C and B vs. C (P < 0.05), but not between A vs. B classes (P > 0.05) (*Table 1*). Representative dynamics of the cortical GS alteration among classes of liver cirrhosis can be seen in *Fig. 8*.

AQP4+ labeling in all studied brain regions of the control cases was related to the membranes of perivascular and parenchymal astrocytic processes of individual astrocytes (*Fig. 5*). AQP4 expression in this group appeared to be the highest in the hippocampus and the lowest in the white matter, 4.26 (4.17; 5.25) %; 1.25 (0.75; 1.34) %, respectively (*Table 1*).

As well as in GS trend, AQP4 expression in cirrhotic groups altered in growing mode and was correlated with liver cirrhosis aggravation. Increased AQP4 expression was associated with immunolabeling of cell body's plasmalemmas and increased numbers of positive cells in all studied regions, which caused moderate-to-weak homogenous staining of neuropil (*Fig. 6*). Group "A" demonstrated increase in AQP4 expression in all studied regions with the highest values in the striopallidum – 2.62-fold and the least in the cerebellum – 1.66-fold rise compared to the control (*Table 1*). In group "B", AQP4 elevation gained maximal indications of 3.73-fold increase in the thalamus, 3.37-fold in the cortex and the least increase in the hippocampus – 2.41-fold compared to the control (*Table 1*). The highest increase of brain AQP4 level was observed in the group "C". The cortical (*Fig. 6*) and thalamic regions showed the most prominent AQP4 elevation compared to the control, 14.45 (11.85; 14.74) %, (4.25-fold increase); 6.21 (5.73; 7.45) %, (4.34-fold increase), respectively. The least AQP4 elevation was related to the cerebellum:

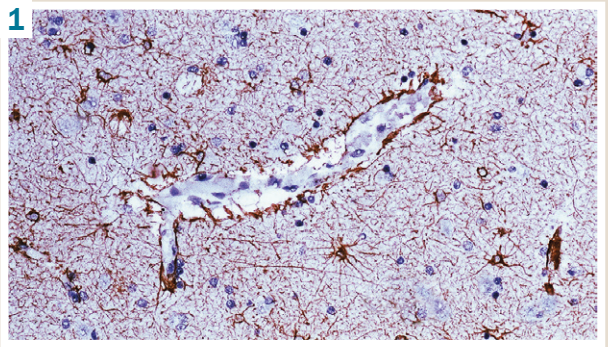


Fig. 1. GFAP cortical expression in a control case (anti-GFAP, clone ASTRO6, Thermo Scientific, USA). ×200.

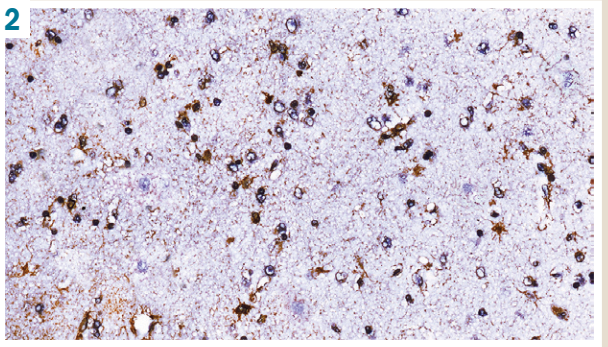


Fig. 2. GFAP cortical expression in a cirrhotic patient of class C died in hepatic coma (anti-GFAP, clone ASTRO6, Thermo Scientific, USA). ×200.

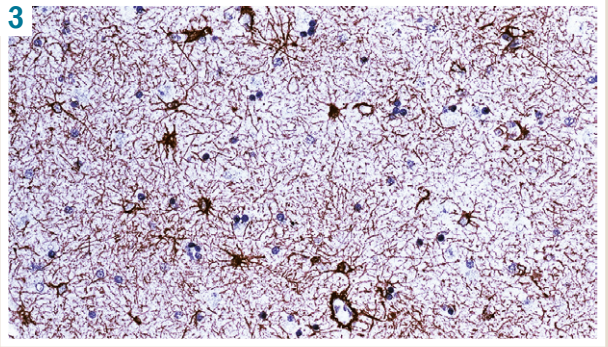


Fig. 3. GS cortical expression in a control case (anti-GS, Thermo Scientific, USA). ×200.

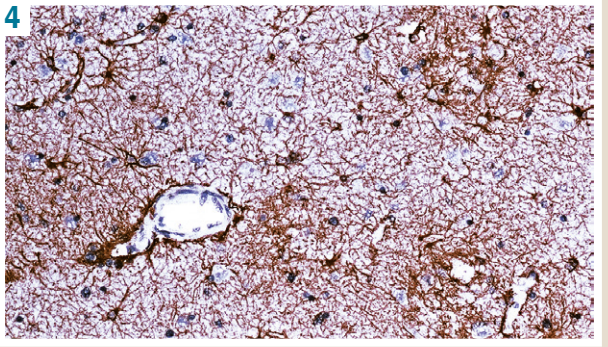


Fig. 4. GS cortical expression in a cirrhotic patient of class C died in hepatic coma (anti-GS, Thermo Scientific, USA). ×200.

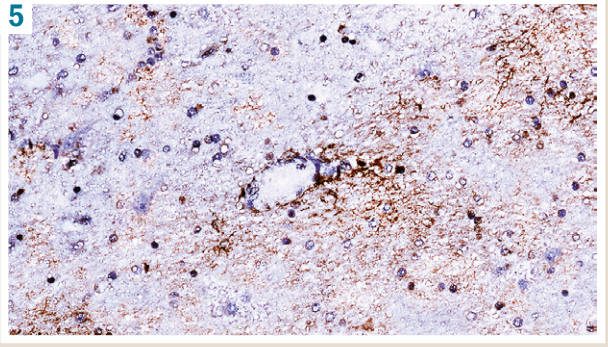


Fig. 5. AQP4 cortical expression in a control case (anti-AQP4, Thermo Scientific, USA). ×200.

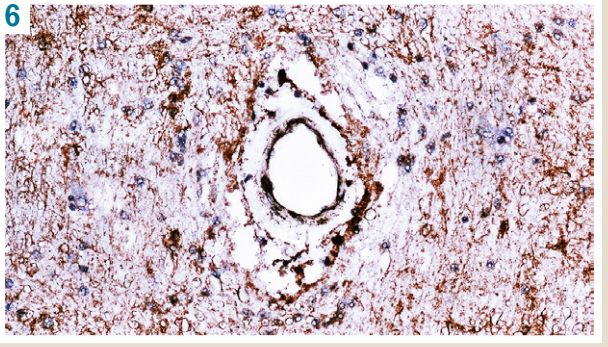
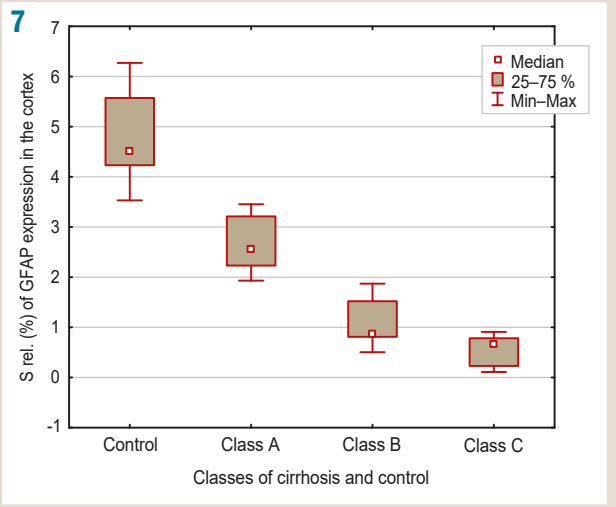
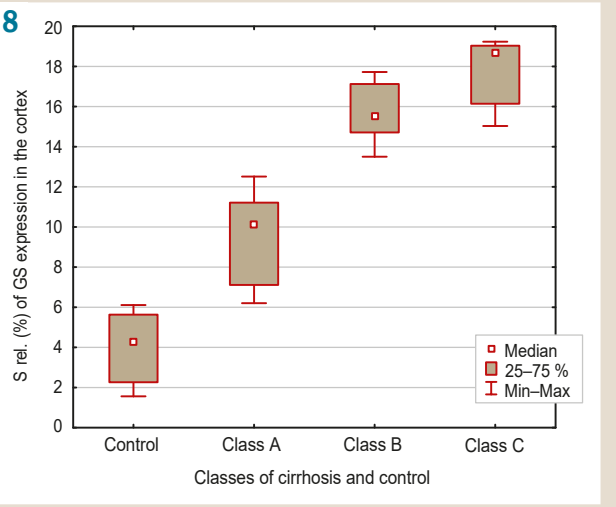


Fig. 6. AQP4 cortical expression in a cirrhotic patient of class C died in hepatic coma (anti-AQP4, Thermo Scientific, USA). ×200.

2.92-fold increase compared to the control (*Table 1*). AQP4 expression differed significantly in all pairs of subsequent cirrhotic classes in the white matter, thalamus, striopallidum and cerebellum, $P < 0.05$. In the cortex and hippocampus, AQP4 levels differed significantly between "A" vs. "B" and "A" vs. "C" ($P < 0.05$), but not between "B" vs. "C" ($P > 0.05$) groups (*Table 1*). Representative dynamics of the cortical AQP4 alteration among classes of liver cirrhosis can be seen in *Fig. 9*.

Correlation analysis of GFAP, GS and AQP4 expression in three classes of liver cirrhosis revealed that in class B there was a direct medium correlation between GS and AQP4 expression ($r = 0.57$), an inverse medium correlation between GS and GFAP ($r = -0.62$) and an inverse strong correlation between AQP4 and GFAP ($r = -0.72$) in the cortical region ($P < 0.05$). In the white matter, similar findings were detected: a direct medium correlation between GS and AQP4 expression ($r = 0.52$), an inverse medium correlation between GS and GFAP ($r = -0.53$) and an inverse medium correlation between AQP4 and GFAP ($r = -0.65$), $P < 0.05$. In the hippocampus: a direct weak correlation between GS and AQP4 expression ($r = 0.42$) and an inverse weak correlation between GS and GFAP ($r = -0.32$), $P < 0.05$. In the thalamus: a direct medium correlation between GS and AQP4 expression ($r = 0.51$), an inverse weak correlation between GS and GFAP ($r = -0.36$) and an inverse weak





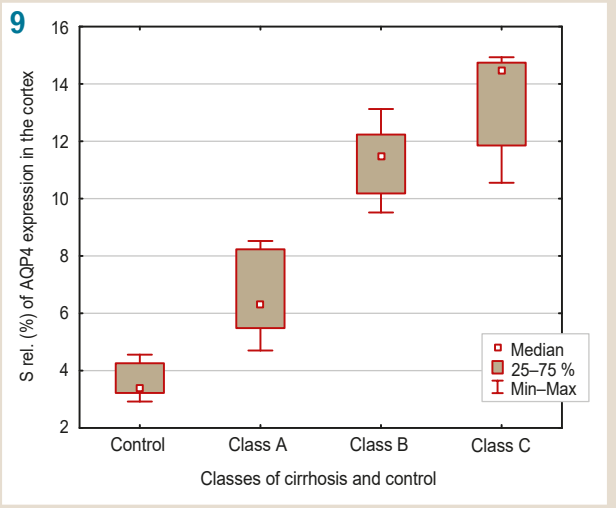


Fig. 7. GFAP expression in the cerebral cortex of patients with cirrhosis of A, B and C classes.

Fig. 8. GS expression in the cerebral cortex of patients with cirrhosis of A, B and C classes.

Fig. 9. AQP4 expression in the cerebral cortex of patients with cirrhosis of A, B and C classes.

correlation between AQP4 and GFAP (r = -0.41), P < 0.05. In the striopallidum: a direct weak correlation between GS and AQP4 expression (r = 0.37) and an inverse weak correlation between AQP4 and GFAP (r = -0.48), P < 0.05. In the cerebellum: a direct weak correlation between GS and AQP4 expression (r = 0.41) and an inverse weak correlation between AQP4 and GFAP (r = -0.36), P < 0.05 (*Table 2*).

In liver cirrhosis class C, a significant correlation was found in all studied brain regions. Thus, the cortex was characterized by a direct medium correlation between expressions of GS and AQP4 (r = 0.64), an inverse strong correlation between expressions of GS and GFAP (r = -0.83) and an inverse strong correlation between expressions of AQP4 and GFAP (r = -0.73), P < 0.05. In the white matter: a direct strong correlation between expressions of GS and AQP4 (r = 0.77), an inverse strong correlation between expressions of GS and GFAP (r = -0.86) and an inverse medium correlation between expressions of AQP4 and GFAP (r = -0.63), P < 0.05. In the hippocampus: a direct medium correlation between expressions of GS and AQP4 (r = 0.64), an inverse strong correlation between expressions of GS and GFAP (r = -0.83) and an inverse strong correlation between expressions of AQP4 and GFAP (r = -0.73), P < 0.05. In the thalamus: a direct medium correlation between expressions of GS and AQP4 (r = 0.67), an inverse strong correlation between expressions of GS and GFAP (r = -0.79) and an inverse medium correlation between expressions of AQP4 and GFAP (r = -0.61), P < 0.05. In the striopallidum: a direct strong correlation between expressions of GS and AQP4 (r = 0.71), an inverse strong correlation between expressions of GS and GFAP (r = -0.81) and an inverse medium correlation between expressions of AQP4 and GFAP (r = -0.65), P < 0.05. In the cerebellum: a direct medium correlation between expressions of GS and AQP4 (r = 0.61), an inverse weak correlation between expressions of GS and GFAP (r = -0.45) and an inverse weak correlation between expressions of AQP4 and GFAP (r = -0.48), P < 0.05 (*Table 2*).

Discussion

The results of the present study revealed the dynamic molecular changes in the brain astrocytes in the conditions of liver cirrhosis of different degree. As early as in class A of liver cirrhosis, studied key astrocytic proteins were altered significantly in a region-dependent manner compared to the control values. GFAP expression appeared to be dramatically and dynamically reduced with aggravation of the cirrhosis degree. In class A, it showed profound decrease in the thalamic region, but in class

Table 2. Correlations between the brain expressions of GFAP, GS and AQP4 in liver cirrhosis of classes A, B, C and in the control group

Class "A"				Class "B"				Class "C"				Control			
Cortex															
	GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4
GFAP	1.00	-0.45	-0.32	GFAP	1.00	-0.62*	-0.72*	GFAP	1.00	-0.83*	-0.73*	GFAP	1.00	-0.25	-0.22
GS	-0.45	1.00	0.23	GS	-0.62*	1.00	0.57*	GS	-0.83*	1.00	0.64*	GS	-0.25	1.00	0.23
AQP4	-0.32	0.23	1.00	AQP4	-0.72*	0.57*	1.00	AQP4	-0.73*	0.64*	1.00	AQP4	-0.22	0.23	1.00
Subcortical white matter															
	GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4
GFAP	1.00	-0.23	-0.27	GFAP	1.00	-0.53*	-0.65*	GFAP	1.00	-0.86*	-0.63*	GFAP	1.00	-0.13	-0.17
GS	-0.23	1.00	0.35	GS	-0.53*	1.00	0.52*	GS	-0.86*	1.00	0.77*	GS	-0.13	1.00	0.25
AQP4	-0.27	0.35	1.00	AQP4	-0.65*	0.52*	1.00	AQP4	-0.63*	0.77*	1.00	AQP4	-0.17	0.25	1.00
Hippocampus															
	GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4
GFAP	1.00	-0.21	-0.28	GFAP	1.00	-0.32*	-0.29	GFAP	1.00	-0.83*	-0.73*	GFAP	1.00	-0.11	-0.18
GS	-0.21	1.00	0.31	GS	-0.32*	1.00	0.42*	GS	-0.83*	1.00	0.64*	GS	-0.11	1.00	0.21
AQP4	-0.28	0.31	1.00	AQP4	-0.29	0.42*	1.00	AQP4	-0.73*	0.64*	1.00	AQP4	-0.18	0.21	1.00
Thalamus															
	GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4
GFAP	1.00	-0.22	-0.27	GFAP	1.00	-0.36*	-0.41*	GFAP	1.00	-0.79*	-0.61*	GFAP	1.00	-0.18	-0.13
GS	-0.22	1.00	0.29	GS	-0.36*	1.00	0.51*	GS	-0.79*	1.00	0.67*	GS	-0.18	1.00	0.22
AQP4	-0.27	0.29	1.00	AQP4	-0.41*	0.51*	1.00	AQP4	-0.61*	0.67*	1.00	AQP4	-0.13	0.22	1.00
Striopallidum															
	GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4
GFAP	1.00	-0.25	-0.28	GFAP	1.00	-0.35	-0.48*	GFAP	1.00	-0.81*	-0.65*	GFAP	1.00	-0.11	-0.18
GS	-0.25	1.00	0.26	GS	-0.35	1.00	0.37*	GS	-0.81*	1.00	0.71*	GS	-0.11	1.00	0.24
AQP4	-0.28	0.26	1.00	AQP4	-0.48*	0.37*	1.00	AQP4	-0.65*	0.71*	1.00	AQP4	-0.18	0.24	1.00
Cerebellum															
	GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4		GFAP	GS	AQP4
GFAP	1.00	-0.22	-0.29	GFAP	1.00	-0.32	-0.36*	GFAP	1.00	-0.45*	-0.48*	GFAP	1.00	-0.12	-0.15
GS	-0.22	1.00	0.32	GS	-0.32	1.00	0.41*	GS	-0.45*	1.00	0.61*	GS	-0.12	1.00	0.31
AQP4	-0.29	0.32	1.00	AQP4	-0.36*	0.41*	1.00	AQP4	-0.48*	0.61*	1.00	AQP4	-0.15	0.31	1.00

Data are presented as Spearman's rank order correlation coefficient (r); ***:** significant correlation between protein expression within one brain region in each of the studied groups ($P < 0.05$).

B, it appeared the lowest in the cortex; in class C – displayed the maximal decline among classes with minimal values found in the cortical and thalamic regions. These results confirmed the findings of earlier animal and human studies describing GFAP loss in the condition of acute and chronic hyperammonemia in such regions as hippocampus, corpus callosum, cerebellum and cortex, which was associated with astrocyte swelling and increased water content in the brain [7,14]. Our results indicated that GFAP level began to fall significantly since the first clinical degree of the cirrhosis and this event involved all 6 studied regions, i. e. the cortex, subcortical white matter, hippocampus, thalamus, striopallidum and cerebellum. These observations expand the knowledge about the brain areas which show obvious reactive changes in astroglia during severe chronic liver disease and are considered to be the primary target for hyperammonemia influence.

Similar to GFAP, GS and AQP4 underwent significant alteration in all studied regions beginning from the class A of cirrhosis, although in an opposite mode. Thus, in the first degree of cirrhosis, GS protein level appeared to be the highest in the cortex, while class B and C were characterized by the maximal increase in the cortex and thalamus compared to the control values. These findings only partially match other observations indicating that chronic liver insufficiency can be accompanied by regional variation of GS activity and strong elevation of GS protein in astrocytic perivascular end-feet in the rat model of portacaval anastomosis [7]. However, the intriguing thing is that most of studies using animal models of chronic liver disease and individual human postmortem studies of the brain cortex from cirrhotic patients consistently describe a substantial reduce in GS activity with no changes in GS protein expression found in selective brain areas including the cortex, hippocampus and cerebellum among other studied regions [7]. The results of our IHC assessment of GS contradict to previously stated mention and can claim that liver cirrhosis and suspected chronic hyperammonemia stimulate gradual increase in the production of GS protein to metabolize excessed brain ammonia. However, we can hypothesize that at some period of the disease, the activity of GS might start to fall appearing even less than normal levels, attributable to a tyrosine nitration of the enzyme [18]. Furthermore, the mismatch in the results could be explained by the presence of acute-on-chronic liver failure cases in B and C classes of cirrhosis with experience of grade III and IV of HE, which could induce protein alterations specific for acute hyperammonemia.

Thus, our previous study of acetaminophen-induced acute liver failure in rats have demonstrated pronounced region-specific increase in GS expression in 5 brain regions, namely the cortex, white matter, hippocampus, thalamus and caudate-putamen, which was remarked by higher protein expression in the cortex of non-survived rats. The latter result was suspected to be an evidence of the negative effect of increased brain GS on disease progression

and decompensation during acute liver failure [19]. There have been already made successful therapeutic attempts to pharmacologically inhibit GS and subsequently attenuate ammonia-induced astroglial cytoskeletal alterations and cell swelling, finished by temporal brain edema relief [18].

However, other studies provide evidence that deletion of GS in the whole mice brain or selectively in the cortex lead to enormous decline of the glutamine and increased ammonia level in the brain, followed by early death of newborns or progressive gliosis, impaired neurovascular coupling, decreased locomotion, spontaneous seizures, sudden death or survival for several months [23]. In patients with insufficient GS activity due to partial mutations in GS gene, it is observed neonatal epileptic encephalopathy, brain atrophy and early death. Similar to this, patients with mesial temporal lobe epilepsy were evidenced to be deficient in glial GS in the epileptogenic hippocampus [18]. Based on the literature data and own observations, we should consequently assume, that GS protein expression and its activity might play a crucial role in the edema formation and synaptic dysfunction in HE, being strongly dependent on the brain ammonia level and the duration of hyperammonemia.

AQP4 immunoreactivity in the brain of cirrhotic patients revealed the similar pattern as GS was increased as early as in the class A of cirrhosis being maximally elevated in the striopallidum and thalamus compared to the controls. In class B, the expression continued growth and appeared to be the most intensive in the thalamus, cortex and striopallidum, while in class C, the highest elevation was in the cortical and thalamic regions compared to the controls. Meanwhile, analyzing AQP4 level in groups B and C of cirrhosis, we can not exclude that the high indicators could be determined by cases of acute-on-chronic liver failure on the moment of patient's death, which might seriously affect the average expression level.

Despite common belief that chronic liver disease is not associated with cerebral edema and intracranial hypertension, studies have evidenced the presence of low-grade edema observed in cirrhotic patients with HE, as well as in 4-week bile duct ligation rats, denoted by increased water content in all brain regions. The latter was shown to be accompanied by increased expression of AQP4 in the cortex, hippocampus, striatum and cerebellum of the rat brain [7]. Our results indirectly confirmed the previous notion of the AQP4 role in edematous changes of the brain in liver cirrhosis, since changes in its immunoreactivity were closely correlated with those of two other astrocytic markers, namely GS and GFAP, which are critical regulators of water content and cellular volume of astrocytes.

Interestingly, the alteration of protein levels occurred heterogeneously in different brain regions, which may indicate a different degree of sensitivity and/or vulnerability of the brain regions or astroglial regional populations to the impact of a complex microenvironment that occurs in conditions of liver failure. Regarding GFAP, it was demonstrated that significantly declined level was observed consistently, from class to class, and different between classes in all studied regions, with the exception of the white matter and thalamus, where already in group B, its decreased levels did not differ from the minimal indicators of group C. GS expression reached the highest levels already in class B and did not differ from the maximal values of class C in certain regions, including the cortex, hippocampus and thalamus; while the striopallidum and cerebellum were characterized by maximal increase in GS only in class C. AQP4 expression, as well as GS showed the highest levels already in class B in the cortex and hippocampus, while in other regions – consequent growth from class to class.

Correlations between levels of GFAP, GS, and AQP4 indicated that, starting only from class B and getting stronger in class C, protein alterations apparently became an obvious with region-dependent relationship. Thus, such interrelation was reflected in the positive correlation between GS and AQP4 expression in all 6 studied regions of the brain, indicating the simultaneity and unidirectionality of reactive changes in these astrocytic molecules, as well as strongly suggesting their important roles in HE pathophysiology and potentially similar effects on the disease progression. The inverse relationship was found in classes B and C between GFAP vs. GS and GFAP vs. AQP4 molecules. Cortical, hippocampal and thalamic expression of GFAP and GS were negatively correlated with each other in both classes B and C, while in the striopallidum and cerebellum, the analogous relationship was found only in class C, indicating its later establishment in these regions. The pair GFAP vs. AQP4 was also marked by the negative correlation which was observed in classes B and C in all studied regions, except the hippocampal region, where it appeared significant only in class C.

Based on the obtained data on the regional heterogeneity of the protein expression changes, we should conclude that among the studied brain areas, the most susceptible to the conditions of chronic severe hepatogenic intoxication were the cortex and thalamus. In these regions, astroglia exhibited the most pronounced reactive alterations in the expression of proteins involved in the fundamental physiological processes, in particular, regulation of cell volume, brain water content and the maintenance of neurotransmitter balance.

Conclusions

1. Astroglial expression of GFAP in liver cirrhosis is characterized by the dynamic decline from class A to class C in the cortex, white matter, hippocampus, thalamus, striopallidum and cerebellum with a maximum decrease in immunoreactivity in the cortex and thalamus (by 6.74- and 6.23-fold, respectively). A significant loss of GFAP expression indicates morpho-functional remodeling of astrocytes, indirectly associated with changes in their shape and volume.

2. Expression of AQP4 in astrocytes in liver cirrhosis is characterized by the gradual growth from class A to class C in all of the above brain areas with the maximum elevation in the cortex and thalamus (4.25 and 4.34 times, respectively). Pronounced increase in AQP4 reflects the hyperactivation of the regulatory mechanisms of astrocytic intra- and extracellular fluid content, including intensive function of the brain glymphatic system.

3. Astrocytic GS expression in liver cirrhosis elevates from class A to class C in all 6 studied brain regions with the maximum increase in the cortex and thalamus (by 4.34 and 4.26 times, respectively). The significant gain in GS, a key enzyme for ammonia metabolism in the CNS, indirectly

points to the brain hyperammonia and neurotransmitter imbalance. Territorial heterogeneity in GS alterations are conditioned by both regional predominance/absence of glutamate neurotransmission and the diverse sensitivity of local astroglial populations to hepatotoxic factors.

4. Regional changes in the expression of the studied molecules (GFAP in the cortex, hippocampus, striopallidum and cerebellum, AQP4 in the white matter, GS in the white matter, thalamus, striopallidum and cerebellum) differ significantly between all three classes of liver cirrhosis, which allows using the obtained data in retrospective pathoanatomical grading of cirrhosis and hepatic encephalopathy.

Prospects for further research. Further study on regional astroglial interaction with microglia is needed to improve our knowledge on the mechanisms of the brain cellular reactivity in human liver cirrhosis. Moreover, additional human and animal studies are essential to elucidate the relationships between liver failure of different degree, brain metabolism, edema formation and clinical manifestations of chronic HE for attempts to find future glia-directed therapies of this challenge.

Funding

This study was conducted in the framework of the scientific research work of Zaporizhzhia State Medical University "Morphogenesis of destructive-reparative processes of the brain in diseases of vascular and toxic-metabolic genesis", state registration No. 0118U004253.

Conflicts of interest: authors have no conflict of interest to declare.
Конфлікт інтересів: відсутній.

Надійшла до редакції / Received: 13.07.2022
Після доопрацювання / Revised: 09.08.2022
Прийнято до друку / Accepted: 22.08.2022

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