

Ammonia level and Alzheimer type 2 astrocytes in the brain of deceased patients with liver cirrhosis of the varying degree

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The aim of the study – comparative analysis of the ammonia level and Alzheimer type 2 astrocytes in the brain cortex and white matter of cerebral hemispheres, hippocampus, thalamus, striatum and cerebellum in the deceased patients with liver cirrhosis of classes A, B and C according to Child–Pugh.

Materials and methods. The study was performed on the brain of deceased 90 patients (65 ± 3 years) suffered from non-alcoholic liver cirrhosis (LC) of classes A (n = 30, group “A”), B (n = 30, group “B”) and C (n = 30, group “C”) according to Child–Pugh score, among which 59 (65.55 %) patients had clinical symptoms of hepatic encephalopathy of I–IV grades. The control group included postmortem brains of 30 patients (59.0 ± 2.5 years) who died from acute cardiovascular insufficiency and did not suffer from liver diseases or intoxication. A retrospective analysis of clinical and laboratory data from case histories was carried out. For histochemical (HC) determination of the ammonia in paraffin sections of the cerebral cortex and white matter, hippocampus, thalamus, striatum and cerebellum we used the protocol with Nessler’s reagent proposed by Gutierrez-de-Juan et al. (2017). In the noted brain regions, the analysis of HC ammonia optical density was performed in five standardized fields of view (×200) of the microscope scope A1 “Carl Zeiss” (Germany) with Jenoptik camera progress Gryphax 60 N-C1”1.0x426114 (Germany) using ImageJ software; in each noted brain region the number of Alzheimer’s type 2 astrocytes (AA2) was counted per twenty standardized fields of view at magnification ×400.

Results. HC method for ammonia detection with Nessler’s reagent according to V. Gutiérrez-de-Juan et al. (2017) reveals region-dependent fine-grained expression of ammonia in the brain neuropil of deceased patients in control and cirrhotic groups. In control patients, a very low HC ammonia expression is observed with higher values in cerebellum, thalamus, and striatum, while still ranked as negative. Increased HC ammonia expression (compared to control) is observed in deceased patients with compensated LC of “A” group in the cortex, thalamus, striatum and cerebellum; in subcompensated LC of “B” group – as well in the white matter and hippocampus. In deceased patients with decompensated LC of “C” group, in cerebellum, thalamus and striatum HC ammonia expression is maximally increased (by 6.18, 5.72, and 5.50 folds, respectively). Significant correlations are present between patients’ postmortem brain HC ammonia expression and the last intravital indicators of the blood total bilirubin, AST, ALT, albumin, leukocytic intoxication index. In compensated cirrhosis, increase in AA2 numbers compared to control is found in thalamus, striatum and cerebellum, which corresponds to AA2-astrocytosis of I degree. In subcompensated cirrhosis, AA2-astrocytosis of moderate II degree is found in the cortex, thalamus and cerebellum; AA2-astrocytosis of I degree – in striatum. In decompensated cirrhosis, pronounced AA2-astrocytosis of III degree is determined in the cortex, thalamus, striatum and cerebellum; moderate AA2-astrocytosis of II degree – in cerebral white matter, and mild AA2-astrocytosis of I degree – in the hippocampus. There is a moderate, strong, and very strong positive relationship between the levels of AA2-astrocytosis and HC ammonia expression in the thalamus, striatum, and cerebellum.

Conclusions. In deceased patients with LC, the neuropil HC ammonia expression in cerebellum, thalamus, striatum, and cerebral cortex directly correlates with the severity of LC according to Child–Pugh, reaching a maximum in LC of class C, and has significant correlations with intravital blood levels of total bilirubin, AST, ALT, albumin, leukocytic intoxication index. With LC progression, AA2-astrocytosis increases significantly in thalamus, cerebellum, striatum and cerebral cortex, which positively correlates with HC ammonia expression in these brain regions.

Ключові слова:
цироз печінки,
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Рівень аміаку й астроцитів Альцгеймера 2 типу в головному мозку померлих хворих на цироз печінки різних ступенів тяжкості

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Мета роботи – порівняльний аналіз рівня аміаку й астроцитів Альцгеймера 2 типу в тканині кори та білої речовини великих півкуль головного мозку, гіпокампу, таламусу, смугастого тіла та мозочка у померлих хворих на цироз печінки класу А, В та С за Чайлд–П’ю.

Матеріали та методи. Дослідили головний мозок (ГМ) 90 померлих хворих (віком 65 ± 3 роки) на неалкогольний цироз печінки (ЦП) класу А, В і С за Чайлд–П’ю. Визначили 3 групи спостережень: «А» (n = 30), «В» (n = 30) та «С» (n = 30). У 59 (65,55 %) хворих груп «А», «В» і «С» виявили клінічну симптоматику печінкової енцефалопатії I–IV ступенів. У групі контролю дослідили ГМ 30 померлих (віком 59,0 ± 2,5 року) від гострої серцево-судинної недостатності, в яких не діагностували захворювання печінки або інтоксикацію. У кожному випадку виконали ретроспективний аналіз клінічних і лабораторних даних історій хвороб. Методом V. Gutiérrez-de-Juan et al. (2017) з використанням реактиву Несслера здійснили гістохімічне (ГХ) визначення вмісту аміаку в парафінових зрізах тканини кори та білої речовини великих півкуль головного мозку (ВПГМ), гіпокампу, таламусу, смугастого тіла та мозочка. У цих відділах ГМ виконали порівняльний

аналіз оптичної щільності ГХ експресії аміаку в п'яти стандартизованих полях зору (при збільшенні $\times 200$) мікроскопа Score A1 «Carl Zeiss» (Germany) з камерою Jenoptik Progres Gryphax 60N-C1 J; у кожному відділі ГМ у двадцяти стандартизованих полях зору зі збільшенням $\times 400$ обрахували кількість астроцитів Альцгеймера 2 типу (AA2).

Результати. Завдяки застосуванню ГХ методу виявлення аміаку в нервовій тканині з використанням реактиву Несслера за V. Gutiérrez-de-Juan et al. (2017) визначили регіон-залежну дрібнозернисту експресію аміаку в нейропілі тканини ГМ померлих контрольної групи та померлих хворих на ЦП. У померлих контрольної групи виявили дуже низьку ГХ експресію аміаку з відносно вищими значеннями в мозочку, таламусі та смугастому тілі, яку за градацією, що застосували, визначили як негативну. Підвищену ГХ експресію аміаку (порівняно з групою контролю) встановили в померлих хворих на компенсований ЦП групи «А» в корі ВПГМ, таламусі, смугастому тілі та мозочку; в померлих пацієнтів із субкомпенсованим ЦП групи «В» – також у білій речовині ВПГМ і гіпокампі. У померлих хворих на декомпенсований ЦП групи «С» у мозочку, таламусі та смугастому тілі ГХ експресія аміаку підвищена максимально (у 6,18, 5,72 та 5,50 раза відповідно). Між посмертним ступенем ГХ експресії аміаку в тканині ГМ та останніми прижиттєвими показниками рівня загального білірубину, АсАт, АлАт, альбуміну, лейкоцитарного індексу інтоксикації в крові хворих виявили значущі кореляційні зв'язки.

У померлих хворих на компенсований ЦП у таламусі, смугастому тілі та мозочку встановили підвищену (щодо контролю) кількість AA2, що відповідала I ступеню AA2-астроцитозу. У померлих хворих на субкомпенсований ЦП AA2-астроцитоз помірного II ступеня виявили в корі ВПГМ, таламусі та мозочку; AA2-астроцитоз I ступеня – в смугастому тілі. У померлих пацієнтів із декомпенсованим ЦП виражений AA2-астроцитоз III ступеня визначили в корі ВПГМ, таламусі, смугастому тілі та мозочку; AA2-астроцитоз помірного II ступеня зафіксували в білій речовині ВПГМ, слабкий AA2-астроцитоз I ступеня – в гіпокампі. Кореляційний аналіз показав наявність середнього, сильного та дуже сильного позитивного зв'язку між середніми кількісними показниками AA2-астроцитозу та ГХ експресією аміаку в таламусі, смугастому тілі та мозочку.

Висновки. У померлих хворих на ЦП груп «А», «В» і «С» ступінь ГХ експресії аміаку в нейропілі тканини мозочка, таламусу, смугастого тіла та кори ВПГМ прямо корелює зі ступенем тяжкості ЦП за Чайлд–П'ю, досягаючи максимуму при тяжкому ЦП класу С, має значущі кореляційні зв'язки з рівнем загального білірубину, АсАт, АлАт, альбуміну, лейкоцитарного індексу інтоксикації в крові хворих. Із прогресуванням тяжкості ЦП спостерігали істотне підвищення ступеня AA2-астроцитозу в таламусі, мозочку, смугастому тілі та корі ВПГМ, що позитивно корелювало зі ступенем ГХ експресії аміаку в тканині цих відділів ГМ.

Among the main manifestations of decompensated liver cirrhosis (LC), along with fatal complications, represented by profuse esophageal variceal bleeding and septic conditions, severe refractory ascites, jaundice and hepatic encephalopathy (HE) are also often observed. The latter is the second most common complication of decompensated LC after ascites and is typical for 30–45 % of patients [1]. HE is characterized by a spectrum of neuropsychiatric disorders from hardly noticeable to a lethal form – hepatic coma [2]; the grade of HE is assessed from 0 to IV according to the West Haven criteria [3]. According to the Child–Pugh cirrhosis scoring [4] patients with subcompensated cirrhosis of class B mostly manifest with “overt” HE II grade, while in patients with decompensated cirrhosis of class C, severe forms of HE III–IV grades prevail [2]. Severe forms of HE reduce the survival rate of patients with liver cirrhosis to 2 years [1].

The central factor in the pathophysiology of HE is the neurotoxic effect of NH_4^+ and NH_3 forms of ammonia, which levels markedly increase in the blood of cirrhotic patients [5]. Diffusible gaseous NH_3 ammonia freely penetrates through the blood-brain barrier (BBB), and in the ionized form NH_4^+ pass BBB through K^+ channels and cellular transmembrane transporters [6]. NH_4^+ and NH_3 , as well as cytotoxic bile acids significantly increase in the blood and the brain tissue [7], while both NH_4^+ and NH_3 are capable to change the pH of the cellular cytoplasm and liquid sectors of the brain significantly [5]. In astrocytes, high ammonia concentration and glutamate are metabolized via glutamine synthetase in osmotically active glutamine, accumulation of which causes astrocytes swelling and subsequent generalized brain edema [8]. Glutamine overloading of astrocytes leads to its hydrolysis in mitochondria followed by releasing of new portions of ammonia (the “Trojan horse” hypothesis

and reactive oxygen species [9,10]. Stimulation of benzodiazepine receptors by ammonia instigates an increase in the activity of gamma-aminobutyric acid (GABA) and leads to a neurotransmitter imbalance with a predominance of GABAergic tone [2]. Violation of the glutamine–glutamate astrocytic shuttle, direct ammonia inhibition of EAAT-1 (GLAST) and EAAT-2 (GLT-1) transporters contribute to the growth of synaptic glutamate followed by persistent depolarization of neuronal membranes, while gaining oxidative stress may lead to neuronal death [9,11].

Despite the prognostic value of HE in assessing the risk of lethal outcome in LC, its clinical diagnosis remains low since 80 % of cirrhotic patients predominately suffer from its covert forms [12]. Currently, a large number of morphological studies of the brain in the experimental liver failure contrasts sharply with single pathomorphological studies of the human brain in cases of HE, coma, or liver failure. The ambiguous relationship between plasma ammonia levels and HE clinical manifestation has been studied extensively [12] whereas pathomorphology of the brain damage in hepatogenic neurotoxicosis is reflected only partially in a few works of 2001–2020, indicating that the main histopathologic hallmark for HE is the detection of the so-called Alzheimer's type 2 astrocytes (AA2) in the brain [5,13–16]. Postmortem histochemical determination of tissue ammonia in different organs was performed in a single study of V. Gutiérrez-de-Juan et al. (2017) [17], which showed its effectiveness in comparison with the standard colorimetric method for ammonia detection. The relationship of pathomorphological signs of hepatogenic brain damage with the level of ammonia in the brain tissue, as well as with the main intravital laboratory parameters in deceased cirrhotic patients, has not yet been elucidated in the literature.

Aim

Comparative analysis of the ammonia level and Alzheimer type 2 astrocytes in the brain cortex and white matter of cerebral hemispheres, hippocampus, thalamus, striatum and cerebellum in the deceased patients with liver cirrhosis of classes A, B and C according to Child–Pugh.

Materials and methods

The brains and livers from 90 deceased patients (65 ± 3 years) suffered from non-alcoholic LC of classes A, B, C according to the Child–Pugh score [4], which composed 3 groups: “A” ($n = 30$, compensated LC), “B” ($n = 30$, sub-compensated LC) and “C” ($n = 30$, decompensated LC), were studied. Among noted 90 patients, 59 (65.55 %) patients had clinical symptoms of HE grades I–IV. Cases with comorbidity with systemic intoxication, endocrine disorders, as well as patients cancerous and alcoholic liver diseases, were excluded from the study. Control group was designed from 30 patients (59.0 ± 2.5 years) who died from acute cardiovascular insufficiency and did not suffer from liver diseases or intoxication. In each case, a retrospective analysis of clinical and laboratory data from case histories was carried out with an emphasis on the dynamics of laboratory blood parameters, presence of HE clinical symptoms and ascites. According to clinical data, LC was verified as viral in 64 (71.12 %) patients, secondary biliary – in 13 (14.44 %) patients, congestive – in 9 (10.00 %) patients, drug-induced – in 2 (2.22 %) observations and as cryptogenic – in 2 (2.22 %) patients. In group A, LC was morphologically represented by macronodular form in 27 (90.00 %) cases and was accompanied by clinical symptoms of I grade HE in 8 (26.66 %) patients. In group B, LC was macronodular in 13 (43.33 %) cases, micronodular in 4 (13.33 %) patients, macromicronodular in 8 (26.66 %) patients, and macronodular-septal in 5 (16.68 %) patients. In group B, 21 (70.00 %) patients with subcompensated LC had symptoms of I–II grades HE. In group C, LC was ranked as micronodular in 23 (76.66 %) patients, macronodular in 2 (6.68 %) patients, and macromicronodular in 5 (16.66 %) patients. In group C, II–III grades HE occurred in 23 (76.66 %) patients, and HE IV degree (hepatic coma according to the Glasgow coma scale [18]) developed in 7 (23.33 %) patients before death.

During the autopsy, specimens of the liver, as well as the cortex and subcortical white matter from the frontal, parietal, temporal and occipital lobes of the brain hemispheres, hippocampus, thalamus, striatum (putamen, globus pallidus, caudate nucleus) and the cerebellum were taken from the deceased of 3 groups and the control group. The choice of these brain regions is conditioned by the previous neuroimaging and experimental studies highlighted the presumable regional selectivity of the brain hepatogenic toxic damage in acute and chronic liver failure [19–21]. Pieces of the brain and liver were fixed in 10 % buffered formalin followed by and paraffin embedding. Serial tissue slices thickened of 4 μm were prepared on a precision rotary microtome HM 3600 (MICROM Laborgerate GmbH, Germany); after deparaffination they were stained with hematoxylin and eosin for a general histopathological assessment.

The levels of ammonia in deparaffinized brain sections were determined histochemically (HC) using Nessler's reagent according to the V. Gutiérrez-de-Juan et al. (2017) method [17]. Aqueous solution of Nessler's reagent (potassium tetraiodomercurate dihydrate, $\text{K}_2\text{HgI}_4 \cdot \text{KOH}$) has pale yellow coloring; in the presence of ammonia it becomes darker, and when high ammonia concentrations, forms small orange-brown granules / precipitates of different density in the tissues [17]. In preliminary studies, for determining the ammonia level in the brain tissue we used 2 options for assessing the HC reaction:

1. determining the relative area of HC-positive (orange-brown) granules to the area of the standardized field of view at magnification $\times 200$ of the microscope Scope.A1 “Carl Zeiss” (Germany) with Jenoptik progress Gryphax 60N-C1”1.0x426114 (Germany) camera using Videotest-Morphology 5.2.0.158 (VideoTest LLC) software;
2. determination of the optical density (staining intensity) of HC-positive granules in conditional units of optical density (CUOD) in a standardized field of view of the same microscope at magnification $\times 200$ using an automatic analysis system and standard plugin color deconvolution-“H DAB” of ImageJ software.

The results of the preliminary studies have shown that in the dynamics of LC decompensation, the rates of optical density of HC-positive granules changed more significantly than their relative area. Therefore, for the quantitative assessment of the brain HC reaction to ammonia, the HC optical density values (measured in CUOD) was chosen. At CUOD values from 0 to 20, the degree of HC ammonia expression in the brain tissue is assessed as negative (“-”); from 21 to 50 – as weak (“+”); from 51 to 100 – as moderate (“++”); from 101 and above – as high (“+++”).

In each case, in the cortex and subcortical white matter of the aforementioned 4 cerebral lobes, hippocampus, thalamus, striatum (putamen, globus pallidum, caudate nucleus) and cerebellum, a comparative analysis of the optical density of HC ammonia expression was performed in five standardized fields of view (SFV) (at magnification $\times 200$) of the microscope Scope.A1 “Carl Zeiss” (Germany) with Jenoptik camera progress Gryphax 60 N – C 1”1.0 x 426114 (Germany) using ImageJ software; in twenty standardized fields of view (mag. of $\times 400$) of each noted brain region, the number of Alzheimer's type 2 astrocytes was counted. Based on our preliminary studies, as well as the data provided by A. N. Agarwal & D. D. Mais (2019), according to the numbers of AA2 in SFV of the microscope (at magnification $\times 400$), we designed 4 degrees of AA2-astrocytosis: 1-5 AA2/ 20 fields of view, $\times 400$ – 0 degree of AA2-astrocytosis (absent); 6–10 AA2/ 20 fields of view, $\times 400$ – I degree of AA2-astrocytosis (weak); 11–20 AA2/ 20 fields of view, $\times 400$ – II degree AA2-astrocytosis (moderate); from 21 or more AA2/ 20 fields of view, $\times 400$ – III degree of AA2-astrocytosis (severe).

The results were analyzed using the Statistica® package for Windows 13.0 (StatSoft Inc., License No. JPZ8041382130ARCN10-J). Correspondence of quantitative indicators to the law of normal distribution was determined using the Kolmogorov–Smirnov test. Results were expressed as median (Me) with range (Q1; Q3) and as mean \pm standard deviation. The Mann–Whitney U-test was used to compare two groups, and the Kruskal–Wallis

Table 1. HC ammonia expression (in CUOD), HC ammonia scale, numbers of AA2 and AA2-score in deceased patients with LC of "A", "B", "C" groups and control

Brain region	Parameter	Group "A"	Group "B"	Group "C"	Control group
Cerebral cortex	CUOD	27.11 (23.82; 28.85)*	29.54 (24.34; 35.12)*	64.23 (53.12; 76.07)*#	18.14 (15.26; 19.53)
	Ammonia scale	+	+	++	-
	AA2 numbers	3.50 (2.40; 5.20)	12.50 (6.30; 14.20)*	21.10 (15.50; 26.20)*#	3.10 (1.20; 4.50)
	AA2-score	0	II	III	0
Cerebral subcortical white matter ⁰	CUOD	11.23 (10.75; 15.81)	12.47 (11.15; 16.25)	21.15 (20.22; 22.73)*#	11.10 (10.34; 2.26)
	Ammonia scale	-	-	+	-
	AA2 numbers	3.60 (2.70; 5.40)	3.70 (2.90; 5.90)	14.50 (7.20; 15.60)*#	3.40 (1.80; 4.30)
	AA2-score	0	0	II	0
Hippocampus	CUOD	18.12 (15.57; 19.37)	22.48 (21.39; 33.79) *	55.43 (52.61; 61.48)*#	17.25 (14.68; 18.72)
	Ammonia scale	-	+	++	-
	AA2 numbers;	2.60 (1.80; 3.40)	3.40 (2.70; 5.20)	6.10 (4.80; 9.50) *	2.10 (1.50; 3.20)
	AA2-score	0	0	I	0
Thalamus	CUOD	26.68 (24.72; 29.35)*	65.47 (51.71; 78.89)*†	110.23 (99.22; 112.35)*#	19.25 (16.58; 19.72)
	Ammonia scale	+	++	+++	-
	AA2 numbers	9.30 (7.40; 10.60)*	15.20 (12.60; 19.90)*†	35.70 (21.50; 54.10)*#	4.10 (3.70; 6.20)
	AA2-score	I	II	III	0
Striatum	CUOD	24.37 (22.68; 28.61)*	55.52 (52.48; 65.57)*†	101.56 (100.48; 103.27)*#	18.46 (15.69; 18.93)
	Ammonia scale	+	++	+++	-
	AA2 numbers	7.60 (6.30; 9.70) *	8.30 (6.70; 10.20) *	25.20 (21.30; 45.20) *#	3.80 (3.20; 5.90)
	AA2-score	I	I	III	0
Cerebellum	CUOD	29.27 (26.48; 31.43)*	67.08 (54.29; 84.27)*†	122.16 (107.37; 131.27)*#	19.74 (18.32; 19.83)
	Ammonia scale	+	++	+++	-
	AA2 numbers	9.10 (7.50; 10.20)*	16.10 (14.60; 20.40)*†	32.40 (20.90; 52.20)*#	3.90 (3.80; 6.10)
	AA2-score	I	II	III	0

*; reliable difference compared to control ($p < 0.05$); †; reliable difference compared to group "A" ($p < 0.05$); #: reliable difference compared to groups "A" and "B" ($p < 0.05$); data are presented as median with lower and upper quartiles – Me (Q1; Q3).

test was used to compare more than two groups. Correlation analysis was performed using Spearman's rank correlation coefficient (r). The results were considered statistically significant at the level of 95 % ($p < 0.05$).

Results

HC ammonia detection method with Nessler's reagent according to V. Gutiérrez-de-Juan et al. (2017) reveals region-dependent, fine-grained, ammonia expression of different optical density in the brain neuropil of deceased cirrhotic and control patients. In the brain of deceased patients from control (did not suffer from liver diseases), in all six studied regions, HC ammonia expression is weak and correspond to "negative expression" according to ammonia scoring we used. The lowest ammonia expression is determined in the cerebral subcortical white matter: 11.10 (10.34; 14.26), CUOD, and the highest – in cerebellum 19.74 (18.32; 19.83) CUOD, and thalamus: 19.25 (16.58; 19.72) CUOD (Table 1, Fig. 1).

In deceased cirrhotic patients of the three main groups, regional heterogeneity of HC ammonia expression is also observed with its gradual proportional increase (from LC class A to class C) in the corresponding brain regions (Table 1, Fig. 1). In compensated cirrhosis, weak HC ammonia expression is observed in the cortex, thalamus, striatum, and cerebellum (Table 1). In deceased patients with subcompensated LC, moderate ammonia expression appears in the thalamus, striatum and cerebellum, which is higher compared to group "A". Weak ammonia expression is observed in the cortex and hippocampus, while negative – in the cerebral white matter (Table 1).

In decompensated cirrhosis, among all the studied brain regions, the highest compared to control, "A" and "B" groups HC ammonia expression is found. The maximally increased, strong HC expression is observed in cerebellum [122.16 (107.37; 131.27), CUOD (6.18 times higher, by 518.84 % compared to control)]; thalamus [110.23 (99.22; 112.35), CUOD (5.72 times higher, by 472.62 %)]; striatum [101.56 (100.48; 103.27), CUOD (5.50 times higher, by 450.16 %)]. Moderate HC expression is noted in the cortex [64.23 (53.12; 76.07), CUOD (3.54 times higher, by 254.07 % compared to control)], and hippocampus [55.43 (52.61; 61.48), CUOD (3.21 times higher, by 221.23 %)]; and weak HC expression is observed in the white matter [21.15 (20.22; 22.73), CUOD (1.90 times higher, by 90.54 % compared to control)] (Table 1).

Histopathological examination of 6 brain regions of 90 deceased cirrhotic patients of groups "A", "B" and "C", revealed astrocytes with specific morphotype, so-called Alzheimer type 2 astrocytes, which are well distinguishable when stained with hematoxylin-eosin, as well as in the HC ammonia reaction (Fig. 2, 3, 4). They have an enlarged, watery, vacuolated nucleus with a prominent nucleolus adjacent to the nuclear membrane as well as peripherally localized punctate chromatin accumulations beside the nucleolemma. The nuclei of AA2 can be very large and at least 2 times the size of the nuclei of neighboring oligodendrocytes, while the cytoplasm of AA2 is represented by an inconspicuous rim ("naked nuclei"). AA2 are often arranged in pairs, sometimes forming astrocytic triplets. In the brain of deceased patients of control group, such astrocytes in different brain regions are observed in single numbers / 20 SFV, which corre-

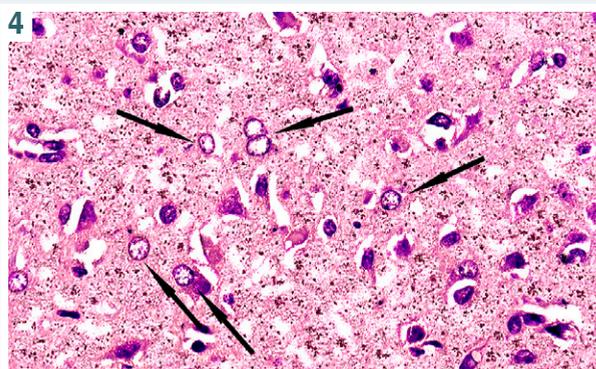
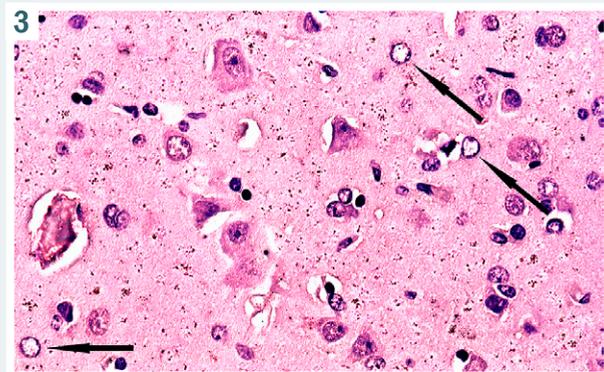
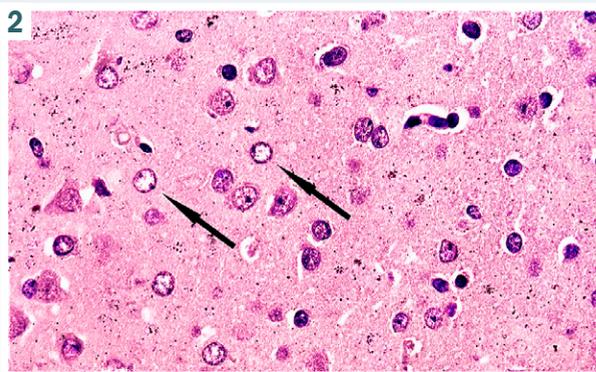
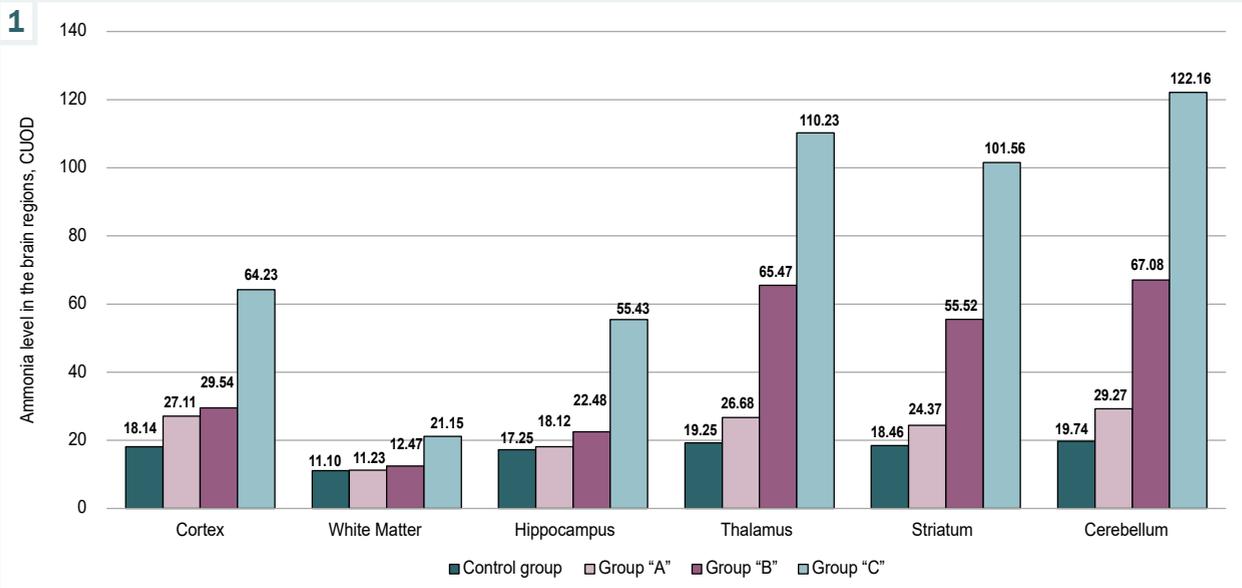


Fig. 1. HC ammonia expression, expressed in CUOD in different brain regions of deceased cirrhotic patients with compensated (group "A"), subcompensated (group "B") and decompensated (group "C") LC and control values.

Fig. 2. Weak HC ammonia expression with presence of AA2-astrocytes (arrows) in the cortex of deceased patient with compensated cirrhosis of group "A". HC reaction with Nessler's reagent. Mag. x200.

Fig. 3. Moderate HC ammonia expression with presence of AA2-astrocytes (arrows) in the cortex of deceased patient with subcompensated cirrhosis of group "B". HC reaction with Nessler's reagent. Mag. x200.

Fig. 4. Strong HC ammonia expression with presence of AA2-astrocytes (arrows) in the cortex of the patient with decompensated cirrhosis of group "C" died in hepatic coma. HC reaction with Nessler's reagent. Mag. x200.

sponds to "0" degree of AA2-astrocytosis according to the scale we used (Table 1).

According to ultrastructure analysis performed as early as 1987 [22], at the first stages of AA2 formation, the cytoplasmic volume of AA2 increases due to mitochondrial and granular / smooth endoplasmic reticulum hyperplasia, appearing of lipofuscin inclusions, accumulation of glycogen in the cytoplasm and the nucleus. Subsequently, a noticeable rarefaction of nuclear chromatin occurs with the formation of clumpy clusters under the nucleolemma. When metabolic potential depletion of AA2 finally reaches its extreme, pronounced hydropic degeneration

of the nucleus, mitochondria and vacuolization of the cytoplasm appear.

With exacerbation of LC, in deceased patients of "A", "B", and "C" groups, a gradual region-dependent increase in numbers of AA2 astrocytes is observed in the following brain regions in descending order: thalamus, cerebellum, striatum, cortex, white matter, hippocampus (Table 1). In compensated LC, increased numbers of AA2 are found in thalamus, striatum and cerebellum, which corresponds to AA2-astrocytosis of weak (I degree). In subcompensated LC, a moderate (II degree) AA2-astrocytosis is noted in the cortex, thalamus and cerebellum, herewith in the two

Table 2. In vivo blood laboratory parameters in patients with liver cirrhosis of groups "A", "B" and "C"

Parameters, units of measurement	Group "A"	Group "B"	Group "C"	Control group
Albumin (Alb), g/L	37.00 (36.30; 45.20)	33.20 (30.50; 35.30)*	25.70 (22.40; 32.20)*	43.00 (36.50; 46.30)
Bilirubin total (Bil), $\mu\text{mol/L}$	21.57 (20.65; 32.84)*	82.41 (46.83; 97.72)*	172.12 (122.52; 315.23)*	11.23 (9.35; 16.43)
AST (aspartate aminotransferase), $\mu\text{mol}/(\text{sec}\times\text{L})$	0.46 (0.38; 0.55)	0.92 (0.82; 1.34)*	2.25 (1.72; 2.93)*	0.23 (0.18; 0.37)
ALT (alanine aminotransferase), $\mu\text{mol}/(\text{sec}\times\text{L})$	0.35 (0.29; 0.71)	0.75 (0.57; 1.12)*	1.74 (1.33; 2.25)*	0.34 (0.20; 0.55)
LII (leukocytic intoxication index by Kalf-Kalif formula), units	0.65 (0.57; 1.47)	0.93 (0.74; 1.78)	1.92 (1.57; 2.97)*	0.52 (0.34; 1.12)

*: significant differences relative to the control ($p < 0.05$); data are presented as median with lower and upper quartiles – Me (Q1; Q3).

latter, the indicators are higher than those in control and group "A" ($p < 0.05$). Weak (I degree) AA2-astrocytosis is determined in the striatum (Table 1).

In decompensated LC, the highest AA2-astrocytosis of III degree is determined in the cortex, thalamus, striatum, cerebellum; moderate (II degree) – in the white matter, wherein indicators are higher than control, "A" and "B" groups values ($p < 0.05$). Weak (I degree) AA2-astrocytosis is observed in hippocampus, where indicators differ only from the control. In patients with decompensated LC, AA2-astrocytosis average values are higher compared to control: in thalamus – 8.71 times; in cerebellum – 8.30 times; in cortex – 6.81 times; in striatum – 6.63 times, in white matter – 4.26 times, in hippocampus – 2.90 times (Table 1).

Correlation analysis revealed moderate, strong, and very strong positive relationship between the average number of AA2 and indicators of HC ammonia expression in the thalamus, striatum, and cerebellum. Thus, in the thalamus from patients of group "A", the correlation coefficient (r) between these parameters is 0.52 ($p < 0.05$), in the group "B" – $r = 0.68$ ($p < 0.05$), group "C" – $r = 0.92$ ($p < 0.05$). In the striatum from patients of group "A" $r = 0.47$ ($p < 0.05$), group "B" – $r = 0.61$ ($p < 0.05$), group "C" – $r = 0.79$ ($p < 0.05$). In the cerebellum of patients of group "A" – $r = 0.75$ ($p < 0.05$), group "B" – $r = 0.86$ ($p < 0.05$), group "C" – $r = 0.93$ ($p < 0.05$). Moderate and weak correlation is observed in the cortex and white matter, as well as in the hippocampus. In the cortex from patients of group "A", the correlation coefficient (r) between these indicators is 0.63 ($p < 0.05$), group "B" – $r = 0.48$ ($p < 0.05$), group "C" – $r = 0.47$ ($p < 0.05$). In the hippocampus from patients of group "A", the correlation coefficient $r = 0.41$ ($p < 0.05$), group "B" – $r = 0.65$ ($p < 0.05$), group "C" – $r = 0.43$ ($p < 0.05$). In the white matter from deceased patients of group "A" – $r = 0.51$ ($p < 0.05$), group "B" – $r = 0.52$ ($p < 0.05$), group "C" – $r = 0.39$ ($p < 0.05$).

An analysis of the latest intravital laboratory parameters from the case histories of cirrhotic patients showed that in compensated patients there is upward trend for the blood total bilirubin, AST, ALT, as well as downward trend for albumin levels. These trends progress in patients with subcompensated LC and reach maximum values in patients with decompensated cirrhosis and hepatocellular insufficiency (Table 2). The leukocytic intoxication index (LII), calculated according to Y. Y. Kalf-Kalif's formula, in patients with compensated and subcompensated cirrhosis tends to increase by 25.00 % and 78.84 %, respectively compared to control; in patients with decompensated cirrhosis, this indicator was increased by 3.69 times (by 269.23 %) compared to control group (Table 2).

Correlation analysis confirmed the presence of positive relationships between the morphological manifestations of elevated tissue ammonia (HC ammonia expression, AA2-astrocytosis degree) in deceased patients of groups "A", "B", "C" and their intravital laboratory parameters of liver failure, which contributed to hyperammonemia.

In deceased patients with compensated LC, a positive weak relationship exists between the ratio of total plasma bilirubin and HC ammonia expression, as well as indicators of AA2-astrocytosis ($\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$) in the cortex, thalamus, striatum and cerebellum.

In deceased patients with subcompensated LC, a positive moderate relationship exists between the plasma total bilirubin, HC ammonia expression, and AA2-astrocytosis in the cortex, thalamus and cerebellum. In the thalamus, a moderate positive correlation takes place for $\text{AST}/\text{AA2}$. Weak positive correlation takes place in the cortex – between $\text{AST}/A_{\text{CUOD}}$; $\text{AST}/\text{AA2}$; $\text{ALT}/\text{AA2}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$; in white matter – $\text{AST}/A_{\text{CUOD}}$; in the hippocampus – $\text{Bil}/\text{AA2}$; in thalamus – $\text{AST}/A_{\text{CUOD}}$; $\text{ALT}/A_{\text{CUOD}}$; $\text{ALT}/\text{AA2}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$; in striatum – $\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$; $\text{AST}/A_{\text{CUOD}}$; $\text{AST}/\text{AA2}$; $\text{ALT}/\text{AA2}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$; in the cerebellum – $\text{AST}/A_{\text{CUOD}}$; $\text{AST}/\text{AA2}$; $\text{ALT}/A_{\text{CUOD}}$; $\text{ALT}/\text{AA2}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$. In the thalamus, a negative weak correlation exists between $\text{Alb}/\text{AA2}$.

In deceased patients with decompensated LC, a very strong positive correlation takes place in the cortex – $\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$; in thalamus – $\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$; in cerebellum – $\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$. Strong negative correlation is noted: in the cortex – $\text{Alb}/\text{AA2}$; in thalamus – $\text{Alb}/\text{AA2}$; in cerebellum – $\text{Alb}/A_{\text{CUOD}}$; $\text{Alb}/\text{AA2}$. Strong positive correlation is present in the cortex – $\text{AST}/A_{\text{CUOD}}$; $\text{AST}/\text{AA2}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$; in thalamus – $\text{AST}/A_{\text{CUOD}}$; $\text{AST}/\text{AA2}$; $\text{ALT}/\text{AA2}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$; in striatum – $\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$; $\text{AST}/\text{AA2}$; in the cerebellum – $\text{AST}/A_{\text{CUOD}}$; $\text{AST}/\text{AA2}$; $\text{ALT}/\text{AA2}$. Moderate positive correlation is observed in the cortex – $\text{ALT}/A_{\text{CUOD}}$; in hippocampus – $\text{Bil}/A_{\text{CUOD}}$; $\text{Bil}/\text{AA2}$; in striatum – $\text{AST}/A_{\text{CUOD}}$; $\text{ALT}/\text{AA2}$; in cerebellum – $\text{ALT}/A_{\text{CUOD}}$; $\text{LII}/A_{\text{CUOD}}$; $\text{LII}/\text{AA2}$. Moderate negative correlation is present in the cortex – $\text{Alb}/A_{\text{CUOD}}$; in thalamus – $\text{Alb}/A_{\text{CUOD}}$; in the striatum – $\text{Alb}/\text{AA2}$.

Discussion

Over the last decade, it has been proven that neurotoxic effect of ammonia is the key factor in the HE pathophysiology. Considering pathological data, HE is classified on three types: type A – occurs in acute liver failure; type B – develops as a result of portosystemic shunting (e. g., transjugular intrahepatic portosystemic shunting); type C – develops with LC [23]. During significant hyperam-

monemia in patients with LC, the level of both ionized NH_4^+ and gaseous NH_3 ammonia forms increases in the blood and brain tissue [5–7]. High ammonia levels in the brain of cirrhotic patients was confirmed using dynamic $^{13}\text{NH}_3$ -positron emission tomography [5].

A large amount of ammonia is produced in the intestine during the degradation of urea, digestion of proteins and the deamination of amino acids. High concentrations of intestinal ammonia are absorbed into the portal vein, and then in the liver, up to 80 % of circulating ammonia is extracted via its conversion to urea in the periportal hepatocytes and the formation of glutamine in the perivenous hepatocytes. A minor part of the circulating ammonia is metabolized in skeletal muscles, where glutamine is formed with the participation of glutamine synthetase [2]. In cirrhotic patients, significant increase in blood ammonia occurs with porto-systemic shunting and decrease in the metabolic activity of hepatocytes [5]. Moreover, in chronic liver failure, increased reabsorption of conjugated bile acids from the intestinal lumen occurs, while increased intestinal pH contributes to the conversion of the predominant intestinal ionized ammonium (NH_4^+) into the easily diffusing gaseous form (NH_3) leading to increased blood and brain ammonia levels and correlating with HE severity [7].

HE more often develops in alcoholic cirrhosis, severe portal hypertension, in combination with metabolic syndrome and kidney disease, as well as when cirrhotic patients take proton pump inhibitors, opiates, GABAergic drugs and benzodiazepines [24]. HE is a prognostic marker of imminent threat of death in cirrhotic patients. When cirrhosis is compensated, the average survival of patients exceeds 12 years, while with its decompensation, determined by the development of HE, jaundice, ascites and variceal bleeding, the average survival declining to 2 years [1].

In HE pathogenesis, the main attention is turned to the systemic toxic effects of ammonia, which disrupts BBB functions, promotes neuroinflammation, astrocytes swelling, affects pH and brain cellular membrane potential, violates neurotransmission, changes intercellular communication and causes clinical manifestations of HE [2,5].

Despite the key role of ammonia in HE, determination of blood ammonia in cirrhotic patients has shown conflicting and heterogeneous results, which can be explained by the incomplete compliance of researchers with the difficult technological protocol for its laboratory determination [12,25]. Number of studies have not confirmed a direct relationship between plasma ammonia and HE development. In some patients with cirrhosis, blood ammonia may be within normal limits, while in other patients with mild, minimal HE or its absence ammonia may be high [26]. By results of other studies [27], in severe forms of HE, blood ammonia rarely remains within conventionally normal values. Probably, given the ambiguity of the aforementioned results AASLD and Choosing Wisely Campaign-2017 protocols does not recommend mandatory testing of blood ammonia to make or exclude the diagnosis of HE [12].

The relationship of neuropathological manifestations of hepatotoxic brain damage with the cerebral ammonia is not reflected in the literature, and its histochemical detection in paraffin sections of various organs is described

only in one study [17]. We have managed to reproduce proposed by V. Gutiérrez-de-Juan et al. in 2017 [17] HC method for ammonia determination in the brain of cirrhotic patients. Our results have showed that among six studied brain regions, the cerebellum, thalamus and striatum are the richest with ammonia. Increased HC ammonia expression (compared to control) is observed in deceased patients with compensated LC, in the cortex, thalamus, striatum and cerebellum; in deceased patients with subcompensated LC, HC ammonia expression is increased in all six studied brain regions. In deceased patients with decompensated cirrhosis, in the cerebellum, thalamus and striatum the level of HC ammonia increases maximally. A significant increase in brain ammonia level in patients with decompensated LC may be due to the presence of the largest number of cases with severe forms of HE including hepatic coma in this group, as well as increased BBB permeability for ammonia due to neurotoxicosis occurred with multiple organ failure in this category of patients. Among the studied brain regions, hippocampus and white matter shows the lowest ammonia values, as well as weak correlations between neuropathological and intravital laboratory parameters. The revealed heterogeneity of ammonia levels in different brain regions is most likely due to the initially different levels of enzymes involved in ammonia metabolism, as well as different levels of glutamate, dopamine, GABA, and other neurotransmitters associated with ammonia metabolism.

Neuropathological changes characteristic for HE in conjunction with *in vivo* increased blood ammonia have been described in a few works. Hyperammonemia causes astrocytes swelling and increase in AA2 numbers [16], stimulates Iba1+ microglia activation [28], disrupts synapse architecture and structure of neurons, contributing to their death [5]. In cirrhotic patients, after several episodes of coma, spongy degeneration can develop in the deep cortical layers, basal ganglia and cerebellum, as well as demyelination of straight and crossed cortical-spinal tracts, leading to paralysis of the lower extremities; deficiency of dopaminergic neurons leading to parkinsonism [5]. Experimental hyperammonemia at rats reduces density of dendritic spines sensorimotor cortical layer V and pyramidal neurons of hippocampal CA1 [29].

We have confirmed the data [14, 16] that hepatogenic damage induces formation of the so-called Alzheimer type 2 astrocytes in significant quantities, which were described for the first time in 1912 by von Hosslin and Alzheimer and since 1936 have been considered as the histopathological hallmark of HE [16]. In our study, in deceased patients with compensated cirrhosis, a significant increase in numbers of AA2 is found in the thalamus, striatum, and cerebellum. In deceased patients with subcompensated cirrhosis, pronounced AA2-astrocytosis is determined in the cortex, thalamus, striatum and cerebellum; moderate AA2-astrocytosis occurs in the white matter, and mild AA2-astrocytosis occurs in the hippocampus. The relationship between AA2-astrocytosis and HC ammonia expression in the same brain regions is confirmed by correlation analysis, which shows the presence of a moderate, strong, and very strong positive relationship between these indicators in the thalamus, striatum, and cerebellum.

AA2-astrocytosis in different brain regions which increases with the severity of LC, could be explained by the peculiarities of the intensive ammonia metabolism in the brain during hyperammonemia. As the excess of brain ammonia is metabolized in astrocytes with synthesis of osmotically active glutamine, the accumulation of the latter causes swelling of astrocytes and their nuclei [8], as well as glutamine hydrolysis in mitochondria with the release of new amounts of ammonia [9]. In our opinion, the presence of AA2 in the brain of cirrhotic patients reflects the transformation of astrocytes, which is characteristic of hyperammonemia due to a persistent high level of osmotically active glutamine in them; therefore, astrocytes swollen due to osmotic imbalance are not strictly specific cells for only HE. It is in line with A. N. Agarwal i D. D. Mais (2019) [16], who emphasized that astrocytes AA2 are regular and highly sensitive findings in HE. Morphologically similar AA2-astrocytosis has also been described in other critical conditions accompanied by acute ion-osmotic imbalance [16,30,31].

Analysis of clinical and laboratory data revealed that the most significant correlations take place between elevated blood levels of total bilirubin, AST, ALT, LII, as well as hypoalbuminemia and the HC ammonia expression, as well as AA2-astrocytosis in the patient's brain. The strongest correlations between these parameters can be traced in the cerebellum, thalamus, striatum, and cortex; a correlation of lesser strength takes place in the white matter and hippocampus. The elevated leukocytic intoxication index in decompensated cirrhotic patients, as well as a significant direct correlation of its level with the HC ammonia expression and AA2-astrocytosis in all six brain regions confirm the contribution of systemic neurotoxicosis and neuroinflammation to the complex pathogenesis of HE in cirrhosis [32].

Conclusions

1. Histochemical method of the ammonia detection in the nervous tissue using the Nessler's reagent (according to V. Gutiérrez-de-Juan et al., 2017) determines an increased region-dependent fine-grained expression of ammonia in the neuropil of different brain regions in deceased patients with liver cirrhosis.

2. In deceased cirrhotic patients, histochemical ammonia expression in the tissue neuropil of the cerebellum, thalamus, striatum, and cerebral cortex directly correlates with the severity of Child-Pugh score, reaching a maximum in severe class C liver cirrhosis, and possessing significant correlations with intravital blood total bilirubin, AST, ALT, albumin, leukocytic intoxication index. In the cerebellum, thalamus, and striatum of the deceased cirrhotic patients of class C, the histochemical ammonia expression is maximally increased (by 6.18, 5.72, and 5.50 times, respectively) in comparison with similar brain regions of deceased patients who did not suffer from liver diseases.

3. With progression of liver cirrhosis, the maximal increase in Alzheimer2-astrocytosis in the thalamus, cerebellum, striatum and cerebral cortex is observed, which directly correlates with histochemical ammonia expression in the same brain regions.

4. An increased histochemical ammonia expression and Alzheimer-2-astrocytosis in the cerebral cortex, thalamus, striatum and cerebellum in deceased patients with intravitaly elevated blood levels of total bilirubin, AST, ALT, leukocytic intoxication index and hypoalbuminemia may be used as the pathomorphological confirmation of hepatogenic toxic damage of the brain.

Prospects for further research. Further study on regional ammonia brain level in other life-threatening somatogenic toxic states, as well as its correlation with central astroglial markers are needed to improve our knowledge on the brain cellular responsibility in patho- and morphogenesis of acute and chronic metabolic encephalopathies.

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