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Chapter 4

Epilepsy as a Pyridoxine-Dependent Condition: Quantitative Urinary Biomarkers of Epilepsy. Family Disorders of Pyridoxine Metabolism

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Additional information is available at the end of the chapter

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Abstract

The affected pyridoxine metabolism is discussed as an inborn genetic sign of epilepsy. In children with different forms of epilepsy and matched healthy controls, the urinary parameters of pyridoxal phosphate–dependent tryptophan degradation were measured by high-performance liquid chromatography (HPLC) method with simultaneous ultraviolet and fluorimetric detection. Concentrations of compounds, which are formed in the course of tryptophan degradation, and correlations between them turned out to be quantitative biomarkers useful for evaluation of patient's condition and monitoring individualized antiepileptic treatment. Accumulation of tryptophan, kynurenine, and neurotoxic 3-hydroxykynurenine, along with reduced kynureninase activity, is characteristic of epileptic patients. Growing progressively worse, epilepsy is accompanied by aggravation of pyridoxal phosphate–dependent disturbances of tryptophan metabolism and further inhibition of kynureninase.

In asymptomatic first-degree relatives of epileptic probands, disorders of pyridoxine metabolism are of the same (or even higher) extent as in probands. Long-term pyridoxine treatment (7–10 mg/kg daily) is suggested as safe and effective protective replacement therapy. The protocols of this study have been approved by the ethics committee of Kaplan Hospital (Israel).

Keywords: epilepsy, epileptic families, tryptophan metabolism, vitamin B6 (pyridoxine)-dependent enzymes

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1. Quantitative urinary biomarkers for evaluation of patient's state and monitoring antiepileptic treatment

1.1. Introduction

Sixty years ago Hunt et al. [1] described pyridoxine-dependent epilepsy (PDE), which until now is considered as a rare (1:100.000) autosomal recessive genetic disorder, occurring in the uterus, or later in infancy, or early childhood. Most often seizures are observed within the first month of life, even within hours of birth. In atypical (late-onset) PDE seizures start later (up to 2 years). After the first year of life autistic features are often revealed. The resistance to conventional antiepileptic drugs (AEDs) and response to vitamin B6 administration (5–10 mg/kg/day) are accepted as the main characteristic features of PDE [2–5].

Vitamin B6 consists of six different vitamers: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphate-esterified forms. Pyridoxal phosphate (PLP) is the active B6 vitamer, which is produced from its precursor vitamers (PN, PM, and PL) by phosphorylation and oxidation (PN phosphate, PLP, and PM phosphate) through the actions of pyridoxal kinase and pyridox(am)ine oxidase (PNPO), respectively (**Figure 1**) [6]. Vitamin B6 is broken down to pyridoxic acid (4PA), which is excreted in the urine.

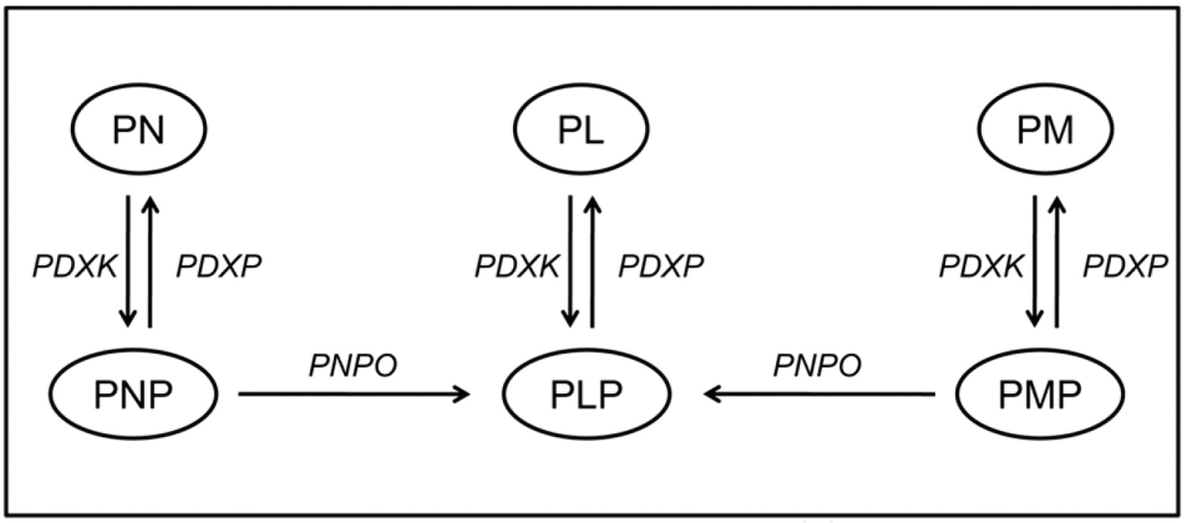

Figure 1. Human vitamin B6 metabolism. PDXK, pyridoxal kinase; PDXR, vitamin B6-specific phosphatase; PNPO, pyridox(am)ine phosphate oxidase.

PLP is a cofactor for numerous enzymatic reactions in the central nervous system (CNS). An inborn abnormality of PLP-dependent GABA synthesis induced by glutamate decarboxylase (GAD) deficiency earlier was postulated as a cause of epilepsy, and lifelong pyridoxine administration was recommended. Later on in the search for the PDE-responsible gene, the primary involvement of the GAD 1 gene on chromosome 2q31 and the GAD 2 on 10p23 was discussed and excluded [7, 8].

At present, PDE is considered as a result of mutations in the ALDH7A1 gene, encoding antiquitin. Antiquitin deficiency in the lysine degradation pathway leads to the accumulation of piperidein-6-carboxylic acid, which inactivates PLP [9, 10]. Recently, several cases of other inborn errors of vitamin B6 metabolism, that is, pyridox(am)ine 5-phospate oxidase deficiency and type 2 hyperprolinemia, have also been described. So, the accumulated data have shown that autosomal recessive pyridoxine-dependent seizures are genetically heterogeneous [11–13].

Nevertheless, neither data accumulation, nor recommendations for pyridoxine administration in early cases of intractable epilepsy [3, 14, 15], or pyridoxine applicability as the first-line drug for infantile spasms [16–19] have changed conventional perception of the strictly limited role of pyridoxine in the pathogenesis of *epilepsy as a whole*.

Meanwhile, disturbances in the metabolism of glutamate, GABA, tryptophan (TRP), serotonin, taurine, dopamine, and norepinephrine, which are synthesized and/or metabolized by PLP-dependent enzymes [20–32], have been repeatedly found in epileptic patients. The increased levels of excitatory amino acids—glutamate, aspartate, and glycine [21–27] along with the reduced levels of inhibitory amino acids and amines—GABA, serotonin, and taurine [28–32] were detected in the plasma, cerebrospinal fluid (CSF), and epileptogenic foci of patients with different forms of epilepsy. Moreover, a moderate increase in the activity of glutamic acid dehydrogenase, the glutamate-synthesizing enzyme, which is specifically inhibited by PLP, has been found in epileptic foci [33].

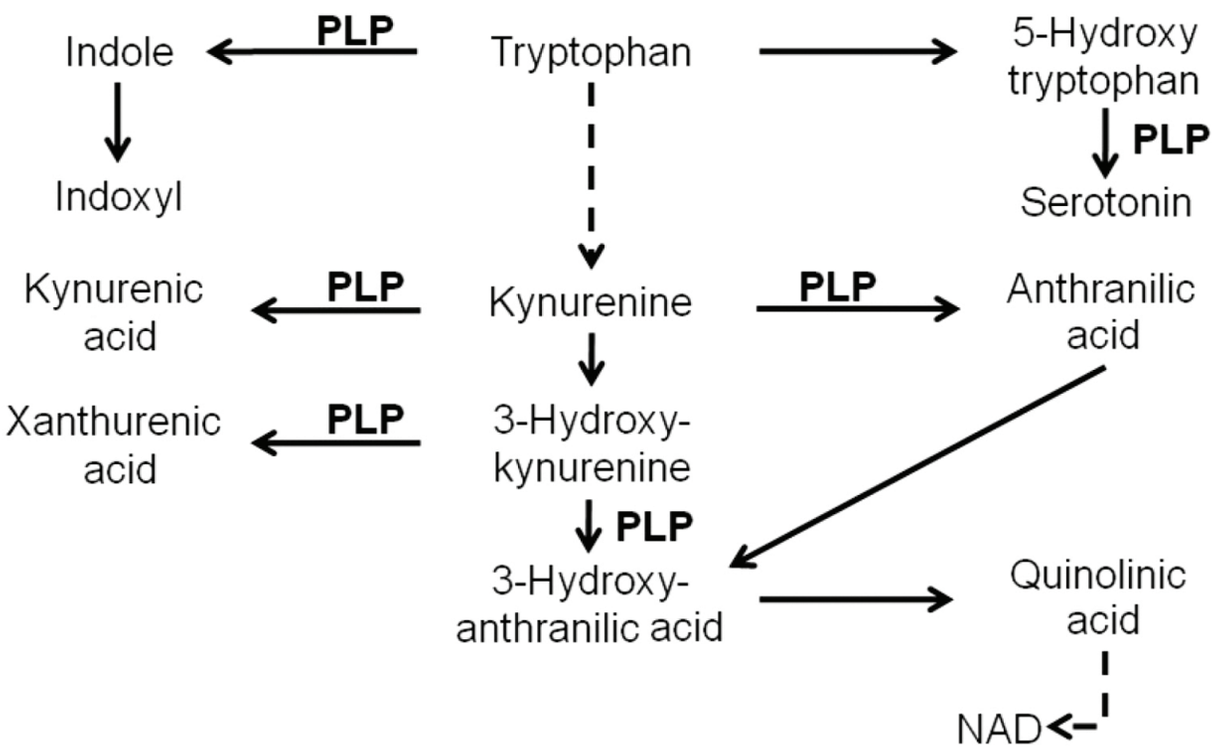

Figure 2. Outline of kynurenic pathway of tryptophan degradation.

These clinical data along with experimental results obtained in genetically epilepsy-prone seizure-naive animals, in comparison with genetically epilepsy resistant [34–37], enable us to hypothesize that an inborn error of pyridoxine metabolism (accentuated by high pyridoxine requirement during early development) is inherent in epilepsy. Being a starting point for neurotransmitter disorders, such an error may be a key determinant of epileptic diathesis. An impairment of GABA (as well as serotonin and taurine)-mediated inhibition along with an enhancement of glutamate (and aspartate)-mediated excitatory transmission evidently facilitates spreading of ictal activity throughout the brain and thereby generation of seizures.

Disturbances of PLP-dependent tryptophan degradation, in particular over-excess of neurotoxic 3-HOKYN, have been repeatedly shown in epileptic patients starting from 50-s [38–41]. Summarizing the data obtained [42–44], we suggested that quantitative correlations between metabolites formed in the course of TRP degradation (**Figure 2**) might be indicative of clinical status in epileptic patients.

Specifically, the ratio of KYN to TRP serves as an index of activity of indoleamine-2, 3-dioxygenase (IDO), the rate-limiting enzyme of TRP degradation, initiating the pathway. (Being heme-containing enzyme, IDO is apparently PLP dependent, inasmuch as heme synthesis is PLP dependent).

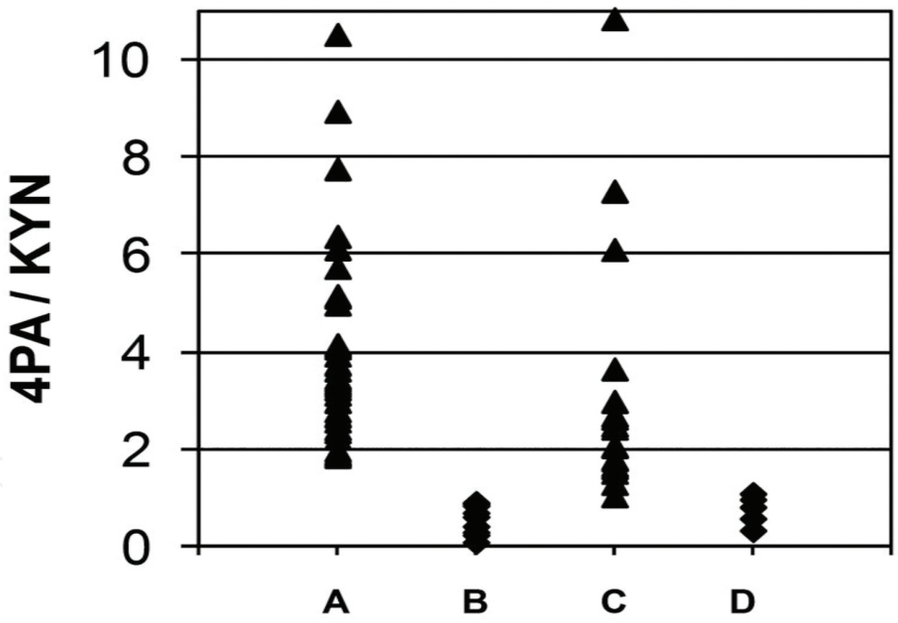

Figure 3. The value of 4PA/KYN ratios in healthy controls (A); patients experienced the first seizure attack (B); AED-treated seizure-free patients (C); partially AED-controlled epileptic patients (D).

The ratio between the levels of 3-HOAA and 3-HOKYN is considered as an index of kynureninase activity, the enzyme of critical sensitivity to PLP supply [45–47]. The ratio between 4-PA and KYN turned out to be an indicator of recently experienced seizure attack (**Figure 3**).

We have used these and other quantitative urinary biomarkers for clarification of patient's state in epilepsy and for tailoring of individual AED treatment.

1.2. Materials and methods

1.2.1. Subjects

Urine samples were analyzed in children of 4–17 years of age with different clinical forms and stages of epilepsy, excluding absence and atonic seizures, healthy in all other respects. Altogether, 109 subjects divided into following groups were comparatively studied:

1. Newly diagnosed epileptic patients, who had experienced their first epileptic attack on the previous day(s) and were never treated with AEDs ($n = 11$);
2. Epileptic patients regardless of the type of epilepsy successfully treated with AED and at present seizure-free for at least 3 months, regardless of the type of epilepsy ($n = 19$);
3. Epileptic patients partially responsive to AED treatment, that is, those having repeated seizure attacks in spite of antiepileptic treatment ($n = 19$);
4. Control group of healthy children matched by sex and age ($n = 37$).

About 270 urine samples were analyzed. Control samples were collected from healthy children in local kindergartens and elementary schools.

1.2.2. Determination of tryptophan and its metabolites in urine by high-performance liquid chromatography with simultaneous ultraviolet and fluorimetric detection

Urinary TRP and its metabolites were determined by high-performance liquid chromatography (HPLC) modified in our laboratory by Rabinkov, Pressman, and Malitsky. In addition to KYN, 3-HOKYN and 3-HOAA detected by Herve et al. [48]; some other TRP metabolites, that is, anthranilic acid (AA), kynurenic acid (KA), indoxyl sulfate (IND), and 4-PA, were also measured [36, 49].

All standards were purchased from Sigma. All solvents were of HPLC grade. The same method of TRP metabolite detection has been used by our collaborators in patients with attention deficit hyperactivity disorder (ADHD), the disease mutually interconnected with epilepsy [50].

1.2.2.1. Sample preparation

Mixed standard solutions (1 mM of each compound) were stored at −80°C for up to 3 months. Urine samples were collected into 20-mL glass scintillation vials and stored in aliquots at −80°C. Samples were acidified by the addition of 100 μL of 2.4 M perchloric acid to 900 μL of urine. After centrifugation (5000 g, 15 min, 4°C), supernatants were filtered (0.22-μL Millipore filter) into HPLC vials and analyzed the same day.

1.2.2.2. Chromatography

Reverse phase HPLC analysis was performed with an Inertsil (C-18, 5 μm) column (250 × 4.6 mm) and Merck Hitachi system equipped with a Quaternary Pump L-7100 and interface D-7000.

Peaks detection and quantification were carried out using a scanning fluorescence detector L-7485 connected to the programmable photodiode array detector L-7450A. Samples were analyzed using the following gradient: 28-min isocratic elution of 100% solvent A, 6-min linear gradient from 100 to 75% of solvent A, 61-min isocratic elution of 75% of solvent A, 2-min linear gradient from 75 to 100% of solvent A, and 8-min isocratic elution of 100% solvent A. Solvent A was 1 M ammonium acetate buffer, pH 5.2. Solvent B was 6% acetonitrile in 1 M ammonium acetate buffer, pH 5.2. The mobile phase was prepared on the day of analysis. Acquisition and processing of chromatograms were performed using HSM software (Merck-Hitachi). Standard compounds showed linearity range from 0.03 to 10 μM. Concentrations were calculated on the basis of peak areas of external standards. TRP, its metabolites, and 4-PA were determined with UV and fluorescence detection at two different excitation and emission wavelengths: 3-HOKYN and KYN were detected by UV absorption at 365 nm and eluted at 14.3, 41.4, and 87.0 min, respectively, while 3-HOAA, 4-PA, AA, TRP, IND, and KA were detected by fluorescence and eluted at 31.6, 58.9, 72.4, 84.5, 92.8, and 99.9 min, respectively.

For 3-HOAA and 4-PA, fluorescence excitation and emission wavelengths were set up as 320/420 nm during the first 60 min of elution; for AA, TRP, IND, and KA the wavelengths were 254/404 nm during the following 45 min of elution.

1.2.3. Statistical analysis

The data were expressed as mean ± SEM. Paired Student's *t*-test was used to assess the difference between groups; $p < 0.05$ was considered as statistically significant difference between values of parameters.

1.3. Urinary biomarkers for detection of the endured first seizure attack

In patients who endured seizures for the first time before admission to the ward, the mean level of TRP, and especially of KYN were sharply increased in comparison with healthy control group, thus providing the elevated mean value of KYN/TRP ratio and decreased value of 3-HOKYN/KYN ratio. The level of KA was twofold elevated, while the IND level was almost twice reduced.

Taken together, these changes in "first seizure attack" children formed a pattern strongly distinguishable from that of healthy controls (**Table 1**). The level of urinary 4-PA was statistically indistinguishable from healthy controls (though in four out of 11 patients it was reduced to 1μM or even lower). The mean value of 4-PA/KYN ratio in children of this group turned out to be almost sevenfold less than in healthy controls (**Table 1** and **Figure 3**) and appeared to be the marker of seizure attack occurred (regardless of its type).

The alterations in the urinary levels of TRP and KYN lead to a rightward shift in the histogram of distribution of the KYN/TRP ratio and a strong leftward shift in the histogram of distribution of the ratio 3-HOKYN/KYN (**Figures 4** and **5**).

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Parameters	Epileptic patients								*P* values between compared groups $^{*}p < 0.1$; $^{**}P \leq 0.05$; $^{***}P \leq 0.01$				
			Non treated		AED treated								
	Group 1: healthy controls (*n* = 37)		Group 2: after first seizure attack (*n* = 11)		Group 3: seizure-free (*n* = 19)		Group 4: partially controlled (*n* = 19)						
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	1–2	1–3	2–3	2–4	3–4
TRP, (μM)	23	2	57	20	36	7	58	15	***	**			
KYN, (μM)	1.7	0.2	5.7	1.4	2.4	0.2	3.3	0.3	***	**	***		**
3-HOKYN, (μM)	1.1	0.1	1.8	0.5	1.1	0.2	4.6	0.8	*			**	****
3-OHAA, (μM)	4.5	0.7	3.5	0.6	4.4	1.0	2.4	0.5					*
AA, (μM)	0.20	0.02	0.26	0.09	0.20	0.06	0.30	0.11					
KA, (μM)	3.3	0.4	6.3	1.1	5.3	1.3	8.4	1.3	***	**			*
4PA, (μM)	6.0	0.9	5.0	1.3	6.8	1.2	5.5	1.0					
IND, (μM)	576	53	294	70	289	50	460	77	**	***			*
4PA/KYN	4.1	0.5	0.6	0.1	3.1	0.6	2.5	0.7	***		***	**	
4PA/3HOKYN	8.42	1.45	3.85	0.94	8.6	1.26	4.12	1.53	*		***		**
4PA/(KYN + 3HOKYN)	2,5	0.35	0.76	0.24	1.94	0.28	1.3	0.35	***		***		
KYN/TRP	0.08	0.005	0.226	0.086	0.116	0.029	0.116	0.034	***	*			
3HOKYN/KYN	0.72	0.08	0.35	0.09	0.56	0.12	1.57	0.29	**			***	***
3HOAA/3HOKYN	6.0	1.1	4.4	1.3	5.9	1.5	0.9	0.2				***	***
KA/3HOAA	2.2	0.6	2.9	0.8	1.8	0.3	4.9	1.2					**
KYN/(3HOAA/3HOKYN)	1.06	0.27	4.16	1.74	0.91	0.19	9.73	2.65	***		**	***	****
KA/(3HOAA/3HOKYN)	2.0	0.5	5.3	2.3	1.5	0.3	20.4	4.8	**		*	**	***
TRP/(3HOAA/3HOKYN)	14	3	38	15	13	3	155	53	**		***	**	****
IND/TRP	29.4	2.8	6.7	1.4	13.1	2.3	14.1	5.4	****	***	**		
IND/KYN	433	50	78	23	164	26	210	42	***	***	**	**	

Table 1. Urinary metabolites of tryptophan and correlations between them in epileptic patients in comparison with healthy controls.

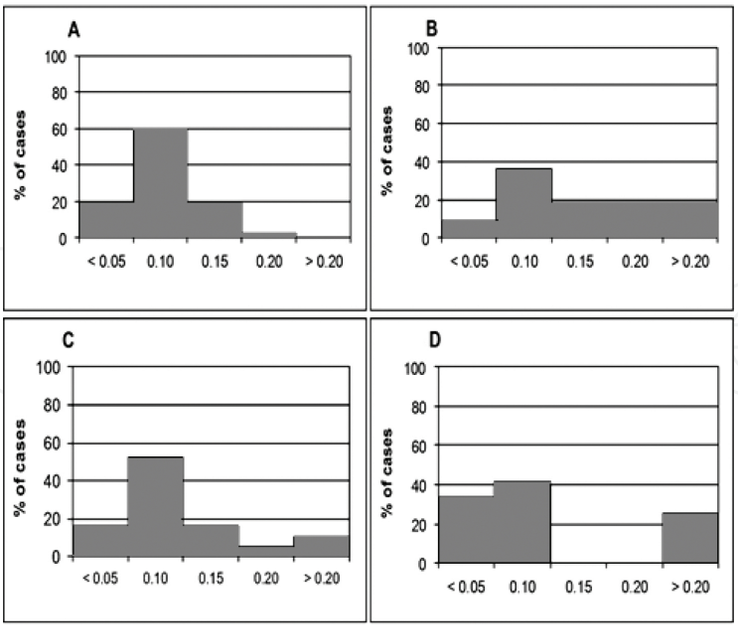

Figure 4. Distribution of KYN/TRP values in epileptic patients, in comparison with healthy controls. Designations: healthy controls (A); patients experienced the first seizure attack (B); AED-treated seizure-free patients (C); partially AED-controlled patients (D).

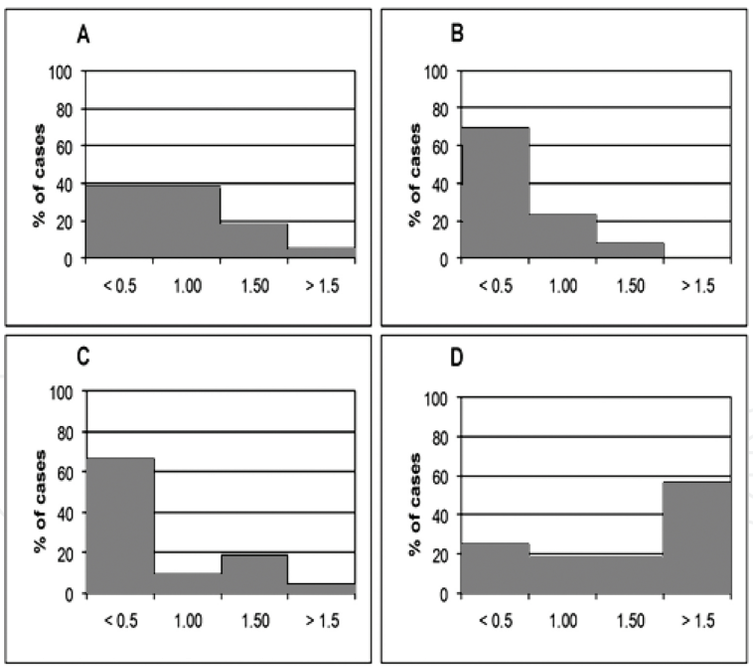

Figure 5. Distribution of 3HOKYN/KYN values in epileptic patients, in comparison with healthy controls. Designations: healthy controls (A); patients experienced the first seizure attack (B); AED-treated seizure-free patients (C); partially AED-controlled patients (D).

At the same time, the histogram of distribution of the 3-HOAA/3-HOKYN ratio tends distinctly to the left (**Figure 6**), reflecting some reduction in kynureninase activity, though the mean value

of this ratio is decreased insignificantly (**Table 1**). The correlations between concentrations of TRP (as well as KYN) and the ratio indicative of kynureninase activity, that is, TRP: (3-HOAA/3-HOKYN) and KYN: (3-HOAA/3-HOKYN), are strongly higher in "first-attack" children than in the control group.

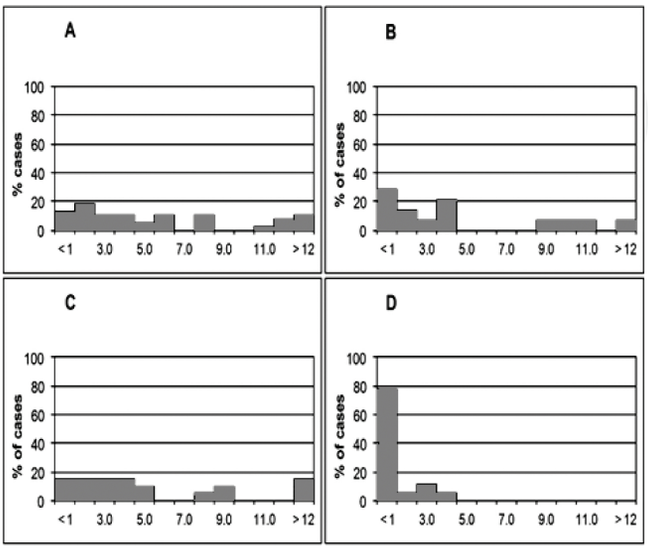

Figure 6. Distribution of 3HOKYN/3HOAA values in epileptic patients, in comparison with healthy controls. Designations: healthy controls (A); patients experienced the first seizure attack (B); AED-treated seizure-free patients (C); partially AED-controlled patients (D).

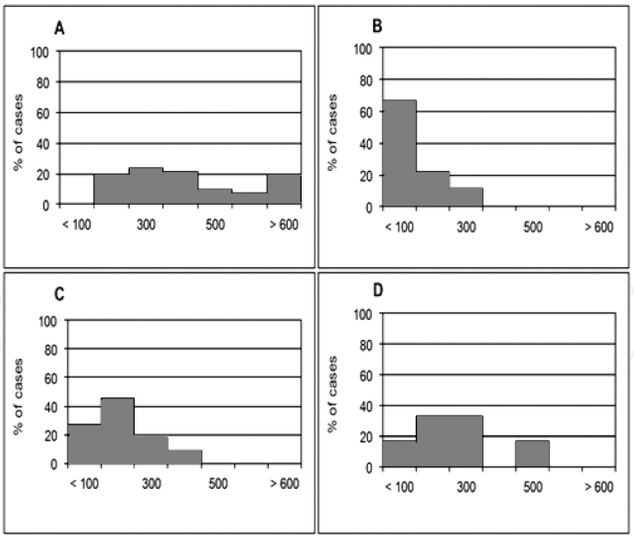

Figure 7. Distribution of IND/KYN values in epileptic patients, in comparison with healthy controls. Designations: healthy controls (A); patients experienced the first seizure attack (B); AED-treated seizure-free patients (C); partially AED-controlled patients (D).

The low value of IND/TRP and especially IND/KYN are also the markers, distinguishing first-attack children from healthy controls. The combination of increased concentrations of TRP and

KYN with decreased concentrations of IND provides drastic diminution in both IND/TRP and IND/KYN ratios in patients who endured the first attack. In approximately 70% of these patients, the IND/KYN ratio is lower than 100, while in healthy children these ratios are always higher than 100; in 60% of the healthy group, these ratios are even higher than 300 (**Table 1** and **Figure 7**).

1.4. Urinary markers in AEDs treated seizure-free patients

In patients well controlled by AEDs (in our study, those who has been seizure-free for at least 3 months) most of the studied parameters are practically similar to those in healthy controls. The mean values of KYN/TRP, 4-PA/KYN, 3- HOKYN/KYN, and 3-HOAA/3-HOKYN ratios coincide with those in the control group (**Table 1**). Though the urinary concentrations of TRP and KYN in seizure-free patients are significantly higher than in the healthy group, the correlations TRP: (3HOAA/3-HOKYN) and KYN: (3HOAA/3-HOKYN) practically coincide with corresponding values in healthy controls.

Histograms of distribution of studied parameters in AED-treated seizure-free children are similar to those in healthy controls (**Figures 4** and **6**), with the exception of histograms of distribution of 3-HOKYN/KYN ratios (**Figure 5**). Similarity between the histogram of distribution of these ratios in seizure-free AED-treated children and the group experienced the first attack signifies that kynureninase activity is not yet restored in AED-treated patients.

The mean concentration of IND in these patients remains as low as in "first-attack" group, but values of IND/TRP and IND/KYN ratios—due to reduction of TRP and KYN levels—are higher than in first-attack group, but far lower than in healthy group. The histogram of the distribution of IND/KYN ratios remains still shifted to the left (**Figure 7**).

1.5. Urinary markers in patients partially responsive to AED treatment

Repeated convulsive attacks, which occur in epileptic patients in spite of AED treatment, result in decreased values of 4-PA/KYN ratio, the marker of recently experienced seizure attacks (**Figure 3**). In three out of five patients, who had seizures shortly before the admission to the hospital, this ratio was less than one, decreasing thereby the mean value of the group (**Table 1**). The fourfold elevated concentration of toxic 3-HOKYN, the twofold elevated ratio of 3-HOKYN/KYN, and the dramatically reduced value of the 3-HOAA/3-HOKYN ratio are the most remarkable signs of the group. The mean value of 3-HOAA/3-HOKYN ratio is reduced to 0.9 and represents only 15% of the corresponding value in seizure-free patients (**Table 1**).

The strongly right-shifted histogram of the distribution of 3-HOKYN/KYN ratios and the strongly left-shifted histogram of the distribution of 3-HOAA/3-HOKYN ratios are apparently the signs of severe disturbances of kynureninase activity in partially AED-controlled patients (**Figures 5** and **6**).

Reduction in the kynureninase activity results also in the accumulation of TRP, KYN, and KA. Accordingly, the correlation of each of these compounds to the 3-HOAA/3-HOKYN ratio, that is, KYN: (3-HOAA/3-HOKYN), KA: (3-HOAA/3-HOKYN), and TRP: (3-HOAA/3-HOKYN), reaches extremely high values in patients partly responsive to AEDs (**Table 1**).

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The intensive inpatient AED treatment distinctly changes the examined parameters. First of all, the value of 4-PA/KYN ratio is increased. The value of 3-HOAA/3-HOKYN ratio is also increased, reflecting an increase in kynureninase activity. Accordingly, values of KYN: (3-HOAA/3-HOKYN), KA: (3-HOAA/3-HOKYN), and TRP: (3-HOAA/3-HOKYN) are significantly diminished. In successful cases, favorable changes, once attained, remain stable (**Figure 8**; patients E and K). In unsuccessful cases, the initial increase in the 3-HOAA/3-HOKYN ratio suddenly reverts back, and the related parameters are accordingly changed (**Figure 8**, patient D).

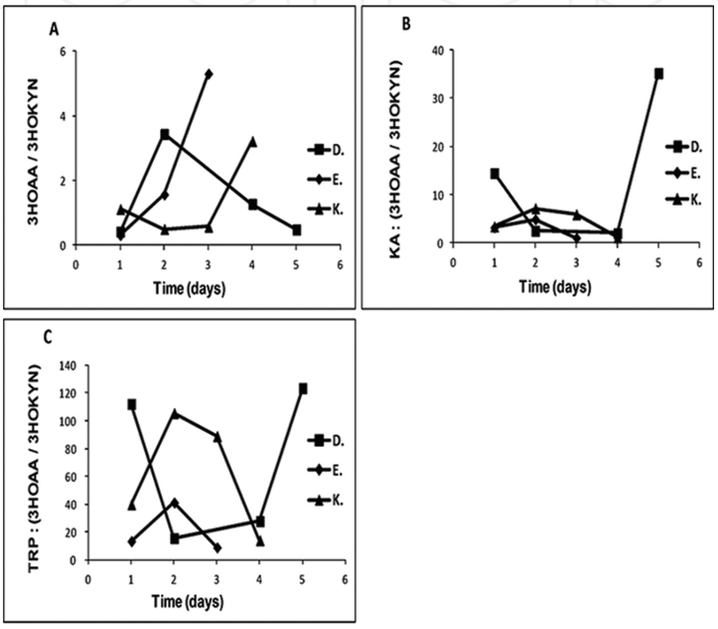

Figure 8. Dynamics of correlations between TRP metabolites under intensive AED treatment in partially AED-controlled patients. Note the difference between successfully (E and K) and unsuccessfully (case D) treated patients.

1.6. Discussion

Disturbances of PLP-dependent TRP degradation revealed in children with different forms of epilepsy confirm the suggestion that **epilepsy as a whole is PLP-dependent disorder**. The data obtained testify that concentrations of compounds formed or metabolized in the course of PLP-dependent TRP degradation, as well as correlations between them, are quantitative urinary biomarkers for the determination of clinical status—from the first seizure attack up to progressively worsening condition. These biomarkers are also indicative for the evaluation of AED treatment effectiveness and its individual monitoring. The parameters reflecting kynureninase activity turned out to be the most sensitive link of this chain.

Once the initial seizure attack has occurred, the drastically increased levels of TRP, KYN, and toxic 3HOKYN, and the drastically reduced level of IND pointed to the disordered PLP-dependent TRP degradation. Low values of IND/TRP and IND/KYN, as well as 4-PA/KYN and 4PA/3-HOKYN ratios, completely change the pattern of TRP metabolites (**Table 1**).

Specifically, the low value of 4-PA/KYN ratio (**Figure 3**) distinguishes an epileptic episode from paroxysmal loss of consciousness of nonepileptic origin. It is important to trace how long this index remains at such a low level after a single seizure episode.

The effective AED treatment normalizes most of the discussed parameters (**Table 1**). The ratio 4-PA/KYN is increased almost up to its control value. We believe that maintaining this ratio within the range between two and four (**Figure 3**) would help to provide adequate seizure control and reduce a risk of pharmacological overtreatment [45, 46]. However, increased levels of TRP, KA, and KYN, reduced concentrations of IND, and diminished IND/TRP and IND/KYN ratios (**Table 1** and **Figure 8**) still clearly distinguish AED-treated seizure-free patients from healthy controls.

The values of IND/KYN and IND/TRP ratios require an additional consideration. Intestinal PLP-dependent tryptophanase of bacterial origin is the key enzyme of alternative IND pathway of TRP degradation, which is inhibited by KYN [20]. A drastic drop in the levels of IND along with increased concentrations of KYN results in diminished IND/KYN and IND/TRP ratios in the studied groups.

Parameters reflecting the activity of kynureninase at the different stages of disease indicate that aggravation of epilepsy is accompanied by expanding inhibition of the enzyme activity [51–53]. The accumulation of toxic 3-HOKYN, along with twofold increase in the 3HOKYN/KYN ratio and sixfold decrease in the 3-HOAA/3-HOKYN ratio, appear to be the most characteristic signs of sharply reduced kynureninase activity in patients partially controlled AEDs (**Table 1**). The decrease in the value of 3HOAA/3HOKYN ratio leads to the accumulation of TRP, KYN, and KA (**Table 1**). The similar pattern is reproduced by kynureninase inhibitors, once even considered as possible anticonvulsants [54].

The intensive inpatient AED treatment of partially controlled patients decreases the 3-HOKYN/KYN ratio and increases the ratio 3-HOAA/3-HOKYN. Stability of attained parameters signifies successful treatment (**Figure 8**). The data obtained indicate that indices of kynureninase activity are the reliable markers for evaluation of clinical status and effectiveness of individual AED therapy.

The effective AED treatment increases alkaline phosphatase (ALP) activity [55–58], the enzyme which dephosphorylates PLP, and thus provides pyridoxal transport through membranes. Intensification of pyridoxine transport normalizes PLP-dependent systems (more details in part 2).

In summary, the suggested quantifiable urinary biomarkers, based on dynamic alterations of TRP metabolites in the course of the disease and antiepileptic treatment, are potentially helpful for

1. Identifying the patients who recently experienced seizure episode regardless of seizure type;
2. Detecting minimal effective doses of AED and gradual improvement of clinical status in the course of AED treatment;

3. Evaluating seizure-free status with greater precision;
4. Identification of inadequate seizure control, and rapid evaluation of the effectiveness of the novel treatment regimen;
5. Tracing the stability of results attained in the course of individualized AED treatment.

Taking into account the overall misdiagnosis rate of epilepsy (26%) and the rate of seizure recurrence after discontinuation of AED treatment ranging from 12 to 66% [59, 60], the use of suggested biomarkers seems to be expedient.

2. Family disorders of pyridoxine metabolism: Urinary TRP metabolites in asymptomatic first-degree relatives of epileptic probands

2.1. Introduction

The results obtained gave us an opportunity to discuss the nature of pyridoxine metabolism derangements in epilepsy. Are they a part of etiology of the disease [61], or consequences of seizures? Namely, are they an inborn error of metabolism, or epiphenomena of seizures, or possibly epiphenomena of seizures superimposed on inherently dysfunctional system of pyridoxine metabolism?

To understand whether dysfunctional vitamin B6 metabolism is an inborn error of metabolism inherent in epileptic families, and thereby a part of etiology of the disease, urinary parameters of vitamin B6–dependent TRP metabolism were HPLC detected in epileptic probands and their first-degree asymptomatic relatives in comparison with healthy controls.

Two non-sanguineous families were studied. Samples of each person were repeatedly analyzed over several (2–8) consecutive days. The mean value of each parameter is presented in **Table 2**.

Family one consisted of AED-treated proband D, an 8-year-old boy with a 3-s/spike-wave epilepsy (the only patient with absence epilepsy included into our study), his asymptomatic 13-year-old sibling brother, their mother affected with bronchial asthma [62], and the healthy stepfather of both children.

Grossly abnormal TRP metabolism in all three closely interconnected relatives, that is, the proband, his sibling (note especially his data), and their mother, clearly distinguished them from healthy controls and their healthy stepfather. Severe hypertryptophanuria and strongly reduced ratio KYN/TRP reflected the low activity of PLP-dependent IDO in all these three relatives. The values of 3-HOAA/3-HOKYN ratios in the proband, his mother, and especially in his asymptomatic sibling brother were 10–25% of the corresponding mean value in healthy controls, coinciding with the mean value of this parameter in epileptic patients ineffectively treated with AED *(see part 1)*. Extremely low values of 3HOAA/3HOKYN ratios along with increased levels of toxic 3-HOKYN testified kynureninase inhibition in all these three interconnected relatives. High levels of urinary 4-PA in siblings apparently meant the elevated PLP

plasma level [63]. The combination of hypertryptophanuria (and increased KA levels) with extremely low values of 3-HOAA/3-HOKYN ratios provides drastically increased correlations between these indices. In each of the relatives from this family ratios TRP: (3-HOAA/3-HOKYN) and KA: (3-HOAA/3-HOKYN) were much higher than in control patients. Low concentrations of IND and low values of IND/TRP ratios in all three interconnected relatives (**Table 2**) attest to familial disruption of alternative pathway of TRP degradation as well.

Family two included AED-resistant proband (Sh-T), a 10-year-old boy with frontal lobe epilepsy and secondary generalized convulsions unsuccessfully treated with carbamazepine, phenytoin, and valproic acid, and his asymptomatic sibling brother of 7 years. Diversity of proband's data made us repeat his analyses every 5 consecutive days and to demonstrate the ranges of them.

Parameters		**Family 1**			**Family 2**	
	Healthy controls (*n* = 37)	**Proband D. (*n* = 8)**	**Proband's mother M. (*n* = 8)**	**Proband's sibling Y. (*n* = 2)**	**Proband Sh.-T (*n* = 5)**	**Proband's sibling (*n* = 1)**
	Average ± SE	**Average ± SE**	**Average ± SE**	**Average**	**Range**	
TRP (μM)	23 ± 2	**135** ± 27	**166** ± 17	**180**	23.6–61.8	25
KYN (μM)	1.7 ± 0.2	1.5 ± 0.23	1.8 ± 0.30	2.8	1.2–2.0	1.7
3HOKYN (μM)	1.1 ± 0.1	1.7 ± 0.41	1.5 ± 0.37	3.6	1.2–2.8	0.80
3HOAA (μM)	4.5 ± 0.7	5.0 ± 2.1	**2.4** ± 0.67	**2.3**	1.3–30.0	1.8
KA (μM)	3.3 ± 0.4	4.3 ± 1.2	5.0 ± 0.95	7.1	2.3–4.6	3.6
4PA (μM)	6.0 ± 0.9	**11.1** ± 2.1	5.3 ± 0.49	**14.3**	**0.6–3.5**	**2.1**
IND (μM)	576 ± 53	**182** ± 38	**365** ± 33	660	446–767	74
4PA/TRP	0.29 ± 0.03	**0.136** ± 0.05	**0.04** ± 0.005	**0.087**	**0.02–0.06**	**0.08**
4PA/KYN	4.1 ± 0.5	**8.3** ± 2.1	3.4 ± 0.46	**8.2**	**0.03–2.69**	**1.3**
4PA/3HOKYN	8.42 ± 1.45	9.4 ± 3.4	**4.5** ± 0.81	**4.2**	**0.3–2.3**	**2.6**
4PA/(KYN + 3HOKYN)	2.5 ± 0.35	4.0 ± 0.96	1.7 ± 0.21	2.7	**0.16–0.85**	**0.86**
KYN/TRP	0.08 ± 0.005	**0.016** ± 0.005	**0.01** ± 0.003	**0.014**	**0.02–0.06**	**0.06**
3HOKYN/KYN	0.72 ± 0.08	**1.14** ± 0.21	0.72 ± 0.14	**1.80**	1.0–2.12	0.48
3HOAA/3HOKYN	6.0 ± 1.1	**2.6** ± 0.87	**1.2** ± 0.21	**0.7**	**0.58–2.7**	**2.3**
KA/KYN	2.5 ± 0.4	2.8 ± 0.64	2.9 ± 0.39	2.8	1.5–3.54	2.2
KA/3HOAA	2.2 ± 0.6	1.9 ± 0.63	3.5 ± 1.1	3.0	0.7–2.6	2.0
KYN/(3HOAA/3HOKYN)	1.06 ± 0.27	1.16 ± 0.48	1.48 ± 0.52	5.03	0.46–3.5	0.73
KA/(3HOAA/3HOKYN)	2.0 ± 0.5	**3.8** ± 1.5	**3.9** ± 1.2	**12.4**	0.9–6.5	1.6
TRP/(3HOAA/3HOKYN)	14 ± 3	**65** ± 17	**106** ± 29	**305**	9.1–57.9	11
IND/TRP	29.4 ± 2.8	**1.8** ± 0.60	**2.3** ± 0.29	**3.4**	**7.9–23.2**	**2.9**
IND/KYN	433 ± 50	**128** ± 21	**225** ± 25	**272**	372–432.8	**45**

Parameters similarly altered in family members are shown in bold.

Table 2. Urinary tryptophan metabolites and correlations between them in epileptic families compared to healthy controls.

In both brothers, the values of 3HOAA/3HOKYN ratios were low, reflecting the low kynureninase activity; the value of this ratio in the asymptomatic brother did not exceed one-third of that in healthy controls (**Table 2**). The IND levels, as well as IND/TRP ratios, were also strongly diminished in both siblings, indicating severe disruption of the alternative route of TRP metabolism.

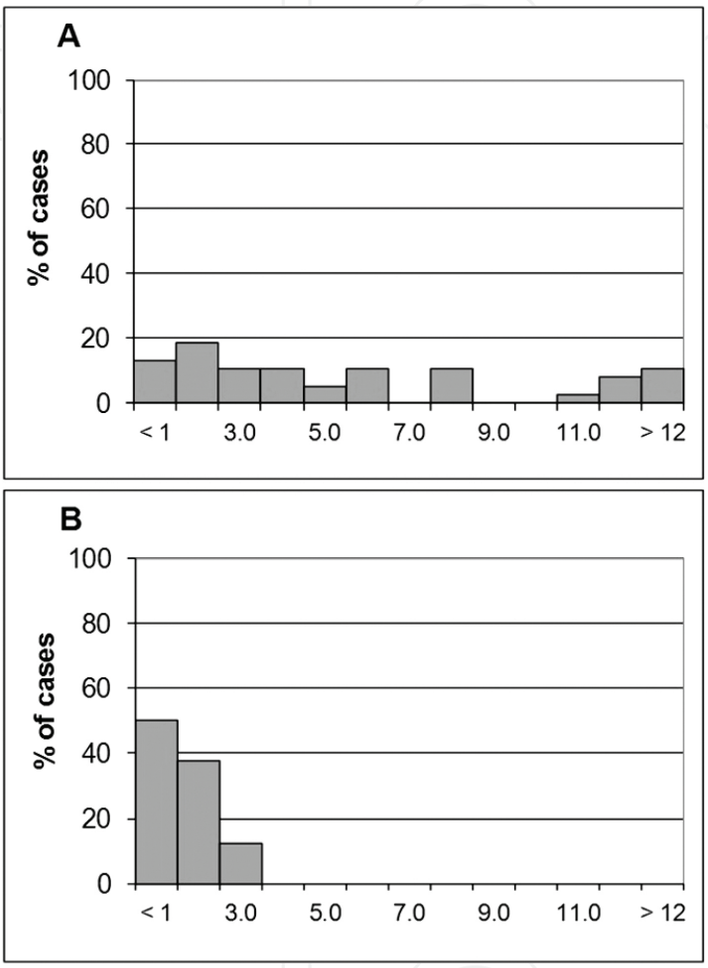

Figure 9. Distribution of 3HOAA/3HOKYN ratios in healthy controls (A) in comparison with epileptic families (B).

2.2. Discussion

Thus, the low activity of kynureninase (mirrored by 3-HOAA/3-HOKYN ratios) and disordered alternative route of TRP metabolism turned out to be the common hidden signs of asymptomatic relatives in both epileptic families, whereas hypertryptophanuria and the elevated level of 4-PA (signifying the increased plasma PLP level) [63] were inherent only in members of the first family (**Table 2**).

Histograms of the distribution of KYN/TRP and 3HOAA/3HOKYN ratios summarized in both families (**Figures 9** and **10**) reveal the reduced activity of both IDO and kynureninase in epileptic families, in comparison with healthy controls.

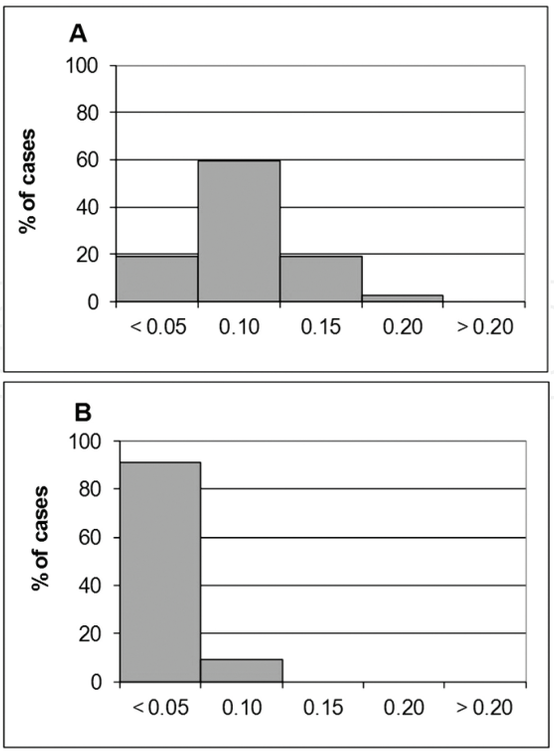

Figure 10. Distribution of the KYN/TRP ratios in healthy controls (A) in comparison with epileptic families (B).

This similarity of studied PLP-dependent disorders in epileptic probands and their asymptomatic first-degree relatives allows considering these disorders as inherent hidden traits of such families. Moreover, in clinically unaffected relatives these disorders are sometimes even more severe than in probands themselves (**Table 2**). The term "endophenotype" [64], as a heritable biochemical sign, which manifests in an individual whether or not illness is active and "co-segregated" with illness within the family, seems the most adequate definition of the state.

Judging by data obtained, *The inherent derangement of pyridoxine metabolism is a part of epilepsy etiology*. Repeated seizure attacks, superimposed on an inherently dysfunctional system of pyridoxine metabolism, may apparently provide extreme diversity of repeated results.

Van Gelder et al. [21–23, 65] were the first to find increased plasma levels of glutamate in first-degree relatives of epileptic patients just as in the patients themselves. The authors, however, considered their finding as a single symptom, rather than the manifestation of pyridoxine metabolism disorders. Later, these results were repeated, and plasma excess of aspartate and glycine along with the reduced level of urinary taurine was found in asymptomatic first-degree relatives of epileptic patients [66, 67], as well as in probands themselves. And again, the combination of biochemical alterations common to epileptic families was not explained, though PLP-dependent metabolism of amino acids pointed to the etiology of these familiar disorders.

We believe that reduced activity of alkaline phosphatase, the enzyme which dephosphorylates PLP and thus provides pyridoxal transport through membranes, may be the main factor of pyridoxine disorders in epileptic patients and their first-degree relatives.

In our experiments carried out in genetically epilepsy-prone and control epilepsy-resistant BALB/c mice (selectively bred from BALB/c strain for susceptibility or resistance to audiogenic seizures) [35, 36, 49], ALP activity in the cortex and hippocampus of seizure-naïve epilepsy-prone mice amounted to only 77.2 ± 6.7 and 74.1± 6.1% of activity inherent in epilepsy-resistant controls (Bresler a. Dolina, unpublished). In agreement with these data, the elevated PLP level was found in the brain of epilepsy-prone DBA/2 mice, in comparison with control epilepsy-resistant animals of the same strain [68].

Recently, it was shown that mice with a splice site mutation in the Akp2 gene for tissue nonspecific isoenzyme of ALP (TNSALP) have approximately 50% of normal plasma ALP activity and possess the elevated PLP plasma level, but do not manifest spontaneous seizures [69]. Unlike them, TNSALP knockout mice have 20-fold elevation of serum PLP level, large reduction in the intracellular brain PLP and lethal convulsions relieved by pyridoxal administration [70, 71].

We believe that disorders of pyridoxine metabolism in epileptic families are the consequences of inborn hypophosphatasia caused by a low activity of TNSALP. Mutations in *ALPL* gene, which encodes TNSALP, are responsible for the reduction of enzymatic activity [71–73]. The clinical spectrum of congenital hypophosphatasia presents a wide variety of phenotypes—from newborn, or infant convulsions controlled by pyridoxine [74–79] to their asymptomatic parents [80–84], whose ALP deficiency may be manifested, for example, by osteoarthropathy and/or odontohypophosphatasia. Diversity of these manifestations may depend on the extent of ALP activity reduction which, in its turn, depends on the variability of mutations in "candidate" ALP genes. Mutations in candidate genes may become the determining factor of pyridoxine metabolism disorders in epileptic families.

In their turn, disruptions of pyridoxine metabolism affect the production of PLP-dependent neurotransmitters. The imbalance of excitatory and inhibitory neurotransmitters becomes the neurochemical background of enhanced familial seizure predisposition.

We believe that *long-term* pyridoxine treatment started at early development (7–10 mg/kg daily) is safe and effective protective replacement therapy for a child born in epileptic families.

3. Conclusion

The pilot clinical trial carried out in children with different forms of epilepsy has confirmed our previous assumption of affected pyridoxine metabolism as an inborn genetic sign in epilepsy [35, 61]. We believe that clinical manifestations of inborn errors of pyridoxine metabolism are a kind of clinical continuum, ranging from severe convulsions resistant to AEDs to more common AEDs correctable forms of epilepsy, and up to remote symptoms in asymptomatic relatives of epileptic probands. This clinical continuum is open to *long-term high dose pyridoxine replacement therapy*. According to our experience, prolonged (over the years) pyridoxine treatment in pharmacological doses (10 mg/kg not exceeding 200 mg/daily) is valuable for different types of epilepsy (excluding—at least at present—absence and atonic

forms). Being started at early stages of the disease and targeted at the stable correction of PLP-dependent metabolic disturbances, such a treatment will be effective by itself, and also as a background for AED management. It seems reasonable to suggest the same **long-term** pyridoxine treatment as a safe and effective protective replacement therapy for a child born in epileptic families.

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Abbreviations

PDE	pyridoxine-dependent epilepsy
PLP	pyridoxal-5-phosphate
AED	antiepileptic drug(s)
GAD	glutamic acid decarboxylase
TRP	tryptophan
KYN	kynurenine
IDO	indoleamine 2, 3-dioxygenase (IDO)
3-HOKYN	3-hydroxykynurenine
3-HOAA	3-hydroxyanthranilic acid
KA	kynurenic acid
IND	indoxyl sulfate
4-PA	4-pyridoxic acid
ALP	alkaline phosphatase
TNSALP	tissue nonspecific isoenzyme of ALP

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