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Changes in tryptophan and kynurenine pathway metabolites in the blood of children treated with ketogenic diet for refractory epilepsy

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ABSTRACT

Purpose: There is growing evidence to support the role of the kynurenine pathway in the anticonvulsant efficacy of ketogenic diets (KDs) in refractory epilepsy. The aim of the present study was to measure blood levels of tryptophan (TRP) and its kynurenine derivatives and correlate them with seizure reduction after starting the KD in children with refractory epilepsy.

Methods: Sixteen children (9 F/7 M; 7.1 ± 5.1 years) with refractory epilepsy were treated with the KDs. Clinical efficacy and metabolic ketosis were monitored throughout the study; blood levels of TRP, kynurenine (KYN), kynurenic acid (KYNA), and 3-OH-kynurenine (3-OH-KYN) were measured at 3, 6, and 12 months on the diet and compared to the pre-KD levels.

Results: Out of 16 children, 14 attained a ≥50% reduction (responders) in seizure frequency 3 months after starting the KD. In the 14 responders, TRP levels decreased numerically (18–25%) but not significantly ($P = 0.077$) compared to the pre-KD control values. KYN levels decreased significantly (30–57%; $P = 0.001$) compared to the pre-KD control levels while KYNA levels significantly increased (38–96%; $P < 0.001$). KYNA/KYN ratios significantly increased (100–323%; $P = 0.003$) while 3-OH-KYN levels ($P = 0.680$) and KYN/TRP ratios ($P = 0.385$) remained unchanged. Higher concentrations of KYNA and lower concentrations of KYN ($P < 0.05$) were found in patients who attained a higher reduction in seizure frequencies on the KD.

Conclusions: We report a pattern of changes in the blood level of kynurenines in patients with refractory epilepsy who started the KD. The results of this study further support the role of specific kynurenines (e.g. KYNA) in the efficacy of the KD in refractory epilepsy.

1. Introduction

A high-fat, low-carbohydrate diet, also called the ketogenic diet (KD), was introduced into clinical practice as an alternative method to treat epilepsy, based on observations that fasting and starvation rendered an anti-seizure effect in epileptic patients [1]. Since then, the classic KD and its later modifications (i.e. the modified Atkins diet (MAD)) and low glycemic index treatment have been used to manage patients with intractable epilepsy. Of note, the term KD currently refers to any dietary therapy which would be expected to result in a ketogenic state of human metabolism [2,3].

Reduced glucose levels and increased ketones levels in the blood are the hallmark biochemical changes during exposure to KDs. Glucose is the main energy source from a carbohydrate-rich diet, but during prolonged exposure to KDs, energy is mainly derived from the oxidation of fatty acids (FAs) in the mitochondria. During a KD, the liver oxidizes FAs at high rates resulting in an overproduction of acetyl-CoA, which is then converted to acetoacetate that can be spontaneously degraded to acetone or metabolically converted to β-hydroxybutyrate (BHB). Consistently, increased levels of these three so-called ketone bodies

Abbreviations: AEDs, antiepileptic drugs; BHB, β-hydroxybutyrate; KAT, kynurenine aminotransferase; KB, keton body; KD, ketogenic diet; KYN, kynurenine; KYNA, kynurenic acid; 3-OH-KYN, 3-hydroxykynurenine; MAD, modified Atkins diet; NMDA, *N*-methyl-D-aspartate; TRP, tryptophan

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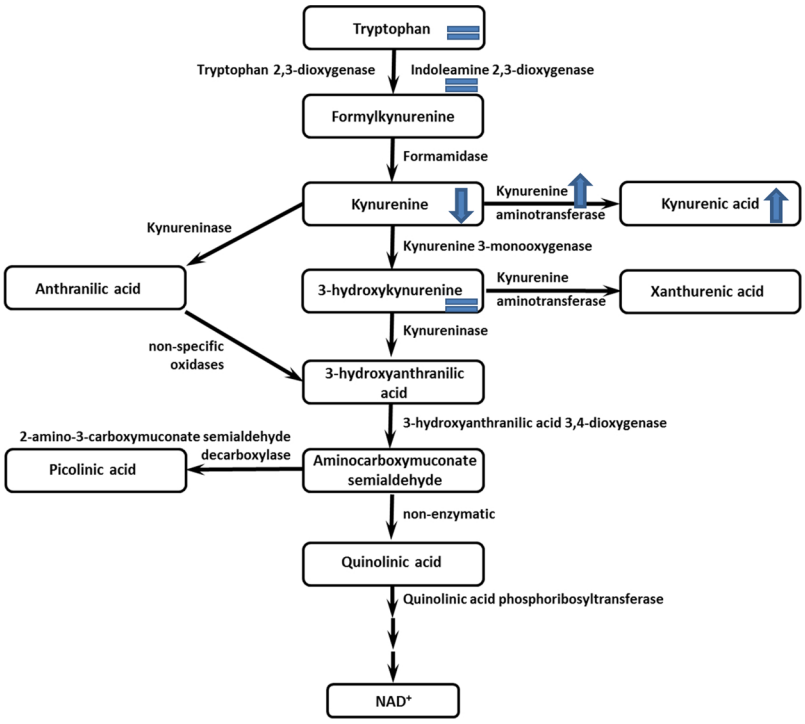

Fig. 1. Schematic diagram illustrating the effects of the KD on kynurenine pathway metabolite contents and enzyme activity. Of note, a small fraction of the ingested essential amino acid tryptophan is used in anabolic processes, whereas the remaining majority (approximately 90%) is metabolized via the kynurenine pathway as shown here. Arrow-up means increase; arrow down means decrease, and equality sign means no change. Kynurenine pathway based on works published earlier [28–30, 34].

(KBs), along with low and stable levels of serum glucose and normal blood gas, create the state of metabolic ketosis provoked by the KD [2]. Apart from metabolic ketosis, changes likely related to the KD's anticonvulsant properties include reduced glycolysis, elevated fatty acid levels, enhanced bioenergetic reserves, increases in γ-aminobutyric acid neurotransmission, decreases in glutamate-mediated toxicity, and effects on antioxidant mechanisms, programmed cell death, and anti-inflammatory processes [4,5].

More recent evidence suggests that the kynurenine pathway is involved in the neuroprotective and anticonvulsant activity of the KD [6–9]. This pathway generates a range of metabolites collectively known as kynurenines, which are involved in a variety of medical conditions such as inflammation, immune response, and several CNS disorders including epilepsy, depression, and diseases associated with neurodegeneration [10,11]. Of note, a small fraction of the ingested essential amino acid tryptophan (TRP) is used in anabolic processes, but the remaining majority (~90%) is metabolized via the kynurenine pathway (Fig. 1). Only approximately half of the KYN in the brain derives from production in situ; the rest comes from the peripheral pool. TRP and its metabolite kynurenine (KYN) can cross the blood-brain barrier, where they are degraded to kynurenic acid (KYNA) in astrocytes and to quinolinic acid in microglia to affect the *N*-methyl-D-aspartate (NMDA) receptors in a pharmacologically opposite fashion [12].

Chronic exposure to the KD has been shown to increase KYNA concentrations in discrete structures in the rat brain [13]. Experimental data indicate that glutamate, the non-selective glutamate receptor agonist, decreases the production of KYNA in bovine retinal slices; this effect is attenuated by acetoacetate and β-hydroxybutyrate, two of three KBs overproduced during the KD [8]. It has also been shown that β-hydroxybutyrate stimulates brain KYNA synthesis in cortical slices and glial cultures [14]. Whether KD-induced modulation of KYNA production would have a clinically meaningful impact in refractory epilepsy is unknown and awaits further verification. In the present study, we sought to investigate changes in blood concentrations of TRP and its derivatives formed along the kynurenine pathway (Fig. 1) – KYN, KYNA and 3-hydroxykynurenine (3-OH-KYN) – in children with refractory epilepsy treated with KDs.

2. Methods

2.1. Participants

This was an open-label, prospective study conducted at the Department of Child Neurology, Medical University, Lublin, Poland. Informed written consent was obtained from all patients' parents or their legal guardians and assent was given by children who were cognitively able to do so. Ethical approval for the study was granted by the University's Bioethical Committee and all research was performed in accordance with its relevant guidelines and regulations.

Twenty children with refractory epilepsy, ages 1–16 years, were prospectively recruited between January 2015 and July 2017. The main inclusion criteria were drug resistant epilepsy with at least two seizures per week during the baseline assessment period, prior treatment failure of at least two antiepileptic drugs (AED), and no previous treatments with the KD. The main exclusion criteria were known or suspected inborn errors of metabolism, a history of hyperlipidemia, or kidney stones. Data from sixteen (16) children (9 F and 7 M) with a mean age ± SD 7.05 ± 5.06 were eventually analyzed. Four patients were excluded from the present study: three because they withdrew after signing the informed consent, and one due to non-compliance.

At the screening visit, each child underwent a detailed clinical evaluation, reporting general medical history and history relevant to the current diagnosis of epilepsy (seizure type, underlying etiology, age at seizure onset, developmental status, and treatment history, etc.). In the analyzed cohort, all patients but one had multiple seizure types (patient 6 had bilateral tonic-clonic seizures only) (Table 1). In addition, a complete physical examination, vital signs assessment, ECG and laboratory / urinary testing were performed. AEDs were continued without dosing changes for at least the first 3 months in all patients. After 3 months on the KD and based on clinical response, an attempt was made to either reduce doses of the concurrent oral AED medications or to wean them off; some patients required an increase in dose and/or an addition of new AEDs. Seizure frequency was determined by parent-reported daily seizure diaries kept for at least one month prior to KD initiation (pre-KD baseline seizure frequency) and continued for one year after starting the KD. For children with multiple seizure types, a

Table 1
Selected demographic characteristics of the patient population. F- female, M- male; Marked in bold are the two patients who were determined as non-responders.

Patients number	Gender	Age at seizure onset	Age at diet initiation	Target seizure type	Underlying etiology	Number of AEDs before diet initiation	Number of AEDs at diet initiation
1	F	13 years	16 years	focal to bilateral tonic-clonic	unknown	7	3
2	F	2 years	2 years 3 months	generalized epileptic spasms	Rett Syndrome/genetic mutation MECP2	2	2
3	M	4 years	6 years 1 month	generalized myoclonic-atonic	myoclonic-atonic epilepsy/Doose Syndrome	7	3
4	M	3 years	16 years 3 months	generalized tonic-clonic	unknown	8	4
5	M	3.5 years	6 years	focal with impaired awareness	unknown	3	1
6	F	1.5 year	15 years 4 months	focal to bilateral tonic-clonic	unknown	5	2
7	F	3 years 3 months	4 years	focal with impaired awareness	unknown	2	0
8	**F**	**second day of life**	**1 year 10 months**	**focal to bilateral tonic-clonic**	**Lissencephaly**	**6**	**2**
9	M	4.5 years	6 years 1 month	focal onset atypical absence	unknown	2	2
10	M	11 months	9 years 11 months	generalized tonic-clonic	unknown	10	2
11	**M**	**3 months**	**1 year 5 months**	**generalized epileptic spasms**	**unknown**	**7**	**3**
12	F	2 months	5 years 3 months	generalized tonic-clonic	unknown	7	2
13	M	2 years 1 month	3 years 3 months	atypical absence	unknown	3	1
14	F	2 years	10 years 5 months	focal to bilateral tonic-clonic	periventricular heterotopia	6	2
15	F	fourth day of life	2 years 6 months	generalized epileptic spasms	genetic mutation/SCN2A	9	2
16	F	1 year	6 years	generalized epileptic spasms	genetic mutation ARX	7	3

target type was identified by the neurologist and used for evaluation of efficacy. The target seizure type was considered the type of seizure(s) that was countable and most consequential (i.e. most impactful on the patient's quality of life). Impact of the KD on seizure control and biochemical parameters were evaluated at 3, 6 and 12 months. Seizure response was categorized on a 4-point scale where 0 = no reduction in the targeted seizure frequency, 1 = < 50% seizure reduction in the targeted seizure frequency, 2 = ≥50% seizure reduction in the targeted seizure frequency, and 3 = > 90% seizure reduction and seizure freedom. Responders were defined as patients experiencing a ≥50% reduction in target seizure frequency, while non-responders were defined as having a seizure reduction < 50% at 3 months after starting the KD [15].

Development of metabolic ketosis in response to the KDs was confirmed by measuring urine levels of acetoacetate (ACA) as well as blood levels of BHB and glucose. Blood levels of TRP, KYN, KYNA, and 3-OH-KYN were measured chromatographically after 3, 6, and 12 months on the diet; pre-KD levels served as base-line controls. Selected demographic patient-level data are presented in Table 1.

2.2. Treatment (diets)

Eligibility for the KD treatment was in concordance with generally accepted guidelines [15]. The diets, either the classic KD or modified KD, were individually assigned to each participant by a dietician considering the child's age and family's preferences, in order to find the most suitable amount of fat, micronutrient intake and ketosis level for achieving maximal seizure reduction. The diets were slowly introduced at home using a non-fasting, gradual initiation protocol.

The classic KD was started with a full calorie ketogenic ratio (fat:protein + carbohydrates by weight) of 0.6:1, and then advanced weekly to 1:1, 2:1, 2.5:1, 3:1 or 4:1 over a 4-week period, at which point the patient achieved optimal seizure control and/or at least moderate metabolic ketosis (i.e. BHB level in serum was > 2 mM and stable glucose in serum ranged from 65 to 80 mg/dL). All participants receiving modified KD were placed on the modified Atkins diet (MAD). The MAD restricted carbohydrates to 10 g daily for the first month (increased by approximately 5 g/month to the limit of 10% carbohydrate by weight/day) and unlimited calories and protein intake. Parents were encouraged to gradually increase fat over 4 weeks. Irrespective of the type of diet, patients also received vitamin and mineral supplements according to the recommended daily allowance for their age. Metabolic outcome was monitored at 1, 3, 6, and 12 months after diet initiation. Throughout the observation period, no clinically meaningful changes in blood laboratory parameters (including those relevant for liver, pancreas and kidney functions, complete blood count, capillary blood gas, electrolytes, and fasting lipids profiles) were noted. The levels of 25-hydroxy-vitamin D as well as free and total carnitine were also monitored. Clinical data summarizing to the KD therapy are depicted in Tables 2 and 3.

2.3. Blood sampling for HPLC

The plasma was obtained by a centrifugation of the blood at 3000 rpm for 30 min (temp. 4 °C). Samples were stored at −80 °C until assayed. Blood plasma was deproteinated with 2 M perchloric acid. Samples were vortexed, kept at 4 °C for 10 min, and centrifuged at 14,000 rpm for 30 min at 4 °C.

2.4. Determination of tryptophan and its metabolites

TRP, KYN and KYNA concentrations were measured according to methods reported elsewhere [16]. Samples were analyzed by a high-performance liquid chromatography (HPLC) system (The UltiMate 3000 Analytical systems (Thermo Fisher Scientific, USA)). The prepared samples were separated on an analytical column (Agilent HC-C18;

Table 2
Clinical characteristics of patients. In the group of responders, data are presented as a number of patients or mean ± SD.

Parameter		Responder's group [mean ± SD]	Non-responder 1	Non-responder 2
Sex		8F/6M	F	M
Age at seizure onset [month]		35 ± **37**	1	3
Target seizures	focal	6	1	spasm
	generalized	8	0	0
Number of targeted seizures before KD		52 ± 53	140	120
Age at KD initiation [month]		96 ± 56	22	17
Diet type	MAD	9	0	0
	classical	5	1	1
Improvement score at 6 month*		2.2 ± 0.4	0	0
Glucose 6 month on KD serum [mg/dL]		73 ± 8	86	92
β-Hydroxybutyrate 6 month on KD [mmol/L]		3.4 ± 1.4	2	1.8
Acetoacetate 6 month on KD (urine) [mmol/L]		2.9 ± 0.8	3	1
Tryptophan before KD [pmol/100 µL]		1695 ± 754	956	1783
Tryptophan 6 months on KD [pmol/100 µL]		1395 ± 655	874	1059
Kynurenine before KD [pmol/100 µL]		82 ± 37	121	91
Kynurenine 6 months on KD [pmol/100 µL]		36 ± 14	56	71
Kynurenic acid before KD [pmol/100 µL]		0.09 ± 0.06	0.12	0.04
Kynurenic acid 6 months on KD [pmol/100 µL]		0.17 ± 0.08	0.19	0.05
3-OH-kynurenine before KD [pmol/100 µL]		19 ± 15	15	19
3-OH-kynurenine 6 months on KD [pmol/100 µL]		21 ± 15	11.2	15

F – female; M – male; KD – ketogenic diet; SD – standard deviation; * cessation of KD.

Table 3
Glucose and ketone bodies during KD. Data are presented as mean ± SD.

	Time on KD [month]	Responders
Glucose (serum) [mg/dL]	3	72.8 ± 7.5
	6	75.9 ± 8.0
	12	75.5 ± 8.6
β-Hydroxybutyrate (serum) [mmol/L]	3	3.4 ± 1.4
	6	3.1 ± 1.8
	12	3.2 ± 2.0
Acetoacetate (urine) [mmol/L]	3,6,12	2.9 ± 0.8

250 × 4.6 mm i.d.; 5 m particle size). The mobile phase was composed of 20 mmol/L NaAc, 3 mmol/L $ZnAc_2$ and 7% acetonitrile and was pumped at a flow-rate of 1 mL/min. The volume of analyte per injection was 100 µL. The wavelength of UV light was set at 365 nm for KYN and 250 nm for TRP; KYNA was quantified fluorometrically (excitation at 344 nm with detection at emission at 398 nm).

3-OH-KYN was analyzed using an electrochemical detector (Thermo Scientific Dionex UltiMate 3000 ECD-3000RS) connected to an analytical cell with the oxidation voltage set at 0.6 V, according to the method reported earlier [17]. Waters Spherisorb S3 ODS2 150 x 2.1 mm column (USA) was perfused with a mobile phase consisting of 2% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM sodium EDTA and 8.9 mM heptane sulfonic acid (flow 0.3 ml/min; the volume per injection was 10 µL). Chromeleon software was used to control HPLC systems and record chromatographic data.

2.5. Statistical analysis

Statistical analyses were performed using the Statistica 12 PL software (StatSoft Poland, Cracow). The results were reported as means ± SD/SEM of raw or relative (i.e., percentages or changes relative to individual baseline) data. The Shapiro-Wilk test was used to test the variables' distribution. A repeated measures ANOVA test with a post-hoc Bonferroni's correction test for multiple comparisons and a Friedman test were performed to assess the changes of variables over time. $\chi 2$ test with Yates correction was used for group comparisons of categorical data. *U* Mann-Whitney test was used for group comparisons of not-normally distributed data. Student's *t*-test was used to compare changes in variables as a function of patients' response on the 4-point seizure-improvement scale. A P-value of less than 0.05 was considered statistically significant.

3. Results

Amongst 16 patients included in the study, nine were girls and seven were boys; seven (43.8%) patients started on the classic KD and nine (56.2%) patients started on the MAD. All patients continued the assigned type of KD throughout the study. The mean age ± SD at the beginning of dietary therapy was 7.1 ± 5.1 years, whereas the mean age at seizure onset was 2.6 ± 3.0 years. All patients but one had multiple seizure types (patient #6 had bilateral tonic-clonic seizures only); 13 patients had focal seizures, 9 patients had generalized seizures, 6 patients had both focal and generalized seizures; 6 patients had focal targeted seizures and 8 had generalized targeted seizures. The mean of the targeted seizure frequency in the baseline period was 52.3 ± 53.1 per month. The classification of the targeted seizures and epileptic syndromes is presented in Table 2.

After four weeks on the diet, all patients developed adequate metabolic ketosis. At the 3-month assessment, there were 14 responders (9 on MAD and 5 on classic KD) and 2 non-responders (both on classic KD) among KD-treated patients. In the group of responders (14 of 16), pharmacological treatment remained unchanged in 7 of 14 patients throughout the study; medication doses were reduced in 2 of 14, while medications were tapered off without seizure relapse in 5 of 14 patients. In the group of non-responders, AED treatment (doses and drugs) was adjusted based on medical need after 3 months on the KDs.

One non-responder patient experienced a drop in BHB levels at 3 months; but in view of the fact that the patient was severely affected and was fed via a gastrostomy tube, non-compliance was an unlikely explanation. The mean age at seizure onset was 2.9 ± 3.2 years among responders and 0.12 ± 0.17 years among non-responders. The two patients categorized as non-responders were diagnosed with severe epileptic encephalopathies in infancy and exhibited multiple seizure types, severe developmental and cognitive delays, and failure to respond to many AEDs (Tables 1 and 2).

Mean age at the diet initiation was 8.0 ± 4.7 years for responders and 1.6 ± 0.2 years for non-responders (Table 2.)

Serum levels of glucose and BHB and urine levels of acetoacetate in responders and non-responders are summarized in Table 3.

Fig. 2 shows blood levels of TRP and its selected metabolites or its ratios pre-KD and at 3, 6, and 12 months on the diet for the 14 responder patients. TRP levels decreased numerically but not significantly compared to the pre-KD control values at 3, 6, and 12 months on the diet (a decrease of 18%, 18%, and 25%, respectively; $F3,18 = 3.21$; $P = 0.077$). KYN levels decreased significantly compared to the pre-KD control levels (a decrease of 30%, 57%, and 35% at 3, 6, and 12 months, respectively; $F3,21 = 8.83$; $P = 0.001$). Relative to the pre-KD values, KYNA blood levels significantly increased by 38%, 80%, and 96% at 3, 6, and 12 months, respectively; $F3,18 = 10.65$; $P < 0.001$). 3-OH-KYN levels remained unchanged throughout the study ($F3,15 = 0.37$; $P = 0.680$). KYN/TRP ratios also remained unchanged ($F3.15 = 1.09$; $P = 0.385$). Relative to the pre-KD values, KYNA/KYN ratios increased

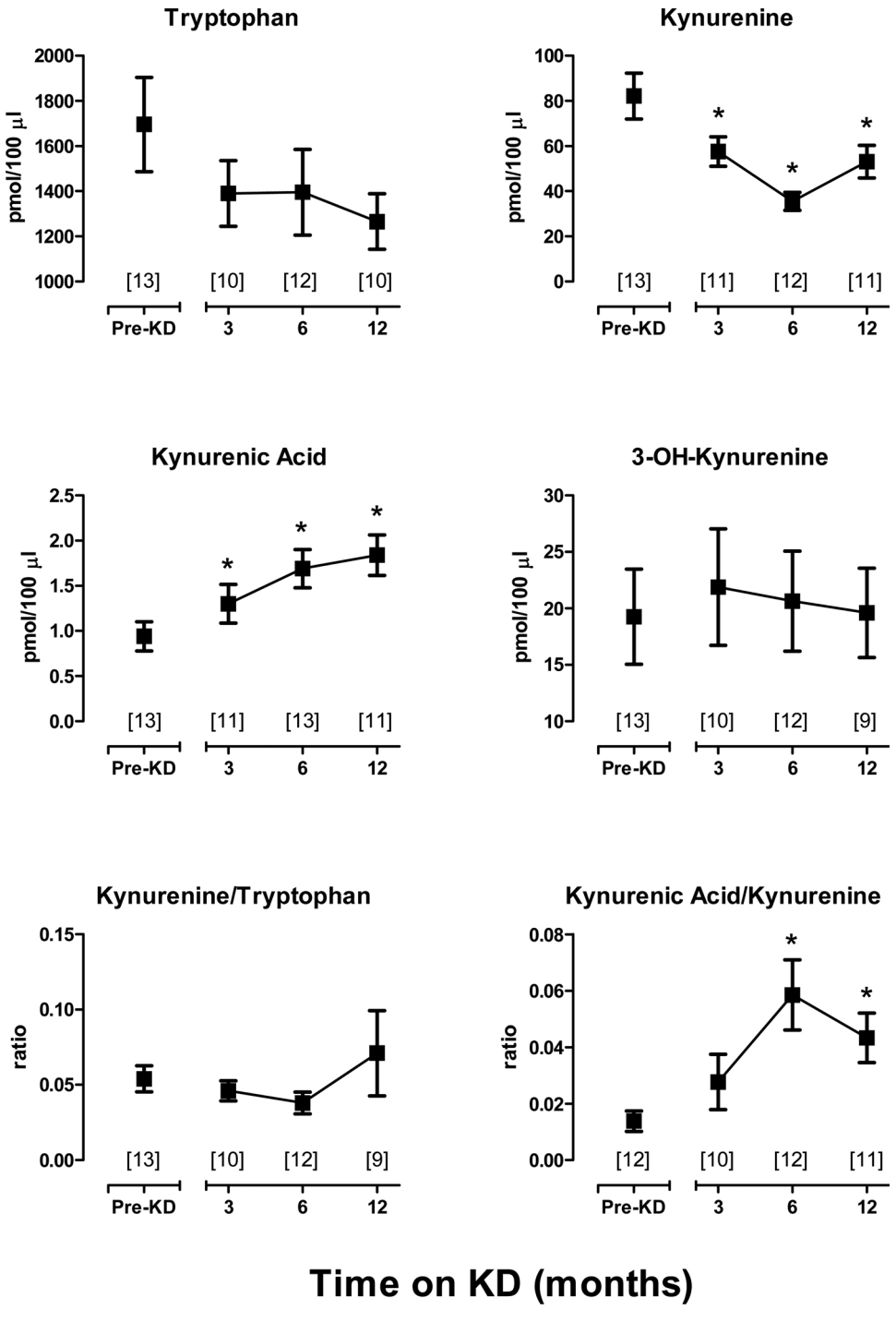

Fig. 2. Blood concentrations of tryptophan, kynurenine, kynurenic acid, 3-OH-kynurenie, kynurenine/tryptophan ratio, and kynurenic acid/kynurenine ratio in patients who attained a greater than 50% reduction in seizure frequency relative to the pre-KD period. Data are presented as a mean ± SEM for the pre-KD baseline measurements and at 3, 6, and 12 months on the KD. Numbers in parentheses indicate the number of patients analyzed per analyte at each time point. *P denotes significant (< 0.05) difference from the pre-KD values (Bonferroni's test after significant ANOVA).

by 101%, 323%, and 214% at 3, 6, and 12 months on the diet ($F_{3,18} = 6.830$; $P = 0.003$). For the two patients categorized as non-responders (Table 2), changes in kynurenines levels followed the same pattern as in those categorized as responders except for 3-OH-KYN (Fig. 2 and Table 2).

Higher ($P = 0.027$) serum concentrations of KYNA were found in patients who had a > 90% seizure reduction or became seizure free (score of 3 on a 4-point seizure reduction scale) relative to those with a 50–90% seizure reduction (score 2) (Fig. 3). Furthermore, larger ($P = 0.048$) serum decreases in KYN were found in patients who met the score 3 criterium on the 4-point seizure reduction scale relative to those who met the score 2 criterium at 3 months on the diet (Fig. 3). Serum levels of TRP and 3-OH-KYN did not differ ($P > 0.05$) between the patients who met the score 3 criterium in comparison to those who met the score 2 criterium while on the KD (data not shown).

4. Discussion

The present study expands our previous work in which we demonstrated that young and adult rats fed according to the KD exhibited

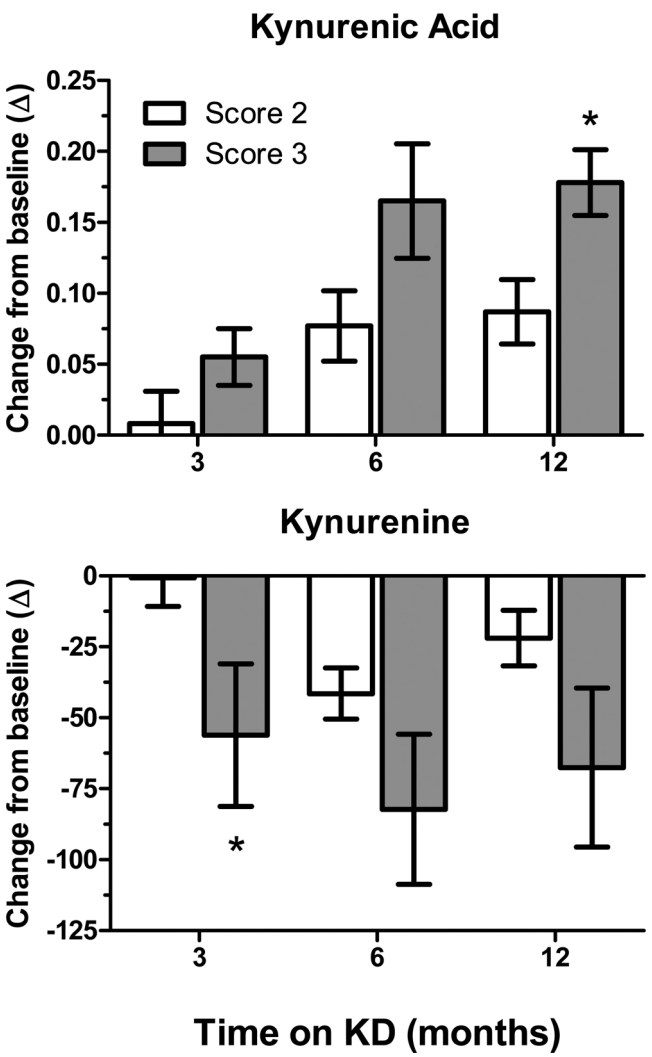

Fig. 3. Comparison of changes in blood concentrations of kynurenic acid and kynurenine in patients who met the criteria for a seizure improvement score of 2 (i.e., 50–90% seizure frequency reduction) or score of 3 (i.e., > 90% seizure reduction frequency or seizure free). Data are presented as the mean ± SEM change (in pmol/100 µL) at 3, 6, and 12 months on the KD relative to the pre-KD baseline in patients meeting the criteria for score 2 (n = 5–8) or score 3 (n = 3–5). *P denotes significant (< 0.05) difference from the pre-KD values (Student's *t*-test).

elevated levels of KYNA in discrete structures of the brain [13]. The data presented here demonstrate that among all kynurenines tested, only KYNA levels were significantly elevated in the blood of children on the KD; levels of TRP and KYN were decreased and levels of 3-OH-KYN were unchanged. Since the product-to-precursor ratio is considered an accurate reflection of enzymatic activity, we calculated KYN/TRP and KYNA/KYN ratios. The KYN/TRP ratio was unchanged, indicating sufficient enzyme activity. However, the KYNA/KYN ratio was significantly enhanced. These findings indicate a metabolic shift toward increased KYNA synthesis in the kynurenine pathway when on the KD (Fig. 1).

The exact molecular mechanism that leads to enhanced KYNA concentrations remains unknown. In the present study, patients with refractory epilepsy attained elevation of ketone bodies in the blood after starting the KD. It was previously reported that BHB enhanced the production of KYNA in the rat brain cortical slices and primary glial cultures [14]. Moreover, in the presence of BHB, the activity of both kynurenine aminotransferases (KAT I and II) was enhanced in cultured glial cells. Thus, it can be speculated that a similar KD-induced increase in KAT activities could be responsible for the increase in KYNA production demonstrated in the present study.

In fact, the activity of kynurenine pathway enzymes is much higher in peripheral tissues than in the brain; therefore, the effect of brain synthesis is negligible in the total blood concentration of metabolites [10]. All substrates pass through portal circulation to the liver, where they are filtered, metabolized, and finally released to systemic circulation. Among all cell types that express kynurenine pathway enzymes, hepatocytes contain all the machinery of tryptophan degradation towards any branch of kynurenine pathway and play a central role in the regulation of the process. Most likely, increased blood level of KYNA could be due to KD-induced liver or muscle KAT activity that is responsible for KYNA formation.

Interestingly, it has been shown recently that skeletal muscles contribute significantly to the metabolism of kynurenines. Especially during exercise, KAT enzymes shift peripheral kynurenine metabolism toward KYNA synthesis [18,19]. As a result, levels of KYNA in the blood are elevated, while KYN levels in the brain are also elevated and reduce stress-induced depressive symptoms. It could be assumed that individuals receiving the KD develop upregulation of KYNA synthesis with a parallel decrease of KYN levels. The same mechanism of KD-induced KAT upregulation cannot be ruled out as a driver of the anticonvulsant effect in our patients. It has also been documented that the KD exerts antidepressant and mood-stabilizing properties [20,21]. Similarly, Lee and coworkers suggested that mice fed a high-fat diet who perform aerobic exercise exhibit a rising KYNA/3-OH-KYN ratio, which is believed to be a meaningful anti-aging indicator [22]. Current understanding of how substrates of the kynurenine pathway change with environmental context is incomplete and prompted the development of mathematical models to help understand their metabolic flux [23].

KYNA is excreted unmetabolized in urine [24,25]. Therefore, the disturbance of kidney function can result in an increase of its blood content. In fact, it was found that plasma KYNA concentration corresponds to the extent of resection of renal tissue and the degree of renal insufficiency in rats [26]. However, decreased excretion of KYNA in investigated children is less probable as monitored renal function was not impaired.

Gut bioavailability of TRP as a substrate for kynurenines was unlikely to be markedly affected by the diet, as TRP's blood levels were comparable pre-KD and during KD (16–25% reduction; $P > 0.05$). In fact, protein intake is limited in the classic KD, which may result in lower TRP levels [15]. However, it cannot be ruled out that the enhanced KYNA concentration found in our study is attributable to increased KYNA absorption from the small intestine as gut microbiota might be altered in response to the KD [27].

KYNA is an antagonist of glutamate receptors and possesses anticonvulsant activity [28–30]. In our study, the content of KYNA in cerebrospinal fluid of KD-treated children was not determined for ethical reasons. In rats chronically exposed to the KD, a significant increase of KYNA was found in the hippocampi of young (3 weeks old) and adult (8–10 weeks old) rats, up to 256% and 363% respectively, compared to controls fed a regular diet [13]. Based on results obtained in rats, it is probable that an increase of KYNA content in serum is accompanied by an increase in brain tissue as well. Thus, a decrease in seizure activity should be expected in children receiving the KD. Indeed, in our study, 14 out of 16 patients responded to the diet therapy. This means that 87.5% of the patients had at least 50% seizure reduction. Lack of anticonvulsant effect of KD in the non-responders (12.5%) might be due to poor compliance, lack of effect, or a combination of the two. Usually, ketosis would develop within one month. However, clinical effects were assessed after 3 months at which full efficacy was achieved as expected [15].

Higher serum concentrations of KYNA were found in patients who attained higher reduction in seizure frequencies (score 3 vs score 2) at 12 months on the KD; furthermore, larger serum decreases in KYN were found in patients with a higher reduction in seizure frequencies. These

results further suggest that KYNA may contribute to the anticonvulsant effect of the KD and that monitoring of blood levels of kynurenines (e.g., KYN and KYNA) may be of value in predicting or monitoring clinical outcomes in response to KDs in certain subsets of patients with refractory epilepsy.

5. Study limitations

The present study has several methodological limitations. First, this was an open-label, single-site study in refractory epilepsy. Regardless of the disease being studied, such settings are prone to creating large expectations of treatment efficacy in patients/care-givers. This, in turn, can inflate treatment effect (in the case of this study, it would be reflected in the magnitude of seizure reduction); however, this potential bias should not affect the objective measures, like plasma levels of kynurenines, collected in the present study. Nevertheless, correlations between clinical responses and plasma levels of kynurenines would have more statistical power and clinical meaningfulness if the study was conducted in a larger population and at several independent clinical sites. Second, the number of patients enrolled was relatively low and the population was heterogeneous. This did not allow for meaningful statistical analyses of subgroups. Even the analysis based on the designation of "responders" versus "non-responders" was not clinically and statistically possible because of the low and imbalanced number of patients in each group. Likewise, outcomes of the analyses performed on changes in kynurenines relative to the magnitude of seizure reduction when on the KD (i.e., score 2 versus score 3 on the 4-point seizure reduction scale) should be interpreted with caution due to a small number of patients per group. Third, we cannot rule out that plasma levels of kynurenines do not correlate with those in the brain due to some brain-specific regulations of the kynurenine pathway in response to KDs. Collecting cerebrospinal fluid to address this question would be unethical in this vulnerable pediatric population. However, even without understanding how exactly brain and plasma levels of kynurenines correlate, research towards identifying potential markers of efficacy in epilepsy (in this study, profiling plasma levels of kynurenines in KD-treated patients with refractory epilepsy) might, like in other therapeutic areas (e.g., oncology), result in better predicting efficacy in certain subsets of patients. Finally, we acknowledge that different analytical approaches to measure kynurenines in response to, for example, the KD [9], may result in different conclusions. It is therefore important to use methodologies, like the one used in the present study, that have been validated across many and varied experimental conditions [16,31–33].

6. Conclusions

We report a pattern of changes in the blood level of kynurenines and their ratios in patients with refractory epilepsy after starting the KD. The results of this study further support a role of specific kynurenines, KYNA in particular, in the efficacy of the KD in refractory epilepsy. Whether levels of a specific kynurenine(s) at pre-KD base-line or when on the KD could serve as predictive or prognostic biomarkers in refractory epilepsy or other neuropsychiatric diseases amenable to KD treatments warrants further studies.

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Author's contributions

I.Z. collected the clinical data. T.K. performed biochemical analyses of the data. T.W.-D. analyzed the data statistically. M.T-B. K.M.-S., M.G., W.AT. and all other authors contributed to the experimental design and the final version of the manuscript.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

Ethical disclosure

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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