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Published in:
Journal of Nutritional Biochemistry

DOI:
10.1016/j.jnutbio.2017.09.020

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Large neutral amino acid supplementation as an alternative to the phenylalanine-restricted diet in adults with phenylketonuria: evidence from adult Pah-enu2 mice☆

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Abstract

Phenylketonuria treatment mainly consists of a phenylalanine-restricted diet but still results in suboptimal neuropsychological outcome, which is at least partly based on cerebral monoamine deficiencies, while, after childhood, treatment compliance decreases. Supplementation of large neutral amino acids (LNAA) was previously demonstrated in young phenylketonuria mice to target all three biochemical disturbances underlying brain dysfunction in phenylketonuria. However, both its potential in adult phenylketonuria and the comparison with the phenylalanine-restricted diet remain to be established. To this purpose, several LNAA supplements were compared with a severe phenylalanine-restricted diet with respect to brain monoamine and amino acid concentrations in adult C57Bl/6 Pah-enu2 mice. Adult phenylketonuria mice received a phenylalanine-restricted diet, unrestricted diet supplemented with several combinations of LNAA or AIN-93M control diet for 6 weeks. In addition, adult wild-type mice on AIN-93M diet served as controls. The severe phenylalanine-restricted diet in adult phenylketonuria mice significantly reduced plasma and brain phenylalanine and restored brain monoamine concentrations, while brain concentrations of most nonphenylalanine LNAA remained subnormal. Supplementation of eight LNAA was similarly effective as the severe phenylalanine-restricted diet to restore brain monoamines, while brain and plasma phenylalanine concentrations remained markedly elevated. These results provide biochemical support for the effectiveness of the severe phenylalanine-restricted diet and showed the possibilities of LNAA supplementation being equally effective to restore brain monoamines in adult phenylketonuria mice. Therefore, LNAA supplementation is a promising alternative treatment to phenylalanine restriction in adult phenylketonuria patients to further optimize neuropsychological functioning.

Keywords: Phenylketonuria; Inborn error of metabolism; Large neutral amino acids; Mouse model; Neurotransmitters; Adults

1. Introduction

Phenylketonuria (PKU; McKusick 261600) is an inborn error of phenylalanine (Phe) metabolism caused by a deficient activity of the enzyme phenylalanine hydroxylase, which normally converts Phe to tyrosine. Left untreated, PKU symptomatology correlates with high blood Phe concentrations and includes severe intellectual disability, seizures and psychiatric problems [1]. Besides a direct toxic effect of high Phe to the brain, cerebral monoaminergic neurotransmitter (monoamine) deficiencies have been hypothesized to contribute to brain dysfunction in PKU [2,3]. Impaired cerebral monoamine synthesis in PKU is due to outcompeted brain uptake of their amino acid precursors tyrosine and tryptophan by high blood Phe concentrations and/or to an inhibitory effect of high brain Phe concentrations on tyrosine and/or tryptophan hydroxylation [4–6].

Since 1953, when the first PKU patient was successfully treated with a Phe-restricted diet, blood Phe reduction has been the primary target of treatment. In practice, this Phe-restricted diet constitutes severe natural protein restriction and supplementation of an amino acid mixture to prevent nutritional deficiencies. The clinical importance of the Phe-restricted diet is undisputed, preventing severe intellectual disability if initiated shortly after birth [1]. In contrast, initiation of a Phe-restricted diet later in life cannot reverse the brain damage that has occurred during the first years of life, but has shown

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https://doi.org/10.1016/j.jnutbio.2017.09.020
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positive effects on behavior [7]. Today, initiation of treatment shortly after birth is enabled through neonatal screening, and treatment is advocated for life.

Notwithstanding the success of the Phe-restricted diet, especially adult PKU patients face two main issues. First of all, in treated adult PKU patients, mood disturbances and emotional distress, slower reaction times, impaired attention, and impaired social cognitive functioning and social skills have been reported [8–12]. These features have been hypothesized to be — at least in part — related to cerebral monoamine deficiencies [13]. Secondly, while treatment is advocated for life [14,15], treatment by diet is socially demanding especially at increased age, so treatment adherence usually declines after childhood [16,17]. Apart from this burdensome dietary treatment, the only other treatment option currently available includes tetrahydrobiopterin that can reduce blood Phe by acting as a chaperone to the phenylalanine hydroxylase enzyme. However, only a minority of PKU patients are tetrahydrobiopterin responsive [18]. Therefore, to improve neuropsychological outcomes, adult PKU patients may require alternative pathophysiology-based treatment strategies that, if possible, are also less socially demanding.

Supplementation of large neutral amino acids (LNAA)s excluding Phe may serve both these aims [4,19]. This is supported by previous clinical studies on LNAA treatment that showed positive effects on behavior and self-injury in late-diagnosed and previously untreated PKU patients, and on electroencephalography activity and cerebral Phe levels in PKU patients on liberalized dietary treatment [20,21]. In addition, LNAA supplementation in young PKU mice has been shown, among others, to improve cerebral serotonin and norepinephrine concentrations by both decreasing brain Phe and increasing brain tyrosine and tryptophan availability [5,6].

To determine whether LNAA supplementation might offer an alternative treatment for the burdensome Phe-restricted diet in adult PKU patients by especially improving cerebral monoamines, the aim of the present study was twofold. Firstly, this study investigated brain and plasma amino acid and monoamine concentrations in adult PKU mice on a Phe-restricted diet compared to PKU and WT control mice. Secondly, this study investigated the effects of an unrestricted diet with various LNAA supplements compared to a Phe-restricted diet on brain monoamine concentrations in relation to brain and plasma amino acid concentrations.

2. Material and methods

2.1. Animals

This study was performed in C57Bl/6 Pah-enu2 (PKU) mice. The Pah-enu2 mouse is a well-established PKU mouse model that resembles the genetics, biochemistry and neurobiology of PKU in humans [22]. As previously described, one of the important advances of this mouse model over PKU patients is the fact that brain concentrations of LNAA:s other than Phe as well as monoamines can be measured directly [5]. Breeding pairs had been kindly provided by Prof. B. Thony from the Division of Clinical Chemistry and Biochemistry, University Children’s Hospital, Zurich, Switzerland. From heterozygous (+/−) mating, we obtained wild-type (WT, +/+), heterozygous and PKU (+/−) mice of both sexes. After weaning at age 4 weeks, genetic characterization was performed by PCR analysis. For many years, PKU patients were diagnosed at birth. However, with the introduction of准确的 prenatal screening procedures, PKU is currently diagnosed in the neonatal period. In the present study, PKU mice were used at age 6.5–8.5 months, animals were included in the experiment, and PKU mice on a Phe-restricted diet compared to PKU and WT control mice. As this diet was different from the diet that the animals received before the experiment, the control mice on an AIN-93M diet were challenged by a dietary change similar to the one on the other experimental diets. The Phe-restricted diet was produced by reducing the amount of casein by 27%, which was compensated for by a synthetic amino acid mixture devoid of Phe plus 20% extra at the expense of cornstarch for the assumed protein conversion factor. The experimental LNAA diets were produced by adding LNAA:s to the AIN-93M diet at the expense of cornstarch on a weight-for-weight basis without restricting the amount of natural protein (in the form of casein). The compositions were based on those in previous experiments on the mechanisms by which LNAA supplementation could improve brain amino acid and monoamine concentrations in young PKU mice [56]. The total amount of added LNAA:s in the LNAA-supplemented diet was equal to the amount of protein in the basal (AIN-93M) diet (214.12 g/kg diet), consisting of various amounts of L-lysine, L-tryptophan, L-valine, L-isoleucine, L-leucine, L-methionine, L-histidine and L-phenylalanine, being, 28%, 17%, 14%, 14%, 6%, 3% and 3%, respectively, of the amount of protein in the basal (AIN-93M) diet (Supplemental Table 1). If compared to a mean natural protein intake in humans of 1 g/kg body weight/d, this corresponds to approximately 280, 170, 140, 140, 60, 30 and 30 mg/kg body weight/d, respectively, in humans. The added amount of tyrosine and tryptophan in the Tyr+Trp-supplemented diet was calculated to be 30% and 10%, respectively, of the amount of protein in the basal (AIN-93M) diet, corresponding to approximately (in total) 300 and 100 mg/kg body weight/d of, respectively, tyrosine and tryptophan in humans. Similarly, the added amount of leucine and isoleucine in the Leu+Ile-supplemented diet was calculated to be 24% and 18%, respectively, of the amount of protein in the basal (AIN-93M) diet, corresponding to approximately 240 and 180 mg/kg body weight/d in humans. Diets were prepared by Research Diet Services B.V. (Wijk bij Duurstede, the Netherlands). Results of amino acid analyses in the various diets are presented in Supplemental Table 1.

2.2. Experimental design

At age 6.5–8.5 months, animals were included in the experiment, and PKU mice were randomly assigned to one of five different dietary treatment groups, balanced for sex and age, while avoiding that littersmates were assigned to the same experimental group. During the first week of dietary treatment, body weight and food intake were measured daily. Hereafter, body weight and food intake were determined weekly.

Dietary treatment was continued for 6 weeks. At the end of the experiment, animals were euthanized by combined heart puncture and decapitation under inhalation anesthetics with isoflurane.

2.3. Experimental diets

The basal diet was AIN-93M [23], which was administered in unadjusted form to the PKU and WT control groups. As this diet was different from the diet that the animals received before the experiment, the control mice on AIN-93M diet were challenged by a dietary change similar to the one on the other experimental diets. The Phe-restricted diet was produced by reducing the amount of casein by 27%. This was compensated for by a synthetic amino acid mixture devoid of Phe plus 20% extra at the expense of cornstarch for the assumed protein conversion factor. The experimental LNAA diets were produced by adding LNAA:s to the AIN-93M diet at the expense of cornstarch on a weight-for-weight basis without restricting the amount of natural protein (in the form of casein). The compositions were based on those in previous experiments on the mechanisms by which LNAA supplementation could improve brain amino acid and monoamine concentrations in young PKU mice [56]. The total amount of added LNAA:s in the LNAA-supplemented diet was equal to the amount of protein in the basal (AIN-93M) diet (214.12 g/kg diet), consisting of various amounts of L-lysine, L-tryptophan, L-valine, L-isoleucine, L-leucine, L-methionine, L-histidine and L-phenylalanine, being, 28%, 17%, 14%, 14%, 6%, 3% and 3%, respectively, of the amount of protein in the basal (AIN-93M) diet (Supplemental Table 1). If compared to a mean natural protein intake in humans of 1 g/kg body weight/d, this corresponds to approximately 280, 170, 140, 140, 60, 30 and 30 mg/kg body weight/d, respectively, in humans. The added amount of tyrosine and tryptophan in the Tyr+Trp-supplemented diet was calculated to be 30% and 10%, respectively, of the amount of protein in the basal (AIN-93M) diet, corresponding to approximately (in total) 300 and 100 mg/kg body weight/d of, respectively, tyrosine and tryptophan in humans. Similarly, the added amount of leucine and isoleucine in the Leu+Ile-supplemented diet was calculated to be 24% and 18%, respectively, of the amount of protein in the basal (AIN-93M) diet, corresponding to approximately 240 and 180 mg/kg body weight/d in humans. Diets were prepared by Research Diet Services B.V. (Wijk bij Duurstede, the Netherlands). Results of amino acid analyses in the various diets are presented in Supplemental Table 1.

2.4. Biochemical analyses

To obtain brain material for biochemical analyses, whole brains were removed and divided into cerebrum and cerebellum. The collected brain samples were all individually snap frozen in liquid nitrogen and stored at −80°C until further preparation. Cerebrum and blood samples were further processed for the analyses of brain and plasma amino acid and monoamine concentrations as described previously [5]. Monoamines and associated metabolites for which brain concentrations were assessed included dopamine, norepinephrine, 3-methoxytyramine and normetanephrine in the catecholamine pathway, and serotonin and 5-hydroxyindoleacetic acid (5-HIAA) in the serotonergic pathway.

2.5. Statistical analyses

Statistical analyses were performed on the data of all animals except for the one mouse that died prematurely. Statistical analyses were performed using the software IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA). All tests were performed two-sided at a significance level of α=0.05. Data are expressed as mean ± standard deviation unless otherwise indicated.

Brain and plasma amino acid and monoamine concentrations were analyzed in two steps. First, brain and plasma biochemistry in PKU mice on a Phe-restricted diet was compared with PKU and WT mice on AIN-93M diet. Secondly, brain and plasma biochemistry in PKU mice on various LNAA treatments and a Phe-restricted diet was compared. Analyses were performed by one-way analysis of variance (ANOVA) and Tukey post hoc analyses on log-transformed data. In case of not normally distributed log-transformed data (assessed by Shapiro–Wilk test) or unequal variances (assessed by Levene’s test), analyses were performed by Kruskal–Wallis tests and Mann–Whitney U post hoc analyses.

The effect of dietary treatment on body weight was analyzed on log-transformed data by repeated-measures ANOVA and Tukey’s post hoc analysis with one between factor (experimental group, 6 levels) and one within factor (time, 6 levels: 1, 2, 3, 4, 5 and 6 weeks). Food intake was analyzed by one-way ANOVA with Tukey’s post hoc analysis.

3. Results

3.1. General health and dietary intake

All experimental diets were well tolerated by the mice. Of all 48 mice included in the experiment, one PKU male mouse on the Leu+Ile-supplemented diet died 3 weeks after inclusion. Postmortem macroscopic pathological examination did not show any pathology. The median age of the mice at the start of the experiment did not significantly differ between experimental groups (P=0.526), being
37 weeks (with a minimum of 29 and a maximum of 37 weeks) for all experimental groups. Body weights during the 6-week dietary treatment are shown as percentages of the body weights at the start of the experiment in Fig. 1 for adult PKU mice on experimental diets as well as for adult WT and PKU mice on AIN-93M diet. At the start of the experiment, body weight of PKU mice (male 22.8±2.9 g; female: 17.6±1.6 g) was lower than that of WT mice (male 29.6±1.5 g; female: 24.4±1.7 g). During the experiment, body weight curves of PKU mice on the Phe-restricted diet or LNAA diet were significantly different from all other experimental groups (P<.05 for the LNAA diet compared to the Leu+Ile diet and P<.001 for all others). In addition, body weight curves of PKU mice on AIN-93M diet were significantly higher than those of PKU mice on the Leu+Ile diet (P<.05) and lower than those of WT mice (P<.01). At the end of the experiment, body weight of female PKU mice on the Phe-restricted diet or LNAA diet no longer significantly differed from that in WT mice (P=.233 and P=.760, respectively). In males, PKU mice on Phe-restricted diet, LNAA diet, Tyr+Trp diet and Leu+Ile diet did not significantly differ from those in WT mice (P=.181, P=.114, P=.057 and P=.114, respectively). Weekly food intake decreased after the first week of dietary treatment for all experimental groups, remaining relatively stable in the later treatment weeks (data not shown). Total food intake, as well as food intake during the first and last week of dietary treatment, did not significantly differ between the experimental groups (P=.066, P=.137 and P=.758; data not shown).

### 3.2. Brain monoamines

Brain monoamine and associated metabolite concentrations in adult PKU mice on experimental diets as well as in adult WT and PKU mice on AIN-93M diet are shown in Fig. 2. In PKU mice on AIN-93M diet, brain concentrations of dopamine, norepinephrine, normetanephrine and serotonin were, respectively, 73% (P<.001), 54% (P<.001), 69% (P<.001) and 37% (P<.01) of concentrations in WT mice on AIN-93M diet. These cerebral monoamine deficiencies were restored on the Phe-restricted diet to concentrations that no longer significantly differed from WT concentrations. In addition, brain 5-HIAA concentrations were partially restored from 16% to 51% of WT concentrations.

When assessing various LNAA-supplemented diets in comparison to the Phe-restricted diet, brain concentrations on the LNAA diet did not...
significantly differ from those on the Phe-restricted diet for any of the investigated monoamines and associated metabolites. In contrast, on the Tyr+Trp diet, brain concentrations of norepinephrine (P<.001) remained significantly lower than on the Phe-restricted diet (Fig. 2B), while concentrations of serotonin and 5-HIAA were significantly lower than on both the Phe-restricted diet and LNAA supplementation (P<.001 for serotonin compared to the Phe-restricted diet and P<.01 for all others; Fig. 2C and F). On the Leu+Ile diet, brain concentrations of dopamine, norepinephrine, serotonin, normetanephrine and 5-HIAA were all significantly lower compared to both the Phe-restricted diet and LNAA supplementation, except for dopamine when compared to LNAA supplementation (Fig. 2A–C, E and F). On the Leu+Ile diet, brain serotonin and 5-HIAA concentrations were also significantly lower than on the Tyr+Trp diet (P<.001; Fig. 2C and F).

3.3. Brain LNAA\s

Brain concentrations of individual LNAA\s in adult PKU mice on experimental diets as well as in adult WT and PKU mice on AIN-93M diet are shown in Fig. 3. In PKU mice, the Phe-restricted diet improved brain concentrations of both Phe and most non-Phe LNAA\s compared to PKU mice on AIN-93M diet. On the Phe-restricted diet, brain Phe concentrations were 65% lower compared to PKU mice on AIN-93M diet (P<.001; Fig. 3A) but were still 238% higher than concentrations in WT mice on AIN-93M diet (P<.001; Fig. 3A). Also, brain histidine concentrations were significantly lower in PKU mice on the Phe-restricted diet than on AIN-93M diet (P<.001; Fig. 3H). Brain concentrations of most other non-Phe LNAA\s were higher in PKU mice on the Phe-restricted diet than on AIN-93M diet, which was statistically significant for tryptophan (P<.05; Fig. 3C) and valine (P<.001; Fig. 3D). However, brain concentrations for tyrosine, valine, isoleucine, methionine, histidine and threonine in PKU mice on the Phe-restricted diet were still significantly lower than in WT mice on AIN-93M diet (Fig. 3B, D, E, G–I).

When assessing brain LNAA concentrations in PKU mice on several LNAA-supplemented diets in comparison to the Phe-restricted diet, brain Phe and most non-Phe LNAA\s were higher on the LNAA-supplemented diets. Brain Phe concentrations were significantly higher on all LNAA-supplemented diets than on the Phe-restricted diet (P<.01; Fig. 3A). Also, brain concentrations of most non-Phe LNAA\s were higher on LNAA-supplemented diets than on the Phe-restricted diet, being statistically significant for methionine on the LNAA diet (P<.01; Fig. 3G) and for histidine on both the LNAA (P<.05) and the Tyr+Trp (P<.001)

![Graphs showing brain concentrations of various LNAA\s](image-url)

Fig. 3. Brain concentrations of (A) Phe, (B) tyrosine, (C) tryptophan, (D) valine, (E) isoleucine, (F) leucine, (G) methionine, (H) histidine and (I) threonine in WT and PKU mice after 6 weeks of receiving various diets. Numbers of mice are n=7 or n=8 for all treatment groups. Untransformed data are expressed as mean ± S.E.M. *P<.05, **P<.01 and ***P<.001 (two-sided) compared to PKU mice on Phe-restricted diet unless otherwise indicated. Statistical analyses were performed in two steps: (a) PKU mice on a Phe-restricted diet were compared with PKU and WT mice on AIN-93M diet, and (b) PKU mice on various LNAA treatments and a Phe-restricted diet were compared. Analyses were performed by one-way ANOVA and Tukey post hoc analyses on log-transformed data. In case of not normally distributed log-transformed data (assessed by Shapiro–Wilk test) or unequal variances (assessed by Levene’s test), analyses were performed by Kruskal–Wallis tests and Mann–Whitney U post hoc analyses.
diets (Fig. 3H). In contrast, brain tryptophan and histidine concentrations were even significantly lower on the Leu+Ile diet than on the Phe-restricted diet ($P < 0.01$; Fig. 3C and H). This was also observed for threonine on all LNAA-supplemented diets ($P < 0.001$ on the LNAA diet and $P < 0.01$ on the Tyr+Trp and the Leu+Ile diet; Fig. 3I).

3.4. Plasma LNAA concentrations of individual LNAAAs in adult PKU mice on experimental diets as well as in adult WT and PKU mice on AIN-93M diet are shown in Fig. 4. The Phe-restricted diet significantly reduced plasma Phe concentrations in PKU mice, while plasma concentrations of non-Phe LNAAAs remained largely unchanged. On the Phe-restricted diet, plasma Phe concentrations were 79% lower than concentrations in PKU mice on AIN-93M diet ($P < 0.001$) but still 716% higher than concentrations in WT mice on AIN-93M diet ($P < 0.001$) (Fig. 4A). Also, plasma threonine concentrations were significantly higher in PKU mice on the Phe-restricted diet than on AIN-93M diet ($P < 0.01$) and no longer significantly differed from WT concentrations (Fig. 4I). In contrast, plasma tyrosine ($P < 0.01$; Fig. 4B) and methionine ($P < 0.01$; Fig. 4G) concentrations remained significantly lower in PKU mice on the Phe-restricted diet than in WT mice on the AIN-93M diet.

When assessing plasma LNAA concentrations in PKU mice on several LNAA-supplemented diets in comparison to the Phe-restricted diet, plasma Phe concentrations were significantly higher on all LNAA-supplemented diets than on the Phe-restricted diet ($P < 0.001$; Fig. 4A). Also, plasma concentrations of most non-Phe LNAAAs were increased upon supplementation. This was statistically significant for valine, isoleucine and methionine concentrations on the LNAA diet ($P < 0.05$ for valine and isoleucine, and $P < 0.01$ for methionine; Fig. 4D, E and G) and for isoleucine and leucine on the Leu+Ile diet ($P < 0.01$ for both; Fig. 4E and F). In contrast, plasma threonine concentrations were significantly lower on all LNAA-supplemented diets than on the Phe-restricted diet ($P < 0.001$ on the LNAA diet, $P < 0.01$ on the Tyr+Trp diet and $P < 0.05$ on the Leu+Ile diet; Fig. 4I).

![Fig. 4](image_url)

Fig. 4. Plasma concentrations of (A) Phe, (B) tyrosine, (C) tryptophan, (D) valine, (E) isoleucine, (F) leucine, (G) methionine, (H) histidine and (I) threonine in WT and PKU mice after 6 weeks of receiving various diets. Numbers of mice are $n=7$ or $n=8$ for all treatment groups. Untransformed data are expressed as mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (two-sided) compared to PKU mice on Phe-restricted diet unless otherwise indicated. Statistical analyses were performed in two steps: (a) PKU mice on a Phe-restricted diet were compared with PKU and WT mice on AIN-93M diet, and (b) PKU mice on various LNAA treatments and a Phe-restricted diet were compared. Analyses were performed by one-way ANOVA and Tukey post hoc analyses on log-transformed data. In case of not normally distributed log-transformed data (assessed by Shapiro–Wilk test) or unequal variances (assessed by Levene’s test), analyses were performed by Kruskal–Wallis tests and Mann–Whitney U post hoc analyses.
4. Discussion

In continuation of our previous studies to elucidate the various biochemical treatment effects of LNAA supplementation as well as its optimal composition in young PKU mice, the present study aimed to assess in adult PKU mice whether LNAA supplementation could serve as an alternative treatment option to the Phe-restricted diet for adult PKU patients. To this purpose, this study compared several LNAA supplements and a Phe-restricted diet — as the common and standard treatment — with respect to brain monoamines and both brain and plasma amino acid concentrations in adult PKU mice, especially showing that (a) a Phe-restricted diet restored brain monoamines, whereas brain concentrations of most non-Phe LNAAAs were still subnormal, and (b) LNAA treatment including supplementation of all non-Phe LNAAAs was similar effective as the Phe-restricted diet to restore brain monoamines, while both brain and plasma Phe concentrations were still markedly elevated.

Before discussing these results in detail, we will first address some methodological considerations. Firstly, the here applied Phe-restricted diet resembled dietary treatment of PKU patients as much as possible, as the included synthetic amino acid mixture is also used for PKU patients.

On the other hand, it should be noted that this mixture was thus originally developed to meet nutritional requirements for humans rather than mice. However, PKU mice on both the LNAA diet and the Phe-restricted diet showed catch-up growth. This does not only support our previous hypothesis that LNAA supplementation may induce net protein synthesis due to a better balance of (large neutral) amino acids [5] but also provides proof that the Phe-restricted diet was nutritionally adequate. Secondly, prior to the start of the experiment, the PKU mice had not received any dietary Phe restriction. This reflects the situation in late-diagnosed and previously untreated PKU patients in whom positive effects of LNAA supplementation on (self-injurious) behavior were previously observed [20]. Thirdly, while previous PKU mouse studies did not show significant gender effects, this study was powered for group sizes of eight animals (containing equal numbers of males and females), and gender effects were no longer included in the statistical analyses.

This study investigated adult PKU mice as a model for adult PKU patients. Previous research showed that the toxicity of high plasma Phe to the brain may be quite different in adulthood compared to infancy and childhood, so adults may require different recommendations on upper plasma Phe concentrations than children [15]. Apart from that, the cerebral symptoms associated with high plasma Phe seem to differ between children and adults. During infancy and childhood, untreated PKU generally results in severe intellectual disability and seizures, while insufficiently treated PKU leads to a lower IQ and impaired executive functioning. Such brain damage is largely irreversible and has especially been attributed to direct brain Phe neurotoxicity. In contrast, in adults, the cerebral problems reported to be associated with high plasma Phe primarily include mood (swings), behavior and social functioning rather than executive functioning [12,24]. These symptoms in adulthood seem to be largely reversible and have been primarily attributed to impaired cerebral monoamine synthesis, as supported by reduced dopamine and serotonin metabolite (homovanillic acid and 5-HIAA) concentrations in CSF of adult PKU patients after discontinuation of dietary treatment [25–27].

While the Phe-restricted diet has been the principal treatment in PKU for almost 65 years, the associated brain biochemical effects cannot be easily investigated in vivo in PKU patients. To our knowledge, this study was the first to investigate the effect of a Phe-restricted diet, consisting of natural protein restriction and supplementation of an amino acid mixture devoid of Phe similar to the Phe-restricted diet that is applied in PKU patients, on both plasma and brain amino acid as well as brain monoamine concentrations in PKU mice. The observed reductions in brain and plasma Phe concentrations in PKU mice on a Phe-restricted diet are in agreement with studies in patients showing significant correlations between plasma and brain Phe concentrations by magnetic resonance spectroscopy [28–30]. Brain concentrations of most non-Phe LNAAAs in PKU mice on LNAA supplementation in the present study were higher than in PKU mice on AIN-93M diet but still significantly lower than in WT mice. Investigating the effect of a Phe-restricted diet on brain non-Phe LNAAAs concentrations in PKU patients has been limited by the fact that, until very recently, only Phe could be determined validly by magnetic resonance spectroscopy, while tyrosine has been reported only once to be measured by this method [31]. A recent study in PKU mice reported reduced concentrations of brain non-Phe amino acids on diets in which protein was restricted by 67% and 78% without additional supplementation of any essential amino acids [32]. The brain amino acid profile therefore most likely primarily reflected profound catabolism as evidenced from the plasma Phe reductions of only 22%–34%, as also suggested by the authors themselves. Other studies in PKU mice on Phe-restricted diets did not report on brain non-Phe LNAA concentrations [33,34]. Moreover, the present study showed that brain monoamine concentrations in PKU mice on the Phe-restricted diet were restored to WT concentrations. This supports the previous observations of increased homovanillic acid and 5-HIAA concentrations in cerebrospinal fluid in adolescent and adult PKU patients following reintroduction of a Phe-restricted diet, which was associated with increased vigilance and reduced reaction times [26,35,36]. Additionally, this supports the results of improved behavior and brain monoamines in (mild) hyperphenylalaninemia compared to classical PKU mice [34].

In contrast to the Phe-restricted diet that aims to improve brain amino acid and monoamine concentrations by lowering plasma Phe concentration, LNAA supplementation aims to restore the disturbed LNAA transport across the BBB without dietary Phe restriction [5,6,21]. Depending on the composition of the LNAAAs being supplemented, LNAA treatment can (primarily) serve to (a) decrease brain Phe, (b) increase brain non-Phe LNAAAs and (c) increase brain monoamine concentrations [6]. In the present study, supplementation of all non-Phe LNAAAs in adult PKU mice was shown to be similarly effective as the Phe-restricted diet to restore brain monoamines to WT concentrations. The observed improvements in brain dopamine, norepinephrine and serotonin concentrations in adult PKU mice on LNAA diet were in agreement with previous studies in young PKU mice [5,6]. Previous studies in PKU patients did not directly compare a Phe-restricted diet to LNAA treatment but did show that a Phe-restricted diet and tyrosine supplementation were both able to increase HVA and 5-HIAA concentrations in cerebrospinal fluid, which was accompanied by a reduced visual reaction time (variability) [35,36]. Considering the composition of LNAA treatment, the present results in adult PKU mice support our previous findings in young PKU mice that brain monoamine concentrations could most effectively be improved by a combination of increasing precursor amino acids and reducing brain Phe [6]. The present data show that such effect can be achieved by both a severe Phe-restricted diet and LNAA supplementation. When comparing plasma LNAA concentrations, the restored brain monoamines and improved brain LNAA profiles on LNAA treatment were not accompanied by improved plasma LNAA profiles, with plasma LNAA concentrations being all significantly higher than under physiological conditions. Possibly, however, the improved balance in plasma (large neutral) amino acid profile has induced net protein synthesis, as supported by the observed catch-up growth in PKU mice on LNAA diet. Induction of net protein synthesis has previously been hypothesized to be the mechanism by which LNAA treatment would reduce plasma Phe concentrations [5]. Such reduction of plasma Phe concentrations on LNAA treatment, however, is not large and certainly does not (fully) explain the observed improvements in brain monoamine concentrations. This implicates that plasma Phe that has thus far successfully been used to monitor treatment...
with either a Phe-restricted diet or tetrahydrobiopterin on its own does not provide an adequate biomarker to monitor LNAA treatment.

The clinical implications of the present study are twofold. Given the importance of cerebral monoamine deficiencies in the behavioral and neuropsychological problems observed in adult PKU patients combined with the social burden posed by a diet for life, the observation in adult PKU mice that LNAA supplementation without any dietary Phe restriction was similarly effective as a severe Phe-restricted diet to restore brain monoamines makes LNAA treatment a promising alternative treatment strategy for adult PKU patients. Although LNAA treatment thus showed to be comparable rather than superior to the Phe-restricted diet, such optimal biochemical control, as achieved in PKE mice on the Phe-restricted diet in the present study, is generally not achieved in adult PKU patients due to issues with treatment compliance [17]. LNAA treatment, on the other hand, is less demanding and thereby expected to be better followed by adult PKU patients. Thereby, LNAA treatment may not only reduce the treatment burden but, even more importantly, may also be more effective to improve neuropsychological outcome in adult PKU patients. To facilitate clinical application of LNAA treatment, attempts should be made to improve the taste and tolerability of the LNAA supplement, while future studies in PKU mice need to elucidate whether higher doses of a particular LNAA, probably influencing the taste again, would be more beneficial. In addition, it should be determined whether and how LNAA supplementation could best be combined with a Phe-restricted diet to (a) improve neuropsychological outcome and (b) liberalize the dietary restrictions. Also, the possible effects of the increased amino acid load posed by LNAA treatment to renal functioning deserve further attention [37]. Moreover, parameters other than plasma Phe that could better reflect brain amino acid and monoamine concentrations and metabolism need to be identified to monitor LNAA treatment.

To conclude, the present study in adult PKU mice compared several LNAA supplements to a Phe-restricted diet — as the current golden standard of treatment — that in principle is very effective but whose clinical effects in adult PKU patients are limited due to low treatment compliance. The current finding in adult PKU mice that LNAA treatment including supplementation of all non-Phe LNAA was equally effective as a severe Phe-restricted diet to restore brain monoamine concentrations makes it a promising alternative treatment strategy for all adult PKU patients who do not fully adhere to the severe Phe-restricted diet.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnuthbio.2017.09.020.

Acknowledgments

The authors express their gratitude to Ms. F. Hormann for her practical support and to Mrs. H.A. Kingma, Mrs. E.Z. Jonkers, Mrs. K. Boer and Mrs. H. Adema for their analytical support. Also, we are grateful to Prof. B. Thöny from the University Children’s Hospital, Zurich (Switzerland), for providing C57Bl/6 Pah-en2 breeding pairs and to Nutricia Research for providing the synthetic amino acid mixtures and funding the experimental diets.

Competing interests

D.v.V. has received speaker’s honoraria from Biomarin. E.A.v.d.Z. has received advisory board fees from Arla Foods. F.J.v.S. has received research grants, advisory board fees and speaker’s honoraria from Merck Serono, Biomarin and Nutricia Research; has received grants from Sobi; has received speaker’s honoraria from Vitalo; and has received advisory board fees from Arla Foods and Applied Pharma Research. All other authors have declared not to have conflicts of interest. All authors have read the journal’s policy on disclosure of potential conflicts of interest.

Funding sources

This research was supported by the National PKU Alliance (USA) and Stofwissenschap (the Netherlands). Experimental diets were provided by Nutricia Research.

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