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Research Paper

Correlations of blood and brain biochemistry in phenylketonuria: Results from the Pah-enu2 PKU mouse

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ABSTRACT

Background: In phenylketonuria (PKU), treatment monitoring is based on frequent blood phenylalanine (Phe) measurements, as this is the predictor of neurocognitive and behavioural outcome by reflecting brain Phe concentrations and brain biochemical changes. Despite clinical studies describing the relevance of blood Phe to outcome in PKU patients, blood Phe does not explain the variance in neurocognitive and behavioural outcome completely.

Methods: In a PKU mouse model we investigated 1) the relationship between plasma Phe and brain biochemistry (Brain Phe and monoaminergic neurotransmitter concentrations), and 2) whether blood non-Phe Large Neutral Amino Acids (LNAA) would be of additional value to blood Phe concentrations to explain brain biochemistry. To this purpose, we assessed blood amino acid concentrations and brain Phe as well as monoaminergic neurotransmitter levels in in 114 Pah-Enu2 mice on both B6 and BTBR backgrounds using (multiple) linear regression analyses.

Results: Plasma Phe concentrations were strongly correlated to brain Phe concentrations, significantly negatively correlated to brain serotonin and norepinephrine concentrations and only weakly correlated to brain dopamine concentrations. From all blood markers, Phe showed the strongest correlation to brain biochemistry in PKU mice. Including non-Phe LNAA concentrations to the multiple regression model, in addition to plasma Phe, did not help explain brain biochemistry.

Conclusion: This study showed that blood Phe is still the best amino acid predictor of brain biochemistry in PKU. Nevertheless, neurocognitive and behavioural outcome cannot fully be explained by blood or brain Phe concentrations, necessitating a search for other additional parameters.

Take-home message: Blood Phe is still the best amino acid predictor of brain biochemistry in PKU. Nevertheless, neurocognitive and behavioural outcome cannot fully be explained by blood or brain Phe concentrations, necessitating a search for other additional parameters.

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1. Introduction

Phenylketonuria (PKU; McKusick 261,600) is an inborn error of metabolism caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH, EC 1.14.16.1), which catalyzes the conversion of phenylalanine (Phe) into tyrosine (Tyr). Consequently, Phe accumulates in blood and brain, the latter being considered the primary cause of PKU brain dysfunction. If left untreated, high blood and brain Phe concentrations result in severe intellectual disability, seizures, psychiatric problems and motor problems [1]. Treatment is therefore based on decreasing blood and brain Phe concentrations by dietary restriction of Phe. This diet can be combined with chaperone treatment of Sapropterin dihydrochloride to enhance the PAH enzyme activity. Unfortunately, this only works in a minority of patients [1,2]. Moreover, enzyme replacement therapy with subcutaneous injections of

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phenylalanine ammonia lyase (PAL) has become a third treatment option, metabolizing Phe into other non-toxic compounds. Even so, this needs to be combined with drugs to suppress an immune response [1,3,4]. Based on the treatment aims, PKU patients are monitored by frequent blood Phe analyses [5]. However, notwithstanding the large number of clinical studies addressing the relevance of blood Phe concentrations to neurocognitive outcome in these patients, blood Phe does not explain the variance in neurocognitive outcome completely [6–11]. Moreover, while a strong relationship between blood Phe and brain Phe concentrations has been reported, the relationship between other blood markers and brain biochemistry (Brain Phe and neurotransmitters) has not yet been thoroughly clarified [12–16]. From a biochemical perspective, especially high brain Phe and low brain monoaminergic neurotransmitter levels seem to be responsible for cognitive and behavioural impairments in PKU [17–19]. To this end, this study will investigate the relationships between blood and brain biochemistry in PKU.

As cerebral biochemistry is difficult to obtain in humans, animal models have provided the most representative data to investigate the association between blood and brain biochemical parameters in PKU [20]. In this study, we will therefore rely on the data of the enu2-PKU mouse model on different dietary treatment groups to investigate: 1) the relationship between plasma Phe and brain biochemistry, and 2) whether other blood non-Phe Large Neutral Amino Acids (LNAA) would be of additional value to blood Phe concentrations to explain brain Phe and monoaminergic neurotransmitter levels.

2. Material & methods

2.1. Animal experiments

In total, biochemical data from 114 *Pah-enu2* (PKU) mice from two mouse background strains (C57Bl/6, $n = 58$, f:m = 29:29) and Black-Tan-Brachyury (BTBR, $n = 56$, f:m = 29:27) were included in this study. These mice were included in five different experiments at the Groningen Institute for Evolutionary Life Sciences (GELIFES) in collaboration with the University Medical Center Groningen (UMCG). Mice were treated with: 'Normal chow' ($n = 59$), 'Phe restricted' ($n = 31$) or 'Phe semi-restricted' ($n = 24$) diets. Further characteristics of the different mouse cohorts are outlined in the supplemental data (Table 1). Data of four out of five experiments have been published previously [21–24]. All procedures and treatments *were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health* [25]. *The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen.*

From the start of dietary treatment, animals were individually housed. Dietary treatments were continued for six weeks. Diets were based on the composition of AIN-93 M (96.2 g/kg Phe derived from casein) [26], which was administered in unadjusted form to the PKU mice on "normal chow". The Phe-restricted and semi Phe-restricted diets were produced by reducing the amount of casein by 75% and 33%, respectively. 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 [21]. Diets were prepared by Research Diet Services B.V. (Wijk bij Duurstede, The Netherlands).

2.2. Biochemical analyses

To obtain brain material for biochemical analyses, whole brains were removed, the cerebrum was isolated, snap frozen in liquid nitrogen, and stored at −80 °C until further preparation [21–24]. Brain and blood samples were further processed for the analyses of brain Phe and plasma amino acid concentrations as described previously [21–24]. From the obtained plasma amino acid measurements, plasma Phe/LNAA ratios were calculated defined as plasma Phe / (Tyr + tryptophan (Trp) + valine + isoleucine + leucine + methionine + histidine (His) + threonine (Thr) concentrations.

2.3. Calculation of LNAA brain influx

Presumptive brain influxes of different individual LNAAs were calculated based on plasma LNAA concentrations measured in mice, using the Michaelis-Menten equation [27]:

$$v = \frac{V_{max}[AA]}{K_m(app)} + [AA]) + K_d(AA).$$

In this equation, v is the brain influx, Vmax the maximal rate of transport across the BBB as determined by the saturation of the transporter, AA the concentration of other LNAA/amino acids at the BBB, K_m the LNAA concentration at which half of the Vmax occurs and K_d the diffusion uptake clearance. When calculating the influx of LNAAs across the BBB, one has to take into account the competition effect. In this equation, $K_m(app)$ is therefore used rather than K_m. $K_m(app)$ is the apparent kinetic constant of the LNAA/Amino acid when taking into account the competition of other LNAAs at the blood brain barrier (BBB). For each LNAA, $K_m(app)$ was calculated using previously published equations and constants from Strauss et al. [28] In contrast, K_mLNAA is the absolute kinetic constant in case of no competition at the BBB. To calculate the $K_m(app)$, the following calculation was used: $K_m(app)^{LNAA} = K_m^{LNAA}\left(1 + \sum \frac{[AA]}{K_m^{AA}}\right)$ [29], with K_m^{AA} being the absolute kinetic constant of all other LNAA/amino acids combined.

2.4. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 26.0. Normal distribution was assessed using Shapiro-Wilk tests, and homogeneity of variances by Levene's test.

To explore the variance in brain biochemistry explained by plasma amino acid concentrations, stepwise multiple regression models with either brain Phe, dopamine, norepinephrine or serotonin as dependent variables and plasma Phe, Tyr, Trp, Valine, isoleucine, leucine, methionine, His, and Thr together as independent variables were made. In addition, to assess the correlations of blood parameters in ratio with blood Phe as well as the brain influxes of individual parameters, several univariate linear regression analyses were done. Univariate regression

Table 1
Linear regression analysis of plasma phenylalanine to brain phenylalanine and monoaminergic neurotransmitters.

Plasma phenylalanine (Phe)								
	B6				BTBR			
	B	β	Adj. R^2	F	B	β	Adj. R^2	F
Brain Phenylalanine (PHE)	0.298	0.936*	0.874	383.93	0.392	0.957*	0.912	591.65
Brain Dopamine (DA)	−0.001	0.563*	0.305	25.56	−0.001	0.299	0.072	5.19
Brain Norepinephrine (NE)	−0.001	0.828*	0.680	119.85	0.000	0.742*	0.553	68.96
Brain Serotonin (5HT)	−0.001	0.854*	0.724	147.62	−0.001	0.776*	0.587	81.88

* $p < 0.005$ (Bonferroni correction).

analyses were done for either brain Phe, dopamine, norepinephrine and serotonin as dependent variable and plasma Phe, Tyr, Trp and Phe/Tyr, Phe/Trp, Phe/LNAA, Phe/Thr and Phe/His ratios, and estimated brain influxes of Phe, Tyr or Trp as independent variable. Probability plots and scatterplots of standardized predictive value against standardized residuals were used to assess distribution of the regression lines. Significance for the multiple regression analyses was assumed using a two-tailed *P*-value of $\alpha = 0.05$. To correct for multiple testing in the univariate analyses, we applied the Bonferroni correction resulting in a significance of a two-tailed P-value of $\alpha = 0.005$ (0.05/11). Regarding the strength of the correlations, we considered: $r < 0.2$ a negligible correlation; $0.2 < r < 0.4$ a weak correlation; $0.4 < r < 0.6$ a weak correlation; $0.6 < r < 0.8$ a strong correlation; and $r > 0.8$ a very strong correlation (Evans 1996) [30]. A factorial ANOVA, testing background, genotype, diets and their interactions, was used to assess differences in blood and brain metabolites between the two mouse strains (B6/BTBR) (supplemental data, Table 2–3). Significant differences were found in plasma Trp ($p < 0.001$), plasma His ($p = 0.001$), brain dopamine ($p < 0.001$) and brain norepinephrine ($p < 0.001$) concentrations between the strains. Therefore, both cohorts were analyzed individually. Differences between mouse strains are further reported by Bruinenberg et al. (2016) [31].

3. Results

3.1. Relation between plasma Phe and brain biochemistry

Linear regressions were calculated to predict brain Phe or monoaminergic neurotransmitter concentrations based on plasma Phe in B6 and BTBR PKU mice (Table 1/ Fig. 1). Plasma Phe accounted for 87.4% (B6) and 91.2% (BTBR) of the variance in brain Phe. In addition, plasma Phe was a significant predictor of brain serotonin and norepinephrine concentrations. Plasma Phe was only a weak to moderate predictor for brain dopamine ($R^2 = 0.305$ (B6); $R^2 = 0.072$ (BTBR)).

3.2. Relation between plasma LNAA, LNAA ratios, brain influxes and brain biochemistry

3.2.1. Multiple regression analyses

To investigate whether plasma non-Phe LNAA would be of additional value to solely plasma Phe concentrations to predict brain Phe and monoaminergic neurotransmitter levels in PKU mice, we performed stepwise multiple regression analyses with either brain Phe, dopamine, norepinephrine or serotonin as dependent variables and plasma Phe, Tyr, Trp, valine, isoleucine, leucine, methionine, His, and Thr together as independent variables (Table 2). The combination of Phe + Thr + His (B6) and Phe + Thr (BTBR) accounted for 93.5% (B6) and 91.2% (BTBR) of the variance in brain Phe, with plasma Phe as the most important predictor for both strains, explaining 87,4% (B6) and 91,2% (BTBR) (Table 2a). For B6 mice, Thr and His could explain an additional 3,8% and 2,4% of this variance, while for BTBR mice, Thr explained an additional 2,2%.

Plasma Phe (B6) and the combination of Phe + Trp (BTBR) accounted for 72.4% (B6) and 63.0% of the variance in brain serotonin, with plasma Phe as the most important predictor for both strains, explaining 72.4% (B6) and 58.7% (BTBR) (Table 2b). For BTBR mice, plasma Trp could explain an additional 4.9% of the variance in brain serotonin.

The combination of Phe + Tyr (B6 + BTBR) accounted for 75.6% (B6) and 58.7% (BTBR) of the variance in brain norepinephrine, with plasma

Table 2
Stepwise multiple regression analysis of brain biochemistry compared to plasma LNAA.

Model	Predictor	Adj. R^2	β	F	P-Value model	VIF	Model	Predictor	Adj. R^2	β	F	P-Value model	VIF
B6 ($n = 56$)							BTBR ($n = 55$)						
(2a) Brain phenylalanine (Phe)													
1	Constant	0.874					1	Constant	0.912				
	P.Phe		0.936**	383.93	0.00	1.000		P.Phe		0.956**	561.38	0.00	1.000
2	Constant	0.911					2	Constant	0.933				
	P.Phe		0.932**	283.19	0.00	1.000		P.Phe		0.912**	376.04	0.00	1.088
	P.Thr		−0.194**			1.000		P.Thr		−0.153**			1.088
3	Constant	0.935											
	P.Phe		0.919**	266.10	0.00	1.008							
	P.Thr		−0.296**			1.427							
	P.His		0.187**			1.431							
(2b) Brain serotonin (5HT)													
1	Constant	0.724					1	Constant	0.587				
	P.Phe		−0.854**	147.62	0.00	1.000		P.Phe		−0.771**	77.88	0.00	1.000
							2	Constant	0.630				
								P.Phe		−0.776**	46.99	0.00	1.013
								P.Trp		0.222*			1.013
(2c) Brain norepinephrine (NE)													
1	Constant	0.680					1	Constant	0.543				
	P.Phe		−0.828**	119.85	0.00	1.000		P.Phe		−0.742**	65.04	0.00	1.000
2	Constant	0.756					2	Constant	0.587				
	P.Phe		−0.877**	87.61	0.00	1.030		P.Phe		−0.667**	39.39	0.00	1.111
	P.Tyr		0.285**			1.030		P.Tyr		0.239*			1.111
(2d) Brain dopamine (DA)													
1	Constant	0.305					1	Constant	0.072				
	P.Phe		−0.563**	25.56	0.00	1.000		P.Phe		−0.299*	5.19	0.00	1.000
2	Constant	0.367					2	Constant	0.233				
	P.Phe		−0.560**	17.20	0.00	1.000		P.Phe		−0.420**	9.19		1.085
	P.Thr		0.268*			1.000		P.Val		0.432**			1.085
3	Constant	0.414											
	P.Phe		−0.254	14.17	0.00	2.677							
	P.Thr		0.680*			4.039							
	P.Met		−0.562*			5.663							

* $p < 0.05$.
** $p < 0.01$.

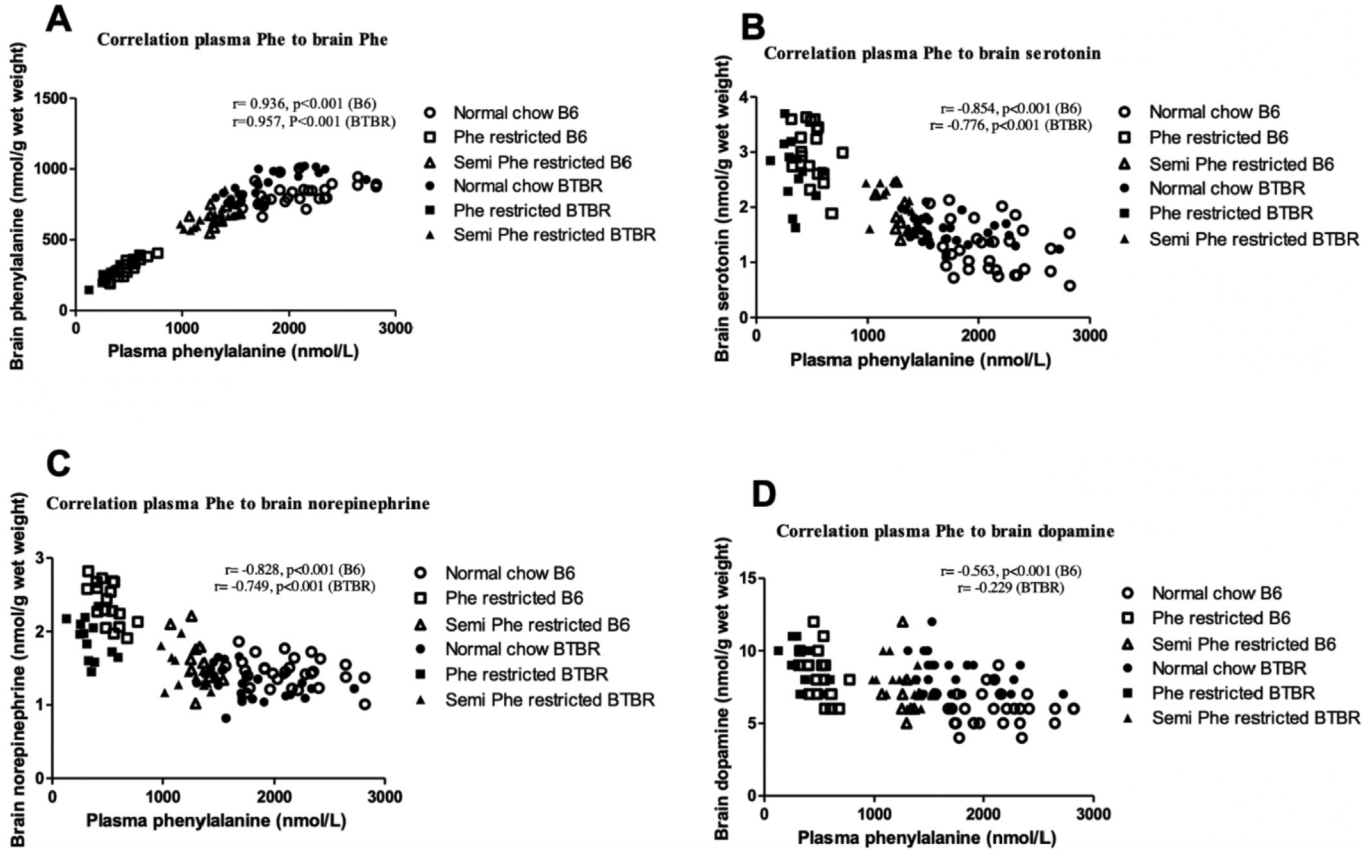

Fig. 1. Correlations of plasma phenylalanine to brain biochemistry.
(a) Plasma Phe was positively correlated to brain Phe ($r = 0.936$, $p < 0.001$ (B6)/ $r = 0.957$, $p < 0.001$ (BTBR)), (b) Plasma Phe was negatively correlated to brain serotonin ($r = -0.854$, $p < 0.001$ (B6)/ $r = -0.776$, $p < 0.001$ (BTBR)), (c) Plasma Phe was negatively correlated to brain norepinephrine ($r = -0.828$, $p < 0.001$ (B6)/ $r = -0.749$, $p < 0.001$ (BTBR)), (d) Plasma Phe was negatively correlated to brain dopamine ($r = -0.563$, $p < 0.001$ (B6)/ $r = -0.295$, $p < 0.001$ (BTBR)).

Phe as the most important predictor for both strains, explaining 68.0% and 54.3% of the variance in brain norepinephrine respectively (Table 2c). Plasma Tyr could explain an additional 7.9% (B6) and 5,1% (BTBR) of this variance.

The combination of Phe + Thr + Met (B6) and Phe + Val (BTBR) accounted for 41.4% (B6) and 23.3% (BTBR) of the variance in brain dopamine, with plasma Phe as the most important predictor for B6 and plasma valine as the most important predictor for BTBR (Table 2d). Although statistically significant, the multivariable regression models for brain dopamine were much weaker than the other correlations found in this study and therefore we did not consider them clinically relevant results.

3.2.2. Linear regression analyses

Acknowledging that Tyr and Trp are the amino acid precursors for dopamine and norepinephrine and Trp is the precursor for serotonin, we also performed univariate linear regression analyses for plasma Tyr and Trp, their ratios and their estimated brain influxes as separate independent variables and brain Phe, dopamine, norepinephrine or serotonin as dependent variables (Supplemental material: Tables 4–7.) Moreover, since results of the multiple regression analysis for brain Phe showed significant effects of plasma Thr and His, regression analyses were done for brain Phe as dependent variable and plasma Phe/Thr or Phe/His ratios as independent variables.

Regarding the regressions between plasma markers and brain Phe levels, significant correlations between all plasma markers and brain Phe ($p < 0.005$), with exception of plasma Tyr in the B6 ($p = 0.354$) and plasma Trp in the BTBR mouse strain ($p = 0.271$) were observed (supplemental table 4). As shown above, the relation was strongest between plasma Phe and brain Phe. Other strong correlations were observed with plasma Phe/LNAA ratio, influx Phe, influx Trp, plasma Phe/Thr ratio and finally plasma Phe/His ratio. Correlations of plasma Tyr, Phe/Tyr ratio and Phe/Trp ratio were weaker in B6 compared to BTBR mice, while the correlation of Plasma Trp was weaker in BTBT compared to B6 mice.

Regarding the regressions between different plasma markers and brain serotonin levels,. all correlations were significant except for brain serotonin and plasma Tyr in both mouse strains (B6,$p = 0.254$/ BTBR, $p = 0.006$) and plasma Trp in the BTBR strain ($p = 0.022$) (Supplemental Table 5). The strongest correlation in both mouse strains was seen with estimated brain influx of Trp ($R^2 = 0.729$ (B6); $R^2 = 0.614$ (BTBR)). Moreover, strong correlations were found between brain serotonin and plasma Phe and estimated brain influx of Phe in the B6 mouse strain. Correlations for plasma Tyr were weaker in B6 compared to BTBR mice, while the correlation in Plasma Trp was weaker in BTBT compared to B6 mice.

Regarding the regressions between brain dopamine and plasma markers, no strong correlations were observed (supplemental Table 6). Regarding brain norepinephrine, all correlations were significant except for plasma Tyr in B6 mice ($p = 0.319$) and plasma Trp in BTBR mice ($p = 0.420$) (Supplemental Table 7). The strongest correlations were observed with influx of Phe ($R^2 = 0.808$ (B6); $R^2 = 0.564$ (BTBR)). In the B6 mouse strain, plasma Phe, plasma Phe/LNAA ratio and influx Trp also showed strong correlations with brain norepinephrine. Correlations for plasma Tyr were weaker in B6 compared to BTBR mice, while the correlation in plasma Trp and influx Phe were weaker in BTBR compared to B6 mice.

4. Discussion

This study started with the observation that variances in neurocognitive and behavioural outcome in PKU cannot be fully explained by blood Phe concentrations. Several other studies have already extensively examined the relationship between plasma Phe and brain Phe concentrations [12,14,16]. In addition, some LNAA studies have looked at the relationship between plasma Phe and plasma non-Phe markers and brain Phe concentrations [21–24]. However, the relationship between plasma markers and brain neurotransmitters, which seems to play an important role in PKU pathophysiology, has not yet been thoroughly examined. Therefore, this study aimed to investigate the different relationships of blood and brain biochemistry in PKU mice of both B6 and BTBR backgrounds. Moreover, we investigated whether adding non-Phe plasma LNAA in addition to plasma Phe could help explain brain Phe and monoaminergic neurotransmitter levels in PKU mice. The main finding of this study is that several plasma amino acid parameters show strong correlations with brain Phe and monoaminergic neurotransmitter levels, but that adding other non-Phe plasma markers to blood Phe does not considerably help explain brain biochemistry in PKU mice.

Before discussing the results, some methodological issues will be addressed. Firstly, the present study consisted of biochemical data obtained in PKU mice. Comparison of transport characteristics for LNAA across the BBB in humans and rats showed that absolute values differed between both species [32]. However, K_m-values for individual LNAA as determined in brain microvascular endothelial cells of humans and rats showed a strong correlation [33]. Thereby, the present data in mice may well reflect the human situation, but validation in PKU patients is necessary for absolute values. Secondly, this study used data of both B6 and BTBR PKU mice strains. To adjust for possible differences in the relationship between plasma and brain biochemistry, separate analyses were done for both strains. The fact that, notwithstanding the differences in both strains, clear correlations in the same direction were observed between brain Phe and plasma biochemistry as well as clear effects of different dietary treatments to these relationships provides further proof for the robustness of the data to answer the present question. Lastly, in addition to the different PKU mice strains, different cohorts with different ages at the start of dietary treatment were used in this study. A difference in age could cause differences in blood and brain amino acids and neurotransmitter concentrations, thereby negatively influencing results. However, no significant effect of age was found on amino acid and neurotransmitter concentrations in this study.

First, results showed that plasma Phe concentrations correlated very strongly to brain Phe concentrations. These results were comparable to correlations between blood Phe and brain Phe concentrations measured by H-MRS in PKU patients ($r = 0.93$, $p < 0.001$) and correlations between blood Phe and CSF Phe concentrations ($r = 0.81$, $p < 0.05$) [12,13,29]. Interestingly, however, the relation of plasma markers to brain Phe in the various PKU treatment groups seemed to differ slightly. As can be seen from Fig. 1, there seems to be a flattening of the curve toward the higher brain Phe concentrations. This suggests that the relation between plasma markers and brain Phe concentrations might not be completely linear. This is in line with observations in PKU patients [34], but has later been questioned by other groups [13]. Moreover, a difference was seen in the correlations in B6 mice compared to BTBR mice. The differences could be the result of different neurodevelopment in both mice strains as described by Bruinenberg et al. [31] It is hypothesized that, as a result, both strains could have different susceptibility toward brain Phe and neurotransmitter depletion, explaining our findings.

Regarding the relationship between plasma Phe and cerebral neurotransmitter levels, our results showed that correlations of plasma Phe to serotonin and dopamine are comparable to results from the study of Pilotto et al.(2019), correlating CSF serotonin to CSF-Phe ($r = -0.84$, $P = 0.002$; $r = -0.86$, $P = 0.001$, respectively), or CSF-Phe to CSF dopamine in PKU patients, which did not reach statistical significance [13]. These findings suggest that dopamine might be less affected in PKU mice and patients than currently thought and raises the question of how relevant dopamine is in the explanation of neurocognitive and behavioural outcome in PKU. Nonetheless, dopamine deficiency has been related to executive functioning deficits in PKU [17], suggesting effects might be regional in the brain rather than diffuse [35]. Since this study assessed whole brain dopamine levels, it would therefore be relevant to look at regional brain dopamine concentrations in areas such as the prefrontal cortex or the hippocampus [17,35]. Another hypothesis could be that the active dopamine is reflected better by its metabolite norepinephrine rather than dopamine itself.

Moreover, we investigated whether non-Phe LNAA in plasma could better explain brain biochemistry, consisting of brain Phe and neurotransmitters, than plasma Phe. Our results showed that Phe (plasma or influx) correlated best for most brain measures (brain Phe, dopamine and norepinephrine). For correlations with brain serotonin, the influx of Trp only gives minimal advantage over plasma Phe. However, measurements of Trp are currently not routinely done in every center, so this measure currently does not provide a superior marker for PKU monitoring. Therefore, Phe still holds the best marker for brain biochemical outcome of all investigated markers, but the other strong correlations of several plasma markers with brain biochemistry illustrate that the relationship of blood and brain biochemistry is complex and should perhaps rather be investigated using models, instead of plasma markers alone.

The less strong correlations between plasma Phe concentrations and neurocognitive and social outcome as found in literature could be explained by several mechanisms. One explanation might be that plasma Phe, as measured in the clinic, does not measure the continuous fluctuations of Phe in the body, indicating that we do not measure true metabolic control in patients [36]. Improving this would ask for a measure comparable to HbA1c in diabetes mellitus in which HbA1c outperforms glucose as prediction method, giving a long-term overview in (variability of) blood glucose concentrations [37]. Using untargeted metabolomics, Vaclavik et al. already found Phe-glucose as possible novel biomarker for PKU, which could be used as a long-term predictor of Phe regulation, because of the hypothesized non-enzymatic formation of Phe-glucose [38]. Another explanation could be the inter-individual differences in brain vulnerability to high brain Phe concentrations in PKU patients as discussed by Leuzzi et al. 2020 [39]. In support, late-diagnosed and untreated PKU patients have been described who have somehow escaped from severe intellectual disability despite high blood Phe concentrations [39,40]. This suggests either a generally lower brain vulnerability to high plasma Phe concentrations (possibly due to changed Phe transport from blood to brain or into the neurons) in some PKU patients or an escape mechanism in one of the metabolic/molecular pathways in the brain mediating the cerebral responses to high brain Phe concentrations that would prevent certain PKU symptoms. In reference to the first hypothesis, some of these unusual patients have been described to have high blood Phe but close to normal brain Phe concentrations [40].[41] Regarding the latter hypothesis, besides brain Phe levels, especially brain monoaminergic neurotransmitters have been suggested to be important for neurocognitive and psychosocial outcome of PKU patients [17,42,43]. Data of our study show that the relation between plasma Phe and brain Phe was stronger than the relation between plasma Phe and brain neurotransmitters, suggesting that the pathogenesis of decreased neurotransmitters is not only based on Phe toxicity, but could in addition be due to the inhibition of the influx of the precursors of the dopamine and serotonin pathway or the direct inhibition of brain Phe on Tyr and Trp hydroxylase activities [44–46].

Results from the multiple regression analyses showed that in B6 mice, in addition to contributions from plasma Phe, Thr and His could explain a small additional portion of the variance in brain Phe. For BTBR mice, Thr explained an additional small portion. Earlier studies showed that there is an inverse correlation between Phe and Thr [47,48] as well as a negative correlation between brain Phe and plasma

His [49]. However, presently, there is no conclusion on the mechanism involved in these relationships. Given that various studies found a relation between Thr and His on Phe, it would be worth investigating this further in the future.

Previous clinical studies suggested that blood Phe/Tyr ratios could be superior to measuring plasma Phe as the sole parameter to explain brain Phe in PKU [50]. Although our results showed some additional explanatory value of Tyr to brain norepinephrine, owing to the fact that Tyr is the precursor for brain norepinephrine, these findings were not substantiated by our results. The discrepancy between the results of previous (clinical) studies and our (mice) study could be due to the fact that clinical studies did not take the diurnal variation of Tyr into account [35,51], Furthermore, ratios such as of Phe/Trp might be of additional value to plasma Phe to explain brain Phe concentrations. As Trp measurement are currently not widely used, studies in PKU patients using Trp or Phe/Trp are generally lacking, while this study could not proof the value of this measure.

For brain serotonin, plasma Trp could explain an additional 4.9% of the variance in brain serotonin in BTBR mice besides plasma Phe. For brain norepinephrine, plasma Tyr could explain an additional 7.9% (B6) and 5.1% (BTBR) besides plasma Phe. These findings can partly be explained by the fact that Trp is the precursor for brain serotonin and Tyr is one of the precursors for brain norepinephrine. Although there seems to be a relation between these plasma measures and serotonin and norepinephrine, the results show that this association cannot fully be illustrated using simple correlations.

Multivariable regression models for brain dopamine in our study were weak. Although adding other plasma markers to the model could slightly increase the strength of the model, they remained much weaker than other correlations found in this study.

Despite the fact that some plasma markers helped explain brain biochemistry for a small part, these contributions were only minor compared to the contributions of plasma Phe, which already explained a large portion of the variance in brain biochemistry. Our results therefore showed that plasma concentrations of other non-Phe LNAA are not of considerabel additional value in understanding brain biochemistry in PKU. Therefore, future studies should 1) evaluate whether we can further strengthen the predictive value of Phe as a biomarker at itself and 2) look for additional markers, outside of the scope of amino acids and LNAA that could eventually help to better explain neurocognitive impairments in PKU. In line with this, Coene et al. proposed using untargeted metabolomics, identifying over 10.000 markers per patient sample, to look for novel biomarkers in PKU [52]. Using this technique, Václavík et al. already identified two possible novel biomarkers in PKU could perhaps be used in monitoring treatment.

In conclusion, this study investigated the relationship between plasma and brain biochemistry in PKU mice and aimed to find other amino acids that could be of additional explanatory value. Results showed that plasma Phe correlated strongly to brain Phe and that adding other non-Phe plasma markers did not considerably help to explain the variance in brain biochemistry in PKU mice. Therefore, brain biochemistry in PKU is still best explained by blood Phe but at the same time neurocognitive and behavioural outcome cannot fully be explained by blood or brain Phe concentrations, necessitating a search for other additional or alternative parameters.

Details of the contributions of individual authors

- Allysa M. Dijkstra: First author; Conception and design, data analyses and interpretation, computed figures and drafted the article and contributed to the final manuscript.
- Ninke van Vliet; helped conception and design, data analyses and interpretation, drafting paper and contributed to the final manuscript.
- Danique van Vliet; Contributed and collected data, data interpretation, helped supervise the project, contributed to the final article.
- Cristina Romani; Revised the article critically and contributed to the final manuscript.
- Stephan C. Huijbregts; Revised the article critically and contributed to the final manuscript.
- Els van der Goot; Revised the article critically and contributed to the final manuscript.
- Iris Hovens; Revised the article critically and contributed to the final manuscript.
- Eddy A. van der Zee; Revised the article critically and contributed to the final manuscript.
- Ido Kema; Revised the article critically and contributed to the final manuscript.
- M. Rebecca Heiner-Fokkema Revised the article critically and contributed to the final manuscript.
- Francjan J. van Spronsen; Conception and design, supervised the project and contributed to the manuscript.

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Ethics aproval

NA.

Patient consent statement

NA.

Documentation of approval from institutional commuttee for care and use of laboratory animals

All procedures and treatments for this study *were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen.*

Availability of data and material

Additional information on correlations can be found in the supplemental data sheet. For raw data supporting the results, please contact the corresponding author.

Declaration of Competing Interest

F.J van Spronsen has been a member of scientific advisory boards for defects in amino acid metabolism of APR, Agios, Arla Food International, BioMarin, Eurocept Int, Lucana, Moderna TX, Nutricia, Rivium, Homoly, and Nestle-Codexis, his institute has received research grants from Alexion, Biomarin, Codexis, Nutricia, SoBi, and Vitaflo, has received grants from patient organizations ESPKU, Metakids, NPKUA, Stofwisselkracht, Stichting PKU research and Tyrosinemia Foundation, and has received honoraria as consultant and speaker from APR, Pluvia, Biomarin, MendeliKABS and Nutricia.

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