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Phenylketonuria and Blood-Brain Barrier Competition Investigated by Magnetic Resonance Spectroscopy

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Introduction

With the advent of localized magnetic resonance spectroscopy (MRS), direct observation of cerebral metabolites and neurochemical pathways in the intact human brain *in vivo* has become possible [1]. Avison *et al.* [2] were the first to detect magnetic resonance (MR) signals from the aromatic phenylalanine (Phe) protons (^{1}H) in the brain of hyperphenylalanemic rabbits. They demonstrated that Phe does not achieve equal concentrations on both sides of the blood-brain barrier (BBB). About a decade ago, quantitation of elevated Phe concentrations in the human brain, $[Phe]_{brain}$, was achieved in patients with phenylketonuria (PKU) [3–6] and, more recently, also in healthy subjects following oral Phe loading [7]. A major motivation to use ^{1}H MRS in PKU has been the expectation that $[Phe]_{brain}$ might be more closely linked to the clinical phenotype than blood Phe levels, $[Phe]_{blood}$. Besides correlating $[Phe]_{brain}$ data with indicators characterizing the outcome in PKU [8–15], cerebral Phe uptake [6–8, 11, 16–21] and potential implications for the treatment of adult patients [21–23] have been other topics of recent research.

Regarding the elevation of $[Phe]_{brain}$, normal levels of the routinely detected cerebral metabolites are typically observed as summarized in Figure 1a and Table 1. These results are corroborated by Pietz *et al.* [26], who reported normal concentrations of NAA, Cr, Cho, and mI in occipital GM and in parietal and frontal WM. We may therefore exclude consistent biochemical alterations secondary to PKU concerning these metabolites and will subsequently focus on findings related to cerebral Phe and its BBB transport.

Single-Voxel MRS for Quantifying Phe

^{1}H MR spectroscopy measurements of $[Phe]_{brain}$ can be performed on clinical scanners operating at a magnetic field strength of 1.5 T or higher. It may conveniently be combined with magnetic resonance imaging (MRI) exam of potential WM abnormalities [27]. Although a conventional birdcage head coil is sufficient, modern multichannel array coils offer a substantial sensitivity advantage. This could be an important benefit in view of a sub-millimolar cerebral Phe concentration even in patients with 'classic' PKU (*i.e.*, $[Phe]_{blood} > 1.2$

mmol/L on a normal protein intake) off diet, which directly translates into a poor signal-to-noise ratio (SNR).

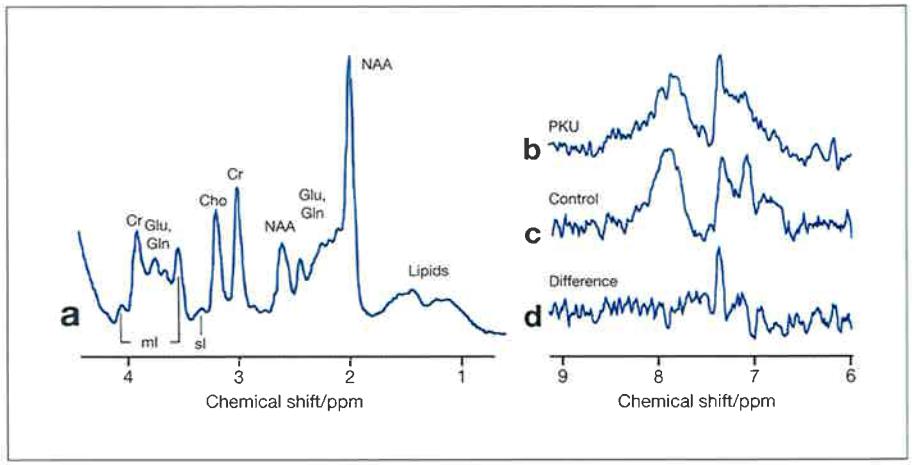

*Figure 1: Brain ^{1}H spectra from a 26-year old male PKU patient ($[Phe]_{blood}$ = 1.40 mmol/L) [17]. The upfield region **(a)** shows resonances from abundant brain metabolites summarized in Table 1. In the downfield region **(b)**, elevated signal intensity is found around 7.3 ppm, which can be identified and integrated after subtraction of a corresponding spectrum from a healthy control subject **(c)**: A single distinct peak at 7.36 ppm remains in the difference spectrum **(d)** which permits assignment to the Phe phenyl protons (Reprinted from **Brain Research** 778, Möller HE, Weglage J, Wiedermann D, Vermathen P, Bick U, Ullrich K. Kinetics of phenylalanine transport at the human blood-brain barrier investigated in vivo, 329–337, 1997, with permission from Elsevier).*

In previous studies, single-shot single-voxel techniques, such as point-resolved spectroscopy (PRESS) [28] or stimulated echo acquisition mode (STEAM) [29–31], proved to be robust methods for recording a Phe signal from a well-defined volume of interest (VOI) in the brain. STEAM is especially suited to realize ultra-short echo times, whereas PRESS achieves a doubled SNR. An exhaustive description of an acquisition and processing scheme for quantifying $[Phe]_{brain}$ was published by Kreis *et al.* [4]. As $[Phe]_{brain}$ is low, selection of a large VOI (≥ 25 mL) is recommended to achieve a sufficient SNR for quantitation within a reasonable acquisition time (< 15 min). In a preliminary study, variations of Phe levels among different brain areas were below significance [17].

While complex multiplets from the α and β Phe protons between 3 and 4 ppm are masked by overlying strong signals of abundant metabolites in the *in vivo* spectra [32], all signals of the chemically and magnetically inequivalent phenyl ring protons collapse into a single peak (≈ 7.36 ppm) at moderate field strengths, which can be used for quantitation (Figures 1b–d). However, also the spectral region between 6.5 and 8.5 ppm is composed of several overlapping peaks, which have only been in part assigned [33]. Difference spectroscopy has therefore been utilized for the unequivocal identification of elevated Phe in previous studies [4–6]. Most efficient for removing the background signal is the use of a control spectrum averaged across subjects from a group of healthy volunteers. This method leads to absolute concentrations if the results are corrected by the normal level, which is approximately 0.05 mmol/L based upon biopsy data [34]. While it was previously assumed that Phe in the blood does not contribute to the brain spectrum [4], a careful recent study indicated that vascular Phe might be completely visible at 1.5 T depending on the localization sequence [35]. This would require a further correction of $[Phe]_{brain}$ measured by MRS whenever blood levels are significantly higher than cerebral levels (*e.g.* in PKU patients at steady state or during oral Phe loading experiments). Another obstacle for quantifying $[Phe]_{brain}$ is the relatively short apparent relaxation time, $T_{2,app}$, which includes both effects from pure spin-spin relaxation and modulation of the echo decay due to *J*-coupling. Estimates from *in vivo* studies in rabbits at 4.7 T [2] and in a PKU patient at 1.5 T [4] are 40 ms and 65 ms, respectively. Choice of a short echo time, $T_E \leq$ 30 ms, is therefore a prerequisite to minimize T_2-weighting of the spectrum.

Metabolite	Concentration [mmol/L]	
	PKU patients	Control subjects
N-acetylaspartate (NAA)	10.18 ± 1.40	10.04 ± 1.72
N-acetylaspartylglutamate	2.35 ± 1.48	2.40 ± 1.12
GABA	1.59 ± 0.48	1.47 ± 0.49
Total choline (Cho)	1.59 ± 0.26	1.51 ± 0.33
Total creatine (Cr)	7.12 ± 0.90	6.76 ± 0.82
Glutamate (Glu)	8.95 ± 1.65	8.93 ± 3.58
Glutamine (Gln)	4.08 ± 1.93	3.89 ± 1.67
Myo-inositol (mI)	5.75 ± 1.02	5.95 ± 0.92
Scyllo-inositol (sI)	0.21 ± 0.11	0.29 ± 0.18
Lactate	0.42 ± 0.18	0.45 ± 0.13
Taurine	1.53 ± 0.51	1.61 ± 0.57

Table 1: Levels of abundant brain metabolites obtained from ^{1}H MRS in 11 treated adult PKU patients (6 male, 5 female, 20–34 years, 67 measurements, Phe levels were $[Phe]_{blood}$ = 0.47–2.52 mmol/L and $[Phe]_{brain}$ = 0.10–0.96 mmol/L) and 11 healthy controls (10 male, 1 female, 25–35 years, 16 measurements). Spectra were recorded from 36-mL voxels centered in the parieto-occipital white matter (WM) with some additional contribution from cortical gray matter (GM). Absolute concentrations (mean ± standard deviation) were determined using LCModel [24, 25] with reference to the brain tissue water signal. Differences between patients and controls were insignificant ($P > 0.05$; t-tests corrected for multiple comparisons).

Intracerebral Phe levels clearly remained below blood concentrations in all MRS studies in PKU patients. McKean [34] reported $[Phe]_{brain}$ = 0.85 ± 0.015 mmol/kg wet weight obtained biochemically at autopsy ($[Phe]_{blood}$ not available), which is at the upper end of the *in vivo* results measured by MRS. Literature results on the brain-blood [Phe] ratio (BBR) seem to deviate to some extent among studies probably due to differences in the quantitation procedures [36]. They become more consistent if basic assumptions are standardized and potential systematic effects in the data acquisition and processing routines are considered [37].

Modeling BBB Phe Transport I: Symmetric Michaelis-Menten Kinetics

The cellular supply of essential amino acids is a function of their plasma concentrations and membrane transport processes. As the surface area of the brain cell membranes by far exceeds that of the brain capillary endothelial membrane, equilibration between interstitial and intracellular spaces occurs comparably fast whereas BBB transport is rate-limiting for cerebral uptake [38, 39]. Dynamic investigations in PKU patients after an oral Phe challenge indicated a delay in the rise of $[Phe]_{brain}$ with respect to that of $[Phe]_{blood}$ [8, 20]. As intra- and extracellular spaces both contribute to the MRS Phe signal, this underlines experimentally that the rate-limiting step is at the BBB. We will therefore assume a single kinetic pool within the brain for modeling transport kinetics as shown in Figure 2. Large neutral amino acids (LNAAs) are transported across the BBB by a common carrier, the LNAA transporter type 1 (LAT1) isoform, which is facilitative, Na^+ independent, and has a much higher affinity (i.e., a lower transport Michaelis constant, K_m) for amino acids as compared to the so-called 'system L' in peripheral tissues [39, 40]. A consequence of the low K_m values is that the BBB LAT1 is normally heavily saturated by the existing concentrations of circulating LNAAs [41, 42], making their brain uptake sensitive to competition.

Due to competition effects, only an apparent Phe transport Michaelis constant according to

$$K_{m,app}^{Phe} = K_m^{Phe}\left(1 + \sum_{LNAA \neq Phe} \frac{[LNAA]}{K_m^{LNAA}}\right) \quad (1)$$

is measured directly *in vivo* [43]. K_m^{LNAA} and K_m^{Phe} are the absolute transport Michaelis constants of a competing LNAA and Phe, respectively, and [LNAA] is the LNAA concentration (either in blood or brain). It is obvious from Equation 1 that $K_{m,app}^{Phe}$ will equal its minimum possible value, K_m^{Phe}, if [LNAA] << K_m^{LNAA} (conditions of minimal competition). The following analysis is based on a symmetric Michaelis-Menten model, which is characterized by $K_{m,app}^{Phe}$, the maximal transport velocity, V_{max}^{Phe}, and the intracerebral Phe consumption rate, CMR_{Phe} [17]. In the most simple scenario, $K_{m,app}^{Phe}$ is assumed to be identical for both transport directions, and CMR_{Phe} is assumed to be constant, which yields:

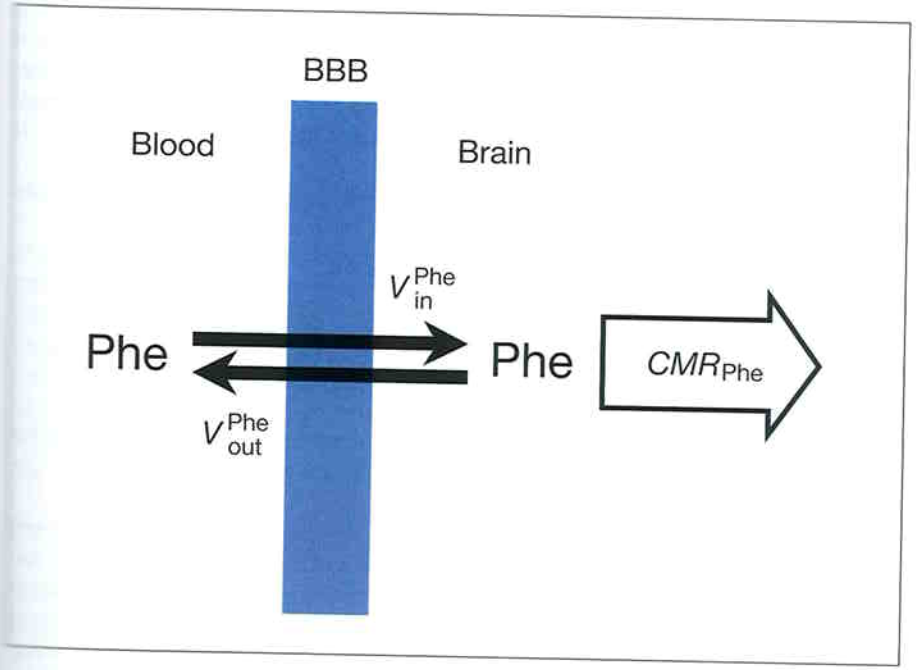

Figure 2: Two-compartment model for cerebral Phe transport, which assumes that the rate-limiting step is located at the BBB. Due to fast equilibration between the intrastitial and intracellular spaces, a single kinetic Phe pool results within the brain water phase. Carrier-mediated unidirectional fluxes across the BBB (V_{in}^{Phe}, V_{out}^{Phe}) are symbolized by the arrows and assumed to follow classic Michaelis-Menten kinetics. The open arrow represents cerebral metabolic Phe consumption at a rate CMR_{Phe}.

$$\frac{d[Phe]_{brain}}{dt} = \frac{V_{max}^{Phe}[Phe]_{blood}}{K_{m,app}^{Phe} + [Phe]_{blood}} - \frac{V_{max}^{Phe}[Phe]_{brain}}{K_{m,app}^{Phe} + [Phe]_{brain}} - CMR_{Phe}. \quad (2)$$

The first term describes influx across the BBB (V_{in}^{Phe}), the second term efflux (V_{out}^{Phe}), and the third term cerebral metabolization. An analogous approach has been used to model BBB glucose transport kinetics [38, 39].

Metabolism, such as incorporation into proteins and peptides (*e.g.*, γ-glutamylphenylalanine) or conversion (*e.g.*, hydroxylation or decarboxylation), provides a drain of cerebral Phe [44]. The above assumption of a constant catabolic flux should be justified regarding that $[Phe]_{brain}$ is increased by an order of magnitude in classic PKU (*i.e.*, CMR_{Phe} approaches its maximum velocity due to enzyme saturation) and is consistent with computer simulations for $[Phe]_{blood} > 1$ mmol/L [45]. The approximation may, however, break down in rare 'atypical' cases with unusually low $[Phe]_{brain}$ despite elevated blood levels as in classic PKU (see below) [8]. A contribution from non-saturable diffusive transport is ignored in Equation 2, which is reasonable for typical *in vivo* $[Phe]_{blood}$ values [42, 46, 47].

Equation 2 can be rearranged for steady-state conditions (*i.e.*, $d[Phe]_{brain}/dt = 0$) according to:

$$[Phe]^{ss}_{brain} = K^{Phe}_{m,app} \frac{\{(V^{Phe}_{max}/CMR_{Phe}) - 1\}\ [Phe]_{blood} - K^{Phe}_{m,app}}{\{(V^{Phe}_{max}/CMR_{Phe}) + 1\}\ K^{Phe}_{m,app} + [Phe]_{blood}}. \quad (3)$$

With Equation 3 an upper limit,

$$[Phe]^{max}_{brain} = K^{Phe}_{m,app}\{(V^{Phe}_{max}/CMR_{Phe}) - 1\}, \quad (4)$$

is obtained at saturating $[Phe]_{blood}$ and a linear regime,

$$[Phe]^{ss}_{brain} \approx \frac{(V^{Phe}_{max}/CMR_{Phe}) - 1}{(V^{Phe}_{max}/CMR_{Phe}) + 1}[Phe]_{blood} - \frac{K^{Phe}_{m,app}}{(V^{Phe}_{max}/CMR_{Phe}) + 1}. \quad (5)$$

at low blood levels (*i.e.*, $[Phe]_{blood} << K^{Phe}_{m,app}\{(V^{Phe}_{max}/CMR_{Phe}) + 1\}$). Experimentally, a linear correlation between brain and blood [Phe] was consistently observed in previous MRS studies at steady state up to $[Phe]_{blood} \approx 1.7$ mmol/L [6, 14, 15, 17, 20].

Unequivocal indications of non-linear behavior due to LAT1 saturation as predicted by the symmetric Michaelis-Menten model were obtained by MRS in hyperphenylalaninemic rabbits [2]. Data from nine PKU patients investigated over a relatively broad range of $[Phe]_{blood}$ are shown in Figure 3 [17]. Due to the poor SNR of the Phe signal, substantial statistical errors (approx. ±0.15 mmol/L) degrade the estimates of $[Phe]_{brain}$ and contribute to the scatter in the data. Fitting the pooled data to Equation 3 yields $K^{Phe}_{m,app} = 0.16 \pm 0.11$ mmol/L and $V^{Phe}_{max}/CMR_{Phe} = 9.0 \pm 4.1$. Note that a general problem of using combined measurements from different patients is that potential inter-individual variations are completely ignored and, hence, the above values should be regarded only as rough estimates.

While valuable information on BBB transport can be derived from MRS at steady-state Phe levels, dynamic experiments, which simultaneously measure the time courses of $[Phe]_{blood}$ (*e.g.*, by enzymatic assay) and $[Phe]_{brain}$ (by MRS) during an oral Phe loading test (typically 100 mg/kg body weight), provide a much more direct approach to extracting kinetic parameters (Figure 4) [8, 11] and may even be used in the normal population [7]. Results from a study in 15 patients with classic PKU study are summarized in Table 2. The averaged kinetic parameters ($K^{Phe}_{m,app} = 0.48$ mmol/L; $V^{Phe}_{max}/CMR_{Phe} = 4.43$) are reasonably consistent with the above steady-state results extracted from the pooled data in Figure 3. Note that no vascular correction has been applied in these studies; hence, Phe influx might be overestimated depending on the amount of visibility of the blood pool [35].

The kinetic parameters estimated with the symmetric Michaelis-Menten model compare well with studies employing different methodology: In rat brain, $K^{Phe}_{m,app} = 0.218 \pm 0.009$ mmol/L was obtained with the *in situ* brain perfusion technique [46]. Theoretical estimates at various concentrations of competing LNAAs yielded $K^{Phe}_{m,app} = 0.44$–1.33 mmol/L [48]. In healthy human subjects, $K^{Phe}_{m,app} = 0.03$–0.58 mmol/L and $V^{Phe}_{max} = 0.0144$–0.0943 mmol/kg/min were measured using the double-indicator method [47].

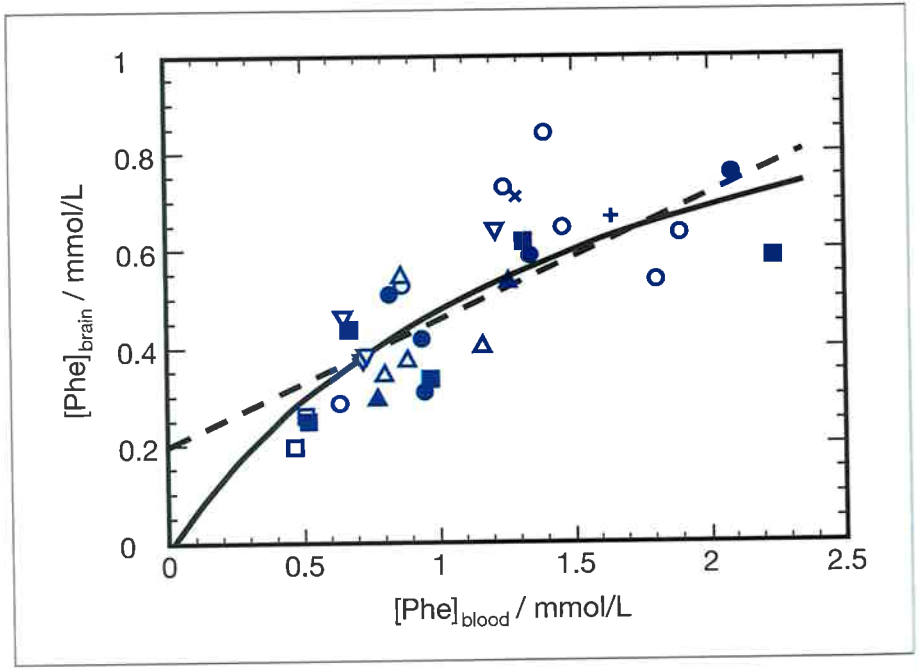

Figure 3: Plot of parieto-occipital brain Phe concentrations versus corresponding plasma levels and results from non-linear least-squares fitting (solid line; r = 0.81) assuming saturable Phe transport described by Equation 3 [17] and from linear regression analysis (dashed line; r = 0.76) according to Equation 5. (Adapted from ***Brain Research*** *778, Möller HE, Weglage J, Wiedermann D, Vermathen P, Bick U, Ullrich K. Kinetics of phenylalanine transport at the human blood-brain barrier investigated in vivo, 329–337, 1997, with permission from Elsevier).*

Parameter	Range	Mean
IQ	77 ... 132	98.4
BBR	0.18 ... 0.58	0.29
$K^{Phe}_{m,app}$ [mmol/L]	0.10 ... 1.03	0.48
V^{Phe}_{max}/CMR_{Phe}	14.00 ... 2.61	4.43

Table 2: Stationary brain/blood ratios of [Phe] (measured under free nutrition at $[Phe]_{blood}$ = 0.90 ... 1.51 mmol/L) and kinetic parameters obtained with dynamic MRS after an oral Phe challenge in 15 patients with classic PKU [11].

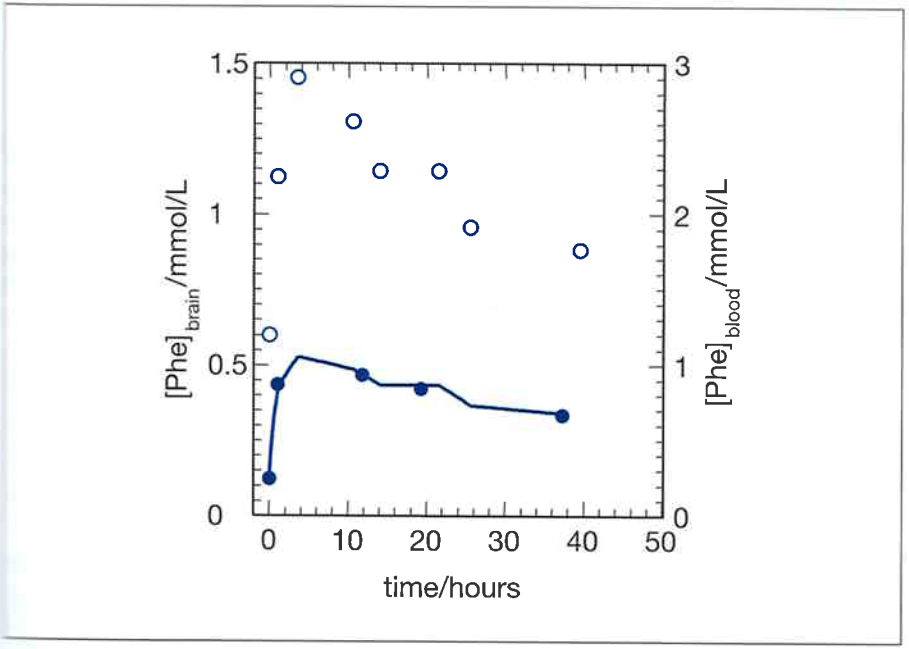

Figure 4: *Dynamic changes of $[Phe]_{blood}$ (open circles) and $[Phe]_{brain}$ (filled circles) after* ***oral Phe*** *loading in an 'atypical' PKU patient and results from fitting the time course of* ***[Phe]brain*** *to Equation 2. [8] (Reprinted from the* ***Journal of Cerebral Blood Flow and Metabolism*** *18, Möller HE, Weglage J, Wiedermann D, Ullrich K. Blood-brain barrier phenylalanine transport and individual vulnerability in phenylketonuria, 1184–1191, 1998, with permission from Lippincott, Williams & Wilkins).*

Modeling BBB Phe Transport II: Asymmetric Michaelis-Menten Kinetics

Although the symmetric Michaelis-Menten model seems to fit the experimental data reasonably well, obvious limitations inherent to this approach indicate further consideration. As there is a concentration gradient across the BBB (*i.e.*, reduced [LNAA] on the brain side), a difference in $K^{Phe}_{m,app}$ is expected to some degree for both transport directions considering Equation 1. Due to the concentration gradient, the efflux of LNAAs must consume energy and cannot be explained exclusively by facilitative transport systems. Recent results indicate an additional Na^+-dependent carrier on the abluminal membrane, which might participate in regulating the LNAA brain content in addition to facilitative transport by the LAT1 at both sides of the endothelial cells [49]. Under such conditions, Equations 2 and 3 might better be modified according to

$$\frac{d[\text{Phe}]_{\text{brain}}}{dt} = \frac{V_{\text{max}}^{\text{Phe}}[\text{Phe}]_{\text{blood}}}{K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{blood}}} - \frac{V_{\text{max}}^{\text{Phe}}[\text{Phe}]_{\text{brain}}}{K_{\text{m,brain}}^{\text{Phe}} + [\text{Phe}]_{\text{brain}}} - CMR_{\text{Phe}}, \quad (6)$$

where $K_{\text{m,blood}}^{\text{LNAA}}$ and $K_{\text{m,brain}}^{\text{LNAA}}$ are the appropriate apparent transport Michaelis constants on either side of the BBB. Note that using a single $V_{\text{max}}^{\text{Phe}}$ is probably still an oversimplification in view of a potential contribution from Na^+-dependent efflux or if the expression of the LAT1 differs for the luminal and abluminal membrane. The corresponding steady-state solution is now

$$[\text{Phe}]_{\text{brain}}^{\text{ss}} = K_{\text{m,brain}}^{\text{Phe}} \frac{\{(V_{\text{max}}^{\text{Phe}}/CMR_{\text{Phe}}) - 1\}\,[\text{Phe}]_{\text{blood}} - K_{\text{m,blood}}^{\text{Phe}}}{\{(V_{\text{max}}^{\text{Phe}}/CMR_{\text{Phe}}) + 1\}\,K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{blood}}}, \quad (7)$$

To study the effect of varying degrees of asymmetry, we performed computer simulations assuming $K_{\text{m,blood}}^{\text{LNAA}}$ = 0.4 mmol/L (a theoretical estimate computed with Equation 1, absolute $K_{\text{m}}^{\text{LNAA}}$ values from rats [41], and average blood LNAA concentrations from a group of PKU patients [21]), $K_{\text{m,brain}}^{\text{LNAA}}$ between 0.1 and 0.3 mmol/L, and $V_{\text{max}}^{\text{Phe}}/CMR_{\text{Phe}}$ between 2 and 10. The synthetic data derived with Equation 7 for typical Phe levels in PKU patients (0.5 mmol/L ≤ $[\text{Phe}]_{\text{blood}}$ = 2.0 mmol/L) could be fitted to Equation 3 without indications of major deviations (correlation coefficients, $r > 0.99$). The advanced asymmetric model with three parameters instead of only two (although a reasonable assumption) did, hence, not improve the statistical significance of the fit. Quantitatively, use of the simple symmetric model to analyze asymmetric BBB Phe transport at steady-state yielded an apparent transport Michaelis constant that was roughly the arithmetic mean of the true values $K_{\text{m,blood}}^{\text{LNAA}}$ and $K_{\text{m,brain}}^{\text{LNAA}}$ and tended to underestimate the ratio $V_{\text{max}}^{\text{Phe}}/CMR_{\text{Phe}}$ by 15–45% depending on the degree of asymmetry. Due to relatively large errors of the MRS data for $[\text{Phe}]_{\text{brain}}$, this level of inaccuracy was already considered in previous estimates of kinetic parameters [8, 11, 17, 19].

While a broad experimental database supports the existence of the linear regime predicted by Equation 5, only few human spectra (n = 4) were recorded at steady-state blood levels exceeding 2 mmol/L where saturation effects should become increasingly evident [5, 14, 17]. The suggestion of saturation of Phe transport at high blood levels [6, 8, 17] has thus been a topic of debate. Without providing a mathematical analysis of the underlying kinetics, other authors proposed either a strict linear correlation between blood and brain Phe [15, 20] or an exponential fit assumed to be due to upregulation of the number of transporters [12, 22]. On the other hand, the existence of saturation effects was confirmed by MRS experiments in the hyperphenylalaninemic rabbit model [2] and by the

possibility to block BBB Phe transport in PKU patients by LNAA supplementation [21]. Reduced cerebral levels of tyrosine, tryptophan, and branched-chain amino acids have been found in a genetic mouse model of PKU [50, 51]. To account for all these observations, a modification of the transport model should (*i*) still be based upon the assumption of LAT1 saturation but (*ii*) further explore possibilities that might lead to a steady-state relation between $[\text{Phe}]_{\text{blood}}$ and $[\text{Phe}]_{\text{brain}}$ that is different from Equation 3.

Upregulation of the number of BBB transporters is equivalent to increasing $V_{\text{max}}^{\text{Phe}}$ but does not lead to a change in the mathematical expression of the transport model. Although this might produce inconsistencies with Equation 3 due to inter-individual variations if pooled steady-state data from different subjects are analyzed, one would not expect to see deviations in single-patient studies where serial MRS experiments are performed during a relatively short period of time.

The fact that Phe influx and efflux are facilitated by the same BBB transporter suggests that the occupancy of the LAT1 by, for example, influxing Phe might affect its availability for effluxing Phe by blocking binding sites and vice versa. Product formation is then no longer unidirectional, and reversible Michaelis-Menten kinetics applies. In analogy to competition with other LNAAs as accounted for by Equation 1, this leads to replacing the apparent transport Michaelis constant by $K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{brain}}$ for influx and by $K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{blood}}$ for efflux [52], which is equivalent to a reduced affinity for cerebral Phe uptake with increasing steady-state Phe levels [53]. If, for simplicity, symmetric transport properties are assumed for both directions, reversible Michaelis-Menten kinetics predict:

$$\frac{d[\text{Phe}]_{\text{brain}}}{dt} = \frac{V_{\text{max}}^{\text{Phe}}[\text{Phe}]_{\text{blood}}}{(K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{brain}}) + [\text{Phe}]_{\text{blood}}} - \frac{V_{\text{max}}^{\text{Phe}}[\text{Phe}]_{\text{brain}}}{(K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{blood}}) + [\text{Phe}]_{\text{brain}}}$$

$$-CMR_{\text{Phe}} = \frac{V_{\text{max}}^{\text{Phe}}\,([\text{Phe}]_{\text{blood}} - [\text{Phe}]_{\text{brain}})}{K_{\text{m,app}}^{\text{Phe}} + [\text{Phe}]_{\text{brain}} + [\text{Phe}]_{\text{brain}}} - CMR_{\text{Phe}}. \quad (8)$$

Equation 8 leads to a purely linear relation between blood and brain Phe at steady state, which is identical to Equation 5 but now applies regardless of the value of $[\text{Phe}]_{\text{blood}}$. A fit to the pooled steady-state data from Möller *et al.* [17] assuming reversible Michaelis-Menten kinetics is also shown in Figure 3. The obtained slope is quite similar to previously published data from multiple patient studies (Table 3) [12, 15]. It leads to a ratio $V_{\text{max}}^{\text{Phe}}/CMR_{\text{Phe}}$ = 1.7 ± 0.2, which is considerably smaller but of the same order as the above estimate from the fit to the simple symmetric model. This discrepancy might point to a potential overestimation of $V_{\text{max}}^{\text{Phe}}/CMR_{\text{Phe}}$ when using the simple symmetric Michaelis-Menten model. However, a meaningful value for the intercept, which should be negative according to Equation 5, was not obtained with linear regression, and a computation of

$K^{Phe}_{m,app}$ is, hence, not possible. Again, this was also observed in previously published studies assuming a linear correlation [12, 15]. Although we cannot exclude the possibility that errors in the quantitation procedure might systematically offset the estimates of $[Phe]_{brain}$, a clear indication of reversible Michaelis-Menten transport kinetics is not obtained. In view of such current shortcomings, the simple symmetric model seems to work equally well to describe BBB Phe transport despite its inherent limitations. Further single-patient steady-state data including measurements at $[Phe]_{blood}$ > 2 mmol/L are highly warranted to clarify if reversible Michaelis-Menten kinetics might be more appropriate.

Slope	Intercept [mmol/L]	r	Reference
0.257 ± 0.041	0.201 ± 0.049	0.76	This work*
0.22 ± 0.02	0.022 ± 0.023	0.93	[17]
0.33	0.034	0.66	[12]

* Data taken from Ref. [17].

Table 3: Results from linear regression analysis of combined steady-state blood and brain Phe data from different patients with PKU.

Individual Brain Vulnerability to Phe

In a number of clinical studies, PKU patients who had similar metabolic control have demonstrated different degrees of cerebral white-matter changes [54], of electroencephalogram (EEG) abnormalities [55], or of intellectual and neuropsychological deficits [56]. Siblings with identical mutations of the phenylalanine hydroxylase gene may have different clinical expressions [57]. Besides such subtle though significant variations, most evident indicators of an individual vulnerability of the brain to elevated $[Phe]_{blood}$ are occasional reports of untreated adults with a biochemical profile consistent with classic PKU who escape brain damage and show normal intelligence [10, 13, 57, 58]. An explanation might be variations in the BBR among individuals secondary to differences in either BBB Phe transport characteristics or cerebral Phe metabolic rates providing a varying degree of 'protection' of the brain [10].

The range of biological variations in the BBR and transport kinetics is still insufficiently known and has been discussed with controversy regarding spectroscopy results [11–13, 15, 59, 60]. The few data available from tracer injections [47] as a methodology complementary to MRS seem to support the hypothesis that significant inter-individual differences in Phe transport characteristics do exist. However, further systematic investigation is strongly advocated to address this important issue and its implication for PKU.

Some support of the hypothesis that the BBR and, potentially, favorable BBB transport conditions may be important for the individual clinical outcome in PKU was obtained from MRS experiments in a group of three highly 'atypical' adult patients with genotypes and

steady-state $[Phe]_{blood}$ (1.15 ± 0.10 mmol/L) of classic PKU but average (n = 2) or borderline normal (n = 1) intelligence quotients (IQ) although they had never adhered to any specific diet (Group 1) [8]. Cerebral Phe was hardly detectable in these individuals ($[Phe]_{brain} \leq 0.15$ mmol/L). A similar observation in an untreated person with PKU achieving normal intellect was also published by Koch *et al.* [13]. The reduction in the BBR was significant ($P < 0.01$) when comparing Group 1 to 'typical' phenylketonurics including a group of four patients who had been untreated in early infancy and were retarded (Group 2a: $[Phe]_{blood}$ = 1.28 ± 0.06 mmol/L; $[Phe]_{brain}$ = 0.60 ± 0.04 mmol/L) and a group of four early treated adults, who were off diet at the time of the examination (Group 2b: $[Phe]_{blood}$ = 1.27 ± 0.09 mmol/L; $[Phe]_{brain}$ = 0.59 ± 0.15 mmol/L). Further results are given in Table 4. Dynamic MRS performed during an oral Phe challenge consistently revealed both a higher $K^{Phe}_{m,app}$ ($P < 0.04$) and a smaller ratio V^{Phe}_{max}/CMR_{Phe} ($P < 0.03$) in the group of 'atypical' cases when compared to the 'typical' PKU Group 2b [8]. Qualitatively, $[Phe]_{brain}$ reached its maximum later in the 'typical' patients whereas both blood and brain [Phe] seemed to relax quicker towards the baseline after the load in the 'atypical' cases [8]. The latter behavior was also observed in normal subjects exposed to an oral Phe challenge [7].

Parameter	Group 1	Group 2a	Group 2b
IQ rage	75 ... 105	50 ... 60*	77 ... 109
BBR	<0.13 ± 0.01	0.47 ± 0.04	0.47 ± 0.12
$K^{Phe}_{m,app}$ [mmol/L]	0.81 ± 0.33	–	0.10 ± 0.04
V^{Phe}_{max}/CMR_{Phe}	2.87 ± 0.33	–	11.3 ± 4.4

* IQ not available in one case due to severe psychomotoric retardation.

Table 4: Stationary brain/blood ratios of [Phe] and kinetic parameters (mean ± S.D.) obtained with dynamic MRS after an oral Phe challenge and the symmetric Michaelis-Menten model, Equation 2, in 'atypical' (Group 1) and two groups of 'typical' PKU patients (Group 2a: untreated in early infancy; Group 2b: early-treated adults) [8].

Cerebral LNAA imbalances may affect neurotransmitter metabolism, and hence, brain function. The different $K^{Phe}_{m,app}$ among both groups might suggest that some individuals have a polymorphism within the LAT1 coding region leading to a high K_m LAT1 [9, 61, 62]. A high BBB transport Michaelis constant desaturates the transporter and makes the brain uptake of LNAA less sensitive to competition [63]. This may minimize the deleterious effects from a hyperphenylananinemic state and would correlate with the observation of an almost normal mental status in the 'atypical' patient group. A similar phenomenon occurs in rabbits or dogs, which are known to have a low-affinity BBB LAT1 and are not subject to competition effects within the physiological concentration range of plasma LNAAs [39, 62]. Conversely, any polymorphism resulting in a reduction in the K_m (*i.e.*, increased affinity) of the LAT1 for substrate LNAAs would be expected to increase competition for binding sites and, hence, the vulnerability of the brain to hyperphenylalanine-

mia. Recently, Boado *et al.* [62] demonstrated that a single nucleotide polymorphism can significantly alter the affinity and capacity of the rabbit LAT1, whereas the phenotype could be restored with a double mutation. Alternatively, one may also speculate that the expression of other transport systems (*e.g.*, the multidrug resistance protein MDR1) at the BBB might contribute to the brain efflux of Phe and participate in regulating the LNAA brain content. Previous computer simulations could not produce strong effects on $[Phe]_{brain}$ upon varying $K^{Phe}_{m,app}$ over a range consistent with Table 4 [45, 48, 64]. More experimental work is thus needed to evaluate if competitive interactions at the BBB alone can explain an 'atypically' low BBR in exceptional cases.

Proton MRS cannot easily distinguish between individual processes (*i.e.*, efflux and metabolism) contributing to the drain of intracerebral Phe because only the ratio V^{Phe}_{max}/CMR_{Phe} is obtained with an acceptable error in view of the relatively poor time resolution during the initial Phe rise after the load [8]. Very rough estimates of the maximum Phe transport velocity and the cerebral Phe metabolization rate from the kinetic analysis yielded $V^{Phe}_{max} \approx 0.12$ mmol/L/min, $CMR_{Phe} \approx 0.046$ mmol/L/min and $V^{Phe}_{max} \approx 0.078$ mmol/L/min, $CMR_{Phe} \approx 0.008$ mmol/L/min for the 'atypical' (Group 1) and 'typical' (Group 2b) patient groups, respectively. Although substantial inaccuracies are likely to affect these data, the estimated range for V^{Phe}_{max} compares reasonably well with results from the double-indicator technique reporting values between 0.0151 and 0.0990 mmol/L/min (units converted assuming a brain density of 1.05 kg/L) in three normal subjects [47]. Regarding this coincidence, we may speculate that the reduced ratio V^{Phe}_{max}/CMR_{Phe} observed in the 'atypical' patients (Table 4) indicates an increased Phe consumption rate rather than depressed Phe influx. High brain Phe metabolic rates could be caused by an enzyme system restoring a more or less normal $[Phe]_{brain}$ via hydroxylation [65]. The existence of an intracerebral isoenzyme of Phe hydroxylase, which was previously suggested [66, 67], is not broadly accepted [68]. Alternatively, there is evidence that various brain areas contain tyrosine hydroxylase, which also acts on Phe [69–72]. Full activity of any of those enzymes should protect the brain to some extent against neurotoxic consequences of permanently elevated $[Phe]_{blood}$.

The PKU patients of the above Groups 1 and 2 may be regarded as extreme examples with respect to both their clinical outcome (*i.e.*, intellectually normal vs. classic clinical phenotype) and the differences in the BBR and kinetic parameters (Table 4), which rises questions about the relevance of such observations for a more general patient population. A comparison with the results from dynamic MRS in the larger sample of 15 early-treated subjects with classic PKU summarized in Table 2 [11] indicates that BBR variations may indeed be more common. This is also corroborated by another study in 29 patients [13]. However, Rupp *et al.* [15] reported intra-individually almost stable BBRs (0.22–0.30) in a group of 17 patients although the same group of authors also proposed indications of a large individual spread of kinetic parameters based on BBR time courses measured after a Phe challenge with and without additional supplementation with competing LNAAs [21].

In dynamic investigations (Table 2), a higher $K^{Phe}_{m,app}$ and a lower V^{Phe}_{max}/CMR_{Phe} ratio were negatively correlated with more severe WM abnormalities on MRI brain scans and (as a trend) positively with IQ scores [11], consistent with the assumption of a beneficial effect from a reduced LAT1 affinity for Phe and efficient intracerebral Phe metabolization. It is interesting to note that $K^{Phe}_{m,app}$ was further negatively correlated with V^{Phe}_{max}/CMR_{Phe}. This could indicate LAT1 upregulation (*i.e.*, increased V^{Phe}_{max}/CMR_{Phe}) under conditions of more severe competition (*i.e.*, highest affinity of the LAT1 for Phe or lowest $K^{Phe}_{m,app}$) as an adaptive response to hyperphenylalaninemia. In the developing rabbit, the level of the transporter protein was found to be stable despite a developmental down-regulation of the BBB LAT1 mRNA [73]. This is consistent with a posttranscriptional mechanism of regulation of BBB LAT1 gene expression and may serve to maintain the constancy of LNAA availability within the developing brain.

Additional support for the hypothesis of a beneficial effect from favorable kinetic parameters was derived from a separate analysis in a subgroup of four pairs of siblings [19]: A lower preload $[Phe]_{brain}$ (but not preload $[Phe]_{blood}$), higher $K^{Phe}_{m,app}$, and lower V^{Phe}_{max}/CMR_{Phe} were associated with a higher IQ score in three of the pairs (Figure 5a–d). Siblings are especially well suited for such investigations because of their identical genotype for PKU and comparable socioeconomic background and diet history.

While there is some evidence that favorable kinetic characteristics provide a varying degree of protection of the brain from uptake or accumulation of the neurotoxin Phe, confounding influences from known determinants of the clinical outcome, such as the quality of diet during the first decade of life, cannot be ruled out (Figure 5e). Little is further known whether individual Phe transport characteristics are independent of age or intra-individually stable over time. In view of the small patient population investigated so far, of potential error sources limiting the accuracy of the extracted kinetic data, and of remaining unexplained inconsistencies among studies, further research (*e.g.*, in the form of collaborative multi-center studies) is absolutely needed.

Utilizing BBB Competition in Therapy of PKU

The exact mechanisms of brain damage and impaired brain function due to elevated Phe levels in PKU are not entirely clear [74]. Besides direct neurotoxic effects of Phe, imbalances of the other LNAAs in the brain due to their inefficient cerebral uptake in the presence of competing high plasma Phe levels with potential consequences for neurotransmitter synthesis (*e.g.*, dopamine) have been discussed for many years as causative factors. Conversely, competition for the LAT1 might also be utilized as a basis for alternative therapy, which aims at lowering Phe influx by elevating the plasma concentrations of the other LNAAs [75]. Researchers have thus looked into alternative approaches to treatment by adding supplements of LNAAs [76, 77]. By using MRS, the effect of such strategies on $[Phe]_{brain}$ can now be evaluated directly with the methods outlined above.

A gradual lowering of $[Phe]_{brain}$ despite unchanged $[Phe]_{blood}$ was documented in PKU patients treated with LNAA supplements in a longitudinal study where repetitive MRS examinations were performed over six months [22, 23]. This observation is corroborated by results obtained in PKU mice [51]. A direct measure of competition effects and quantitative markers of the efficiency of such therapeutic strategies can be obtained from dynamic MRS investigations. Pietz *et al.* [21] recorded $[Phe]_{brain}$ during an oral Phe challenge in PKU patients with and without additional supplementation with seven competing LNAAs. Without supplementation, cerebral Phe increased after the Phe load similar to the dynamic studies outlined above, while EEG recordings revealed a slowing of activity. In contrast, Phe influx was completely blocked with concurrent LNAA supplementation and EEG abnormalities were no longer observed. Such studies might be used in the future to provide valuable quantitative information on the effects of single LNAAs or specific LNAA mixtures, which have been suggested to supplement diet therapy or which might be used in adolescent or adult patients after relaxation or termination of a strict diet.

Conclusions

Proton MRS of the brain is an excellent, non-invasive diagnostic modality for monitoring $[Phe]_{brain}$. It can be used repetitively (*e.g.*, for dynamic investigations of transport kinetics) without harm to the patient. While there is no indication to apply MRS routinely in young children who usually follow treatment strictly, it may be a useful modality in the assessment and management of adult patients who find lifelong diet compliance burdensome and socially limiting. Identifying patients at risk of developing neurological problems or those for whom discontinuation of diet might be safe in the long term would be extremely useful.

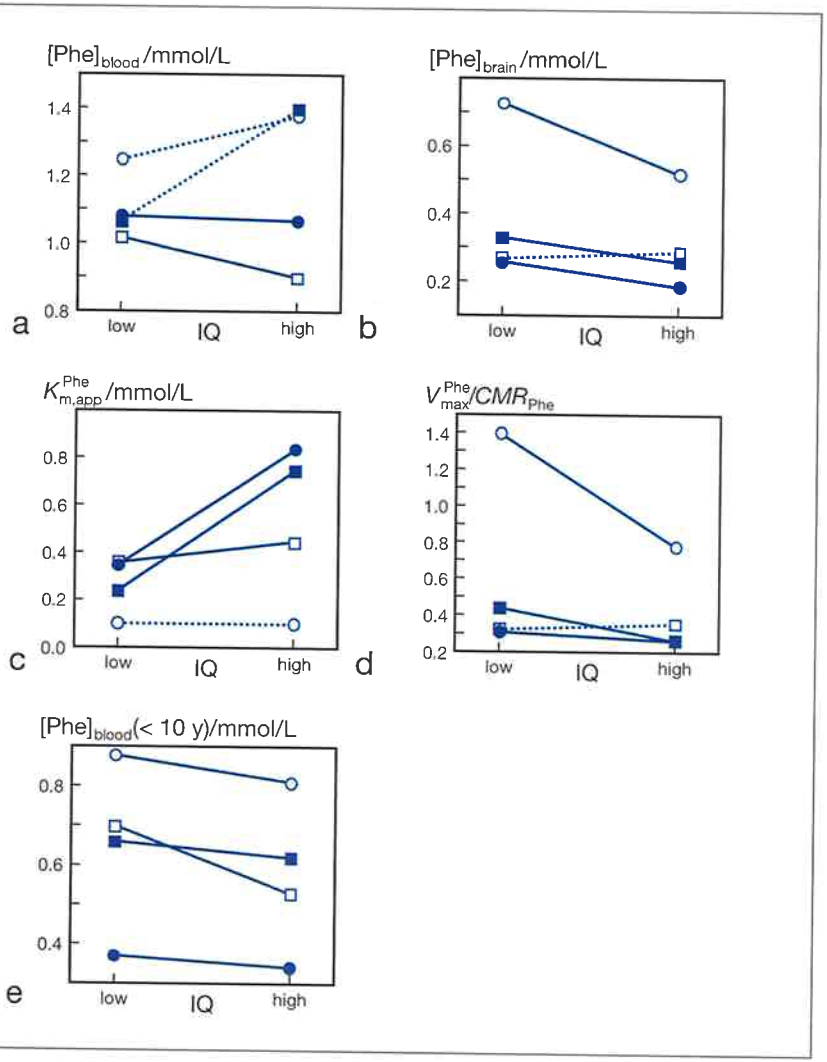

Figure 5: Concurrent Phe levels in ***(a)*** *blood and* ***(b)*** *brain and of kinetic parameters* ***(c)*** *K_m and* ***(d)*** *V^{Phe}_{max}/CMR_{Phe} as determined in oral Phe loading MRS experiments in four pairs of siblings with classic PKU and correlation with the intellectual performance [19]. Different symbols denote different pairs. Siblings were classified as having the 'higher' or 'lower' IQ in each pair. The average IQ difference was 10.3 points with individual scores between 86 and 112 in the 'higher IQ' group and between 77 and 106 in the 'lower IQ' group.* ***(e)*** *Correlation of long-term mean blood Phe levels during the first decade of life with the IQ score.*

The results of currently available studies indicate that BBB transport kinetics can be characterized quantitatively to some extent providing a step towards a functional characterization of the LAT1 in individual patients. Specifically, MRS permits to study competition effects in the transport of LNAAs across the human BBB. Preliminary findings suggest that inter-individual variations in the kinetics of Phe uptake or of its cerebral metabolism exist, leading to different brain concentrations of the neurotoxin Phe at comparable blood levels. Such variations seem to contribute to the pathogenesis of PKU and might be factors of the outcome. In the search for alternative treatments, dynamic MRS has a potential for evaluating and optimizing strategies based upon competition effects. However, despite initial encouraging results, the current data base from previous MRS studies is still quite small. Future work is awaited to assess the range of biological variations in the extracted parameters and to investigate the extent by which BBB amino acid transport contributes to the vulnerability of the brain in PKU or may be exploited for treatment.

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Abbreviations

BBB = blood-brain barrier; BBR = brain/blood ratio of [Phe]; Cho = total choline; Cr = total creatine; EEG = electroencephalogram; Glu = glutamate; Gln = glutamine; GM = gray matter; IQ = intelligence quotient; LAT1 = large neutral amino acid transporter type 1; LNAA = large neutral amino acid; mI = *myo*-inositol; MR = magnetic resonance; MRI = magnetic resonance imaging; MRS = magnetic resonance spectroscopy; NAA = *N*-acetylaspartate; Phe = phenylalanine; PKU = phenylketonuria; PRESS = point-resolved spectroscopy; sI = *scyllo*-inositol; SNR = signal-to-noise ratio; STEAM = stimulated echo acquisition mode; VOI = volume of interest; WM = white matter.

Symbols

CMR_{Phe} = cerebral Phe metabolization rate; K_m = transport Michaelis constant; K_m^{LNAA} = LNAA transport Michaelis constant; K_m^{Phe} = Phe transport Michaelis constant; $K_{m,app}^{Phe}$ = apparent Phe transport Michaelis constant; $K_{m,blood}^{Phe}$ = apparent Michaelis constant for Phe transport from blood into brain; $K_{m,brain}^{Phe}$ = apparent Michaelis constant for Phe transport from brain into blood; [LNAA] = LNAA concentration; n = number of cases; P = error probability; $[Phe]_{blood}$ = blood Phe concentration; $[Phe]_{brain}$ = brain Phe concentration; $[Phe]_{brain}^{max}$ = maximum brain Phe concentration; $[Phe]_{brain}^{ss}$ = steady-state brain Phe concentration; r = correlation coefficient; t = time; T_2 = spin-spin relaxation time; $T_{2,app}$ = apparent spin-spin relaxation time; T_E = echo time; V_{in}^{Phe} = influx velocity; V_{max}^{Phe} = maximal Phe transport velocity; V_{out}^{Phe} = efflux velocity.

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Laboratory Diagnosis of Phenylketonuria

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Introduction

Phenylketonuria (PKU) is a metabolic disorder that usually results from a deficiency of a liver enzyme known as phenylalanine hydroxylase (PAH, EC.1.14.16.1). This enzyme deficiency leads to elevated levels of the amino acid phenylalanine (Phe) in the blood and in other tissues, and accumulated phenylketones. The untreated state is characterized by mental retardation, microcephaly, delayed speech, seizures, eczema, behavior abnormalities, and other symptoms. A partial deficiency of PAH results in non-PKU hyperphenylalaninemia and a lower degree of blood phenylalanine elevation without phenylketone accumulation. Both forms of hyperphenylalaninemia, which account for the vast majority of cases, are autosomal recessive disorders caused by mutations in the PAH gene. Rarely, mutations in other genes that are necessary for the synthesis or recycling of the tetrahydrobiopterin cofactor of PAH also result in hyperphenylalaninemia (see corresponding chapter).

The introduction of systematic neonatal screening for PKU was based on the prediction by Bickel and co-workers that dietary control of phenylalaninemia in PKU patients might prevent the mental retardation [1]. They wrote: *"It is reasonable to presume that the best results of dietetic treatment of phenylketonuria will be obtained if treatment is started in infancy and particularly in the neonatal period"*. The first pilot schemes used the ferric chloride test (Phenistix®) which was aimed at detecting "phenylketones" in urine; however, it rapidly became evident that the test had its limits due to the fact that the appearance of phenylketones in urine can be delayed.

Robert Guthrie, an American microbiologist, was very concerned with the prevention of mental retardation, and especially by PKU, since his niece had been diagnosed with PKU only at 15 months of age with the ferric chloride test. All his efforts were concentrated on conceiving a simple, reliable, robust and cheap method that would be suitable for mass screening. As a bacteriologist, he adapted a bacteriological method (bacteriological inhibition assay; BIA [2]), initially designed to screen for different antimetabolites in the blood of patients who were treated for cancer, for the measurement of phenylalanine in blood. In addition, he also considered that a mass screening should be based on a simple method to collect the blood (dried blood sample, DBS): *"While I recognize how pivotal the phenylalanine assay was for the development of newborn screening, I have always considered the filter paper blood specimen to be my most important contribution"* [3].

PKU and BH_4 – Advances in Phenylketonuria and Tetrahydrobiopterin
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