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ARTICLE

Phenylalanine Effects on Brain Function in Adult Phenylketonuria

Andrea Pilotto, MD, Carl M. Zipser, MD, Edytha Leks, MD, Dorothea Haas, MD, Gwendolyn Gramer, MD, Peter Freisinger, MD, Eva Schaeffer, MD, Inga Liepelt-Scarfone, PhD, Kathrin Brockmann, MD, Walter Maetzler, MD, Claudia Schulte, Bio, Christian Deuschle, Ann Kathrin Hauser, Georg F. Hoffmann, MD, Klaus Scheffler, MD, Francjan J. van Spronsen, MD, Alessandro Padovani, MD, PhD, Friedrich Trefz, MD, and Daniela Berg, MD

Correspondence
Dr. Pilotto
pilottoandreae@gmail.com

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Abstract

Objective

To evaluate the relationship between circulating phenylalanine and brain function as well as neuropsychiatric symptoms in adults with phenylketonuria.

Methods

In this prospective cross-sectional study, early-treated patients with phenylketonuria older than 30 years and age- and sex-matched controls were included. Extensive neurologic evaluation, neuropsychological and behavioral testing, sensory and motor evoked potentials, and MRI were performed. CSF concentrations of neurodegenerative markers were evaluated in addition in a subset of 10 patients.

Results

Nineteen patients with phenylketonuria (median age 41 years) with different phenylalanine levels (median 873 μmol/L) entered the study. They showed higher prevalence of neurologic symptoms, cognitive and behavioral abnormalities, autonomic dysfunction, alterations in neurophysiologic measures, and atrophy in putamen and right thalamus compared to controls. In CSF, patients with phenylketonuria exhibited higher β-amyloid 1-42 ($p = 0.003$), total tau ($p < 0.001$), and phosphorylated tau ($p = 0.032$) levels compared to controls. Plasma phenylalanine levels highly correlated with the number of failed neuropsychological tests ($r = 0.64$, $p = 0.003$), neuropsychiatric symptoms ($r = 0.73$, $p < 001$), motor evoked potential latency ($r = 0.48$, $p = 0.030$), and parietal lobe atrophy.

Conclusions

Our study provides strong evidence for a correlation between phenylalanine levels and clinical, neuropsychological, neurophysiologic, biochemical, and imaging alterations in adult patients with phenylketonuria.

From the Neurology Unit (A. Pilotto, A. Padovani), Department of Clinical and Experimental Sciences, University of Brescia, Italy; Department of Neurodegeneration (A. Pilotto, I.L.-S., K.B., W.M., C.S., C.D., A.K.H., D.B.), Hertie Institute of Clinical Brain Research (A. Pilotto, C.M.Z., I.L.-S., K.B., W.M., C.S., C.D., A.K.H., D.B.), Department of Neurology and Stroke (C.M.Z.), Department of Biomedical Magnetic Resonance (E.L., K.S.), and German Center for Neurodegenerative Diseases (I.L.-S., K.B., C.S., C.D., A.K.H., K.S.), University of Tübingen, Germany; Parkinson's Disease Rehabilitation Centre (A. Pilotto), FERB ONLUS, S. Isidoro Hospital, Trescore Balneario, Italy; Department of Pediatrics (D.H., G.G., G.F.H., F.T.), Division for Neuropediatrics and Metabolic Medicine, University of Heidelberg; Department of Pediatrics (P.F., F.T.), Reutlingen Hospital; Department of Neurology (E.S., W.M., D.B.), University-Hospital-Schleswig-Holstein, Campus Kiel, Christian-Albrechts-University Kiel; and Division of Metabolic Diseases (F.J.v.S.), Beatrix Children's Hospital, University Medical Center Groningen, University of Groningen, the Netherlands.

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Glossary

Aβ = β-amyloid; **AD** = Alzheimer disease; **AHB** = abductor hallucis brevis; **BDI** = Beck Depression Inventory; **CMCT** = central motor conduction time; **CWD** = cumulative years without diet; **ETP** = early-treated patients with phenylketonuria; **HC** = healthy control; **MEP** = motor evoked potential; **MoCA** = Montreal Cognitive Assessment; **NMSS** = Nonmotor Symptom Scale; **NPI** = Neuropsychiatric Inventory; **p-tau** = phosphorylated tau; **PD** = Parkinson disease; **Phe** = phenylalanine; **PKU** = phenylketonuria; **t-tau** = total tau; **SEP** = somatosensory evoked potential; **TMS** = transcranial magnetic stimulation; **TMT-A** = Trail-Making Test part A; **TMT-B** = Trial-Making Test part B; **VBM** = voxel-based morphometry; **WMH** = white matter hyperintensities.

Phenylketonuria (PKU) is the most common monogenetic metabolic disorder of amino acid metabolism, with an estimated frequency of 1 in 10,000 newborns. It is characterized by mutations within *PAH* genes, leading to deficient conversion of phenylalanine (Phe) to tyrosine. Phe blood concentrations are increased and Phe crosses the blood–brain barrier, causing detrimental effects through phenylpyruvate on brain development and function.[1]

Starting a Phe-restricted diet right after screening at birth has led to an increased life expectancy of affected patients and improvement of global cognitive function being within the normal range, with the first early-treated patients with PKU (ETPs) now entering their middle ages. This entails a new era for prognostic markers as well as treatment targets for PKU in adulthood.[2–5]

Several series in children and young adults showed a correlation between Phe levels and neuropsychological outcomes[3] as well as imaging alterations.[6,7] In adulthood, however, when complete maturation of the brain can be assumed, the relationship between Phe and brain pathology is less evident.[8–11] Preclinical studies suggested that brain aging may increase the risk for neurologic decline in ETPs given the presence of metabolic abnormalities, Phe aggregation, oxidative damage, and biogenic amine deficits.[12,13] For this reason, current guidelines for adult PKU suggest plasma Phe target levels of <600 μmol/L[14] and <360 μmol/L[15] in Europe and the United States, respectively. These targets were set to limit neurologic/psychiatric complications but they are still under discussion, as they are not supported by large clinical studies in adult PKU.[16]

We used an extensive protocol to characterize this target population, including clinical, neurophysiologic, CSF, and imaging assessments, in order to evaluate differences in ETPs compared to age-matched controls and correlation with Phe levels.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

All participants signed written informed consent for the study. The study was approved by the Ethics Committees of the Universities of Tübingen and University of Heidelberg (S-517/2014). The study conforms to The Code of Ethics of the World Medical Association.

Inclusion Criteria and Selection of Patients With PKU

Adult ETPs were contacted by the metabolic centers in Tübingen, Heidelberg, and Reutlingen and via website with support of the German Phenylketonuria Society (DIG-PKU) and PKU board (pku.eu/PKU_Info/PKU_Board.html). The study procedure was explained via a telephone call. If patients agreed to participate in the study, they were invited to come to the study center at the University of Tübingen in order to receive the complete assessment performed by a neurologist (A. Pilotto). Age- and sex-matched healthy controls (HCs, n = 25) were recruited from students and coworkers at the University of Tübingen and underwent the same clinical assessments and neuropsychological, physiologic, and MRI protocols.[17] Inclusion criteria were (1) ETPs older than 30 years of age and (2) medical history data about severity of PKU (maximum pretreatment Phe blood levels in the newborn period or genotype of the *PAH* gene). Exclusion criteria were (1) the presence of any disability that may prevent the patient from completing the informed consent form or other study requirements, (2) participation in clinical trials with new investigational compounds or therapies within 4 weeks prior to baseline visit, (3) alcohol or drug dependency or abuse (except for nicotine), and (4) specific contraindications for 3T MRI: pacemaker, other stimulation device or metal, and large tattoos.

Neurologic Assessments and Biochemical Markers

A detailed medical history was taken including former and current concomitant diseases as well as medication; for each participant, cumulative years without diet (CWD) were calculated, defined as self-reported number of years without any Phe-restricted diet or Phe plasma tests after age 18 years. The neurologic assessment included an evaluation of cranial nerves, motor, sensory, cerebellar, and basal ganglia–related function. In case mild parkinsonian signs were detected, they were graded using the motor part of the Movement Disorders Society–sponsored version of the Unified Parkinson's Disease Rating Scale.[18] All patients underwent measurement of plasma Phe levels, blood cell count, renal and liver function measures, vitamin D, vitamin B_{12}, and folate levels, to exclude common non-PKU-related causes for neurologic symptoms.

Cognitive and Behavioral Assessment

The following cognitive domains were evaluated[19]: global cognition (Montreal Cognitive Assessment [MoCA]), executive function (Trial-Making Test part B [TMT-B], phonemic and semantic fluencies, Tower of London), attention/working memory (digit span, Trail-Making Test part A [TMT-A], test D-2), visual performance (Rey-Osterrieth figure copy and clock drawing test), memory (short story of the Wechsler logic memory subtest, Rey-Osterrieth figure recall), language (token test, Boston Naming Test, and semantic fluency) and ideomotor apraxia (Berlin Apraxia Test). The neuropsychological deficits were scored as abnormal if they were below 1 SD compared to age-, sex-, and education-matched controls. The number of pathologic test results for each domain and in total were defined for each participant. Behavioral disturbances and depression were tested using Neuropsychiatric Inventory (NPI) and Beck Depression Inventory (BDI-II).

Additional Nonmotor Symptoms

Presence and severity of common nonmotor symptoms, which are known to occur in prodromal and clinical phases of, e.g., Parkinson disease (PD) and Alzheimer disease (AD) (such as hyposmia and autonomic and sleep deficits), were evaluated using Sniffin' Sticks[20] and questionnaires such as the REM Sleep Behavioural Disorder Screening Questionnaire and the Nonmotor Symptom Scale (NMSS).[18,21]

Motor and Sensory Evoked Potentials

Participants were seated during motor evoked potential (MEP) recording. Transcranial magnetic stimulation (TMS) applied to elicit MEP in the lower extremities was delivered using a Neurosoft Evidence 9000 MS stimulator connected to a round-coil with a diameter of 150 mm (Neurosoft Company, Ivanovo, Russia) with a biphasic current waveform. Central motor conduction time (CMCT) was calculated using the F-wave approach.[22] The optimal coil position over the feet area of the left M1 area for eliciting MEPs in the right abductor hallucis brevis muscle (AHB), for right M1 stimulation respectively in the left AHB, was determined as the site where TMS at a suprathreshold intensity consistently produced the largest MEPs—stimulation output was between 80% and 100% of maximal stimulator output. Somatosensory evoked potentials (SEPs) were maintained from the tibial nerve, recording from Cz with a single cup-electrode. Stimulation intensity was slightly higher than the one that was evoking a motor response and approximately 150 trials were averaged. SEPs and F-wave examination were performed on Dantec Keypoint neurophysiology equipment (Natus Medical Inc., San Carlos).

Neurodegenerative CSF Markers and Biochemical Investigations

CSF markers levels were evaluated in 10 ETPs and 21 age- and sex-matched neurologically healthy controls of whom CSF was stored in the Neuro-biobank of Tübingen. Specific contraindications for lumbar puncture were thrombocytopenia (<80,000/mL) or clinical evidence for increased bleeding probability, including ongoing anticoagulant therapy. Lumbar puncture was performed between 8:00 and 10:00 AM after night fasting. CSF was centrifuged at 4,000*g* for 10 minutes at 4°C and stored at −80°C within 60 minutes after collection until analysis. All samples showed normal routine CSF diagnostics (leukocytes $<4 \times 10^6$/L; immunoglobulin G index CSF/plasma <0.6). CSF concentrations of β-amyloid (Aβ) 1-42, total tau (t-tau), and phosphorylated tau (p-tau) were measured using commercially available ELISA kits (INNOTEST, Fujirebio, Belgium).[23] The measurements were performed by board-certified laboratory technicians who were blinded to the clinical data.

MRI Structural Imaging Analyses

Anatomical T1-weighted MRI was acquired using a 3T scanner (PRISMA, Siemens AG Erlangen, Germany) with a 20-channel array head coil. In order to obtain a high-resolution anatomical image of each participant's brain, an anatomical T1-weighted 2D magnetization-prepared rapid gradient echo sequence was used (repetition time/inversion time/echo time 2,300/900/4.18 ms, flip angle 9°, field of view 256 × 256 mm^2, matrix 256 × 256, 176 slices, voxel size 1 × 1 × 1 mm^3). We used the CAT 12 toolbox (Computational Anatomy Toolbox 12) of the structural brain mapping group (Jena University Hospital, Germany), which is implemented in Statistical Parametric Mapping 12 (Institute of Neurology, London, UK) for voxel-based morphometry (VBM) analysis.[24] For white matter hyperintensities, a global burden was calculated using Fazekas scale for periventricular and deep white matter lesions.[25] An optimized method of VBM segmentation was applied using customized templates and prior probability maps with diffeomorphic anatomical registration exponentiated lie algebra (Dartel).[25] Images were smoothed with an 8-mm full-width at half-maximum smoothing kernel.

Statistical Analyses

Clinical and demographic characteristics as well as neurologic and plasma Phe level comparisons between ETPs and HCs were performed using the Mann-Whitney test and the χ^2 test for continuous and dichotomous variables, respectively. The neuropsychological and behavioral test comparisons were performed with nonparametric Mann-Whitney testing on age/sex- end education-adjusted scores.

The correlation between (1) cognitive/behavioral tests, (2) nonmotor symptoms, (3) MEP/SEP latencies, and (4) CSF neurodegeneration markers and Phe plasma levels as well as CWD were evaluated by using partial correlation adjusted for the effect of age, sex, and educational level by Pearson test, significance set by $p < 0.05$.

Imaging Analyses Statistical Design

VBM analysis was used to define the regions differing significantly (1) between ETPs and HCs and (2) between patients with PKU with actual plasma Phe levels above and below 3

different cutoffs: the suggested 360 μmol/L and 600 μmol/L and the median Phe value of the cohort. The VBM analyses were corrected for the effect of age, sex, and educational level by using *t* test and analyses of variance, respectively. A linear regression model adjusted for the effect of age, sex, and educational level was applied to evaluate the positive and negative correlation between gray matter atrophy and actual plasma Phe as well as CWD. Cluster threshold was set at 100 voxels for both analyses.

Data Availability

All the data presented (including clinical, biomarker, and imaging findings) will be available in a trusted data repository in an anonymized form upon reasonable request by any qualified investigator. Any data not published within the present article are available or in a public repository.

Results

Nineteen adult (median age 41 years, range 30–45 years, 12 [63.2%] female) ETPs (i.e., treated with Phe-restricted diet before 1 month of age) entered the study. Ten patients never stopped the diet while the other 9 patients presented different treatment histories and a number of cumulative years without diet ranging from 3 to 24 years, not starting before 18 years of age. Clinical findings, dietary aspects, as well as neurologic and neuropsychological measures are summarized in tables 1 and 2. Fourteen patients reported Phe-restricted diet (with different Phe levels at the time of examination, as outlined in table 2), while 7 patients reported not following a disease-specific diet. One patient following the diet was also treated with sapropterin dihydrochloride (Sapropterin; BioMarin Pharmaceutical Inc., Novato, CA), a synthetic formulation of BH4, a cofactor for PAH reducing Phe concentrations. None of the patients was treated with selective serotonin or noradrenaline reuptake inhibitors, and none of the patients had a previous diagnosis of attention-deficit/hyperactivity disorder or of major psychiatric disease. In general, the patients with PKU showed high educational levels, were socially integrated and active in their lives, as confirmed by partners and caregivers, and were aware of the importance of metabolic control in adulthood. Patients with PKU exhibited more depressive episodes during lifetime (28.6% vs 4%, $p = 0.04$) with no differences in comorbidities (table 1). No significant differences between patients with PKU and controls were observed concerning educational level and family history for tremor/parkinsonism or dementia. Plasma Phe levels at the time of examination were significantly higher in patients with PKU and ranged from 57 to 2,100 μmol/L (median 873 μmol/L) vs 33–64 μmol/L (median 44 μmol/L) in controls.

Table 1 Demographic and Clinical Characteristics of Early-treated Patients With Phenylketonuria (ETPs) and Healthy Controls (HCs)

	HCs (n = 25)	ETPs (n = 19)	*p* Value
Demographics			
Age, y	34 (30–40)	41 (35–44)	0.139[a]
Sex, female	60 (15)	63.2 (12)	0.719
Educational level, y	15 (13–18)	14 (11–17)	0.262[a]
Comorbidities			
Hypertension	0.0 (0)	15.8 (3)	0.230
Migraine	4.0 (1)	21.1 (4)	0.340
Lifetime depression	4.0 (1)	31.5 (6)	0.042
Family history			
Dementia	8.0 (2)	16.7 (3)	0.603
Tremor	8.0 (2)	5.6 (1)	1.000
Blood analyses			
Phe plasma level, μmol/L	44 (38–50)	873 (644–1,115)	<0.001[a]
Neurologic examination			
Hyperreflexia	0.0 (0)	52.6 (10)	<0.001
Tremor	0.0 (0)	21.1 (4)	0.039
Bradykinesia/ hypokinesia	0.0 (0)	17.6 (3)	0.104
Rigidity	0.0 (0)	0.0 (0)	1.000
Slowed saccades	0.0 (0)	31.6 (6)	0.020
Supranuclear gaze palsy	0.0 (0)	5.9 (1)	0.486
MDS-UPDRS-III total score	0 (0–0)	0 (0–2)	0.38[a]
Nonmotor symptoms			
Sniffin' Sticks, total score	12 (11–12)	10 (9–11)	<0.001[a]
Hyposmia	0 (0)	15.8 (3)	0.230
RBDSQ, total score	2.0 (2.0–3.0)	3.0 (2.0–5.0)	0.08[a]
RBD	0 (0)	15.8 (3)	0.230
NMSS, total score	0 (0–0)	2.0 (1.0–5.0)	<0.001

Abbreviations: MDS-UPDRS-III = Movement Disorders Society–sponsored version of the Unified Parkinson's Disease Rating Scale part III; NMSS = Nonmotor Symptom Scale; Phe = phenylalanine; RBD = REM sleep behavioral disorder; RBDSQ = REM Sleep Behavioral Screening Questionnaire.
Data are median (interquartile range) or (%) n.
p Values were calculated by Fisher exact test or [a] Mann-Whitney *U* test.

Neuropsychiatric Symptoms

Neurologic Examination

The neurologic examination showed a higher prevalence of hyperreflexia in patients with PKU compared to controls (27.6% vs 0%, $p < 0.001$; note that all ETPs had negative pyramidal signs), kinetic tremor (21.1% vs 0%, $p = 0.039$), and slowed horizontal saccades (31.6% vs 0%, $p = 0.020$). Three ETPs (17.6%) but no HCs presented with bradykinesia and hypokinesia ($p = 0.09$).

Table 2 Clinical Characteristics of Patients Listed by Phenylalanine Levels in Plasma (Phe-PL)

ID	*PAH* mutations	Age, y/sex	CWD	Phe-PL, μmol/L	Phe level in CSF, μmol/L	MP	MoCA	EX	ATT	VS	MEM	LAN	BDI	MEP	SEP
1	p.R408W/ IVS10nt-11g>a	38/F	18	2,100		+	29	2	3	0	2	1	28	−	+
18	p.R158Q/ p.R158Q	45/M	10	1,312		+	19	3	1	0	2	1	17	−	−
5	p.P281L/ p.R408W	44/M	0	1,284		−	30	3	2	0	1	1	12	−	−
15	p.R408W/ IVS12nt1	46/M	0	1,150		−	24	3	2	0	2	1	14	+	+
16	p.F55fs/ IVS7+1G>A	43/M	24	1,115	226	+	27	3	2	0	1	1	5	−	−
12	p.F39L/p.R252W	37/F	3	1,092		−	23	3	3	0	2	0	0	+	−
13	IVS+1G>A/ IVS12+1G>A	33/F	11	1,051		+	27	3	2	0	2	1	39	+	+
17	p.R261Q/ IVS7+3g>c	35/F	0	1,043		−	28	3	1	0	1	1	0	+	−
4	p.R158Q/ p.R158Q	36/F	0	978		−	28	3	2	0	2	1	3	+	+
21	p.S349P/ p.L348V	32/M	6	873	233	−	30	1	0	0	2	0	0	−	−
9	p.G239V/ IVS10nt-11g>a	43/F	0	861		−	28	1	3	0	1	1	3	−	−
22	p.R408W/IVS10-3C>T	31/F	0	821	196	−	26	1	2	1	0	1	2	−	−
7	p.R261Q/ p.R408W	30/M	0	770	145	−	30	1	2	0	0	0	0	−	−
3	p.L48S/p.Y387H	44/F	0	760[a]	194[a]	−	29	2	2	0	2	1	1	−	
19	p.R261Q/p.G272	45/M	5	598	153	−	28	1	0	0	0	0	1	−	+
11	p.L48S/p.R408W	39/F	12	567	97	−	27	3	2	0	1	1	2	+	−
6	p.R408W/ p.R408W	45/F	0	340	57	+	30	1	1	0	1	0	0	−	−
20	p.R158W/c.753_754delTC	41/F	15	138		+	28	1	2	0	1	0	4	−	−
14	p.P281L/ IVS12+1G>A	42/F	0	57	116	−	22	3	3	0	2	1	0	+	−

Abbreviations: + = abnormal; − = normal; ATT = attention domain; BDI = Beck Depression Inventory; CWD = cumulative number of years without diet; EX = executive domain; LAN = language domain; MEM = memory domain; MEP = motor evoked potential; MoCA = Montreal Cognitive Assessment; MP = mild parkinsonism or tremor; *PAH* = phenylalanine hydroxylase gene; SEP = somatosensory evoked potential; VS = visuo-spatial domain.
The table summarizes demographics and clinical characteristics, including the number of tests with deficits within the different cognitive domains. Memory, executive, visuospatial, and language domains were assessed by 2 tests; attention was assessed by 4 tests (see Methods for further details).
[a] Plus sapropterin dihydrochloride treatment (Kuvan).

Cognitive and Behavioral Assessment

ETPs presented with poorer performances than controls in 7 out of 14 tests of the battery, assessing executive (TMT-B and semantic fluency, $p < 0.001$), attentional (TMT-A, $p = 0.003$, digit span, and test D2, $p < 0.001$) and memory (short story and Rey-Osterrieth figure recall, both $p < 0.001$) domains (table 3). Global cognition assessed by MoCA also showed lower performance in ETPs compared to HCs ($p = 0.002$).

ETPs presented with more/more severe depressive and behavioral symptoms (BDI-II, $p = 0.006$; total NPI, $p < 0.001$). Specifically, ETPs showed higher prevalence of agitation ($p = 0.005$), depression ($p = 0.002$), anxiety ($p < 0.001$), apathy ($p < 0.001$), and irritability ($p < 0.001$). All the differences in cognitive testing between ETPs and HCs were confirmed in covariance analyses using BDI-II as cofactor, to adjust for presence and severity of depressive symptoms.

Table 3 Cognitive and Behavioral Measures in Early-Treated Patients With Phenylketonuria (ETPs) and Healthy Controls (HCs)

	HCs (n = 25)	ETPs (n = 19)	*p* Value
Global cognition			
MoCA	30 (29–30)	28.0 (26.0–29.0)	0.002
Executive function			
Phonemic fluency	21 (20–23)	16 (13–23)	0.03
Semantic fluency	34 (30–36)	25 (17–29)	<0.001
Trail-Making Test part B	41 (32–46)	70 (58–90)	<0.001
Tower of London	18 (17–19)	16 (14–18)	0.085
Attention/working memory			
Digit span	19 (18–21)	14 (12–16)	<0.001
Trail-Making Test part A	20 (18–25)	29 (23–40)	0.003
Test D2	258 (240–275)	144 (95–177)	<0.001
Visuospatial function			
Rey-Osterrieth figure, copy	36 (35–36)	36 (34–36)	0.986
Clock drawing	10 (10–10)	10 (10–10)	1.000
Memory			
Short story recall	20 (18–22)	13 (11–18)	<0.001
Rey-Osterrieth recall	30 (28–32)	21 (15–29)	<0.001
Language			
Token test	36 (36–36)	36 (36–36)	0.27
Boston Naming Test	15 (15–15)	15 (14–15)	0.146
Apraxia			
BAXT ideomotor apraxia	35 (33–35)	33 (30–36)	0.457
Behavioral assessment			
BDI-II	0 (0–2)	2 (0–12)	0.006
NPI	0 (0–1)	7 (2–19)	<0.001

Abbreviations: BAXT = Berlin Apraxia Test; BDI-II = Beck Depression Inventory; MoCA = Montreal Cognitive Assessment; NPI = Neuropsychiatric Inventory.
Data are median (interquartile range).
p Values were calculated by Mann-Whitney *U* test.

Additional Nonmotor Symptoms

ETPs showed worse performance in Sniffin' Sticks assessment ($p < 0.001$) and higher NMSS score compared to HCs ($p < 0.001$). Specifically, ETPs exhibited higher frequency of tenesmus ($p = 0.017$), urinary loss ($p = 0.007$), nycturia ($p = 0.001$), memory impairment and loss of interest (both $p = 0.017$), concentration difficulties ($p = 0.007$), insomnia ($p = 0.003$), and "dream alterations or movement during sleep" ($p = 0.007$).

Association Between Phe Levels and Symptoms

For the correlation between actual Phe levels and symptoms, we excluded patient 14, as she presented with a low plasma (57 μmol/L) but high CSF Phe level (116 μmol/L), which is most likely due to the known delayed exchange of Phe into the CSF[9] and a restriction of Phe intake shortly before the examination.

Circulating Phe Levels Did Not Correlate With CWD in Partial Correlation Analyses

Higher plasma Phe levels were associated with higher total number of pathologic cognitive tests ($r = 0.67$, $p = 0.003$, figure 1A) and also with higher number of deficits in the following domains: attention ($r = 0.517$, $p = 0.028$), executive function ($r = 0.472$, $p = 0.048$), and language ($r = 0.660$, $p = 0.003$).

Higher plasma Phe levels correlated with the severity of impairment in short story recall ($r = -0.71$, $p = 0.001$). Plasma Phe levels correlated significantly with presence and severity of depressive symptoms as assessed with the BDI-II ($r = 0.58$, $p = 0.010$) and neuropsychiatric symptoms as assessed with the NPI ($r = 0.72$, $p = 001$).

The CWD showed a significant correlation with the NPI total score ($r = 0.51$, $p = 0.030$) but not with any of the cognitive tests.

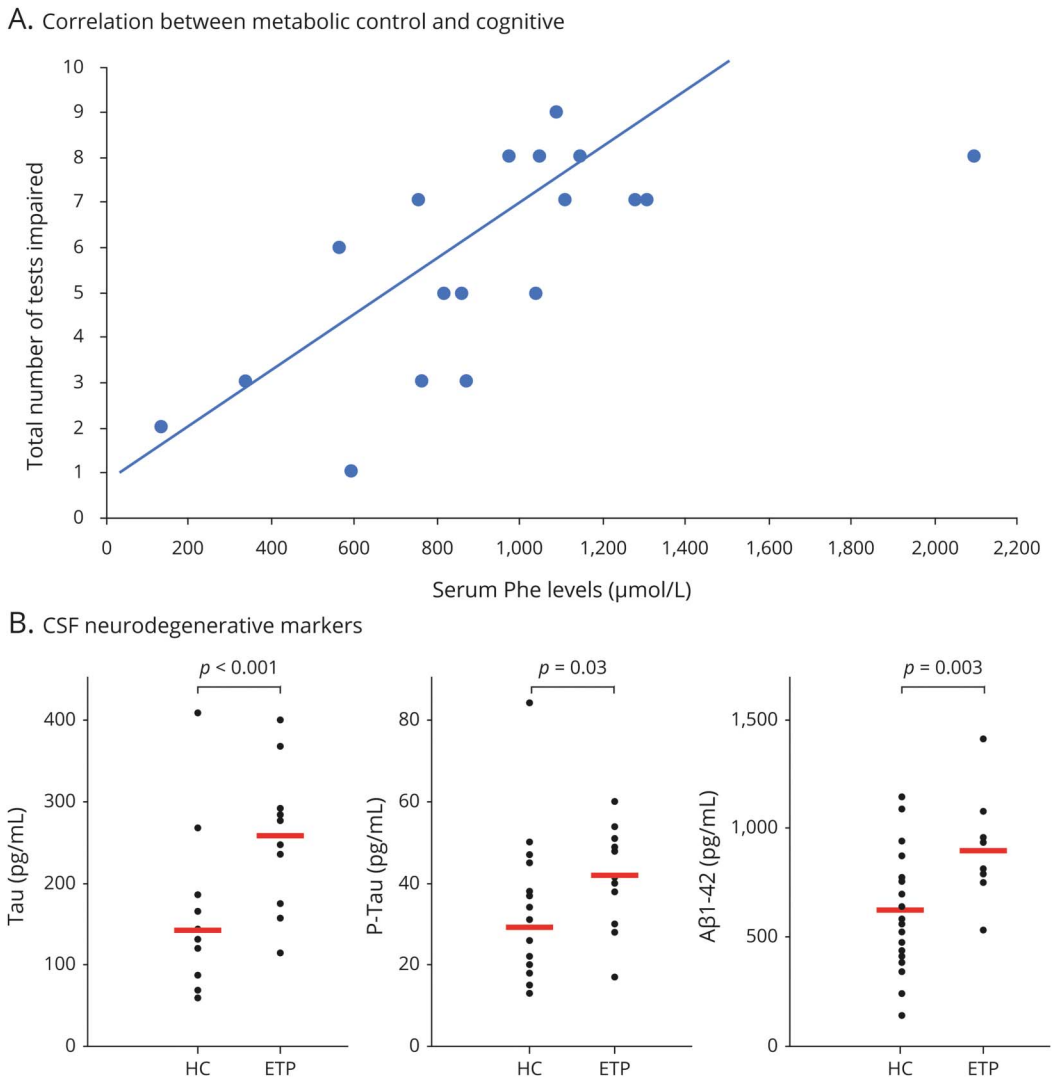
Exploratory Analyses of Application of Current Guidelines' Phe Cutoff Levels

Only 4 ETPs presented with plasma Phe levels below the newly suggested cutoff level of <600 μmol/L[14]; among them, 3 patients showed a level below <360 μmol/L. ETPs presenting with plasma Phe levels above and below the 600 μmol/L cutoff were comparable regarding age and educational levels. ETPs presenting Phe <600 μmol/L exhibited no differences in NPI scores but lower number of pathologic cognitive tests ($p = 0.018$) and better performances in language domain ($p = 0.008$) and semantic fluency ($p = 0.03$), compared to ETPs >600 μmol/L. ETPs with Phe >360 μmol/L showed higher NPI scores ($p = 0.05$) and a trend for higher numbers of pathologic tests ($p = 0.06$) compared to ETPs below this cutoff.

Neurophysiologic Measures

The CMCT was comparable between ETPs and HCs; 7 ETPs and 1 HC presented with pathologic MEP latency ($p = 0.053$; table 4). The median SEP latencies did not significantly differ between ETPs and HCs; however, 5

Figure 1 Phenylalanine (Phe) Levels, Cognitive Alterations, and CSF Neurodegenerative Markers in Early-Treated Patients With Phenylketonuria (ETPs)



(A) Correlation between cumulative number of cognitive tests impaired and plasma Phe levels. (B) CSF neurodegenerative markers in patients with phenylketonuria and age-matched healthy controls (HCs). Aβ = β-amyloid; p-tau = abnormal phosphorylated form of tau protein; t-tau = total tau protein.

Table 4 Neurophysiologic Measures, White Matter Hyperintensities (WMH), and CSF Neurodegenerative Markers in Early-treated Patients With Phenylketonuria (ETPs) and Healthy Controls (HCs)

Neurophysiology measures	HCs (n = 15)	ETPs (n = 19)	*p* Value
CMCT R, ms	12.4 (11.2–14.6)	12.2 (9.8–13.7)	0.464[a]
CMCT L, ms	13.5 (10.9–15.0)	11.6 (9.7–13.2)	0.052[a]
Abnormal MEP	6.7 (1)	36.8 (7)	0.053
SEP latency R, ms	41.1 (39.1–42.6)	41.3 (37.1–44.6)	0.983[a]
SEP latency L, ms	41.2 (39.6–42.9)	40.6 (38.0–44.9)	0.677[a]
Abnormal SEP	0 (0)	26.3 (5)	0.027
WMH burden	HCs (n = 21)	ETPs (n = 19)	
Score, periventricular lesions	0 (0–0)	0 (0–1)	0.307
Score, deep	0 (0–0)	0 (0–1)	0.692
CSF neurodegenerative markers, pg/mL	HCs (n = 21)	ETPs (n = 10)	
$A\beta_{1\text{-}42}$	583 (399–766)	811 (765–952)	0.003[a]
t-Tau	121 (86–155)	248 (206–289)	<0.001[a]
p-Tau	31 (21–31)	41 (34–50)	0.032[a]

Abbreviations: Aβ = β-amyloid; CMCT = central motor conduction time; MEP = motor evoked potential; p-tau = abnormal phosphorylated form of Tau protein; SEP = somatosensory evoked potential; t-tau = total tau protein.
Data are median (interquartile range) or (%) n.
p Values were calculated by Fisher exact test or [a]Mann-Whitney *U* test.

ETPs presented with abnormal SEP ($p = 0.027$). MEP and SEP latencies showed positive correlations with Phe levels ($r = 0.48$, $p = 0.03$; and $r = 0.51$, $p = 0.03$, respectively). No significant correlation was found between CWD and neurophysiologic measures. All 7 ETPs with abnormal MEP and all but 1 with abnormal SEP presented with plasma Phe levels >600 μmol/L.

CSF Neurodegenerative Markers

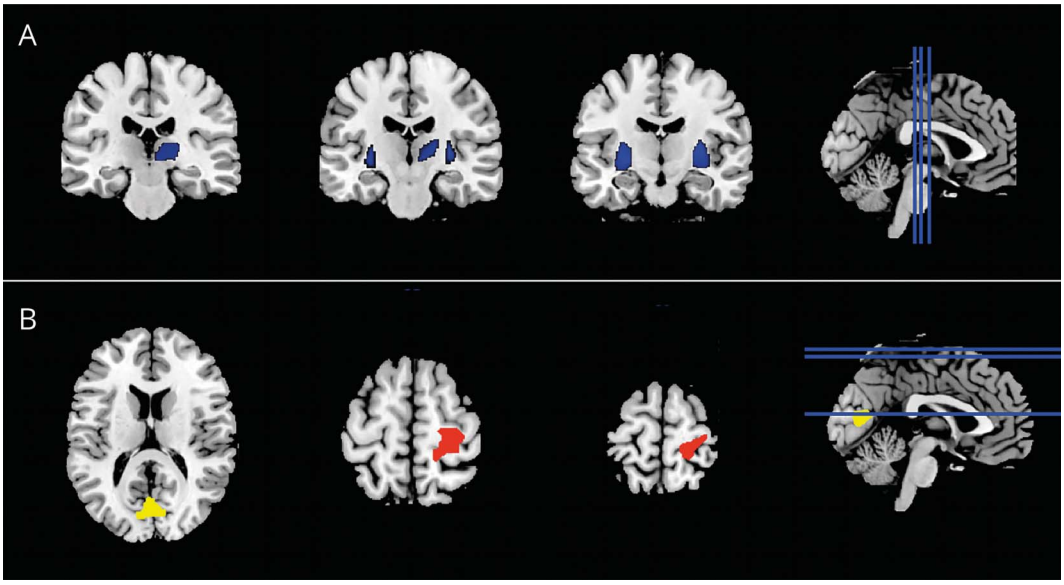
Ten ETPs (mean age 38.2 ± 5.8 years, 6 female) underwent CSF analyses and were compared to samples obtained from 21 age-matched controls (different from the clinical control cohort, see Methods; mean age 40.7 ± 9.2). ETPs showed significantly higher $A\beta_{1\text{-}42}$ (811 pg/mL vs 583 pg/mL, $p = 0.003$), higher t-tau (248 pg/mL vs 121 pg/mL, $p < 0.001$),

Table 5 Brain Atrophy in Early-Treated Patients With Phenylketonuria (ETPs) vs Healthy Controls (HCs) and Correlation With Metabolic Control

Localization	Cluster size, number of voxels	pseudoT	MNI coordinates (x, y, z)
HCs vs PKU			
Right putamen[a]	580	5.88	30, −15, 8
Left putamen[a]	550	5.20	−22, 12, −38
Right thalamus	435	4.45	12, −20, 0
Phe negative correlation			
Right parietal lobe BA3	115	5.37	20, −38, 69
CWD negative correlation			
Right occipital lobe, cuneus BA23	177	4.89	2, −74, 12

Abbreviations: BA = Brodmann area; MNI = Montreal Neurologic Institute; Phe = phenylalanine; PKU = phenylketonuria.
The analyses were performed by using Statistical Parametric Mapping 12. All analyses have been adjusted for the effect of age, sex, and educational level, threshold set at $p < 0.001$ uncorrected, with a minimum cluster size 100 voxels.
[a] Survived at family-wise error correction.

Figure 2 Brain Atrophy in Early-Treated Patients With Phenylketonuria (ETPs) vs Healthy Controls (HCs) and Correlation With Metabolic Control



Results are represented on a multislice viewing implemented by MRIcron. (A) Clusters of gray matter atrophy (blue) in ETPs vs healthy controls including bilateral putamen and left thalamus. (B) Atrophy in gray matter regions significantly correlated to increasing numbers of years without diet (yellow-occipital lobe) and phenylalanine levels (red, parietal lobe).

and higher p-tau values (41 pg/mL vs 31 pg/mL, $p = 0.03$) (figure 1B). t-Tau levels correlated with CWD (0.029, $r = 0.720$), whereas no correlations were found between CSF tau, p-tau, and $A\beta_{1\text{-}42}$ markers and CSF/plasma Phe levels after adjusting for the effect of age and sex.

Imaging

No relevant structural abnormalities, e.g., lacunes or parenchymal defects, were detected in any of the investigated participants. Based on visual inspection, T2-weighted images of ETPs did not show prominent white matter hyperintensities (WMH) and visual rating was similar between ETPs and HCs.

The age-/sex-/education-corrected results of VBM analyses on gray matter atrophy are presented in table 5 and figure 2. ETPs showed atrophy in the right and left putamen (both surviving at family-wise error correction) and right thalamus (significant only at $p = 0.001$ uncorrected threshold) compared with matched controls.

Plasma Phe levels and CWD correlated significantly with atrophy within the right parietal lobe (Brodmann area 3) and right occipital lobe (Brodmann area 23), respectively.

Discussion

This study presents the first exhaustive overview of functional, biochemical, and morphologic changes of the nervous system in adult ETPs. Importantly, our results argue in favor of a consequent and continuous metabolic control of Phe concentrations also in adults.

The sample included ETPs with a median age of 41 years and was highly heterogeneous in terms of treatment history and metabolic control in adulthood, as highlighted by the different actual plasma Phe levels ranging from 57 to 2,100 μmol/L.

Lifetime depression was the most common comorbidity in adult PKU, in line with disease registries[26,27] and potentially related to low serotonergic levels in adulthood.[9]

The neurologic examination confirmed the high frequency of hyperreflexia and kinetic tremor reported recently,[10] with no relevant effect on normal activities of daily living. Of interest, we observed slow saccades in almost one-third of the patients, and 3 of these rather young adults presented with mild parkinsonian signs. The question whether bioaminergic, particularly dopaminergic,[9,28,29] deficits may explain this phenomenon needs to be investigated in more detail.

Compared to controls, the neuropsychological assessment revealed lowered performances in the whole PKU cohort in several tests assessing attention and executive function but also in memory, consistent with previous reports about younger patients with PKU.[10,30,31] The behavioral assessment highlighted the high prevalence of depressive symptoms in adult ETPs, together with other neuropsychiatric features such as anxiety, agitation, apathy, and irritability.

Our study shows strong correlations between actual plasma Phe levels and the number of impaired neuropsychological domains as well as the severity of neuropsychiatric symptoms in adult ETPs. The cognitive differences were confirmed using

the newly proposed cutoff of 600 μmol/L,[14] whereas the CWD and the cutoff of 360 μmol/L correlated only with severity of neuropsychiatric symptoms. This suggests that behavioral abnormalities may be triggered by chronic Phe elevations through adulthood, in line with findings reported on mood by Ten Hoedt et al.[33] and Anjema et al.[34] and on social skills by Jahja et al.[35] Taken together, these data argue strongly for a consequent and continuous metabolic control of Phe in ETPs in adulthood as Phe levels seem to have an important effect on neurocognitive symptoms.

We detected higher prevalence of sleep disorder in ETPs and 3 patients presented with probable REM sleep behavioral disorder, a condition associated with a long-term risk of 50%–80% for developing an α-synucleinopathy.[35,36] Several of our patients with PKU also presented with urinary dysfunction, rare in controls in this age, another important prodromal PD symptom.[21] The severity of nonmotor symptoms correlated strongly with both actual plasma Phe levels and CWD, suggesting a potential effect of Phe toxicity in central and peripheral nervous system, which is supported by neurophysiologic measures (see below). The study also found olfactory dysfunction in ETPs, the most important early symptom for PD but also AD and other neurodegenerative diseases.[20] In PKU, however, an alteration in olfaction might be due to different diet regimen during development resulting in different food acceptance and test behavior.[37]

Motor and sensory evoked potential assessment showed that alterations in both motor and sensory pathways are present in up to one third of ETPs, even in the absence of neurologic symptoms. All but one patient with abnormal neurophysiologic measures presented with Phe values higher than the proposed 600 μmol/L. At the same time, we observed normal MEP and SEP in several patients with many years without any diet but in good metabolic control at the time of examination. These findings suggest that neurophysiologic abnormalities are at least partially reversible, as already demonstrated in children.[38] Further studies focusing on MRI-T2 WMH (more common but not significantly in ETPs than in HCs, based on qualitative visual inspection) or diffusion tensor imaging sequences may help to confirm our findings and to better understand the relationship of our neurophysiologic measures with WMH abnormalities.[39]

Our study evaluated CSF neurodegenerative markers for both amyloid and tau pathology. Amyloid-like pathology was suggested to occur more commonly in PKU,[13] and the authors hypothesized that Phe may aggregate, comparably to Aβ, with cytotoxic effects in vitro. Our in vivo findings with increased CSF $A\beta_{1\text{-}42}$ levels are not supportive for the presence of Aβ-related AD pathology in ETPs. One possible explanation of significantly higher $A\beta_{1\text{-}42}$ levels in ETPs compared to age-matched controls may be the effect of Phe on blood–brain function, possibly facilitating the transport of $A\beta_{1\text{-}42}$ to the CSF.[40] Another explanation could be that accumulation of Phe (and associated metabolites) in the brain alters the normal $A\beta_{1\text{-}42}$ levels in CSF.[12]

Furthermore, we found a significant increase in t-tau and p-tau in CSF of ETPs, which are markers of neuronal or axonal damage in AD and other neurodegenerative diseases.[41,42] Overall, these results argue for brain alterations associated with neuronal damage at different levels and speed if compared to healthy individuals of the same age. We also found an interesting correlation between the cumulative number of years without diet and tau CSF levels. Thus, future studies need to investigate larger cohorts over longer periods with serial Phe levels possibly including promising peripheral neurodegenerative markers including neurofibrillary light chains.[43]

Structural brain imaging using VBM found striking atrophy in bilateral putamen and right thalamus in the whole ETPs cohort. These findings are at least partly in contrast with previous reports that reported no subcortical alterations in presence of extensive[6] or slight cortical alterations.[44] It should be mentioned, however, that several earlier studies included late-treated patients and did not correct for the effect of education, which is often different from controls,[6,7] and all previous studies focused on younger populations far from experiencing effects of brain aging or potential neurodegeneration.

An enlargement of the putamen was described in young patients with PKU[7] and might represent compensatory effects in this region, potentially leading to subsequent atrophy, as recently suggested for monogenic AD.[45] We could also speculate that chronic bioamine depletion, recently described in adult patients with PKU,[9,28] might lead to complex dysfunction in bioaminergic circuits, in which putamen and thalamus are important hubs.[46]

The atrophic regions we found correlated with Phe included the parietal precuneus, a brain region integrating information from temporal, occipital, and parietal lobes associated with early phases of AD.[47] Thus, the association between the atrophy of this specific area with plasma Phe levels might reflect the interaction of the time-dependent changes in the human brain and chronic brain damage secondary to insufficient metabolic control in adulthood.

The main limitation of the study is the relatively small sample size of the cohort, with the exclusion of patients with significant psychiatric diseases, as well as malnutrition or vitamin deficits potentially contributing to cognitive deficits. On the one hand, this helped us collect a comprehensive and easily interpretable dataset that is built on a cohort of highly motivated participants with good educational and social skills. On the other hand, we probably excluded (more) severely affected patients with PKU, with the potential consequence that the effect of Phe levels on brain damage in a naturalistic adult PKU sample may be substantially underestimated in our study results. Another limitation is the lack of lifetime history data evaluating metabolic control over the years and a standardized measure of cognitive performances during young age to examine in more detail the contribution of young and adult age.

This study is the first presenting an exhaustive overview of neuropsychiatric symptoms, neurodegenerative markers, and neurophysiologic and imaging alterations in ETPs continuously treated during brain development. It provides first evidence for a high and direct association between brain function and metabolic control in adulthood and supports the guidelines for diet in adult patients with PKU.

These pilot results should motivate larger multicenter studies evaluating different plasma Phe cutoff values in order to identify the best treatment target in this population. Moreover, longitudinal follow-up is needed to evaluate progression of brain changes and the interaction of Phe metabolism with white matter alterations and neuronal damage, including neurodegeneration.

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Disclosure

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Appendix Authors

Name	Location	Contribution
Andrea Pilotto, MD	University of Tübingen, Germany	Study concept and design, acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content
Carl M. Zipser, MD	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Edytha Leks, MD	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content

Continued

Appendix *(continued)*

Name	Location	Contribution
Dorothea Haas, MD	University of Heidelberg, Germany	Acquisition of data, revising the manuscript for content
Gwendolyn Gramer, MD	University of Heidelberg, Germany	Acquisition of data, revising the manuscript for content
Peter Freisinger, MD	Reutlingen Hospital, Germany	Acquisition of data, revising the manuscript for content
Eva Schaeffer, MD	University of Kiel, Germany	Acquisition of data, revising the manuscript for content
Inga Liepelt-Scarfone, PhD	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Kathrin Brockmann, MD	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Walter Maetzler, MD	University of Kiel, Germany	Acquisition of data, revising the manuscript for content
Claudia Schulte	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Christian Deuschle	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Ann Kathrin Hauser	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Georg F. Hoffmann, MD	University of Heidelberg, Germany	Interpretation of data, revising the manuscript for content
Klaus Scheffler, MD	University of Tübingen, Germany	Acquisition of data, revising the manuscript for content
Francjan J. van Spronsen, MD	University of Groningen, the Netherlands	Interpretation of data, revising the manuscript for content
Alessandro Padovani, MD, PhD	University of Brescia, Italy	Interpretation of data, revising the manuscript for content
Friedrich Trefz, MD	University of Heidelberg, Germany	Study concept and design, interpretation of data, revising the manuscript for content
Daniela Berg, MD	University of Tübingen, Germany	Study concept and design, interpretation of data, revising the manuscript for content

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Phenylalanine Effects on Brain Function in Adult Phenylketonuria

Andrea Pilotto, Carl M. Zipser, Edytha Leks, et al.

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