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## New findings about neuropathological outcomes in the PKU mouse throughout lifespan

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### ABSTRACT

Phenylketonuria (PKU, OMIM 261600) is a genetic disorder caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). If left untreated, PKU leads to systemic phenylalanine (Phe) accumulation, which can result in irreversible brain damage and intellectual disabilities. In the last 60 years, early and strict dietary restriction of phenylalanine (Phe) intake proved to prevent the severe clinical phenotype of untreated PKU. While the specific mechanisms through which phenylalanine causes brain damage are still poorly understood, preclinical models have been deeply explored to characterize the neurotoxic effect of Phe on neurodevelopmental processes. At the same time, that on the aging brain still needs to be explored. In the brain of untreated PAH<sup>Enu2(-/-)</sup> mouse, we previously reported a reduction of myelin basic protein (MBP) during postnatal development up to 60 PND. Later in the diseased mouse's life, a spontaneous and persistent restoration of MBP was detected. In this present longitudinal study, ranging from 14 to

540 post-natal days (PND) of untreated PAH<sup>Enu2(-/-)</sup> mice, we further investigated: a) the long-life consistency of two Phe-related brain metabolic alterations, such as large neutral amino acids (LNAA) and biogenic amine neurotransmitters' depletion; b) the outcome of locomotor functions during the same life span; c) the integrity of myelin as assessed ex vivo by central (hippocampus) and peripheral (extensor digitorum longus-sciatic nerve) action potential conduction velocities. In contrast with the results of other studies, brain Leu, Ile, and Val concentrations were not significantly altered in the brain PAH<sup>Enu2(-/-)</sup> mouse. On the other hand, 3-O-Methyldopa (3-OMD, a biomarker of L-DOPA), serotonin, and its associated metabolites were reduced throughout most of the considered time points, with consistent reductions observed prevalently from 14 to 60 PND. Normal saltatory conduction was restored after 60 PND and remained normal at the last examination at 360 PND, resulting nonetheless in a persistent locomotor impairment throughout a lifetime. These new findings contribute to laying the foundations for the preclinical characterization of aging in PKU, confirming neurotransmitter defects as consistent metabolic traits. LNAAs have a minor role, if any, in brain damage pathogenesis. Transient myelin synthesis failure may impact brain connectivity during postnatal development but not nervous signal conduction.

## **KEYWORDS**

Phenylketonuria, Neurotransmitters, Myelin Integrity, Motor Skills, LNAA

### **1. INTRODUCTION**

Phenylketonuria (PKU) is an inborn error of phenylalanine (Phe) metabolism due to pathogenic variants on the phenylalanine hydroxylase gene (*PAH*). *PAH* defect causes early postnatal accumulation of neurotoxic amino acid Phe in biological fluids and tissue that irreversibly damages the immature brain, leading to neurodevelopmental derangement, intellectual disabilities, and neurological impairment. Early and strict dietary restriction of Phe intake in the last 60 years proved to prevent the clinical phenotype of untreated PKU [1]. Although Phe accumulation is well established as the cause of PKU phenotype, the pathogenesis of the developing brain damage is not well known, and the consequences of Phe exposure during adulthood and in the elderly remain to be at all explored.

Brain depletion of dopamine and serotonin is the most consistent biochemical alteration detected in preclinical and clinical studies of PKU brain [2–6]. A direct inhibitory effect of Phe on tyrosine and tryptophan hydroxylase, the limiting enzymes of biogenic amine synthesis, has been demonstrated [7–10]. A further potential pathogenetic effect of high Phe is the competition with the other large neutral amino acids (LNAAs) for the same L-type carrier (LAT-1) at the concentration of the blood–brain barrier (BBB), which has been demonstrated in preclinical models [11,12] and clinical context [13]. While contributing to the reduced biogenic amine synthesis by limiting dopamine and serotonin precursors in nervous tissue (tyrosine and tryptophan, respectively) [14,15], the depletion of LNAA might also result in a derangement of amino acid homeostasis affecting protein synthesis in the nervous tissue [12,13,15–19]. Supplementation of LNAAs has been suggested as an adjunctive therapy for PKU patients who were unable to adhere to a low Phe diet strictly [12,20–23] and proved to reduce blood and brain Phe, improving the synthesis of the neurotransmitters in the brain [11,12]. Although the supplementation of LNAA corrects some biochemical alteration due to high concentrations of Phe [12], it does not imply that a depletion of LNAA occurs in the brain as a result of high blood Phe concentrations [11,13].

The linkage between these biochemical modifications and neuropathological [24] and neuroimaging [25] alterations detected in PKU is presently conjectural. White matter gliosis and hypomyelination, lower expression of myelin proteins [18,26–28], and derangement of dendritic spine density and maturation in gray matter [29] have been consistently reported in preclinical and clinical *ex vivo* studies [27]. Neuropathological and neurocognitive alterations detected in preclinical studies may be prevented by early treatment of the disease [28], while the long-term clinical and neuropathological outcomes of the untreated disease in preclinical models have not been systematically studied. We recently found an unexpected normalization of myelin protein synthesis (i.e. MBP; Myelin-associated glycoprotein, MAG; Myelin oligodendrocyte glycoprotein, MOG; anillin, ANLN) with aging in the untreated ENU2 PKU mouse, which could not be ascribed to a concurrent marginal reduction of blood Phe [18]. This observation prompted us to further explore the long-life consistency of the above biochemical markers of the disease in the brain of ENU2 mice. Moreover, to probe whether the late restoration of myelin's protein synthesis resulted in normal nervous conduction, we also explored the propagation velocity of action potentials both in the CNS and in the peripheral nervous system (PNS) of ENU2 mice.

## 2. MATERIAL AND METHODS

### 2.1 ANIMALS

BTBR mice have been raised in the animal facility of the Biochemistry and Biotechnology section of the Department of Biomolecular Sciences of the University of Urbino Carlo Bo. PAH<sup>Enu2(-/-)</sup> (ENU2) and PAH<sup>Enu2(+/-)</sup> (+/+ and +/-, healthy control mice; CTR) male and female mice used in this study were obtained from mating between heterozygous animals belonging to the BTBR strain. Male and female mice were equally distributed alongside any experiment. The animals were housed in standard cages, from 3 to 6 mice per cage, with a light-dark cycle of 12 hours and under controlled conditions of temperature ( $22 \pm 1^\circ\text{C}$ ), humidity (60%) and air change (every 12 hours). Genetic characterization was performed as described [30]. All mice were fed with a Teklad global 18% protein rodent diet (Teklad, Harlan Laboratories Inc., Madison, WI) and water *ad libitum*. All the experiments were conducted in accordance with the European legislation (2010/63/EU), with the Italian national legislation (DL26/2014) which governs the use of animals for research and with the guidelines of the National Institute of Health on the use and care of laboratory animals (Authorization n° 486/2017-PR).

### 2.2 ASSESSMENT OF BRAIN AMINO ACIDS

The concentration of twenty-two amino acids, including LNAAs (Met, Ile, Leu, Val, Thr, His, Phe and Tyr), was assessed in the brain from ENU2 and controls at various time points (14-60-180-540 PND; 4-6 animals per time point, respectively). Trp was not included since it did not meet the necessary requirements for the quantitative analysis, probably due to the presence of an interfering compound. Frozen whole brain samples at various time points (14-60-180-540 PND) were weighted and homogenized in 0.1 N HClO<sub>4</sub> (perchloric acid, Carlo Erba) containing 6 mM Na-metabisulphite (Carlo Erba) and 1mM ethylenediaminetetraacetic acid (EDTA, Carlo Erba), to have a ratio of 1:100 ml/mg. Homogenates were then centrifuged at 10000 *g* for 20 min at 4°C. Supernatants were collected and transferred to the 1200 series HPLC system (Agilent Technologies,

CA, United States). The analysis was performed as previously published [31] with minor modifications. Amino acids were derivatized with two different reagents, o-phthalaldehyde-3-mercaptopropionic acid (OPA)(Agilent Technologies, CA, United States) for primary and 9-fluorenylmethylchloroformate (FMOC)(Agilent Technologies, CA, United States) for secondary amino acids. The derivatization reactions were carried out in the needle of an autosampler at room temperature. In sequence 5 µl of 0.4N, pH 10.2 borate buffer (Agilent Technologies, CA, United States), 1 µl of OPA and 3 µl of sample were drawn and mixed; next 1 µl of FMOC was drawn and mixed. The resulting mixture was injected in the column [32]. Chromatographic separation was accomplished with Hypersil AA ODS 2.1 x 200 mm (5 µm) column coupled with an ODS Hypersil ODS guard column 20 × 4 mm I.D. (Agilent Technologies, CA, United States). Mobile phase A was 20mM sodium acetate buffer (pH 7.2) containing triethylamine 0.044% (v/v), while mobile phase B was a solution of 100mM sodium acetate buffer (pH 7.2), acetonitrile and methanol (1:2:2, v/v/v) (Sigma-Aldrich, MO, United States). A gradient elution was applied (Supplementary Material - **Table S1**). The flow rate was 0.45 mL/min and the column temperature was 38 °C. For the fluorescence detection of primary amino acids excitation and emission wavelengths were 340 nm and 450 nm and for secondary amino acids were 260 nm and 315 nm [32].

### 2.3 ASSESSMENT OF BIOGENIC AMINES IN THE BRAIN

For neurotransmitter metabolites determination, frozen whole brain samples at various time points (14-60-180-540 PND) were weighted and homogenized in 0.1N HClO<sub>4</sub> (Carlo Erba) containing 6mM Na-metabisulphite (Carlo Erba) and 1mM EDTA (Carlo Erba), to have a ratio of 1:100 ml/mg. Homogenates were then centrifuged at 10000 g for 20 min at 4°C. Subsequently, to 50 µL of supernatant were added 2 µL of 0.5M NaOH, 10 µL of 1% (w/v) ascorbic acid, 10 µL of 1% (v/v) formic acid, 20 µL of water and 10 µL of internal standard mix solution containing 100nmol/L deuterated 5-hydroxytryptamine (d4-5-HT), 75 nmol/L deuterated 5-hydroxyindoleacetic acid (d5-5-HIAA), 45 nmol/L deuterated 5-hydroxytryptophan (d3-5-HTP) and 125 nmol/L deuterated 3-O-methyl-DOPA (d3-3-OMD). Five µL were injected and analyzed by HPLC-ESI-MS/MS. The LC-MS/MS system consisted of a Waters ACQUITY UPLC coupled with a Waters Xevo TQMS mass spectrometer (Waters Corp., Manchester,UK). Chromatographic separation was performed using a Waters ACQUITY UPLC HSS T3 C18 column (2.1 x 150 mm, 100 Å, particle size 1.8 µm) with a

Waters ACQUITY UPLC HSS T3 1.8  $\mu\text{m}$  VanGuard 3/Pk (2.1 x 5mm) precolumn. Mobile phase A was composed by water with 0.1% (v/v) formic acid, while mobile phase B was composed by acetonitrile containing 0.1% (v/v) formic acid. The ESI-MS/MS instrument was operated in positive electrospray ionization mode. The acquisition method used was multiple reaction monitoring (MRM). The MRM transition was 177.0 $\rightarrow$ 160.1 m/z for 5-HT, 221.3 $\rightarrow$ 162.1, m/z for 5-HTP, 212.1 $\rightarrow$ 153.1 m/z for 3-OMD and 192.2 $\rightarrow$ 146.1 m/z for 5-HIAA. Instrument was operated at a capillary voltage of 1.5 kV and at a temperature of 150  $^{\circ}\text{C}$ . A stream of purified nitrogen was used as desolvation gas (750 L/Hr) and cone gas (20 L/Hr). The desolvation gas temperature was set at 525  $^{\circ}\text{C}$ . Purified argon was used as collision gas. The control of the instrument and the data analysis were carried out through Waters Masslynx software V4.1.

#### 2.4 ACTION POTENTIAL CONDUCTION VELOCITY ASSESSMENT

The propagation velocity of the action potentials (APs) along an axon has been measured both in the Central Nervous System (CNS) [33] and in the Peripheral Nervous System (PNS) [34] by using electrophysiological techniques.

The action potential conduction in the CNS was evaluated on mouse hippocampus slices obtained as previously described [35]. Briefly, after ENU2 and CTR mice (aged 60 and  $\geq$ 360 PND) were sacrificed by decapitation, brains were quickly removed and parasagittal, 400-micrometer-thick brain slices were cut. The slices were allowed to recover neuronal activity and subsequently recorded while being continuously perfused with oxygenated artificial cerebrospinal fluid (aCSF) [35]. Then, in accordance with literature [33], the AP conduction velocity was assessed in the axons of Schaffer collaterals located within the CA1 region of the hippocampus. Concisely, recording micropipettes and bipolar stimulating electrodes were placed in the stratum radiatum of CA1 and, to evaluate the propagation velocity of APs triggered by the stimulating electrode, the latency between the stimulation artifact and the peak of the fiber volley in the extracellular field post-synaptic potential (fEPSP) response has been analyzed. The conduction velocity ( $V_c$ ) in  $\mu\text{m}/\text{ms}$  was calculated using the formula  $V_c = d/t$ , where "d" represents the linear distance between the stimulating and recording electrodes, ranging from 200  $\mu\text{m}$  to 400  $\mu\text{m}$ , and "t" corresponds to the time taken for the AP propagation.

The evaluation of the peripheral nervous conduction velocity was carried out on the *extensor digitorum longus* (EDL) muscle-sciatic nerve preparation dissected as previously described [36]. In

brief, after the animal killing by decapitation, the left EDL muscle, together with the sciatic nerve, was removed from ENU2 and CTR mice (60 and  $\geq 360$  PND) and put into the Ringer's solution [36]. Muscle cells were impaled with an intracellular micropipette filled with 3 M KCl (tip resistance 10–20 M $\Omega$ ), (+)-tubocurarine was added to the bath in the recording chamber, to prevent muscle action potential, and end-plate potentials (EPPs) were evoked by single stimulation at 1-Hz frequency. To evaluate the AP conduction velocity in the peripheral nervous system, the nerve was stimulated at two sites with a distance of 7 mm and the nerve conduction velocity was calculated as the distance between the two stimulating electrodes divided by the difference in the onset latency of each EPPs.

## 2.5 BEHAVIORAL TESTS

Motor skills and anxiety-like behavior of young adult and aged ENU2 mice (60 -  $\geq 360$  PND; n: 10-12 for each age) were evaluated through Open Field Test (OFT). Test was performed in a sound and light attenuated room, putting mice in a round arena with a white Plexiglas floor and gray Plexiglas walls to prevent their escape. The arena was 80 cm in diameter and 30 cm in height. Mice were introduced in a sector of the OFT and left to explore the apparatus for 5 min. Distance moved, velocity and immobility were analyzed as index of motor integrity, time/frequency in the center of arena as index of anxiety and self-grooming as index of stereotypies. All mice were individually tested and behaviors were videotaped by means of a camera placed above the apparatus and connected to a recorder placed outside the room. Videos were analyzed by Video-based EthoVision System (Noldus, The Netherlands) to record, collect, and analyze data.

## 2.6 STATISTICAL ANALYSES

Statistical analyses were performed with GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA, USA) using two-way ANOVA in case of more than two groups to be measured (considering genotype and age as independent variables). Student T-test was used for the saltatory conduction evaluation. P-value threshold for significance was set to  $p < 0.05$ . The data are shown as Mean  $\pm$  SEM.

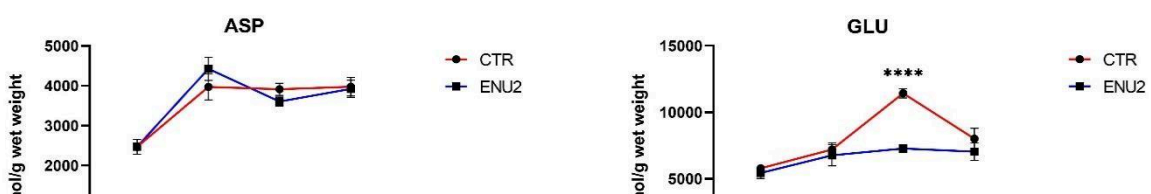
## 3. RESULTS

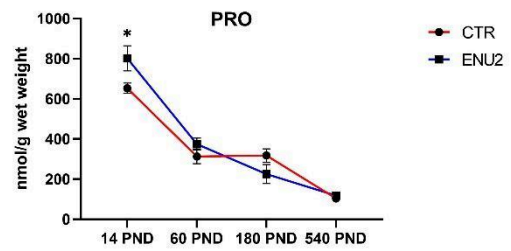
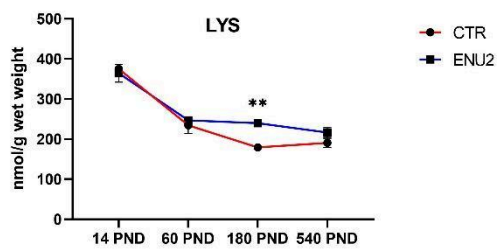
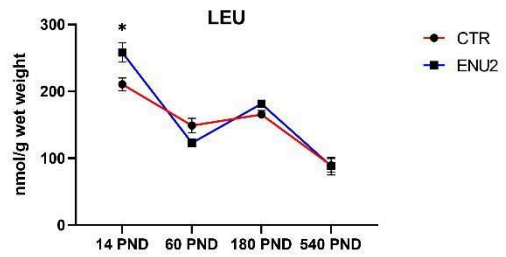
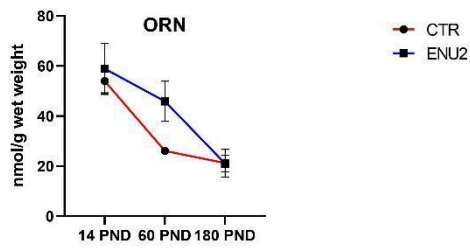
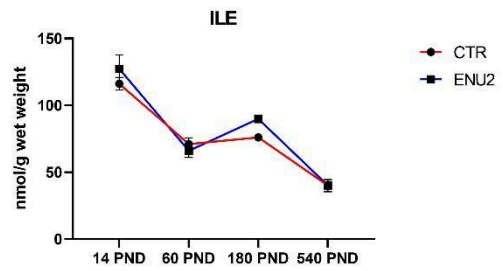
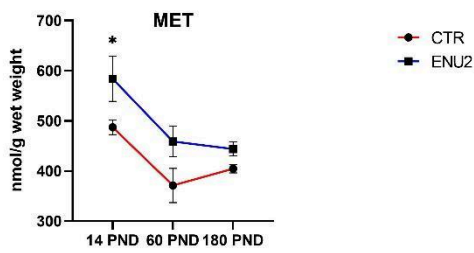
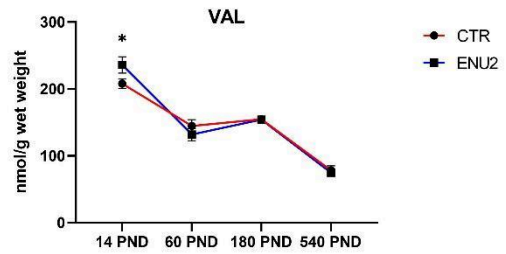
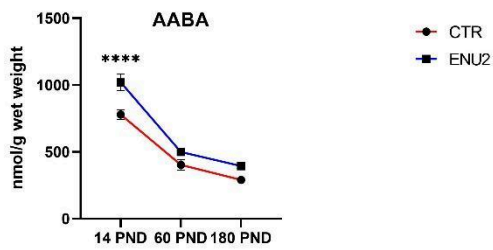
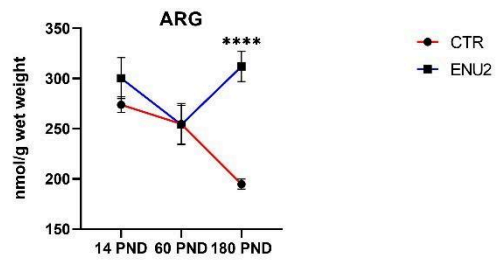
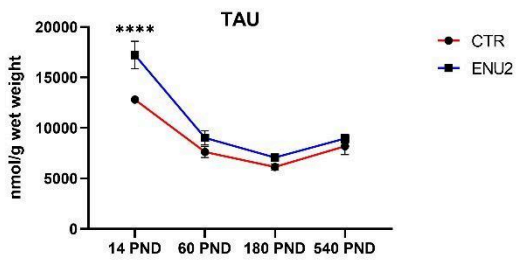
### 3.1 BRAIN AMINO ACIDS CONCENTRATIONS DIFFER DURING ENU2 MICE LIFESPAN

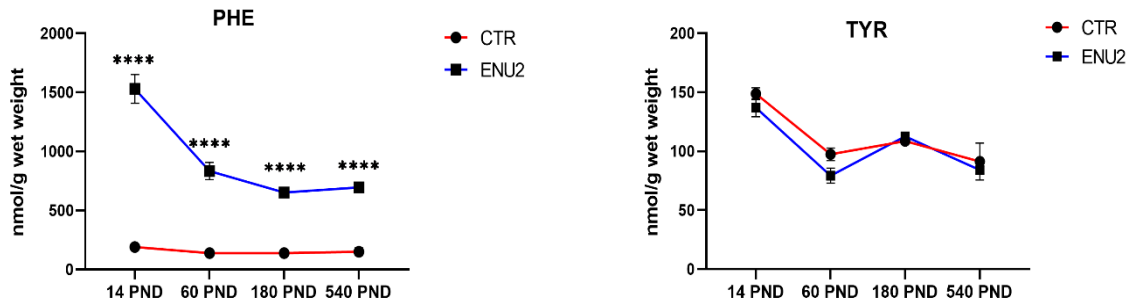
Brain amino acid concentrations are reported in Supplementary Material **Table S2**. The described Phe and Tyr quantification were already obtained from our previous work, as part of the possible role of Phe concentrations over the epigenetic changes observed in ENU2 brain during lifetime [18].

Brain Phe was consistently higher than in controls at any time point. However, from 14 to 60 PND, as previously reported [18], the values decreased by half, and continued to decline until 540 PND, while no significant variation could be detected in healthy controls. Concerning the other amino acids, the concentrations of Asp, Asn, Cit, Thr, Orn, and Ile overlapped with those of control along the entire follow-up; Gly was the only amino acid significantly higher than in control at any time point; Tau, AABA, Met, Pro, Val, and Ile concentrations were higher than controls only at 14 PND; Ser and His remained higher until 180 PND, then overlapped with the control's values. Glu and Gln declined significantly later at 180 and 540 PND, respectively (**Fig. 1**). In all, ruling out Phe, the whole pool of LNAAs (**Fig. 2**) revealed no differences in comparison with healthy mice, with a general trend towards a decline of the pool with aging.

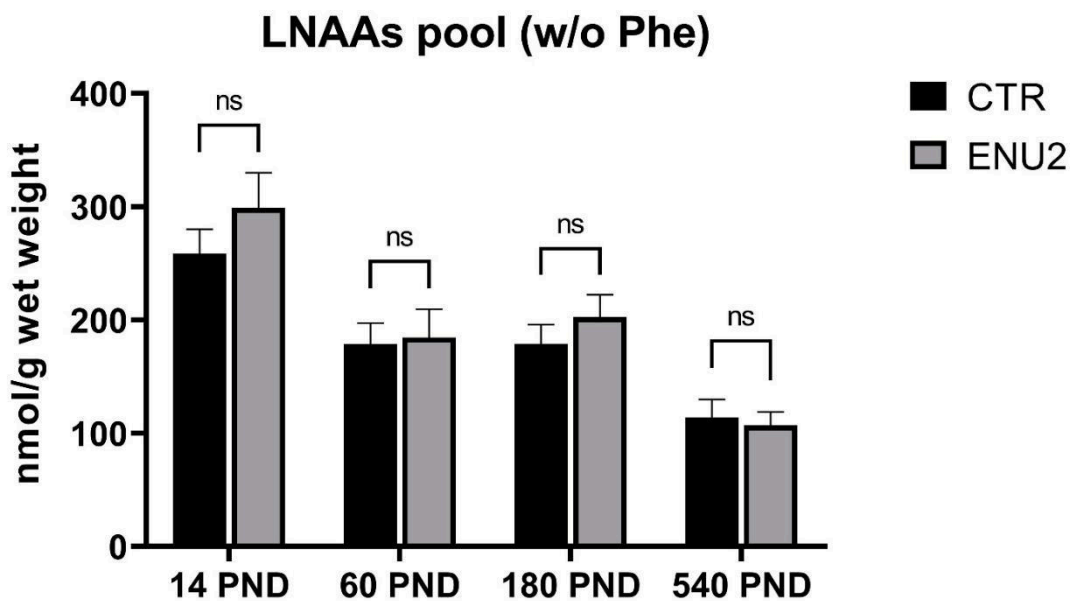
According to the present results, we cannot confirm the reduction of LNAA in untreated ENU2 mouse at any time-point of our longitudinal exploration. For Phe and Tyr concentrations in the peripheral blood, please refer to our previous work or to the Supplementary Material section (**Table S3**) [18].







**Fig.1: Brain amino acid concentrations in CTR and ENU2 during mice lifespan.** Significance has been calculated only between the two conditions per each time point. Data are expressed as Mean ± SEM (n=4-6 per each time point). Two-way ANOVA followed by Bonferroni's multiple comparison test, \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001.

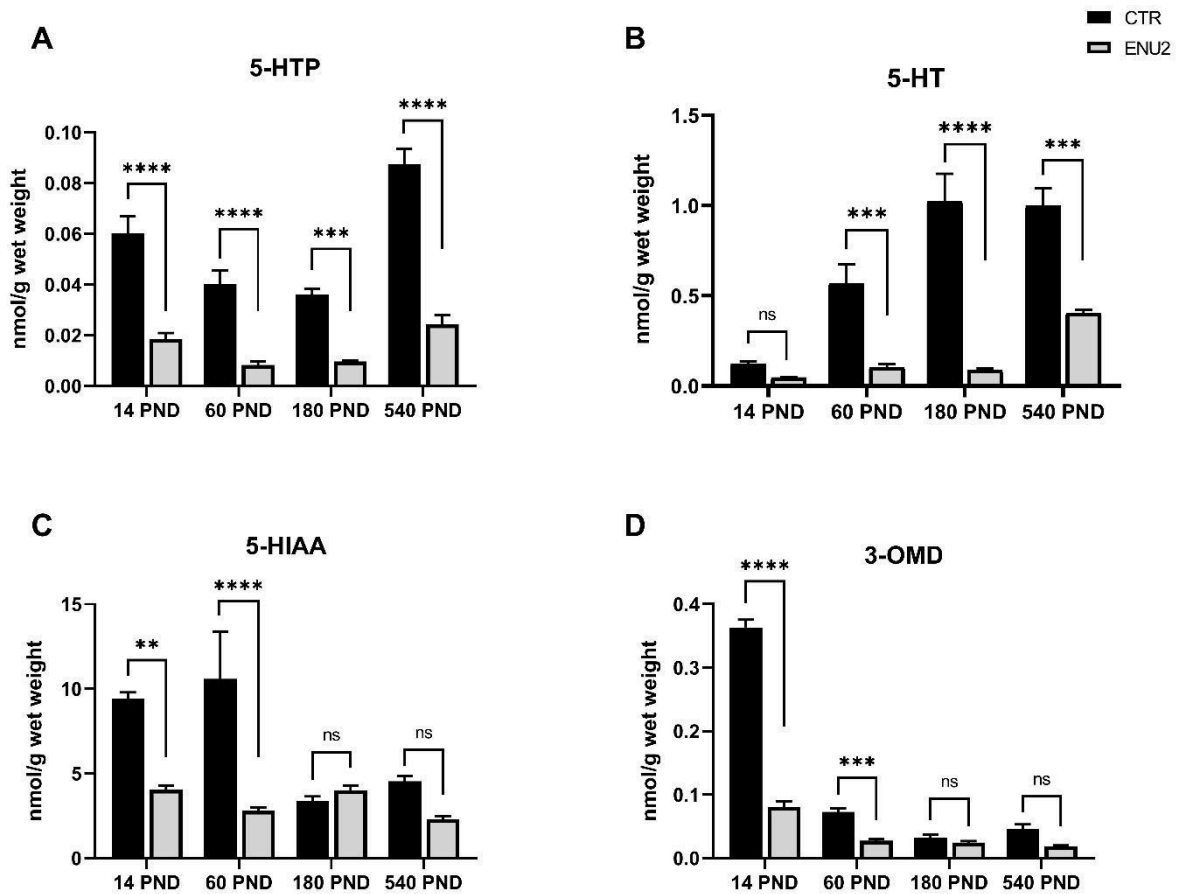


**Fig. 2: Cerebral LNAAs pool profile during mice growth.** The results were obtained through mean of the sum of all the 7 LNAAs (Met at 540 PND was lacking). No statistical differences were found between CTR and ENU2 mice. Two-way ANOVA followed by Bonferroni's multiple comparison test.

### 3.2 MONOAMINERGIC NEUROTRANSMITTERS ARE CONSTANTLY IMPAIRED DURING ENU2 MICE LIFESPAN

Monoaminergic neurotransmitters and related metabolites were lower in ENU2 than in CTR at each time point examined. For the serotonin (5-HT) the difference was significant starting from the second assessment at 60 PND, (**Fig. 3B**), while serotonin's precursor 5-HTP was significantly lower than in CTR in all the measurements (**Fig. 3A**). Serotonin and L-DOPA catabolites (5-HIAA and 3-OMD, respectively) were significantly lower than in CTR at PND 14 and 60 (**Fig. 3C** and **3D**) but overlapped to control values later. Interestingly, the pattern of neurotransmitters' decline in the brain with aging is similar in both normal and affected mice (**Fig. 3C** and **3D**) [37].

The observed metabolic profile suggests an impairment of tyrosine and tryptophan hydroxylase activity rather than the last step of biogenic amine synthesis, catalyzed by the enzyme aromatic L-amino acid decarboxylase (AADC).

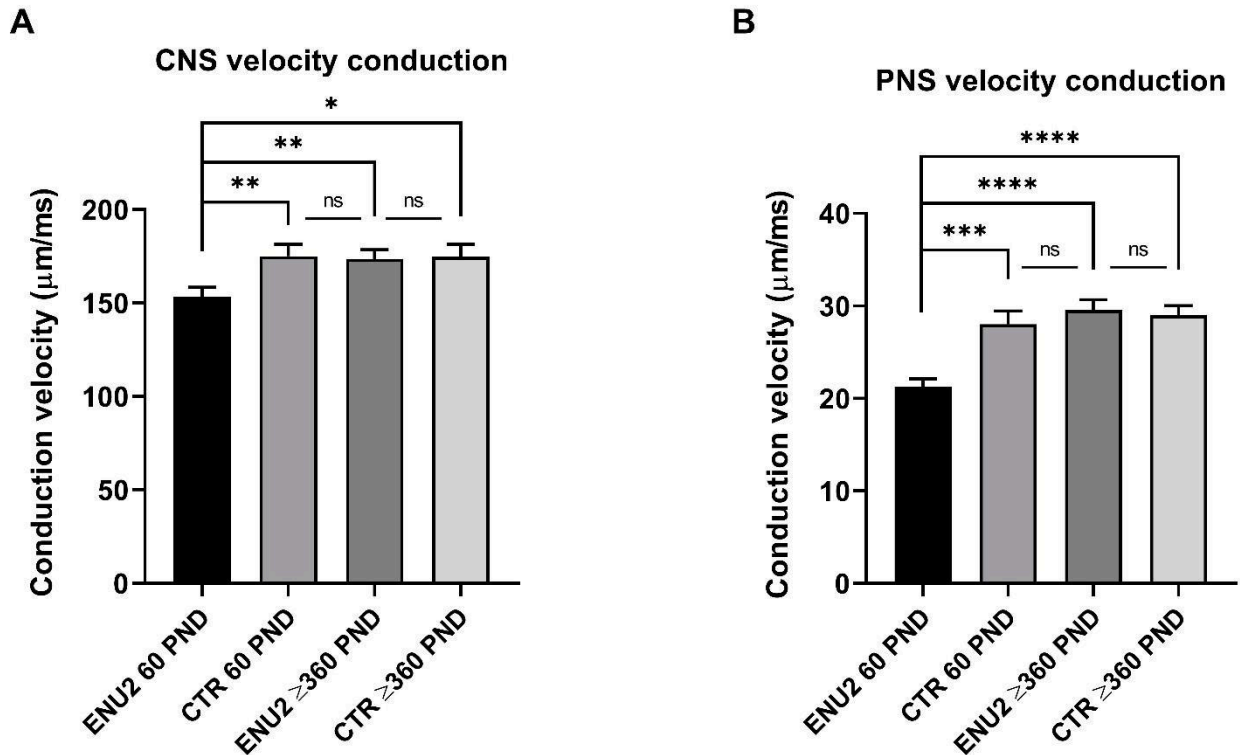


**Fig. 3: Brain concentrations of (A) 5-HTP, (B) 5-HT, (C) 5-HIAA and (D) 3-OMD in CTR and ENU2 brains during aging.** Neurotransmitters follow a fluctuating trend in the evaluated time points, even if they are generally lower in the ENU2 brains compared to CTR. Data are expressed as Mean  $\pm$  SEM (n=4-6 per each time point). Two-way ANOVA followed by Bonferroni's multiple comparison test was applied only between CTR and ENU2 NTs concentrations of the same time point, \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001. 5-HTP: 5-hydroxytryptophan; 5-HT: 5-hydroxytryptamine/serotonin; 5-HIAA: 5-hydroxyindoleacetic acid; 3-OMD: 3-O-methyl-DOPA.

### 3.3 MYELIN PROTEINS RECOVERY RESTORES MYELINATION AND SALTATORY CONDUCTION IN AGED ENU2 MICE

To assess the conduction velocity of action potentials in the Schaffer collaterals - CA1 pathway, hippocampal slices from ENU2 and CTR mice at 60 and  $\geq 360$  postnatal days were electrophysiological recorded. Field responses were induced, and the latency of the fiber volley at a known distance was evaluated. As shown in **Fig. 4A**, the results demonstrated a significantly lower AP conduction velocity in ENU2 mice at 60 PND compared to age-matched CTR, which was

no longer found in ENU2 mice at  $\geq 360$  PND where the conduction velocity of AP was similar in both groups of mice and no different from CTR at 60 PND. AP conduction velocity evaluation in the



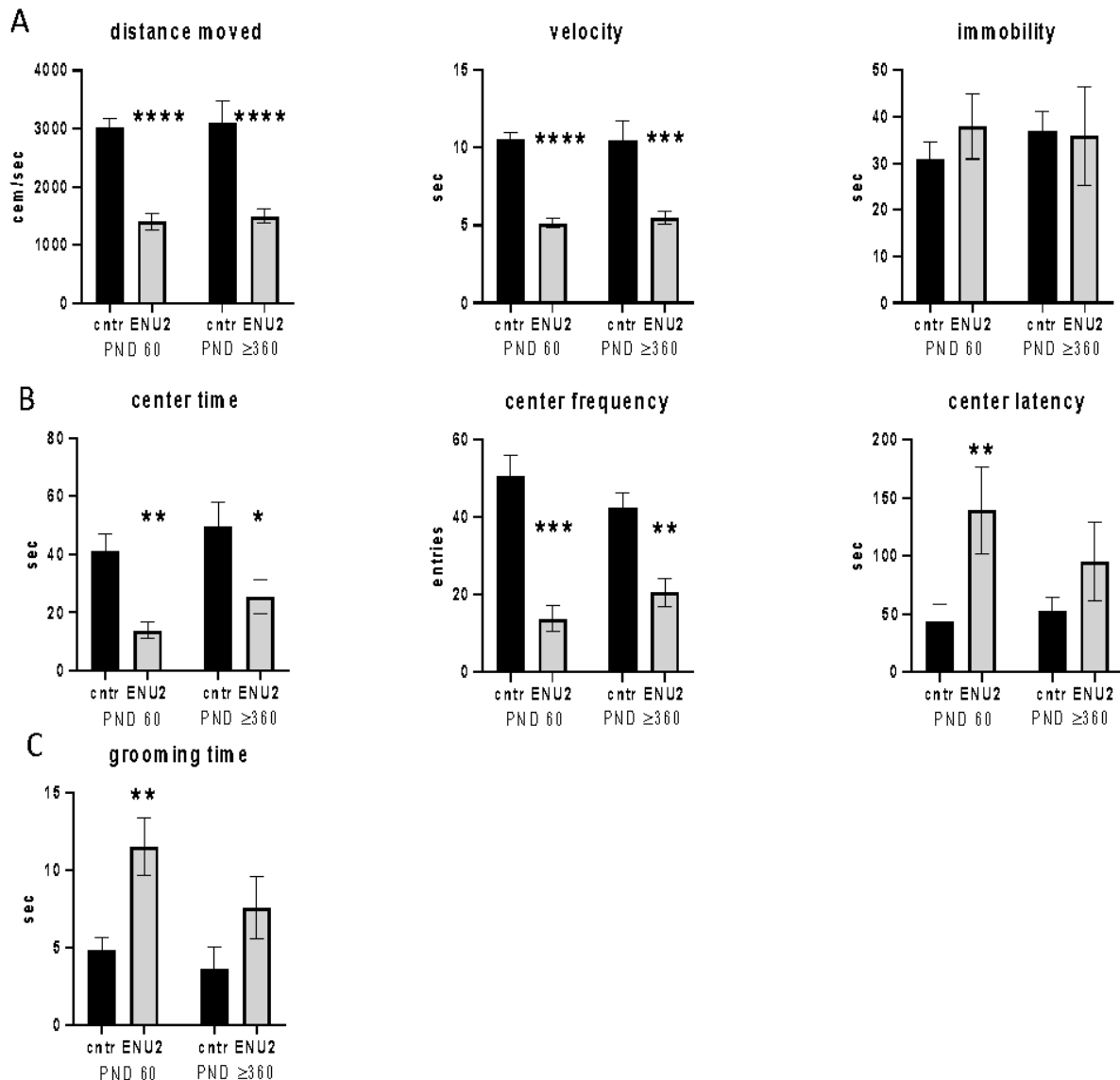
peripheral nervous system was performed by recordings the latency of end-plate potentials following nerve stimulation at two known distances. Similarly to hippocampus results, experiments conducted on the EDL muscle-sciatic nerve preparation revealed a significant reduction in conduction velocity in ENU2 mice at 60 PND compared to same-age controls, which was restored in ENU2 mice at  $\geq 360$  PND (**Fig. 4B**).

**Fig. 4: Evaluation of the conduction velocity of action potentials in CNS (A) and PNS (B) of young adult (60 PND) and aged ( $\geq 360$  PND) CTR and ENU2 mice.** Young adult ENU2 show a decreased velocity of conduction in both CNS and PNS compared to CTR, while aged ENU2 show a recovery of the myelin signal conduction functionality. Data are expressed as Mean  $\pm$  SEM (n=3 per each time point). Student T-Test between ENU2 60 PND and other conditions/time points was applied, \*p<0.05; \*\*\*p<0.001; \*\*\*\*p<0.0001.

### 3.4 MOTOR IMPAIRMENT REMAINS IN ENU2 MICE DESPITE MYELINATION RETRIEVAL

Young adult and aged ENU2 mice (60 -  $\geq 360$  PND) were subjected to OFT in order to check if the observed myelin proteins and propagation of action potential recovery during adulthood could bring to a behavioral rescue. As reported in **Fig. 5A**, OFT test depicted lower locomotor activities for the young adult and aged ENU2 mice, both in terms of distance moved (cm) and velocity (cm/s). Furthermore, the reduced frequency and time spent in the center of the arena as well as the increased latency to reach it that was observed in ENU2 represent an index of increased

anxiety concentrations in ENU2 young adult and old mice (Fig. 5B). Fig. 5C shows that ENU2 young adult mice spent significantly more time in self-grooming compared with controls (difference lessened with the age), confirming the anxious phenotype and pointing out the presence of stereotypical behavior. Interestingly, most of the differences present among ENU2 and healthy controls persist at later stages, indicating that the myelin recovery in adulthood is not sufficient to restore the behavioral phenotype.



**Fig. 5: OFT test in the young adult and aged ENU2 mice compared to CTR.** ENU2 mice display lower locomotor activity in both ages (A) and reduced frequency and time spent to the center of arena (B). ENU2 mice show also more self-grooming time, significant only in the 60 PND group (C). Two-way ANOVA followed by Duncan's multiple comparison test (n=10-12), \*\*\*p<0.001; \*\*\*\*p<0.0001.

#### 4. DISCUSSION

In our previous work, we found a defective expression of proteins (MBP, MOG, MAG, ANLN) implied in myelination processes and building in the young adult ENU2 mouse brain (14-60 PND). Unexpectedly, myelin proteome was completely restored later with aging [18]. These new findings opened the route to the present work aimed at investigating the biochemical phenotype of ENU2 mice during adulthood and the elderly, focusing on the outcome of brain amino acids, including LNAA pool and neurotransmitters in the brain. On the preclinical ground, we have previously described the pattern of cognitive impairment in untreated young PAH<sup>Enu2(-/-)</sup> mice [28,38] while the present study focused solely on locomotor activity.

The measurement of the concentrations of 20 amino acids in the brain of ENU2 mice revealed different trends over time according to different amino acids. Ser, His, Gly, Ala and Met were significantly higher in the ENU2 mouse from 14 to 180 PND (Met at 14 PND), with His that confirms its higher concentration in the ENU2 brain compared to CTR [39,40]. On the other hand, Thr, Val, Ile and Leu were unaltered concerning controls, thus not confirming the hypotheses of LNAAs depletion in the brain of PKU mice and, probably, patients [12,21,41]. Curiously, Ser, His, Gly, Ala and Met concentrations decreased during ageing, reaching the same concentrations observed in the CTR at 540 PND. Four of the above-mentioned amino acids (Ser, His, Met and Gly) plays a key role in the one-carbon (1C) metabolism, providing methyl donors for DNA and protein methylation and thus ensuring a correct epigenetic profile [42–45]; this can be one of the reasons for the epigenetic alterations observed in the ENU2 mouse brain [46,47], which in turn can lead to variations on the protein and miRNAs expression [18,46]. In addition, also oxidative stress can induce epigenome re-patterning [48]. To this extent, it is noteworthy that the increase of His and Gly in the ENU2 mouse from 14 to 180 PND confirms what was recently observed by Dobrowolski *et al.* supporting the cerebral oxidative stress in PKU mice [49]. Indeed, His is involved in the homocarnosine pathway and Gly in the glutathione one, both contributing to anti-oxidative response. Experiments are ongoing to characterize some biochemical and metabolomic aspects of oxidative stress in PKU mice, also aimed to successively evaluate possible therapeutic approaches to ameliorate residual early and late onset of PKU neurological phenotypes.

Gln was always higher in the CTR mouse (except at 180 PND), with its precursor Glu following this trend only at 180. Importantly, the concentrations of many amino acids at 14 PND were significantly higher compared to other time points, potentially due to the increased amino acid uptake that occurs at this age [39]. Together, these data are discordant with the literature

[11–13,22,39]; nonetheless, two works from Van Vliet *et al.* on young adult (age: 60 PND) and aged ENU2 mice (age: 300 and 480 PND) reported similar values as those obtained in the present work [40,41]. Unfortunately, we could not include Trp in our pool of amino acids. In summary, in this preclinical model of PKU, there is no evidence of altered homeostasis of LNAA in the brain, nor defect of neurotransmitter synthesis that can be ascribed to the related amino acids precursors or consequent alteration of protein synthesis.

As expected [4,12,41,50], biogenic amine neurotransmitters were consistently lower than controls in the ENU2 mouse brain, which can be explained by the detrimental effect of Phe on the functionality of the enzymes Trp and Tyr hydroxylase [51–53]. Indeed, 5-HT and its precursor (5-HTP) were persistently downregulated in the ENU2 mouse. In addition, 5-HIAA, the main catabolite of 5-HT, was lower than controls until 60 PND and overlapped to control levels afterwards. A similar trend was observed for 3-OMD, the product of L-DOPA O-methylation by the enzyme catechol-O-methyltransferase (COMT; EC 2.1.1.6), a potential indirect marker of tyrosine hydroxylase activity [54]. These results are concordant with the literature, also concerning the detected variable evolution of the chemicals in the healthy mouse, which can rely on the age intervals taken in the exam [55,56]. Moreover, these findings are particularly interesting because, contrary to what observed for LNAAs and proteome, neurotransmitter deficit was always observed in the ENU2 mouse model in all the considered time points despite the lower Phe concentrations detected at later stages [18], suggesting that concentrations of blood Phe around 600  $\mu\text{M}$  are not sufficient to normalize biogenic amines, or that other unknown factors may influence biogenic amine synthesis [8].

To this concern and to check if the recovery of the myelin sheath proteins detected in older mice [18] could bring positive locomotory consequences and myelination retrieval, we analyzed CTR and ENU2 mice through OFT test at 60 and  $\geq 360$  PND, together with the action potential conduction velocity assessment for the same time points. Starting from the latter, we found that axonal saltatory conduction was completely retrieved in the aged ENU2 mice in both CNS and PNS, reaching the same concentrations observed for CTR. The increase in MBP concentrations previously observed in PKU mice  $\geq 360$  PND [18] concurs with both the restoration of action potential propagation velocity in the central and peripheral nervous systems and 3-OMD concentration recovery, supporting a link between myelination and dopamine in PKU mouse [26]. However, we should not overestimate the value of these catabolites: for example, in the present study, brain serotonin depletion was found at each time point, while 5-HIAA, like 3-OMD,

normalizes after 60 PND. We cannot rule out a similar behavior of dopamine with respect to 3-OMD. To date, no longitudinal studies of CSF neurotransmitters have been performed in adult patients with PKU. In 8 early treated subjects aged 13 to 20 years, Burlina et al. [57] found homovanillic acid (HVA) (and 5-HIAA reduced in 6/8 and 7/8, respectively. In older patients (aged 31-45 years), HVA and 5-HIAA were found to be reduced in 2 out of 10 and 6 out of 10 subjects, respectively [6].

At 60 PND, as expected, locomotory deficiencies were detected in the ENU2 mouse and, despite myelination recovery, were also present at  $\geq 360$  PND both in terms of velocity and exploration time. Moreover, we confirm a greater anxiety for the diseased, as reported by Seibenhener and Wooten [58]. The persistence of motor disorders despite normalizing myelin synthesis and functioning could be ascribed to early and irreversible derangement of connectivity during its critical period of development. However, the persistent defect of serotonergic- and dopaminergic-mediated connectivity may play a role. A recent study on early treated C57Bl/6-*Pah*<sup>enu2/enu2</sup> mice with pegvaliase, detected worsening in the Open Field behavior after one month of treatment discontinuation and restoration of basal performance with the reintegration of the therapy. The detrimental and potentially irreversible effect of early exposure to high Phe concentrations on the cognitive development of ENU2 mice has been well characterized [29,38]. Interestingly, while 5-HTP administration between 14 and 21 PND improves cognitive performances and increases the number of mature dendritic spines in frontal cortical pyramidal neurons in adult ENU2, deficits in locomotor activity remained unaffected by the treatment [29]. Finally, late treated (8 weeks) C57Bl/6-*Pah*<sup>enu2/enu2</sup> shows improvement of open field activity and nesting behavior, but no effect on cognitive impairment [8]. Cumulatively, these observations suggest a possible pathological effect of dopamine depletion on locomotor dysfunction, and of early myelination derangement on irreversible cognitive impairment.

Concerning human PKU, a further cumulative damaging effect of Phe exposure in adulthood is suggested by the cognitive assessment of early treated PKU patients [25,59–61] and the relevant improvement of cognitive functioning by lowering blood Phe concentrations to almost normal blood concentration [62].

## 5. CONCLUSIONS

In conclusion, early neurotransmitter depletion and myelination derangement contribute to the derangement of postnatal brain development in ENU2 mice. We confirm the spontaneous

restoration of myelin synthesis and function, while brain neurotransmitter depletion remains a permanent biochemical consequence of PKU. Finally, the altered homeostasis of brain amino acids plays a minor role, if any, in the pathogenesis of the disease in this preclinical model of PKU, thus paving the way to completely reconsidering their actual impact on PKU cerebral outcomes.

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## 7. AUTHORS' CONTRIBUTIONS

**Alessandro Bregalda:** Conceptualization, Validation, Investigation, Methodology, Visualization, Formal Analysis, Data Curation, Writing - Original Draft. **Claudia Carducci:** Methodology, Investigation, Data Curation, Writing – Review and Editing. **Tiziana Pascucci:** Validation, Investigation, Formal Analysis, Writing – Review and Editing. **Patrizia Ambrogini:** Investigation, Methodology, Visualization, Formal Analysis, Data Curation, Writing – Review and Editing. **Stefano Sartini:** Investigation, Methodology. **Francesca Pierigè:** Investigation, Methodology. **Emanuele di Carlo:** Validation, Methodology, Formal Analysis, Writing – Review and Editing. **Elena Fiori:** Validation, Methodology, Formal Analysis, Writing – Review and Editing. **Donald Ielpo:** Validation, Methodology, Formal Analysis. **Marica Pagliarini:** Methodology, Visualization, Data Curation, Writing - Review and Editing. **Vincenzo Leuzzi:** Conceptualization, Investigation, Writing - Review & Editing. **Mauro Magnani:** Supervision, Resources, Project Administration, Funding Acquisition, Writing - Review & Editing. **Luigia Rossi:** Conceptualization, Supervision, Resources, Project Administration, Funding Acquisition, Writing - Review & Editing.

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## 9. DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article and available from the corresponding author on reasonable request.

## 10. DECLARATIONS

### 10.1 CONFLICT OF INTEREST

Mauro Magnani and Luigia Rossi hold shares in EryDel SpA, a company with interests in the technology of RBC-based drug delivery. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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