



## REVIEW ARTICLE

# Newly validated biomarkers of brain damage may shed light into the role of oxidative stress in the pathophysiology of neurocognitive impairment in dietary restricted phenylketonuria patients

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Despite a strict dietary control, patient with hyperphenylalaninemia or phenylketonuria may show cognitive and/or behavioral disorders. These comorbid deficits are of great concern to patients, families, and health organizations. However, biomarkers capable of detecting initial stages of neurological damage are not commonly employed. The pathogenesis of phenylketonuria is complex in nature. Increasingly, the role of oxidative stress has gained acceptance and biomarkers reflecting oxidative damage to the brain and easily accessible in peripheral biofluids have been validated using mass spectrometry techniques. In the present review, the role of oxidative stress in the pathogenesis of phenylketonuria and hyperphenylalaninemia has been updated. Moreover, we report on newly validated brain-specific lipid peroxidation biomarkers and inform on their relevance in the detection and monitoring of neurological damage in phenylketonuric patients. In preliminary studies, a correlation between lipid peroxidation biomarkers and neurological dysfunction in patients with PKU was reported. However, there is a need of adequately powered trials to confirm the validity of these biomarkers for early detection of brain damage, initiation of treatment, and reliably monitor evolving disease both in phenylketonuria and hyperphenylalaninemia.

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## INTRODUCTION

Phenylketonuria (PKU; OMIM # 261600) is an autosomal recessive inborn error of L-phenylalanine (Phe) originated by variants of the *PAH* gene encoding for the enzyme complex phenylalanine hydroxylase (PAH; EC # 1.14.16.1) located mainly in liver and kidney.<sup>1</sup> At present, different mutations in the *PAH* gene located in the chromosome 12q have been described.<sup>2</sup> PKU is one of the most frequent inherited metabolic diseases with an incidence that ranges from 1/10,000 to 1/15,000 people in Europe.<sup>3</sup> Pathophysiologic alterations (summarized in Fig. 1) in PKU lead to increased concentrations of Phe and other neurotoxic substances such as phenylpyruvic acid in blood and brain.<sup>4,5</sup> As a consequence, undiagnosed PKU patients develop severe neurocognitive and developmental impairment. The main anatomical findings in magnetic resonance imaging (MRI) presented by PKU patients include damage to corpus callosum, striatum, cortical alterations, and hypomyelination.<sup>6</sup> However, newborn screening (NBS) for PKU has enabled early diagnosis, treatment of this condition and prevented severe mental retardation.

The mainstay of the management of PKU resides in a Phe-restricted diet in combination with Phe-free L-amino acid supplements to maintain Phe plasma levels within strict ranges.<sup>1</sup> Fulfillment of the dietary restriction significantly reduces blood

Phe concentration at any age and is therefore the best surrogate measure and the most effective means of achieving the primary goal of treatment which is normal neurocognitive and psychosocial functioning. The American and European guidelines recommend a periodic neuropsychological and behavioral evaluation of PKU patients<sup>3,4</sup> (Table 1).

## PATHOPHYSIOLOGY

Mechanisms of brain damage in PKU

The mechanisms of neurological damage in PKU are still not well understood. Figure 1 shows the detrimental effects of high Phe and low LNAA levels in the brain.<sup>7</sup> Of note, early-diagnosed PKU patients with Phe serum levels within recommended ranges may suffer from neurocognitive alterations reinforcing the hypothesis that additional factors besides Phe serum level control are involved in the pathogenesis of the disease. A metabolomic approach to explore PKU pathogenesis confirmed the involvement of different physiopathological mechanisms, such as protein synthesis, energy metabolism, and oxidative stress.<sup>8</sup> Remarkably, experimental and clinical studies have described severe reduction in the amount of myelin, alterations in the axonal conduction and in the speed of the synaptic transmission in untreated patients.<sup>9</sup>

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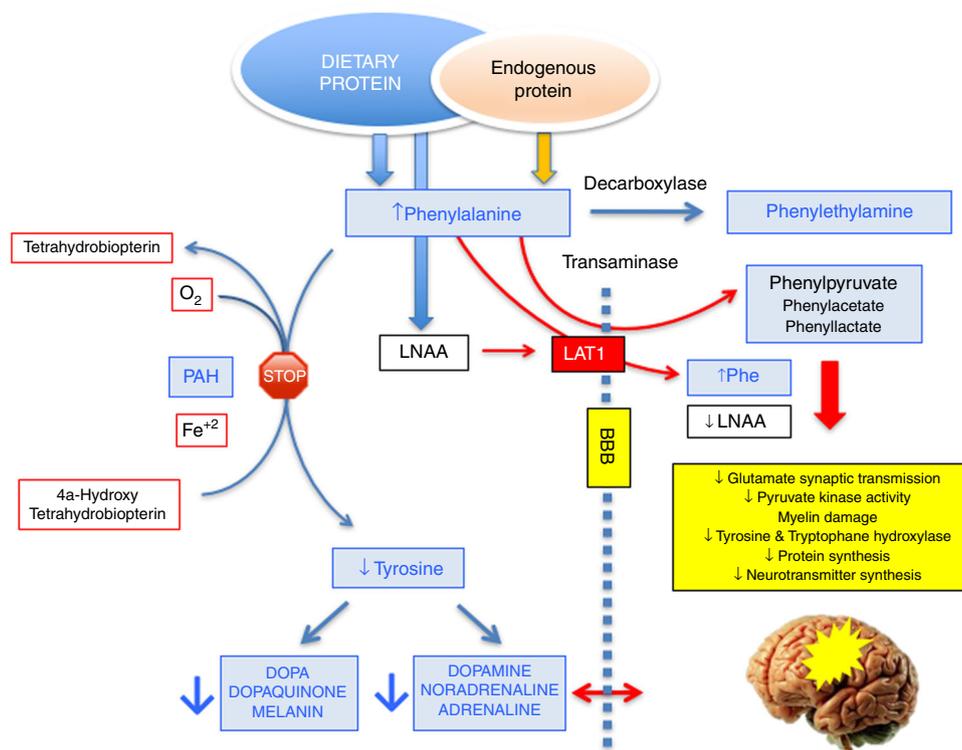
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**Fig. 1** Under normal circumstances, Phenylalanine (Phe) coming from the dietary contribution and endogenous protein is metabolized to Tyrosine (Tyr) by Phenylalanine hydroxylase (PAH) with the concurrence of Tetrahydrobiopterin, oxygen, and iron. In addition, Phe is converted by the action of Phe-decarboxylase to Phenylethylamine. However, patients with Phenylketonuria (PKU) lack PAH, and as a consequence Phe plasma levels increase achieving toxic levels in the brain. Phe in excess is converted in Phenylpyruvate, Phenylacetate, and Phenyllactate that are highly toxic for the brain. Phe competes with the other large neutral aminoacids (LNAA) for the same L-type carrier (LAT-1) to cross the blood–brain barrier (BBB). In addition, circulating Tyr decreases and subsequently the synthesis of metabolites such as Dopamine, Noradrenaline, and Adrenaline diminishes. The consequence of these metabolic alterations is a protracted brain damage. PAH Phenylalanine hydroxylase, LNAA L-neutral amino acids, LAT1 amino acid transporter across the blood brain barrier, BBB blood brain barrier, Phe Phenylalanine

Moreover, using diffusion tensor imaging technology Hood et al., found microstructural white matter lesions in a group of patients aged 6–18 years with early and continuously treated PKU and normal IQ that correlated with multiple index of Phe control.<sup>10</sup> Furthermore, they also found that white matter mediated the relationship between executive abilities and Phe levels suggesting that white matter integrity could be a sensitive marker of neurocognitive dysfunction.<sup>11</sup>

The mechanisms postulated to explain the neurological damage in PKU (Fig. 1) include first saturation of the LNAA transporter to the brain that causes alterations of the regulation of brain metabolism and proteins and neurotransmitters' synthesis. Thus, PKU patients are more susceptible to presenting symptoms caused by dopamine deficiency such as Parkinson's disease.<sup>12</sup> Second, there is a decrease of complexes I–III, II, pyruvate, and creatine kinases activities in the respiratory chain of the mitochondria that generate reactive species (RS) and alter energy metabolism.<sup>13</sup> Third, the production of RS together with a reduction of antioxidant defense levels secondary to dietary treatment leads to a pro-oxidant redox status.<sup>5,14</sup> Brain is especially vulnerable to oxidative stress due to the metabolic rate and oxygen consumption, high polyunsaturated fatty acid content highly vulnerable to free radicals, high iron concentration that favor Fenton chemistry, low level of antioxidant defenses, and the presence of excitatory aminoacids.<sup>15</sup>

The aim of the present study is to provide the reader with the available information regarding the relevance of oxidative stress as a pathophysiologic mechanism contributing to ongoing brain

damage in PKU patients. Moreover, despite optimal dietary control there may be a pro-oxidant activity that can be detected with recently develop mass spectrometry methods, and as a consequence an updated monitoring protocol that would include serial determinations of specific non-invasive biomarkers of brain lipid peroxidation damage.

#### Redox regulation and oxidative metabolism

Oxidative stress plays an essential role in the regulation of the biological systems. Oxidative stress is referred to as an imbalance between the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their clearance by the antioxidant defense system, either by excess production or deficiency of neutralization of ROS/RNS, and it has been associated with some pathological conditions. Oxidative stress causes structural and functional damage to cells, generating new oxidized products that can be chemically reactive and damaging other macromolecules (proteins, lipids, and DNA). Stepwise reduction of oxygen will cause the generation of the most common ROS, such as anion superoxide, hydrogen peroxide, and hydroxyl radical among others. Moreover, the combination of anion superoxide with nitric oxide leads to the formation of peroxynitrite, the most relevant RNS. Both superoxide and hydroxyl ROS are very aggressive free radicals that interacting with target molecules and the antioxidant defense system will determine the redox status of the cellular compartments. Hydrogen peroxide is not a free radical and its biological activity is fundamentally as a signaling molecule within the cells and intercellular cross-talk.<sup>15–17</sup>

**Table 1.** Classification of patients with phenylketonuria (PKU) or hyperphenylalaninemia (HPA) according to clinical and psychological assessment

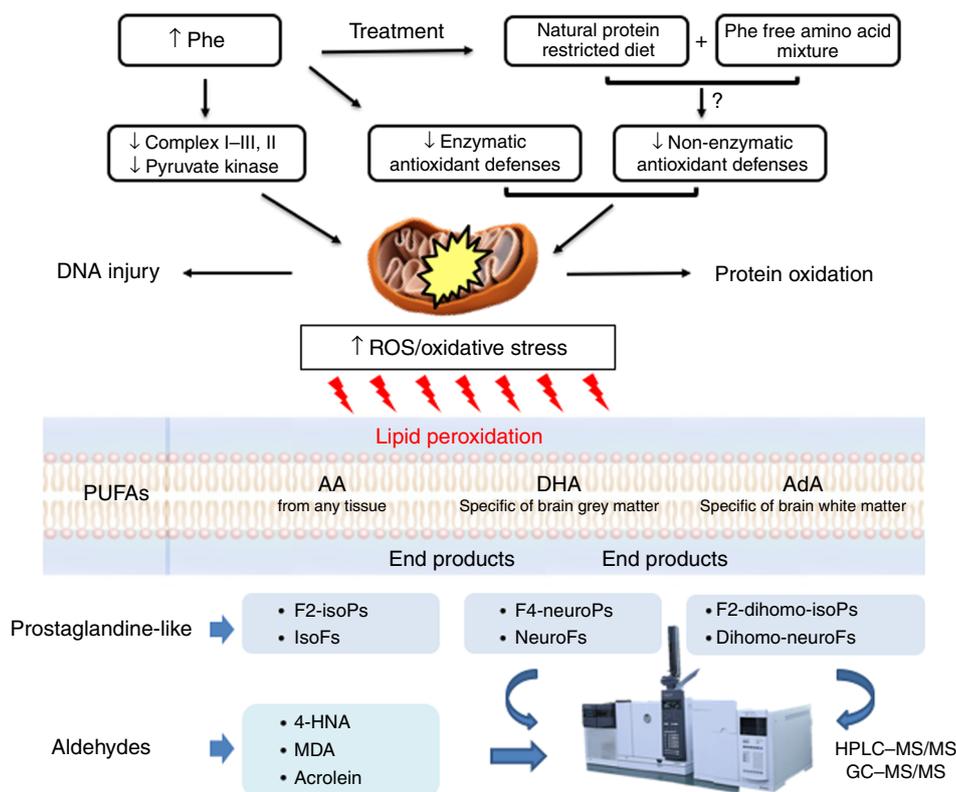
Clinical assessment		Classification			
		PKU	HPA		
Neonatal screening (plasma Phe concentration, $\mu\text{mol L}^{-1}$ )		>360	150–360		
Treatment		Yes	No		
European Guidelines (61) (plasma Phe concentration, $\mu\text{mol L}^{-1}$ )	0–12 years old	120–360	—		
	>12 years old	120–600	—		
American Guidelines (plasma Phe concentration, $\mu\text{mol L}^{-1}$ ) <sup>4</sup>		120–360	—		
Plasma Phe levels monitoring	0–1 years old	Weekly	Monthly		
	1–12 years old	fortnightly	Annually		
	>12 years old	monthly	Biannually		
	<12 years old	Only when indicated	—		
Psychological testing <sup>61</sup>	Neurocognitive assessment	At 12 and 18 years old	Testing at age 12 years. IQ, perception or visuospatial functioning, executive functions and motor control.	—	
		>18 years old	Testing at age 18 years. IQ, perception or visuospatial functioning, executive functions and motor control.	—	
		<12 years old	Annual clinical assessment	—	
	Adaptive issues function examination (e.g., clinically relevant behavioral problems)	At 12 and 18 years old	Screening at age 12 years. Annual clinical assessment	—	
		>18 years old	Screening at age 18 years. Annual clinical assessment	—	
		<12 years old	Annual clinical assessment or discussion once during childhood: PKU-specific quality of life	—	
	Investigations of psychosocial functioning and wellbeing, and health-related quality of life	At 12 and 18 years old	Annual clinical assessment or discussion once during adolescence: PKU-specific quality of life	—	
		>18 years old	Annual clinical assessment or discussion once during adulthood: PKU-specific quality of life	—	
		<12 years old	At onset of psychiatric symptoms	—	
	Psychiatric examination	At 12 and 18 years old	At onset of psychiatric symptoms	—	
		>18 years old	At onset of psychiatric symptoms	—	
		<2.5 years	Bayley Scales <sup>62</sup> as clinically indicated	—	
Psychological testing <sup>3</sup>	Developmental and intellectual	2.5–6 years	Weschler Preschool and Primary Scale (WPPSI-III) <sup>63</sup> every 3 years or as clinically indicated	—	
		>6 years	Weschler Abbreviated Scale (WASI-II) <sup>64</sup> as clinically indicated	—	
		>3 years	Behavior Rating Inventory of Executive Functioning (BRIEF) <sup>65</sup> at least every 2-3 years or as clinically indicated	—	
	Executive functioning	>3 years	Behavior Rating Inventory of Executive Functioning (BRIEF) <sup>65</sup> at least every 2-3 years or as clinically indicated	—	
		3–18 years	Behavioral Assessment Scale for Children (BASC-2) <sup>66</sup> at least every 2-3 years or as clinically indicated	—	
	Behavioral/emotional problems	Adults	Beck Depression Inventory (BDI-II) <sup>67</sup> and/or the Beck Anxiety Inventory (BAI) <sup>68</sup> at least every 2-3 years or as clinically indicated	—	
		>3 years	Adaptive Behavior Assessment System (ABAS-II) <sup>69</sup> at least every 2-3 years or as clinically indicated	—	
	Adaptive skills	>3 years	Adaptive Behavior Assessment System (ABAS-II) <sup>69</sup> at least every 2-3 years or as clinically indicated	—	
		Adults	Adaptive Behavior Assessment System (ABAS-II) <sup>69</sup> as clinically indicated	—	
	IQ intelligence quotient				

**Oxidative stress and PKU**

Recent studies have shown an increased oxidative stress in PKU patients.<sup>18</sup> It has been hypothesized that oxidative stress could be secondary to an increased plasma Phe level that would cause alterations in brain energy metabolism and trigger the generation of RS.<sup>19</sup> Moreover, as shown in Fig. 2, dietary protein restriction can reduce antioxidant defenses, both endogenous enzymatic (SOD, CAT, GPx) and non-enzymatic (glutathione, NADPH, albumin, uric acid, coenzyme Q, bilirubin, melatonin) or exogenous (vitamin E, C,  $\beta$ -carotene), and transition metals (Cu, Se, Zn, Mn). In fact, PKU patients with deficiencies in Se,<sup>20</sup> CoQ10,<sup>21</sup> L-carnitine,<sup>22</sup> and Zn have been widely described in the literature.

**Experimental evidence**

A body of evidence in animal models of hyperphenylalaninemia has shown that elevated concentrations of Phe and neurotoxic derivatives are directly responsible for increased brain oxidative damage or indirectly causing mitochondrial dysfunction or alteration of the antioxidant defense system. Intraperitoneal administration of high doses of Phe to developing Wistar rats caused a substantial reduction in the activities of succinate dehydrogenase and respiratory chain complexes I and III, respectively, thus interfering brain energy metabolism.<sup>23</sup> Preissler et al. incubated astrocytes cultures from newborn Wistar rats with different concentrations of phenylalanine for 72 h. They were able to show that free radical production, alteration of overall



**Fig. 2** The gold standard treatment for patients with Phenylketonuria (PKU) accepted by international organizations is the dietary protein restriction to lower Phenylalanine (Phe) plasmatic levels. Increased Phe will alter specific components of the Krebs cycle and antioxidant enzymes gene expression. Moreover, dietary protein restriction also contributes to the reduction of enzymatic and non-enzymatic antioxidant defenses. Altogether these factors contribute to generate a pro-oxidant status that will cause oxidative damage to protein, DNA, and lipids. Peroxidation of polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA), docosahexanoic acid (DHA) and adrenic acid (AdA) leads to the formation of prostaglandin-like byproducts that can be determined by mass spectrometry and used as biomarkers of damage to the central nervous system. Phe Phenylalanine, ROS reactive oxygen species, IsoPs Isoprostanes, IsoFs Isofurans, NeuroPs Neuroprostanes, NeuroFs Neurofurans, HNA hydroxy-nonenal, MDA malondialdehyde, HPLC high-performance liquid chromatography, GC gas chromatography, MS/MS tandem mass spectrometry

mitochondrial function and diminished cell viability directly correlated with the concentration of Phe in the cell culture media. Hence, Phe was able to induce free radical production and cause astroglia dysfunction or death.<sup>24</sup> In developing rats Fernandes et al.<sup>25</sup> found that Phe concentrations similar to those found in the brain of PKU patients elicited lipid peroxidation and oxidative damage to proteins in the hippocampus and cerebral cortex. Of note, oxidative damage was prevented by the addition of free radical scavengers such as  $\alpha$ -tocopherol or melatonin.

Additional studies have shown that high Phe concentrations can also alter the antioxidant system. Glutathione in its reduced form (GSH) is the most relevant non-enzymatic free radical scavenger and responsible for the redox equilibrium in the cell cytoplasm.<sup>15</sup> The reduced to oxidized glutathione ratio (GSH/GSSG) is kept within physiologic ranges by the action of a set of enzymes including glutathione peroxidase, glutathione reductase, glutathione-S-transferase or glutathione synthase.<sup>26</sup> In a rat model of hyperphenylalaninemia, Barros Moraes et al.<sup>27</sup> found lower activity of the glutathione redox cycle enzymes and consequently GSH concentration was significantly lower. Moreover, in another study with a similar design, levels of Phe and neurotoxic derivatives correlated with decreased activities of superoxide dismutase and catalase. Interestingly, the addition of lipoic acid, a potent antioxidant, partially prevented the effects of Phe upon the antioxidant enzymatic activities.<sup>28</sup>

#### Clinical studies

Numerous clinical studies have confirmed the presence of alterations in the oxidative metabolism in patients with PKU at different ages especially depending on the adherence to the nutritional program and vitamin/mineral supplementation.<sup>29</sup> Recently, several observational studies performed in pediatric patients aged from 5 to 15 years and diagnosed from PKU soon after birth the presence of oxidative stress was analyzed.<sup>19,30,31</sup> In a small trial, Deon et al.<sup>30</sup> aimed to evaluate oxidative damage to biomolecules, antioxidant defenses and pro-inflammatory cytokines in plasma and urine of late diagnosed patients under dietary restriction that already presented neuromotor dysfunction. Interestingly, urinary isoprostanes and di-tyrosine reflecting lipid peroxidation and protein oxidation respectively were significantly increased as compared to normal controls. Moreover, there was a decrease in the total antioxidant capacity of PKU patients and a significant correlation could be established between urinary isoprostanes and IL-1 $\beta$  reflecting a coexistence of oxidative stress and inflammation in patients with established PKU.<sup>30</sup> However, the reduced number and the advanced clinical deterioration in the PKU patients limited the applicability of the results for a screening program and/or for assessing the effectiveness of an intervention. More recently, Kumru et al.<sup>19</sup> compared glutathione peroxidase activity, and Q10 and L-carnitine levels study in early diagnosed PKU patients (<1 month) who showed poor or good adherence to dietary restriction according to the Phe plasma levels at 2 years of age. Results showed that patients with poor adherence were

**Table 2.** Analytical studies related to oxidative stress biomarkers associated to neurological damage in patients with Phenylketonuria (PKU) or Hyperphenylalaninemia (HPA)

Author, Year, ref.	Patients characteristics	Biomarkers	Sample	Analytical technique
Artuch et al. <sup>21</sup>	PKU (n = 30), HC (n = 30)	CoQ10	Serum	HPLC with ultraviolet detection
Artuch et al. <sup>70</sup>	Good metabolic control PKU (n = 24) Poor metabolic control PKU (n = 22) HC (n = 58)	CAT activity, Se, CoQ10	Plasma and erythrocytes	Atomic absorption spectrophotometry for Se, chromatography for CoQ10 and spectrophotometry for CAT
Colomé et al. <sup>37</sup>	PKU (n = 58), HPA (n = 30), HC (n = 58)	Tocopherol, retinol, Se, GSH-Px, MDA	Plasma	HPLC with electrochemical detection, and HPLC with ultraviolet detection
Darling et al. <sup>71</sup>	PKU and HPA (n = 73); HC (n = 25)	Se	Serum	Atomic absorption spectrophotometry
Deon et al. <sup>30</sup>	PKU (n = 10), HC (n = 10)	15-F <sub>2t</sub> -isoprostane, di-tyrosine, TAR, cytokines (IL-1β, IL-6, IL-10)	Urine plasma	Enzyme-linked immunoassay, fluorescence, and chemical assay
Gassió et al. <sup>72</sup>	PKU (n = 36), HC (n = 29)	Se, retinol, tocopherol, CoQ10, MDA	Plasma erythrocytes	
He et al. <sup>73</sup>	PKU (n = 40), HC (n = 40)	TAR, GSH, MDA, 8-isoprostane	Plasma	Spectrophotometric assay
Sanayama et al. <sup>18</sup>	PKU (n = 40), HC (n = 30)	TBARS, TAR, GSH-Px, CAT, SOD	Plasma erythrocytes	Enzyme-linked immunoassay and spectrophotometric assay
Schulpis et al. <sup>38</sup>	Good metabolic control PKU (n = 20) Poor metabolic control PKU (n = 24) HC (n = 40)	TAR	Plasma	HPLC and spectrophotometric assay
Schulpis and Kariyannis <sup>74</sup>	Good metabolic control PKU (n = 13) Poor metabolic control PKU (n = 12) HC (n = 23)	S-100B	Serum	Immunoluminometric assay
Schulpis et al. <sup>39</sup>	Good metabolic control PKU (n = 24) Poor metabolic control PKU (n = 25) HC (n = 24)	TAR, 8-OHdG	Plasma serum	Spectrophotometric assay and immunoassay
Sierra et al. <sup>75</sup>	PKU (n = 42), HPA (n = 28), HC (n = 45)	GSH-Px	Plasma and erythrocytes	Spectrophotometry
Sirtori et al. <sup>33</sup>	PKU (n = 20), HC (n = 20)	TBARS, TAR, GSH-Px, CAT, SOD	Plasma and erythrocytes	Spectrophotometry and chemiluminescence
Sitta et al. <sup>35</sup>	Good metabolic control PKU (n = 7) Poor metabolic control PKU (n = 7) HC (n = 7)	TBARS, TAR, CAT, SOD, GSH-Px	Plasma and erythrocytes	Spectrophotometry and chemiluminescence
Sitta et al. <sup>40</sup>	Good metabolic control PKU (n = 8) Poor metabolic control PKU (n = 10) HC (n = 17)	DNA damage	Blood	Commet assay
Sitta et al. <sup>41</sup>	Early diagnosed PKU (n = 10) Lately diagnosed PKU (n = 10) HC (n = 10)	TBARS, TAR, GSH-Px, Protein Carbonyl Content, GSH, sulfhydryl content.	Plasma and erythrocytes	Spectrophotometry for enzyme activity, chemiluminescence, fluorescence for GSH
Sitta et al. <sup>34</sup>	PKU (n = 18) before and after Se and LC treatment, HC (n = 18)	TBARS, GSH-Px, Protein Carbonyl Content, sulfhydryl oxidation, CAT, SOD	Plasma and erythrocytes	Spectrophotometry, liquid chromatography electrospray tandem mass spectrometry for L-carnitine, and atomic absorption spectrophotometry for Se
Tavana et al. <sup>76</sup>	PKU (n = 20), HC (n = 50)	Prooxidant-antioxidant balance (PAB)	Serum	Spectrophotometric assay
van Bakel et al. <sup>32</sup>	PKU (n = 24), HPA (n = 10), HC (n = 42)	Se, TAR, SOD activity, GSH-Px activity, GSH	Plasma	Electrothermal atomic absorption spectrophotometry for Se, spectrophotometry for enzymes activity, fluorescence for GSH
Wilke et al. <sup>36</sup>	PKU (n = 15), HC (n = 30)	Se, GSH-Px, MDA	Plasma and erythrocyte	Electrothermal atomic absorption for Se, spectrophotometry for enzyme activity, and fluorometry for MDA

\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; n.s. not significant differences between groups  
 HC Healthy Control, SOD superoxide dismutase, MDA malondialdehyde, TBARS thiobarbituric acid reactive substances Se Selenium, LC L-carnitine, GSH-Px glutathione peroxidase, TAR total antioxidant reactivity, MDA maleic dialdehyde, 8-OHdG 8-hydroxy-2-deoxyguanosine

prone to oxidative stress while those with good adherence did not show any differences when compared with normal controls. Although this study included early diagnosed patients and Phe levels were strictly controlled, the reduced number of patients did not allow for generalization of results among PKU patients. Moreover, no information regarding the neurodevelopmental status at 2 years of age of these infants was provided in the study.<sup>19</sup> The most recent study published<sup>31</sup> obviated some of the limitations of the previous studies. A total of 60 PKU patients were included and compared with 30 normal controls. Patients were classified according to the levels of Phe with 30 patients pertaining to the hyperphenylalaninemic group (Phe 600 to >1200 μmol L<sup>-1</sup>) and 30 patients in the Phe-free amino acid admixture group (Phe < 120 μmol L<sup>-1</sup>). The study included the determination of a broad array biomarkers of oxidative stress, a wide panel of amino acids and anti-inflammatory markers derived from sialic acid. Both PKU groups showed a significant pro-oxidant status as compared with the normal controls. However, treatment with phenyl-free amino acid mixture had significant influence on specific amino acid levels and especially on α-amino adipic acid

and phyloquinone. The authors propose to include both these biomarkers together with Phe to better control PKU status and regulate amino acid supplementation.<sup>31</sup> Limitations to this study include a lack of information regarding the neurological and motor status of these patients and the lack of specificity of some oxidative stress markers especially in relation to brain oxidative damage. In view of this, we still need adequately powered studies in the pediatric age that would include patients classified according to Phe levels, highly specific biomarkers related to brain damage (see below) and neurodevelopmental status.

#### Assessment of oxidative stress in biofluids

Oxidative stress has been assessed determining the end-products of the oxidant action of RS to proteins, DNA or lipids in PKU serum samples. Different approaches have been employed to assess the level of these byproducts (summary of analytical methods in Table 2). Hence, the ability to attenuate RS has been measured using plasma total antioxidant reactivity (TAR), erythrocyte glutathione peroxidase (GSH-Px) activity<sup>32,33</sup> or the activities of other antioxidant enzymes, such as catalase or superoxide

dismutase in red blood cells by means of spectrophotometry or chemiluminescence.<sup>34,35</sup> Furthermore, the determination of plasma antioxidant cofactors, such as selenium (Se, cofactor of the enzyme GSH-Px) by means of electrothermal atomic absorption spectrophotometry,<sup>36</sup> as well as, retinol, tocopherol and coenzyme Q10 (CoQ10) by HPLC has also provided information about the antioxidant status in PKU patients.<sup>9,21,37</sup> Specifically, decreased serum CoQ10 concentrations were determined in PKU patients by means of HPLC and UV detection.<sup>21</sup> Long-term low Se and CoQ10 levels may impair health.

In general, decreased levels of antioxidant enzymes and cofactors, and increased levels of oxidized lipids, proteins, and DNA have been found in PKU and HPA patients as compared to healthy controls. In addition, within the PKU subgroups, lower plasma TAR determined by HPLC and spectrophotometry, while higher plasma 8-OH-dG levels obtained using spectrophotometric immunoassay, as well as higher blood DNA damage levels determined by comet assay were found in poorly controlled as compared to adequately controlled PKU patients.<sup>38–40</sup> Seemingly, lower GSH-Px activity determined in erythrocytes by spectrophotometry was found in late as compared to early diagnosed PKU.<sup>41</sup> Finally, PKU patients treated with supplements of L-carnitine and Se exhibited higher SOD and GSH-Px activities reflecting an enhanced antioxidant response capacity as response to the therapy.<sup>32,34</sup>

#### Genetic studies

Recently, the level of ROS in whole blood and antioxidant gene expression profile in leukocytes of adult patients with PKU was evaluated using flow cytometry and real-time PCR respectively and compared with normal controls. The molecular antioxidant signature of white blood cells of PKU patients showed a global decrease of gene expression of relevant antioxidant enzymes such as *PRDX1*, *GPX4*, *GLRX3*, *SOD1*, *PRDX2*, *TXN2*, *PRDX4*, *GLRX5*, *GPX7*, and *SOD3*. Interestingly, a correlation between gene expression and plasma levels of Phe was established. These results support the idea of a strong connection between metabolic alterations and redox status in patients with uncontrolled PKU.<sup>42</sup> However, monitoring of oxidative stress in newborn infants with classical PKU renders extremely difficult. In this regard, the analysis of the demethylation of an allele of the promoter region of *GPX3* activated by oxidative stimuli using DNA extracted from blood leukocytes in a newborn with classical PKU and Phe > 465  $\mu\text{mol L}^{-1}$  after the second day of life has shed light upon the influence of time and Phe concentration upon the early initiation of oxidative stress in newborn infants. This approach could be used as an early epigenetic marker for an extracellular monitoring of increased RS production in newborns with PKU.<sup>43</sup> Ongoing oxidative stress in patients with a poor metabolic control would drive the transition from AMP activated protein kinase (AMPK)/PGC-1 $\alpha$ /Forkhead box O (FOXO3a)-linked repair responses toward pro-inflammatory responses controlled by the m-TOR/HIF-1 $\alpha$ /NF- $\kappa$  axis thus favoring chronic disease progression and neurodegeneration.<sup>44</sup>

#### New brain-specific lipid peroxidation biomarkers

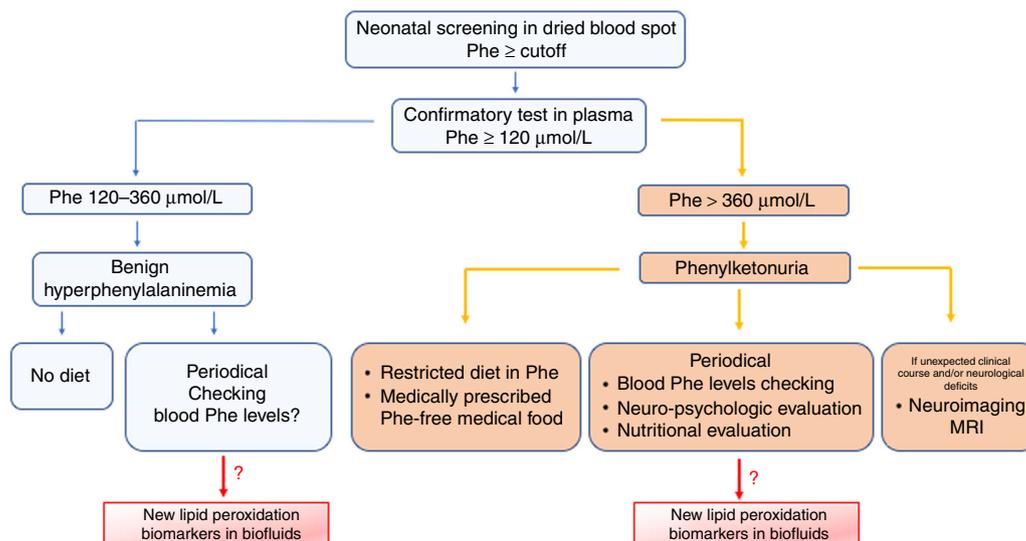
The first studies that analyzed lipid peroxidation showed significantly higher levels of thiobarbituric acid reactive substances (TBARS) determined by means of spectrophotometry in plasma samples from PKU patients than from controls, especially in patients with poor metabolic control suggesting a correlation between high Phe levels and oxidative stress.<sup>35</sup> Sanayama et al. also determined oxidative stress biomarkers in serum samples, and found significantly higher TBARS, acrolein-lysine and malondialdehyde (MDA) levels by means of enzymatic immunoassay and spectrophotometry in PKU patients than in controls.<sup>18</sup> Hence, they established a correlation between high Phe plasma levels, increased lipid peroxidation markers, brain damage, and neuropathological disorders.<sup>9,18</sup>

In recent experimental and clinical studies using lipidomic platforms, the composition of brain tissue has been extensively analyzed (for a review on brain lipid composition go to ref. <sup>45</sup>). The CNS is especially rich in two major polyunsaturated fatty acids (PUFA) specifically adrenic acid (AdA; 22:4  $\omega$ 6) and docosahexanoic acid (DHA; 22:6  $\omega$ 3). Studies in primates have shown that AdA is especially abundant in the white matter; however, it can also be found in the synaptic vesicles that are mainly located in the gray matter. DHA is an essential component of neuronal membranes that are present mostly as cell bodies and dendrites in gray matter and as axons in white matter.<sup>46</sup> PUFAs are extremely vulnerable to oxidation both enzymatic and non-enzymatic. The use of mass spectrometry coupled to high-performance liquid chromatography and/or gas chromatography has provided the means to extensively determine PUFA peroxidation byproducts (Table 2). The in vivo lipid peroxidation biomarkers isoprostanes (IsoPs), constitute a family of compounds generated by free radical-catalyzed oxidation of different PUFAs, including arachidonic acid (AA) and eicosapentanoic acid that lead to the formation of F2-IsoPs and F3-IsoPs, respectively (Fig. 2). In the same way, neuroprostanes (NeuroPs) and dihomono-isoprostanes (dihomo-IsoPs) are formed by autooxidation of PUFAs, docosahexanoic acid (DHA) and adrenic acid (AdA), respectively. At higher oxidation grade, lipid peroxidation of AA and DHA yields isofurans (IsoFs) and neurofurans (NeuroFs), respectively. The high specificity of these metabolites has been acknowledged and at present they constitute the gold standard for the assessment of brain oxidative damage by free radicals (Fig. 2).<sup>45–48</sup>

In the last few years development of strategies for synthesis of pure PUFAs metabolites to be used as analytical standards together with mass spectrometry has led to onset a reliable methodology for analytical determination of these compounds.<sup>47,49</sup> Deon et al. found significantly elevated levels of F2-IsoPs determined by enzyme-linked immunoassay in urine samples from PKU patients with late diagnosis and under dietary treatment compared to controls, but no correlation was found between Phe and F2-IsoPs levels.<sup>30</sup> In general, the levels of these compounds were higher in PKU patients, so they were considered satisfactory neurological damage biomarkers in PKU. However, little information regarding more CNS-specific metabolites such as F2-dihomo-isoPs, F4-NeuroPs, or F3-NeuroPs have been reported in patients with PKU. Nevertheless, significantly increased levels were found in urine and plasma samples from patients with other neurological diseases, such as Alzheimer Disease by means of UPLC coupled to tandem mass spectrometry,<sup>49</sup> or Rett's Syndrome.<sup>50</sup> In addition, studies carried out in newborns with severe metabolic acidosis,<sup>51</sup> and preterm newborns<sup>52</sup> showed higher levels of these compounds in cord serum and plasma samples, respectively, that may be associated to adverse long-term outcomes.

#### Updated analytical methodology

In recent years reliable analytical methods based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to determine biomarkers in biological samples have been validated.<sup>53–55</sup> Determination of F<sub>2</sub>-IsoPs from AA oxidation is considered the reference index in the evaluation of in vivo lipid peroxidation; however, given its ubiquitous distribution AA does not inform exclusively on brain tissue peroxidation unless determined in the cerebrospinal fluid. On the other hand, F<sub>4</sub>-NeuroPs and neuroFs are considered specific markers of neuronal damage and F<sub>2</sub>-dihomo-isoPs are considered sensitive and specific markers of oxidative damage to myelin.<sup>48,56</sup> Recent studies have validated following the stringent requirements of the FDA the corresponding analytical methods to determine this family of isoprostanooids in different biological samples (urine, plasma, saliva) specially to facilitate non-invasive determination in preterm infants in whom invasive access is restricted.<sup>57–60</sup>



**Fig. 3** Flow diagram for screening, diagnosis, dietary control, and monitoring of Phenylketonuria

Different sample treatment procedures were developed depending on the sample matrix in order to improve the analytes extraction. In this sense, solid-phase extraction was used for pre-concentration and clean-up urine and plasma samples,<sup>53–55,59,60</sup> while liquid–liquid extraction was used for saliva samples.<sup>58</sup> In general, these analytical methods are characterized by high throughput, sensitivity, selectivity, precision, and accuracy.

#### PKU monitoring proposal

Brain composition and metabolism is highly vulnerable to oxidative stress. Thus, both oxidative stress and brain Phe and neurotoxic derivatives' accumulation contribute to the neurological dysfunction (mental retardation, psychomotor delay, decreased cognitive functions, increased risk for neurodegenerative processes) in PKU. Clinical studies have focused on two types of oxidative stress determinations including enzymatic and non-enzymatic antioxidant defenses,<sup>14,18–22,32</sup> and biomolecules resulting from oxidative damage.<sup>14,18,30,33,35,38,39,41</sup> Antioxidant determinations (total antioxidant status, beta-carotene, coenzyme Q, glutathione peroxidase (GSHPx), catalase, superoxide dismutase, coenzyme Q10 (Q10), Q10/cholesterol, L-carnitine) have been determined using spectrophotometry and chemical and immune assays for other antioxidant substances. However, these techniques are characterized by limited sensitivity and specificity. Lower antioxidant levels<sup>14,18,19,21,41</sup> or not differences at all<sup>19,32,35</sup> were found in PKU patients with poor adherence. In addition, no correlation was found between antioxidant status and Phe blood levels,<sup>18,35</sup> explaining the considerable number of PKU patients with good diet adherence that present neurological damage. Regarding the biomolecules oxidative damage determinations (TBARS, isoprostane, malondialdehyde, 8-OHdG, di-tyrosine), they are mainly carried out by immunoassays,<sup>18,30,39</sup> fluorometry,<sup>30,32,36</sup> or chemiluminescence techniques,<sup>33,35,41</sup> which usually show low reproducibility and sensitivity; while few works used liquid chromatography with electrochemistry or UV detection,<sup>21,38</sup> which their application is limited to compounds with specific chemical characteristics.

Nowadays, mass spectrometry detection is considered a reliable, robust and high sensitivity technique which could be coupled to gas chromatography or liquid chromatography, depending on the analytes' characteristics. The corresponding validated analytical methods show the advantages of high reproducibility, selectivity and throughput. In this sense, mass spectrometry constitutes a very useful tool in the future PKU monitoring.

Our proposal for PKU monitoring (Fig. 3) is based on the incorporation of non-invasive sequential determinations (e.g., saliva, urine) of brain-specific oxidative stress biomarkers using validated analytical methods based on mass spectrometry to the routine measurement of Phe levels and neuropsychological and nutritional evaluations, providing clinicians with objective and reproducible results that would help them to establish an individualized therapeutic approach.

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#### ADDITIONAL INFORMATION

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