Aging and Disease
www.aginganddisease.org
Volume 6, Number 5; 390-399, October 2015
http://dx.doi.org/10.14336/AD.2015.0827

Review Article

Phenylketonuria Pathophysiology: on the Role of Metabolic Alterations

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[Received July 16, 2015; Revised August 25, 2015; Accepted August 27, 2015]

ABSTRACT: Phenylketonuria (PKU) is an inborn error of phenylalanine (Phe) metabolism caused by the deficiency of phenylalanine hydroxylase. This deficiency leads to the accumulation of Phe and its metabolites in tissues and body fluids of PKU patients. The main signs and symptoms are found in the brain but the pathophysiology of this disease is not well understood. In this context, metabolic alterations such as oxidative stress, mitochondrial dysfunction, and impaired protein and neurotransmitters synthesis have been described both in animal models and patients. This review aims to discuss the main metabolic disturbances reported in PKU and relate them with the pathophysiology of this disease. The elucidation of the pathophysiology of brain damage found in PKU patients will help to develop better therapeutic strategies to improve quality of life of patients affected by this condition.

Key words: brain; hyperphenylalaninemia; metabolic alterations; phenylalanine; phenylketonuria

First described in 1934 by Abjörn Følling, phenylketonuria (PKU; OMIM # 261600) is an autosomal recessive inborn error of L-phenylalanine (Phe) metabolism. Phenylketonuric patients present with high Phe concentrations in their tissues and hyperphenylalaninemia (HPA) due to total or partial deficiency of phenylalanine hydroxylase (PAH; EC # 1.14.16.1) activity, as well as low tyrosine (Tyr) concentrations [1]. Phe is an essential amino acid obtained exclusively by the diet or by proteolysis. It is crucial for protein synthesis, as well as for the synthesis of Tyr and its derivatives, such as dopamine, norepinephrine, and melanin [1,2]. The major metabolic Phe pathway involves its hydroxylation to Tyr by PAH, found mainly in liver, but also in kidney [3,4]. Alternative pathways are less significant and include Phe transamination in its alanine side-chain forming phenylpyruvic acid (PPA), that is catalyzed by the enzyme phenylalanine (histidine) transaminase (EC # 2.6.1.58), found mainly in liver. Since this reaction is activated by substrate, it becomes more relevant in the events of high Phe blood levels [5]. Phe can also undergo decarboxylation to phenylethylamine, although at low rates, by the action of phenylalanine decarboxylase (EC # 4.1.1.53) [6] (Figure 1).

There are many mutations in the *PAH* gene (localized in the chromosome 12q) already described [7-9]. Rigorous Phe-restrict diet is the cornerstone of therapeutic PKU management [10,11]. Improved clinical findings are ascribed to dietary therapy in phenylketonuric patients, particularly decreasing aberrant plasma Phe levels [12,13]. Nevertheless, some putative side effects have been associated to this therapy, including zinc, selenium and iron deficiencies [14]. Copper, zinc [15], magnesium [16], and selenium [17] plasma levels in PKU treated children are significantly decreased, as compared to non-PKU children.

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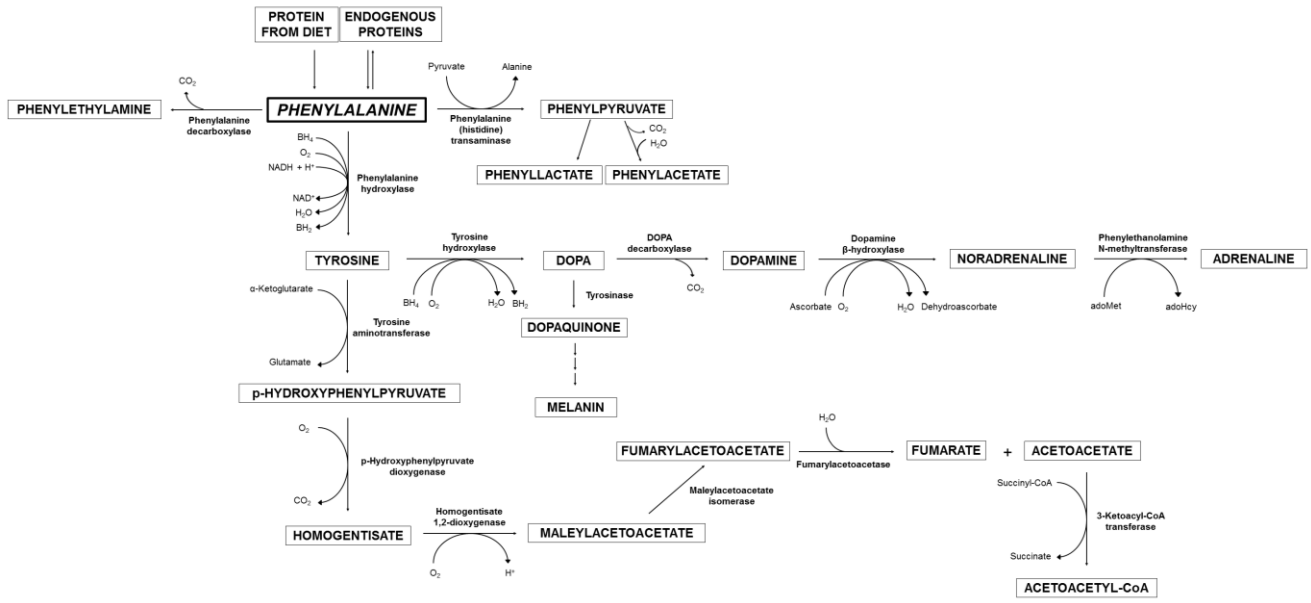

Figure 1. Phenylalanine metabolism. Most of phenylalanine obtained from diet or endogenous proteolysis is hydroxylated producing tyrosine by phenylalanine hydroxylase, which is deficient in PKU. Additional routes include transamination to phenylpyruvate and decarboxylation in order to synthesize phenylethylamine.

The main findings presented by phenylketonuric patients are severe neurological damage, including corpus callosum, striatum, and cortical alterations and hypomyelination, that result in intellectual deficit and neurodegeneration [18-21]. However, the pathophysiology underlying the brain damage is not well understood yet. The main hypothesis is that Phe and its metabolites act as neurotoxins in the brain. Some pathomechanisms involving metabolic alterations are proposed and will be discussed below.

Oxidative stress

Oxidative stress is defined as the lack of balance between reactive oxygen/nitrogen species production and the antioxidant system [22]. Such imbalance may induce oxidative damage to proteins, lipids, or DNA. In fact, oxidative stress has been associated with the pathophysiology of several neurodegenerative diseases, including Parkinson's and Alzheimer's disease, epilepsy, and demyelination [22-30]. It has been demonstrated that the brain tissue is particularly vulnerable to oxidative stress due to high O_2 consumption, high tissue concentrations of iron, low level of antioxidant defenses, the presence of excitatory amino acids and dopamine metabolism, which generate hydrogen peroxide [22].

Over the last years, oxidative damage to macromolecules has been investigated in HPA animal models and biological samples from PKU patients. It was demonstrated that high Phe levels are associated with DNA, protein, and lipid damage, as well with decreased antioxidant defenses in phenylketonuric patients. In this scenario, DNA damage was reported in peripheral blood from PKU patients *in vivo* and *in vitro* in a dose-dependent manner [31,32]. In addition, protein and lipid oxidative damage, measured by carbonyl formation and sulfhydryl oxidation, and thiobarbituric acid-reactive species (TBA-RS) and malondialdehyde (MDA) content, respectively, were described in plasma and erythrocytes from PKU patients [31,33-36]. Decreased antioxidant defenses, both enzymatic and non-enzymatic, were also found in these subjects. Low levels of plasma total antioxidant status and reactivity, L-carnitine, beta-carotene and coenzyme Q_{10}, and altered catalase [CAT], superoxide dismutase [SOD] and glutathione peroxidase [GPx] activities in PKU patients samples indicate an impairment of antioxidant defense that could result in oxidative stress [31,33,35,36]. Some of these findings were reversed by L-carnitine and selenium supplementation [37].

HPA animal models also provide relevant evidence for the elucidation of the role of oxidative stress in the pathophysiology of PKU. Several in vivo and in vitro studies show impairment of the antioxidant enzymes CAT, SOD and GPx, as well decreased non-enzymatic antioxidants levels, including alterations in glutathione

(GSH) metabolism in rat brain tissues. In this scenario, high Phe concentrations also increases sulfhydryl oxidation, TBA-RS and MDA levels, and 2',7'-dichlorofluorescein (DCFH) oxidation, indicating protein and lipid oxidative damage and increased production of reactive species of oxygen, respectively [38-43]. Most of these findings were prevented by supplementation with lipoic acid, melatonin, alpha-tocopherol and/or ascorbic acid, which are potent and well-known antioxidants [22]. Furthermore, *in vitro* and *in vivo* DNA damage was found both in patients [32,44] and animal models [45] and it was proposed that it is associated with oxidative damage.

In vitro experiments demonstrated that the Phe-derived metabolites PPA, phenyllactic (PLA), and phenylacetic (PAA) acids, which also accumulate in PKU, affect antioxidant enzymes. While PLA and PAA increase SOD activity in rat brain tissue, PPA decreases glucose-6-phosphate dehydrogenase (EC # 1.1.1.49) in the same tissue [43,46], indicating a possible indirect mechanism by which Phe leads to oxidative stress in PKU. The main findings on disruption of redox homeostasis reported in patients and animal models of PKU are summarized in Table 1.

Table 1. Landmark studies showing oxidative stress in phenylketonuria.

Reference	Sample	Findings
Schulpis et al., 2005	PKU Patients Blood	↓ TAS ↑ 8-OH-DG
Sitta et al., 2009	PKU patients leucocytes; normal Individuals leukocytes	↑ DNA damage index *in vivo* ↑ DNA damage index *in vitro*
Sirtori et al., 2005	PKU patients plasma and erythrocytes	↓ TAR, GPx ↑ TBA-RS
Sitta et al., 2009	PKU Patients Blood	↓ TAR ↑ TBA-RS
Sitta et al., 2009	PKU patients plasma and erythrocytes	↓ GSH, GPx, TAR, SH ↑ TBA-RS, protein carbonyl
Sanayama et al., 2011	PKU patients plasma and erythrocytes	↓ TAR, GPx, beta-carotene, Q10 ↑ TBA-RS, MDA-modified LDL, CAT, SOD
Ercal et al., 2002	Mice brain and red blood cells	↑ MDA, G6PD, CAT ↓ GSH/GSSG, NADPH
Kienzle Hagen et al., 2002	Rat brain	↑ chemiluminescence ↓ TRAP, CAT, GPx
Martinez-Cruz et al., 2002	Rat brain and cerebellum	↑ Ehrlich adducts, MDA, GSSG, HO-1 ↓ GPx, GR, MAPK 1/2
Fernandes et al., 2010	Rat brain	↑ TBA-RS ↓ SH, GSH
Moraes et al., 2010	Rat brain	↓ CAT, SOD, GPx, G6PD, GSH, TRAP ↑ TBA-RS, ROS
Moraes et al., 2013	Rat brain	↑ TBA-RS, protein carbonyl, SOD, ROS ↓ CAT
Deon et al., 2015	Normal individuals plasma; PKU patients plasma and urine	↑ DNA damage index ↑ 8-OH-DG
Simon et al., 2013	Rat brain and blood	↑ DNA damage index *in vivo* ↑ DNA damage index *in vitro*
Rosa et al., 2012	Rat brain	↓ G6PD

CAT – catalase; GPx – glutathione peroxidase; G6PD – glucose 6-phosphate dehydrogenase; GSH – reduced glutathione; GSSG – glutathione disulfide; GR – glutathione reductase; HO-1 – hemeoxygenase-1; LDL – low density lipoprotein; MAPK 1/2 - Mitogen-activated protein kinases; MDA – malondialdehyde; NADPH - nicotinamide adenine dinucleotide phosphate; PKU – phenylketonuria; Q10 – coenzyme Q10; ROS – reactive oxygen species; SH – sulfhydryl group; SOD – superoxide dismutase; TAR - total antioxidant reactivity; TAS - total antioxidant status; TBA-RS – thiobarbituric acid-reactive species; TRAP - total radical-trapping antioxidant potential 8-OH-DG - 8-hydroxy-2-deoxyguanosine.

Neurotransmitters metabolism

Neurochemical and behavioral studies have demonstrated that animals fed with diets rich in Phe present decreased brain serotonin levels and impaired behavior in some problem-solving tasks [47]. Recently, it has also been demonstrated that these patients are more susceptible to neurological symptoms caused by cerebral dopamine deficiency, such as Parkinsonism [2]. Indeed, several studies have shown that high Phe concentrations is associated with decreased serotonin, dopamine, and norepinephrine levels in human and murine PKU [48-52]. The decrease of these neurotransmitters levels could be related to the effect of high Phe concentration on amino acids transport through the blood-brain barrier (BBB) (such as Tyr and tryptophan – Trp) or on enzymes involved in neurotransmitters synthesis [48]. It is important to stress out that large neutral amino acid (LNNA) transporter has high affinity for Phe, which competes with other amino acids to cross BBB [53], consequently reducing the amount of Trp and Tyr available for neurotransmitter synthesis [52,54,55].

Phe is also a competitive inhibitor of Tyr hydroxylase (EC # 1.14.16.2) and Trp hydroxylase (EC # 1.14.16.4), important enzymes for the brain synthesis of the neurotransmitters dopamine and serotonin, respectively [56-60]. Phe metabolites also inhibit 5-hydroxytryptophan decarboxylase/dopa decarboxylase (EC # 4.1.1.28), another enzyme involved in neurotransmitters metabolism [61]. Such effects collaborate to the impairment of catecholamines in the brain found in PKU patients and animal models. Table II summarizes the main findings on the alterations of neurotransmitters metabolism in patients and animal models of PKU.

Table 2. Landmarks studies showing impaired neurotransmitter metabolism in phenylketonuria.

Reference	Sample	Findings
Güttler and Lou, 1986	PKU patients urine and CSF	↓ dopamine, serotonine, HVA, 5-HIAA
Yano et al., 2013	PKU patients urine and serum	↓ melatonin, 6-sulfatoxymelatonin, dopamine
Pascucci et al., 2002	Mouse brain	↓ serotonin, Trp, 5-HT
Landvogt et al., 2007	PKU patients	↓ fluoro-L-dopamine uptake
Sawin et al., 2014	Mouse brain	↓ catecholamines, serotonin
Harding et al., 2014	Mouse brain	↓ TH
Justice and Hsia, 1965	Mouse brain	↓ 5THD

5-HIAA - 5-hydroxyindoleacetic acid; 5-HT - 5-hydroxytryptophan; 5HTD - 5-hydroxytryptophan; CSF – cerebrospinal fluid; HVA - homovanillic acid; TH – tyrosine hydroxylase; Trp – triptophan; Tyr - tyrosine

Protein synthesis

Investigating Phe effect towards rat brain protein synthesis, Agrawal and coworkers [62] identified an inhibition of [^{35}S]methionine incorporation rate into proteins in 8- and 18-day-old rat brain, as well as decreased [^{35}S]methionine and [^{14}C]leucine transport into the brain acid-soluble pool in 18-day-old rats. *In vitro* studies performed in a cell-free system derived from hamster brain suggested that the failure in protein synthesis caused by Phe might to be due to impairment in the process of initiation [63]. Indeed, it was reported a negative correlation between cerebral protein synthesis rate and supra-physiological plasma concentrations of Phe (200–500 μM) [64]. In addition, it was documented that increased plasma Phe concentration was inversely related to cerebral protein synthesis rate, possibly via impaired LNAA transport across BBB [65] and decreased Tyr incorporation into protein in central nervous system in PKU patients [66]. Saturation of L-amino acid transporter at BBB by increased plasma Phe seems to be a contributing but not pivotal factor to the decreased protein synthesis observed in genetic PKU murine models [67].

By using positron emission tomography to study brain of PKU patients after an intravenous L-[1-(^{11})C]-Tyr administration, Hoeksma and colleagues [68]

demonstrated a significant negative correlation between plasma Phe levels and the cerebral protein synthesis rate. This implies that Phe levels should be lower than 600–800 μmol/L to prevent the disruption of protein synthesis.

Gropper and coworkers [69] showed that the mean plasma immunoglobulin A (IgA) and immunoglobulin G (IgG) concentrations in PKU children were significantly decreased as compared to values from similar aged healthy children, a finding that could be secondary to a poor nutritional status of Phe-restrict diet therapy. Additionally, Imperlini and colleagues [70] compared cerebral protein expression of homozygous PKU mice to that of their heterozygous counterparts by using an electrophoretic technique and identified 21 differentially expressed proteins, four of which were over-expressed (including *Glu2/3* and *NR1*) and 17 under-expressed (*e.g. Ckb*, *Dpysl2*, *Eno1*, *Pgam1*, *Pkm*, and *Syn2*). Due to the significant role of these proteins on brain physiology, the authors concluded that such pattern of expression may be related to the processes underlying PKU brain dysfunction, namely decreased synaptic plasticity, impaired neurotransmission, and impaired brain bioenergetics. Increased levels of Phe and related metabolites were also associated with impaired biosynthesis of antioxidants enzymes in PKU, connecting the impaired protein synthesis induced by Phe to disruption of redox homeostasis [71]. Despite all these findings, it is still to be clarified whether the decreased protein synthesis is due to an intrinsic toxic effect of high Phe levels in tissues or due to a poor nutritional status of PKU patients under rigorous Phe-restrict diet therapy.

Lipid metabolism

Several studies have shown that lipid metabolism is impaired in PKU, which could collaborate to the hypomyelination found in patients. In this scenario, Nagasaka and colleagues [72] demonstrated that phenylketonuric patients have altered serum lipoprotein levels, including lower levels of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and apolipoprotein A-I/A-II and B. These low cholesterol levels may be explained by the impairment of cholesterol synthesis due to down-regulated expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC # 1.1.1.34), the rate controlling enzyme in the cholesterogenesis, as observed in a knock out murine model of PKU [73]. Furthermore, it has been demonstrated that Phe, PPA and PAA inhibit *in vitro* the activities of mevalonate 5-pyrophosphate decarboxylase (EC # 4.1.1.33) and HMGR in chicken liver at concentrations similar to those found in PKU patients [74]. Oxysterols and vitamin D levels, both important metabolites from cholesterol, are also found decreased in serum from phenylketonuric adult patients [75].

In addition, it has been shown that docosahexaenoic acid (DHA) (an essential omega-3 long-chain polyunsaturated fatty acid) levels are lower in plasma phospholipids [76] and in blood samples from phenylketonuric patients [59], and such deficit could collaborate to the neural damage found in this disease [77]. Moreover, PKU patients presented lower concentrations of eicosapentaenoic acid, DHA, and arachidonic acid (AA), as compared with healthy controls [78]. In this context, it has been suggested that Phe and its metabolites affect long-chain polyunsaturated fatty acids biosynthesis by inhibiting a deoxygenize reaction, impairing DHA and AA synthesis [79].

Bioenergetics

Brain energy metabolism alterations play an important role in the pathophysiology of many inborn errors of metabolism [80-83]. In this context, energy metabolism impairment was reported in HPA animal models and patients. Significant decrease of succinate dehydrogenase (EC # 1.3.5.1) and mitochondrial respiratory chain complexes I–III activities were detected in cerebral cortex of rats subjected to experimental HPA [84], as well as decreased serum ubiquinone-10 (Coenzyme Q) concentrations in phenylketonuric patients [85]. On the other hand, Kyprianou and colleagues [86] showed no significant difference in mitochondrial respiratory chain complex I (EC # 1.6.5.3) activity in astrocytoma cells between PKU patients with or without tremor, suggesting that Phe neurotoxicity towards PKU patients does not involve the parameter of mitochondrial respiratory function. Respiratory chain complex II-III activity was also not altered in blood mononuclear cells from phenylketonuric patients [87].

Creatine kinase (CK; EC #2.7.3.2) activity, a key enzyme for maintenance of ATP homeostasis [88], is significantly inhibited *in vitro* by Phe and in cerebral cortex of rats subjected to experimental HPA. These results suggest another putative pathological mechanism through which Phe induces neurometabolic alterations in PKU patients [89].

Berti and colleagues [90] showed that bilateral administration of creatine or pyruvate into hippocampus significantly prevented the cognitive impairment triggered by Phe administration in rats in the open field apparatus, indicating that cognitive impairment found in phenylketonuric patients might be secondary to energy failure. Significant beneficial effect of creatine and pyruvate administration was confirmed by the prevention of adenylate kinase (EC # 2.7.4.3), mitochondrial and cytosolic CK activities impairment in cerebral cortex and

hippocampus from pregnant and lactating rats receiving high Phe administration, corroborating the hypothesis of impaired energy metabolism on Phe neurotoxicity [91].

Phe and its metabolites also interfere on ketone bodies metabolism through inhibition of 3-hydroxybutyrate dehydrogenase (EC # 1.1.1.30) and 3-oxo-acid CoA-transferase (EC # 2.8.3.5) activities in brain of suckling rats [92]. In addition, PPA inhibited pyruvate plus malate oxidation in human and rat skeletal muscle possible due to inhibition of pyruvate dehydrogenase complex (EC # 1.2. 4.1, EC # 2.3.1.12, EC # 1.8.1.4) activity, which could collaborate to the increased lactate levels found in PKU patients [93]. Furthermore, Phe and PPA inhibited pyruvate kinase (EC # 2.7.1.40) and hexokinase (EC # 2.7.1.1) activities in adult human and fetal brain samples [94], as well as in brain of rats submitted to HPA experimental model [95,96].

Considering that metal ions (*e.g.* zinc, iron, and magnesium) are key components of metabolic enzymes [97], acting as regulatory entities on such proteins [15,98], and that they are deficient in PKU patients (as already mentioned), it cannot be ruled that the energy dysfunction observed in PKU may be ascribed to metal ion imbalances that disturb the activity of crucial enzymes of intermediary metabolism. Taken together, these data indicate that disturbances in cell bioenergetics homeostasis may contribute to Phe neurotoxicity observed in PKU.

Calcium Homeostasis

Calcium homeostasis is crucial for brain function and its disregulation in PKU was suggested by several works. In this context, parathyroid hormone (hormone that regulates calcium metabolism), osteocalcin, and dehydrocholecalciferol were found increased in serum samples from phenylketonuric infants, but calcitonin was found reduced. These alterations were not reverted by Phe-restricted diet [99]. In another study, Yu and colleagues [100] demonstrated that Phe alters intracellular free calcium concentrations by modulating plasma membrane Ca^{2+}-ATPase in cortical neurons.

Final considerations

Although the pathophysiological mechanisms of the brain damage found in phenylketonuric patients are not clearly understood yet, there are many evidences of metabolic alterations both in patients and in animal models. Such alterations include bioenergetics deficit, oxidative stress, impairment of lipid and protein metabolism, and disruption of calcium homeostasis and neurotransmitter synthesis in the brain (Figure 2). Taken together, these metabolic disturbances might collaborate to the cognitive dysfunction and brain pathology of PKU.

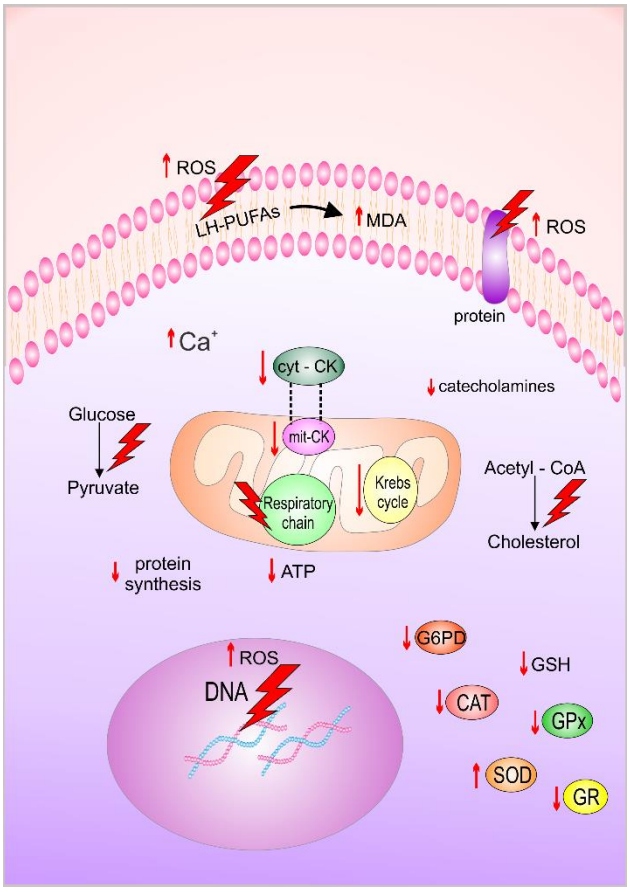

Figure 2. Metabolic alterations involved in the pathophysiology of the brain damage found in phenylketonuric patients. Phenylalanine and its metabolites elicit oxidation of lipids, proteins, and DNA by increasing ROS production and decreasing antioxidant defenses. Bioenergetics is also impaired due to decreased glucose oxidation and alterations of activities of enzymes such as respiratory chain complexes, Krebs cycle enzymes, and creatine kinase. Toxic metabolites also decrease protein, neurotransmitter, and cholesterol synthesis and alter Ca^{2+} metabolism. ATP, adenosine triphosphate; CAT, catalase; cyt-CK, cytosolic creatine kinase; DNA, desoxiribonucleic acid; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; MDA, malondialdehyde, mit-CK, mitochondrial creatine kinase; PUFAS, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase.

It is important to stress out that Phe-restricted diet is effective in preventing the brain damage but the recovery of settled damage is not reached with current therapy. In this scenario, due to the improvement of newborn screening in the past years, the life expectancy of

phenylketonuric patients has increased. It was reported that deterioration of intelligence does not appear to aggravate after adolescence, but psychiatric problems frequently does [101]. Adult PKU patients present decreased brain glucose metabolism in the prefrontal and visual cortices [102], but the metabolic profile and clinical findings characteristic of adult patients are still to be revealed in PKU. That said we believe that the knowledge of the exact mechanisms underlying PKU pathophysiology is pivotal to allow the establishment of more effective therapeutic strategies for phenylketonuric patients at the different stages of life.

Acknowledgements

The authors would like to thank Jéssica de Assis Goularte for the beautiful picture produced specially for this article. PFS, GCF, ELS and FC receive personal grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). FM and JHC receive fellowships from Fundação de Apoio à Pesquisa Científica e Tecnológica do Estado de Santa Catarina (FAPESC) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), respectively.

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