Hyperphenylalaninemia Correlated with Global Decrease of Antioxidant Genes Expression in White Blood Cells of Adult Patients with Phenylketonuria

Charlotte Veyrat-Durebex • Christelle Debeissat •
Hélène Blasco • Franck Patin • Hélène Henique •
Patrick Emond • Catherine Antar • Valérie Gissot •
Olivier Herault • François Maillot

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Abstract Background: Several studies have highlighted disturbance of redox homeostasis in patients with phenylketonuria (PKU) which may be associated with neurological disorders observed in patients, especially during adulthood when phenylalanine restrictive diets are not maintained. The aim of this study was to assess the antioxidant profile in a cohort of PKU patients in comparison to the controls and to evaluate its relation to biochemical parameters especially phenylalaninemia.

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The authors “Olivier Herault” and “François Maillot” contributed equally.

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C. Veyrat-Durebex (✉) • H. Blasco • F. Patin • C. Antar
Laboratoire de biochimie et biologie moléculaire, hôpital Bretonneau
CHRU de Tours, Tours, France
e-mail: charlotte.veyrat@live.fr; charlotte.veyratdurebex@chu-angers.fr

C. Veyrat-Durebex • H. Blasco • F. Patin • P. Emond • C. Antar
INSERM U930, Université François Rabelais Tours, Tours, France

C. Debeissat • O. Herault
CNRS UMR 7292 - équipe LNOx, Université François Rabelais
Tours, Tours, France

C. Debeissat • O. Herault
Service d'hématologie biologique, CHRU de Tours, Tours, France

V. Gissot
INSERM CIC 1415, CHRU de Tours, Tours, France

H. Henique • F. Maillot
Service de médecine interne, CHRU de Tours, Tours, France

H. Henique • F. Maillot
INSERM U1069, Université François Rabelais Tours, Tours, France

Methods: We measured RNA expression of 22 antioxidant genes and reactive oxygen species (ROS) levels in white blood cells of 10 PKU patients and 10 age- and gender-matched controls. We also assessed plasma amino acids, vitamins, oligo-elements, and urinary organic acids concentrations. Then we evaluated the relationship between redox status and biochemical parameters.

Results: In addition to expected biochemical disturbances, we highlighted a significant global decrease of antioxidant genes expression in PKU patients in comparison to the controls. This global decrease of antioxidant genes expression, including various isoforms of peroxiredoxins, glutaredoxins, glutathione peroxidases, and superoxide dismutases, was significantly correlated to hyperphenylalaninemia.

Conclusion: This study is the first to evaluate the expression of 22 antioxidant genes in white blood cells regarding biochemical parameters in PKU. These findings highlight the association of hyperphenylalaninemia with antioxidant genes expression. New experiments to specify the role of oxidative stress in PKU pathogenesis may be useful in suggesting new recommendations in PKU management and new therapeutic trials based on antioxidant defenses.

Abbreviations

BMI	Body mass index
CAT	Catalase
Ct	Cycle threshold
GLRX	Glutaredoxin
GPX	Glutathione peroxidase
GSR	Glutathione-disulfide reductase
Phe	Phenylalanine

PKU	Phenylketonuria
PRDX	Peroxiredoxin
ROS	Reactive oxygen species
RQ	Relative quantification
SOD	Superoxide dismutase
TXN	Thioredoxin

Introduction

Phenylketonuria (PKU, OMIM #26160) is an autosomal recessive disease caused by the deficiency of phenylalanine hydroxylase activity leading to an accumulation of phenylalanine (Phe) and its metabolites in blood and tissues of affected patients (Krause et al. 1985; Mazzola et al. 2013). Neonatal newborn mass-screening of this disorder allows early diagnosis and consequently a better and earlier dietary management leading to a normal development of PKU children (Groselj et al. 2014). In some countries, Phe-restricted treatment was generally not maintained over the second decade of life, after the development period (de Baulny et al. 2007). However, evidences suggest that high plasma Phe concentrations can cause neurological and psychological disorders in adulthood (Waisbren et al. 2007; ten Hoedt et al. 2011; Sharman et al. 2012; Bilder et al. 2013; Okano and Nagasaka 2013; Weglage et al. 2013). Thus, maintaining a Phe-restricted diet in adult PKU patients is still debated (Demirkol et al. 2011; ten Hoedt et al. 2011; Trefz et al. 2011; Hanley 2013; Mutze et al. 2016). Several hypotheses about the pathophysiology of brain injury in PKU have been discussed but the mechanisms remain unclear and seem to be multifactorial (Bone et al. 2012).

Oxidative stress was observed in several diseases such as neurological diseases (Halliwell 2006) and some inborn errors of metabolism including PKU (Sirtori et al. 2005; Sitta et al. 2006; Deon et al. 2015), maple syrup urine disease (Barschak et al. 2006), tyrosinemia (Bird et al. 1995), urea cycle defects, homocystinuria (Streck et al. 2003; Vanzin et al. 2011), and organic acidurias (Wajner et al. 2004). In PKU, accumulation of Phe and/or its metabolites may lead to a disturbance of redox homeostasis (Artuch et al. 2004; Mazzola et al. 2013). Some studies showed an increase of lipoperoxidation markers (Sanayama et al. 2011) and DNA damages (Sitta et al. 2009), or a reduction of various antioxidant compounds, especially selenium, coenzyme Q10, or antioxidant system of glutathione (Menzel et al. 1983; Sitta et al. 2006; Sanayama et al. 2011). Decrease of antioxidant compounds was also observed in animal and in vitro models of PKU (Ercal et al. 2002; Fernandes et al. 2010; Mazzola et al. 2011; Moraes et al. 2014). Imbalance in redox homeostasis in PKU is

now admitted, but its link with metabolic disturbances observed in the disease remains unclear. Understanding and characterization of oxidative mechanisms in PKU are essential for management and treatment of the disease. It remains crucial to assess whether oxidative unbalance may be associated with plasma Phe concentration, and potentially controlled by a diet limiting the plasma Phe concentration and/or antioxidants supplementation strategy, which may be useful for PKU management and patients' outcome (Mazzola et al. 2013).

A better understanding of the relations between PKU biochemical markers and oxidative stress may provide a new light on pathophysiological mechanisms and open new therapeutic perspectives. Thus, the aim of the study was to characterize the antioxidant status in PKU by quantifying the expression of major antioxidant genes in white blood cells and by assessing the link of antioxidant status with biochemical markers.

Methods

Patients and Controls

Blood and urinary samples were obtained from ten untreated PKU adult patients and ten sex- and age-matched healthy controls. Patients and controls were recruited from September 2014 to February 2015 in the University Hospital of Tours. All PKU patients were diagnosed at birth. For each patient, clinical data including information on diagnosis of PKU, gender, age, age of dietary management cessation, treatment, smoking status, pollutant exposure, height, and weight were collected. Body mass index (BMI) was calculated for each included subject. PKU patients did not undergo Phe-restricted diet at time of sample collection or study. Controls were selected in accordance to age and sex following the inclusion of each PKU patient. This study was approved by the ethics committee of University Hospital of Tours (CPPRB, for Comité de Protection des Personnes) and registered on ClinicalTrials.gov (study ID number PHAO14/FM-StressOX-PCU, "Antioxidant Signature in Adults Patients with Phenylketonuria"). Informed consent was signed by all patients and controls prior to inclusion.

Blood and urinary samples were all collected in the morning fasting. Urines were directly stored at -80°C until organic acids analysis. Blood biochemical parameters were measured upon arrival at the laboratory following routine recommendations. For the measurement of oxidative stress parameters, a gentle lysis of red blood cells was performed after samples' collection. Reactive oxygen species level was directly assessed on leukocytes and leukocytes samples

used for antioxidant genes expression were stored in Trizol[®] at -80°C until RNA extraction.

Biochemical Tests

Biochemical tests were performed in routine laboratory using nationally validated reference methods (with validated or ongoing accreditation).

Plasma concentrations of amino acids were determined using ion exchange chromatography with post-column derivation by ninhydrin. Relative urinary concentrations of organic acids were assessed using gas chromatography coupled with mass spectrometry (GC-MS) (Supplementary Methods).

Blood vitamins A, C, and E concentrations were determined by High Performance Liquid Chromatography (HPLC) with UV detection at 245 nm using assay kits provided by Chromsystems (Chromsystems Instruments & Chemicals GmbH).

Plasma concentrations of selenium were measured by inductively coupled plasma–mass spectrometry, and plasma concentrations of zinc and copper by inductively coupled plasma–atomic emission spectrometry.

Oxidative Stress Parameters Measurement

Reactive Oxygen Species (ROS) Measurement

The level of reactive oxygen species (ROS) in leukocytes was evaluated by flow cytometry using the cell permeant reagent CM-H₂DCFDA (chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate) (Life Technologies). This reagent is oxidized in 2',7'-dichlorofluorescein (DCF), a highly fluorescent component, by intracellular ROS, especially H₂O₂.

Heparinized whole peripheral blood samples were incubated for 10 min at 37°C in lysis buffer (125 μM EDTA, 10 mM KHCO₃, 155 mM NH₄Cl) for gentle lysis of red blood cells and washed twice with X-Vivo10[™] (Lonza). Cells were then incubated for 10 min at 37°C with 9.6 $\mu\text{mol/L}$ of CM-H₂DCFDA, washed twice with cold phosphate buffered saline, and DCF level was analyzed by flow cytometry (Gallios[™], Beckman Coulter). Data analysis was performed with FlowJo software. Results are expressed as geometric mean of fluorescence intensity.

Antioxidant Genes Expression Profile

We assessed the expression of 22 antioxidant genes (26 transcripts) by real-time PCR in white blood cells of PKU patients and controls.

Total cellular RNA was isolated from leukocytes with Trizol[®] (Invitrogen) according to the manufacturer's

instructions and after gentle lysis of red blood cells (protocol above). RNA quality were checked on Agilent 2100 bioanalyzer (Applied Biosystems) using Agilent RNA 6000 Nano kit (Agilent Technologies) according to manufacturer's instructions.

RNA reverse-transcription was performed with SuperScript[®] VILO coding DNA (cDNA) Synthesis Kit (Invitrogen) according to manufacturer's instruction. cDNA samples obtained were diluted in H₂O and stored at -20°C . Real-time Polymerase Chain Reaction (PCR) was performed according to Universal Probe Library Technology with Light Cycler 480 Probe Master (Roche Diagnostics) and Real-Time Ready Custom Panel pre-coated plates (Roche Diagnostics). We used previously described antioxidant profile (Herault et al. 2012). cDNA specificity is brought by Universal Probe Library technology that uses short hydrolysis probes recognizing amplicon of a specific primer pair. Primer sequences and specifically associated probes are available on request. Three microliters of cDNA samples were mixed with 5 μL master mix in a final volume of 10 μL , distributed in pre-coated plate and run 10 min at 95°C , 10 s at 95°C , and 30 s at 60°C for 45 cycles, and finally 10 s at 40°C on Light Cycler 480 (Roche). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *B2M* (β -2-microglobulin), and *ACTB* (β -actin) were used as reference genes. The geometric mean of GAPDH, B2M, and ACTB cycle threshold (Ct) was used to normalize samples according to ΔCt method ($\text{Ct}_{\text{target}} - \text{mean Ct}_{\text{reference}}$). Relative quantification (RQ) was calculated as $\text{RQ} = 2^{-\Delta\Delta\text{Ct}}$ with $\Delta\Delta\text{Ct} = (\Delta\text{Ct}_{\text{PKU patients}} - \Delta\text{Ct}_{\text{healthy controls}})$. The numerical value of the Ct is inversely proportional to the amount of amplicon in the reaction (i.e., the lower the Ct, the greater the amount of amplicon). Results of real-time PCR assays are presented as fold change of genes expression in white blood cells of PKU patients in comparison to controls and were considered relevant for a fold change >2 (Schmittgen and Livak 2008).

Statistical Analysis

The Wilcoxon-Mann-Whitney non-parametric test was used to compare mean age, body mass index (BMI), biochemical measure values, and redox parameters between PKU patients and controls. We evaluated the relation between biological parameters and molecular antioxidant status as well as clinical data using Spearman's rank correlation coefficient (Spearman's rho). Differences were considered significant by comparing *p*-value to a threshold value which was calculated according to Benjamini and Hochberg procedure for multiple comparisons (Hochberg and Benjamini 1990; Benjamini et al. 2001).

Statistical analysis was performed with JMP statistical software version 7.0.2 (SAS Institute).

Results

Subjects

Statistical analyses were performed on data from nine PKU patients and their nine matched controls. Data from one patient and his matched control were excluded from the analysis because the patient was receiving treatment by tetrahydrobiopterin (BH4).

The mean age for the PKU patients was 31 years (± 6) and was not significantly different from that of the controls ($p = 0.7186$). BMI of PKU patients was significantly higher than BMI of controls (26 ± 4 vs 22 ± 4 kg/m² with $p = 0.0283$). All patients followed a Phe-restrictive diet in childhood and stopped it at the average age of 6 years (age from 5 to 13 years). There is no significant differences in smoking status or pollutant exposure between controls and patients (p -values of χ^2 -test respectively 0.53 and 0.28). Patients' characteristics were given in supplementary results (Table S1). Among the ten patients initially included in the study, three patients were the subject of a more detailed analysis because of their disease particularity: (1) Patient-2 received BH4 treatment (this patient was excluded from global statistical analysis); (2) Patient-9 presented a permanent moderate hyperphenylalaninemia; (3) Patient-10 was classified as an atypical PKU.

Biochemical Parameters

As expected we observed higher plasma Phe concentrations and lower plasma tyrosine concentrations in PKU patients compared to controls ($p = 0.0003$ and $p = 0.0026$, respectively) (Table S2). PKU patients showed significantly lower plasma concentrations of several amino acids: threonine, glutamine, proline, alanine, α -amino-butyric acid, valine, methionine, isoleucine, leucine, and arginine (Table S2).

Using the routine diagnostic procedure, we measured urinary relative concentrations of 30 organic acids by GC-MS (Table S3). We observed a negative correlation between plasma concentrations of Phe and urinary concentrations of glycerate (ρ Spearman = -0.75 and $p = 0.0002$), suberate (ρ Spearman = -0.64 and $p = 0.0024$), and 3-hydroxy-glutarate (ρ Spearman = -0.63 and $p = 0.0028$). We also showed a positive correlation of plasma Phe and urinary 2-hydroxy-phenylacetate (ρ Spearman = 0.83 and $p < 0.0001$), 2-keto-glutarate (ρ Spearman = 0.69 and $p = 0.0007$), 3-hydroxy-3-methyl-glutarate (ρ Spearman = 0.68 and $p = 0.001$), 4-hydroxy-phenylpyruvate (ρ Spearman = 0.61 and $p = 0.0046$), isovaleryl-glycine (ρ Spearman = 0.59 and $p = 0.0058$), 3-hydroxy-butyrate (ρ Spearman = 0.54 and $p = 0.0145$), and 3-hydroxy-isovalerate (ρ Spearman = 0.54 and $p = 0.0141$) (p -values

were compared to threshold p -values calculated according to Benjamini-Hochberg formula).

Analysis of plasma concentrations of vitamins A, E, and C and trace-elements (selenium, copper, zinc) revealed no significant differences between PKU patients and controls (Table S2).

Redox Parameters

We observed a trend to higher ROS levels in monocytes of PKU patients when compared to controls without any statistical significant differences in all white blood cells subpopulations (monocytes, lymphocytes, and granulocytes) (Fig. 1).

Fold change of antioxidant genes expression in PKU patients compared to controls is illustrated in Fig. 2. PKU patients presented a global decrease of main antioxidant genes (Fig. 2). We observed a two- to threefold decreased expression of the following antioxidant genes in PKU patients when compared to healthy controls: superoxide dismutase 1 (*SOD1*), glutaredoxin 2 and 3 (*GLRX2* transcript 2 and *GLRX3*), glutathione peroxidase 1 and 4 (*GPX1* and *GPX4*), and peroxiredoxins 1, 2, 4, and 5 (*PRDX1*, *PRDX2* transcript 3, *PRDX4*, and *PRDX5*) (Fig. 2 and Table 1). We did not observe difference in the expression of the reference genes, relative to the amount of RNA, between patients and controls (*GAPDH* $p = 0.2568$, *B2M* $p = 0.2265$, *ACTB* $p = 0.2265$). Detailed analysis of antioxidant profile in PKU patients highlighted that the decrease of antioxidant genes expression of patients 2, 9, and 10 (respectively treated by BH4, permanent moderate hyperphenylalaninemia, and atypical PKU) was less pronounced as compared to the average typical PKU patients (Fig. S1).

Relationship Between Metabolic Factors and Antioxidant Status

We observed that high plasma concentrations of Phe were associated with low expression of various antioxidant genes (*PRDX1*, *GPX4*, *GLRX3*, *SOD1*, *PRDX2* transcripts 1 and 3, *TXN2*, *PRDX4*, *GLRX5*, *GPX7*, and *SOD3*) (Table 1). We observed significant positive correlation between plasma concentrations of amino-butyric acid and expression of various antioxidant genes (correlation of low plasma amino-butyric acid with low expression of *SOD1*, *GPX4*, *GPX1*(2), *PRDX5*, *SOD3*, *PRDX1*, *PRDX4*, *GLRX5*, *TXN2*, *GLRX2*(2), and *GLRX3* with p -values < 0.005). We also observed significant correlation between various antioxidant genes expression and plasma tyrosine and branched-chain amino acids (data not shown). Observed variations in urinary concentrations of organic acids also seemed to be correlated

Fig. 1 ROS production in subpopulations of white blood cells. Relative ROS production in white blood cells of PKU patients and controls was determined using the CM-H₂DCFDA dye in flow

cytometry. ROS levels in three subpopulations of leukocytes (monocytes, granulocytes, and lymphocytes) are shown as mean fluorescence intensity in cells (error scales are standard error of the mean)

with the decrease of expression of some antioxidant genes (p -values <0.01).

No significant correlation was observed between age or BMI and biochemical parameters or antioxidant profile (data not shown).

Discussion

This preliminary study is the first to assess the gene expression profile of 22 major antioxidant enzymes in white blood cells of adult PKU patients and to analyze their relation with the metabolic status. These data highlight a lower antioxidant arsenal in leukocytes associated with hyperphenylalaninemia.

PKU patients of this study were representative of the adult PKU population with high phenylalaninemia and low tyrosinemia (Krause et al. 1985). As expected we observed a positive correlation between plasma Phe concentrations and urinary concentrations of Phe-derived organic acids (2-hydroxy-phenylacetate, 4-hydroxy-phenylpyruvate). As previously observed, PKU patients of this study presented a higher BMI compared to controls (Belanger-Quintana and Martinez-Pardo 2011; Burrage et al. 2012; Robertson et al. 2013). This high BMI could be due to an unbalanced diet of PKU patients due to habits acquired during restrictive diet

in childhood (Robertson et al. 2013), but this hypothesis is controversial (Rocha et al. 2012). We did not highlight correlation suggesting that the unbalanced oxidative status that we observed in PKU patients was related to their higher BMI.

We observed a decrease of several amino acids correlated to hyperphenylalaninemia, especially branched-chain amino acids. These lower plasma concentrations of branched-chain amino acids were already described in children with PKU (Antonozzi et al. 1987), and a deficiency of branched-chain amino acids, tyrosine, and tryptophan transport across the brain–blood barrier was described in PKU mice due to a competition with Phe transport (Matalon et al. 2003). A defect in central nervous system protein synthesis has been highlighted in PKU and correlated with plasma Phe concentrations in PKU patients, and experiments in animal or in vitro models (de Groot et al. 2013; Schuck et al. 2015). We showed a significant decrease of plasma amino-butyric acid in PKU patients, which is correlated to plasma Phe and to the decreased expression of some antioxidant genes. Amino-butyric acid is an amino acid not taking part in protein synthesis but is considered as a nonspecific biomarker of liver, malnutrition, or protein catabolism (Chiarla et al. 2011). We highlighted an increase of urinary 3-hydroxy-3-methylglutarate in hyperphenylalaninemia ($p = 0.0018$). This result is

Fig. 2 Molecular antioxidant profile of PKU patients compared to healthy controls. RNA isolated from white blood cells of PKU patients and controls was reverse transcribed, and a real-time PCR using a custom panel was performed on cDNA in order to assess the expression of main antioxidant genes. Results of real-time PCR assays are presented as fold change ratio of genes expression in white blood cells of PKU patients compared to controls. Genes of the antioxidant profile are presented in order from most to least expressed in X-axis,

according to their ΔC_t values (versus mean C_t references). Data are presented as mean fold change ratio of genes expression in PKU patients compared to controls (error scales are standard error of the mean). Red scale represented the twofold change threshold. *Significant correlations between high concentrations of Phe and low expression of antioxidant gene (considered significant with a p -value inferior to threshold value calculated using Benjamini-Hochberg formula for multiple comparison correction)

consistent with previous experiment showing that high concentration of Phe inhibits the activity of 3-hydroxy-3-methylglutaryl-CoA reductase linked with a deficit of antioxidant coenzyme Q_{10} involved in mitochondrial respiratory chain (Hargreaves 2007). Moreover, an increase of oxidative damages was described in patients with 3-hydroxy-3-methylglutaric aciduria (Dos Santos Mello et al. 2015). We also observed significant correlations between plasma Phe concentrations and various urinary organic acids. A recent study reported 19 differentially expressed compounds in urine of PKU patients (Xiong et al. 2015). These results bring up an underlying global metabolic disturbance in PKU which needs more exploration.

The focus of this clinical study was to evaluate antioxidant status of PKU patients in relation to their metabolic condition. Our preliminary results showed a

molecular antioxidant signature of PKU white blood cells characterized by a global decrease in expression of antioxidant genes (Fig. 2). Nine of these antioxidant enzymes had more than a twofold decreased gene expression in white blood cells of PKU patients as compared to controls. We noted that 11 genes had a decreased expression significantly correlated with high plasma concentrations of Phe after multiple testing correction. These data support results of previous studies highlighting disturbance in redox system with reduction of total antioxidant status or unbalance of prooxidant-antioxidant system in blood samples of PKU patients (Sitta et al. 2009; Sanayama et al. 2011; Preissler et al. 2016; Tavana et al. 2016). Several plasma amino acids and urinary organic acids seem to be correlated with antioxidant expression profile. Despite the use of restrictive multiple testing

Table 1 Comparison of antioxidant genes expression profile in PKU patients and controls and correlation with Phe concentrations

ΔCt (mean $\pm$ SD)	PKU patients	Controls	Expression fold change compared to controls	Correlation with high plasma Phe	
				ρ spearman's	p -value
<i>SOD2</i>	0.43 $\pm$ 0.26	0.53 $\pm$ 0.20	-1.36	0.20	0.4504
<i>GLRX1</i>	0.016 $\pm$ 0.004	0.016 $\pm$ 0.005	-1.11	0.13	0.6124
<i>GSR</i>	6.18 $\pm$ 0.50	5.85 $\pm$ 0.31	-1.35	0.35	0.1660
<i>PRDX3</i>	6.23 $\pm$ 0.31	5.94 $\pm$ 0.28	-1.26	0.44	0.0798
<i>PRDX5</i>	6.49 $\pm$ 0.63	5.76 $\pm$ 0.43	-1.83	0.54	0.0242
<i>PRDX1</i>	7.10 $\pm$ 0.65	6.18 $\pm$ 0.68	-2.13	0.66	0.0038^a
<i>TXN</i>	7.21 $\pm$ 0.67	7.14 $\pm$ 0.62	-1.17	-0.04	0.8848
<i>SOD1</i>	7.44 $\pm$ 0.61	6.41 $\pm$ 0.92	-2.18	0.66	0.0039^a
<i>GPX7</i>	8.23 $\pm$ 0.62	7.83 $\pm$ 0.48	-1.48	0.60	0.0107^a
<i>GPX1 (transc. 1)</i>	8.80 $\pm$ 1.08	8.15 $\pm$ 0.57	-2.19	0.32	0.2123
<i>GPX4</i>	8.80 $\pm$ 0.78	7.88 $\pm$ 0.44	-2.20	0.65	0.0048^a
<i>PRDX2 (transc. 1)</i>	8.86 $\pm$ 0.68	8.30 $\pm$ 0.48	-1.69	0.73	0.0008^a
<i>CAT</i>	9.41 $\pm$ 0.56	9.17 $\pm$ 0.70	-1.34	0.05	0.8371
<i>GLRX3</i>	10.43 $\pm$ 0.76	9.66 $\pm$ 0.81	-2.06	0.58	0.0137^a
<i>TXN2</i>	9.64 $\pm$ 0.48	9.30 $\pm$ 0.36	-1.36	0.58	0.0137^a
<i>GLRX2 (transc. 2)</i>	10.90 $\pm$ 1.07	9.93 $\pm$ 0.37	-2.66	0.51	0.0359
<i>PRDX4</i>	10.44 $\pm$ 0.92	9.70 $\pm$ 0.36	-2.07	0.57	0.0166^a
<i>GPX1 (transc. 2)</i>	10.87 $\pm$ 0.66	10.23 $\pm$ 0.73	-1.76	0.41	0.1048
<i>PRDX2 (transc. 3)</i>	12.58 $\pm$ 0.27	11.34 $\pm$ 1.05	-2.26	0.75	0.0005^a
<i>GLRX5</i>	12.32 $\pm$ 0.75	11.68 $\pm$ 0.76	-1.85	0.64	0.0060^a
<i>PRDX5 (transc. 2)</i>	12.38 $\pm$ 1.04	11.42 $\pm$ 0.76	-2.61	0.44	0.0788
<i>GPX3</i>	11.27 $\pm$ 0.49	11.02 $\pm$ 0.67	-1.14	0.26	0.3208
<i>PRDX6</i>	16.46 $\pm$ 0.38	16.32 $\pm$ 0.53	-1.17	0.41	0.1060
<i>GLRX2 (transc. 1)</i>	17.06 $\pm$ 0.93	17.37 $\pm$ 2.88	-1.14	0.32	0.5068
<i>GPX2</i>	19.30 $\pm$ 1.20	19.00 $\pm$ 2.36	-1.73	0.54	0.0242
<i>SOD3</i>	25.81 $\pm$ 0.56	25.05 $\pm$ 0.44	-1.81	0.65	0.0050^a

Genes are ranked in order from most to least expressed, according to their ΔCt values

Ct cycle threshold, ΔCt difference between mean reference gene Ct and gene of interest Ct , *PKU* phenylketonuria, *SD* standard deviation, *transc* transcript. Differences in comparison of gene expression between PKU patients and controls were considered relevant with fold change $> \pm 2$

^a Correlation between high concentrations of Phe and low expression of antioxidant gene using Spearman's correlation coefficient (ρ Spearman) was considered significant with a p -value inferior to threshold value calculated using Benjamini-Hochberg formula for multiple comparison correction

corrections, this result strengthens the idea of a link between metabolic disturbances and redox status in PKU. The low-noise disturbance of global metabolism linked with antioxidant status in PKU patients observed in this study highlighted the potential benefit of the maintenance of plasma Phe concentrations in adult PKU patients. These results need more detailed exploration of both metabolic and oxidative disturbance observed here in order to improve care of adult PKU patients.

Detailed analysis of data of three patients with disease particularity highlighted a less obvious decrease of antioxidant genes expression. These data strengthen the main observation of this study that high plasma Phe concentrations

are linked to a decrease of antioxidant profile. Only patient-2 receiving BH4 treatment was excluded from statistical analysis. Patients 9 and 10 were the subject of a special analysis in a second time. The inclusion of the data from these two patients in the analysis may introduce a bias. These results, although interesting, need to be confirmed in a larger cohort in order to compare various subpopulations of PKU and hyperphenylalaninemia.

Figure 3 illustrates the results of our study compared with results of several studies on PKU patients, in vitro and in vivo models. As in the present study, several studies on PKU patients, adults and children with or without restrictive diet, highlighted a decrease of GPx enzyme in various

Fig. 3 Schematic representation of main results of the study (*black arrows*) compared with results of several studies on PKU patients, in vitro and in vivo models (*grey arrows*). As in this study, several studies highlighted a decrease of GPx enzyme in various tissues in PKU (Menzel et al. 1983; Reilly et al. 1990; Sierra et al. 1998; Sitta et al. 2006; Sanayama et al. 2011). The combination of a decrease in expression of GPX and GSR may suggest a reduced activity of the GSH antioxidant system. The observation of a global decrease of expression of antioxidant enzymes could be related with previous findings showing a lower total antioxidant status (TAS) in PKU

patients compared to controls (Sanayama et al. 2011). The reduced expression of antioxidant systems that we observed could lead to a defect in maintenance of the redox homeostasis in response to elevation of ROS levels (superoxide and hydroxyl anions especially) responsible of cellular damages previously observed in PKU (Ercal et al. 2002; Sitta et al. 2009; Sanayama et al. 2011). *GPX* glutathione peroxidase, *GSR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *SOD* superoxide dismutase, *CAT* catalase, *TXN* thioredoxins, *GLRX* glutaredoxins, *PRDX* peroxiredoxins

tissues (Menzel et al. 1983; Reilly et al. 1990; Sierra et al. 1998; Sitta et al. 2006; Sanayama et al. 2011). The reduced expression of antioxidant systems that we observed could lead to an inefficient response to oxidative stress and by consequences to elevation of ROS levels (superoxide and hydroxyl anions especially) responsible for cellular damages previously highlighted in PKU (Ercal et al. 2002; Sitta et al. 2009; Sanayama et al. 2011) and which may lead to brain injury observed in the disease. In the present study, ROS production in PKU patients trends to increase in monocytes but interindividual variability and lack of statistical power prevent us from concluding. Evaluation of intracellular ROS in erythrocytes, which have a lower turnover than monocytes, might be a better model for highlighting increased ROS generation. Moreover, expression of some antioxidant genes of this study is under the control of the Nrf2 transcription factor (nuclear factor erythroid 2-related factor 2) (Ma 2013). It would be interesting to study the Nrf2 pathway in PKU pathophysiology.

Despite the reduced size of our cohort, an interesting link was highlighted between biochemical parameters and a large molecular antioxidant profile. It might be interesting to extend this study by performing a wide range of

oxidative stress markers, especially markers of cellular damages such as 8-hydroxy-2'-deoxyguanosine (DNA damages), malondialdehyde (lipid peroxidation), and carbonylated proteins (Sitta et al. 2009; Sanayama et al. 2011), in order to validate assumptions from our data and previous studies, and to evaluate their relation with biological markers and clinical data in a longitudinal larger cohort of PKU patients. Analysis of disease phenotype, age (adults vs children), gender, metabolic status or diets, and environmental oxidative stress factors which may introduce a bias (i.e., self-medication treatments, life-style, or activities) could also bring new perspectives in PKU pathophysiology understanding. Furthermore, the assessment of a characteristic molecular antioxidant signature of different subcategories of PKU patients, using the antioxidant profile of the present study, could open perspectives on the use of this signature in disease monitoring or in the prognosis of disease complications, such as neurological deteriorations. Thus, it might be interesting to compare the blood antioxidant profile with cognitive functions in order to characterize the potential role of oxidative stress in neurological troubles observed in PKU. Studies on large neural amino acids supplementation including tyrosine in PKU mice model showed reduced brain Phe concentrations

(van Vliet et al. 2016). Large neural amino acids supplementation could be an interesting alternative dietary treatment in patients with PKU (Andersen and Avins 1976). Physical exercise has been shown to improve brain function by preventing oxidative stress in PKU (Mazzola et al. 2016). Several antioxidant supplementations have been evaluated and may be a complement or an alternative to a restrictive diet for adolescent and adult PKU patients with poor compliance (Mazzola et al. 2013). In this way, supplementation with L-carnitine and selenium seems to prevent oxidative stress observed in hyperphenylalaninemia (Sitta et al. 2011). Antioxidant treatments have been widely considered in several pathologies where oxidative stress is involved, and the necessity of more studies on this topic in PKU seems essential.

These findings highlight the association of hyperphenylalaninemia with antioxidant genes expression and emphasize the importance of the restricted protein diet in PKU, which is fundamental for the metabolic control of phenylalanine and, consequently, the redox status in these patients.

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Take-Home Message (Synopsis)

This preliminary study highlights a lower antioxidant arsenal in leukocytes associated with hyperphenylalaninemia using assessment of gene expression of 22 major antioxidant enzymes in adult PKU patients.

Conflict of Interest

All authors declare that they have no conflicts of interest with the contents of this chapter.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study. Proof that informed consent was obtained are available upon request.

Author Contributions

C.V-D designed and performed experiments, analyzed data, and wrote the manuscript; C.D performed and supervised

redox status experiments and gave technical and intellectual support on redox study; H.B designed experiments, supervised the writing of the manuscript, and gave intellectual support and conceptual advice; F.P performed experiments; H.H collected clinical data; P.E supervised biochemical parameters assays; C.A performed biochemical experiments; V.G recruited healthy controls and participated in the design of study; O.H gave technical and intellectual support on redox status interpretation and supervised the writing of the manuscript; F.M designed and supervised the study, recruited PKU patients, and supervised the writing of the manuscript. All authors provided conceptual advices and comments on the manuscript.

Author who serves as guarantor: François Mailliot.

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Details of Ethics Approval

This study was approved by the ethics committee of University Hospital of Tours (CPPRB, for Comité de Protection des Personnes) and registered on [ClinicalTrials.gov](https://clinicaltrials.gov) (study ID number PHAO14/FM-StressOX-PCU, "Antioxidant Signature in Adults Patients with Phenylketonuria"). Informed consent was signed by all patients and controls prior to inclusion.

References

- Andersen A, Avins EL (1976) Lowering brain phenylalanine levels by giving other large neutral amino acids. A new experimental therapeutic approach to phenylketonuria. *Arch Neurol* 33 (10):684–686
- Antonozzi I, Carducci C, Vestri L, Manzari V, Dominici R (1987) Plasma amino acid values and pancreatic beta-cell function in phenylketonuria. *J Inher Metab Dis* 10(1):66–72
- Artuch R, Colome C, Sierra C et al (2004) A longitudinal study of antioxidant status in phenylketonuric patients. *Clin Biochem* 37 (3):198–203
- Barschak AG, Sitta A, Deon M et al (2006) Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease. *Metab Brain Dis* 21(4):279–286
- Belanger-Quintana A, Martinez-Pardo M (2011) Physical development in patients with phenylketonuria on dietary treatment: a retrospective study. *Mol Genet Metab* 104(4):480–484
- Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I (2001) Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 125(1–2):279–284
- Bilder DA, Burton BK, Coon H et al (2013) Psychiatric symptoms in adults with phenylketonuria. *Mol Genet Metab* 108(3):155–160

- Bird S, Miller NJ, Collins JE, Rice-Evans CA (1995) Plasma antioxidant capacity in two cases of tyrosinaemia type 1: one case treated with NTBC. *J Inher Metab Dis* 18(2):123–126
- Bone A, Kuehl AK, Angelino AF (2012) A neuropsychiatric perspective of phenylketonuria I: overview of phenylketonuria and its neuropsychiatric sequelae. *Psychosomatics* 53(6):517–523
- Burridge LC, McConnell J, Haesler R et al (2012) High prevalence of overweight and obesity in females with phenylketonuria. *Mol Genet Metab* 107(1–2):43–48
- Chiarla C, Giovannini I, Siegel JH (2011) Characterization of alpha-amino-n-butyric acid correlations in sepsis. *Transl Res* 158(6):328–333
- de Baulny HO, Abadie V, Feillet F, de Parscau L (2007) Management of phenylketonuria and hyperphenylalaninemia. *J Nutr* 137(6 Suppl 1):1561S–1563S. Discussion 1573S–1575S
- de Groot MJ, Hoeksma M, Reijngoud DJ et al (2013) Phenylketonuria: reduced tyrosine brain influx relates to reduced cerebral protein synthesis. *Orphanet J Rare Dis* 8:133
- Demirkol M, Gizewska M, Giovannini M, Walter J (2011) Follow up of phenylketonuria patients. *Mol Genet Metab* 104(Suppl): S31–S39
- Deon M, Sitta A, Faverzani JL et al (2015) Urinary biomarkers of oxidative stress and plasmatic inflammatory profile in phenylketonuric treated patients. *Int J Dev Neurosci* 47(Pt B):259–265
- Dos Santos Mello M, Ribas GS, Wayhs CA et al (2015) Increased oxidative stress in patients with 3-hydroxy-3-methylglutaric aciduria. *Mol Cell Biochem* 402(1–2):149–155
- Ercal N, Aykin-Burns N, Gurer-Orhan H, McDonald JD (2002) Oxidative stress in a phenylketonuria animal model. *Free Radic Biol Med* 32(9):906–911
- Fernandes CG, Leipnitz G, Seminotti B et al (2010) Experimental evidence that phenylalanine provokes oxidative stress in hippocampus and cerebral cortex of developing rats. *Cell Mol Neurobiol* 30(2):317–326
- Groselj U, Tansek MZ, Battelino T (2014) Fifty years of phenylketonuria newborn screening – a great success for many, but what about the rest? *Mol Genet Metab* 113(1–2):8–10
- Halliwel B (2006) Oxidative stress and neurodegeneration: where are we now? *J Neurochem* 97(6):1634–1658
- Hanley WB (2013) Optimal serum phenylalanine for adult patients with phenylketonuria (PKU). *Mol Genet Metab* 110(3):199–200
- Hargreaves IP (2007) Coenzyme Q10 in phenylketonuria and mevalonic aciduria. *Mitochondrion* 7(Suppl):S175–S180
- Herauld O, Hope KJ, Deneault E et al (2012) A role for GPx3 in activity of normal and leukemia stem cells. *J Exp Med* 209(5):895–901
- Hochberg Y, Benjamini Y (1990) More powerful procedures for multiple significance testing. *Stat Med* 9(7):811–818
- Krause W, Halminski M, McDonald L et al (1985) Biochemical and neuropsychological effects of elevated plasma phenylalanine in patients with treated phenylketonuria. A model for the study of phenylalanine and brain function in man. *J Clin Invest* 75(1):40–48
- Ma Q (2013) Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol* 53:401–426
- Matalon R, Surendran S, Matalon KM et al (2003) Future role of large neutral amino acids in transport of phenylalanine into the brain. *Pediatrics* 112(6 Pt 2):1570–1574
- Mazzola PN, Terra M, Rosa AP et al (2011) Regular exercise prevents oxidative stress in the brain of hyperphenylalaninemic rats. *Metab Brain Dis* 26(4):291–297
- Mazzola PN, Karikas GA, Schulpis KH, Dutra-Filho CS (2013) Antioxidant treatment strategies for hyperphenylalaninemia. *Metab Brain Dis* 28(4):541–550
- Mazzola PN, Bruinenberg V, Anjema K et al (2016) Voluntary exercise prevents oxidative stress in the brain of phenylketonuria mice. *JIMD Rep* 27:69–77
- Menzel H, Steiner G, Lombeck I, Ohnesorge FK (1983) Glutathione peroxidase and glutathione S-transferase activity of platelets. *Eur J Pediatr* 140(3):244–247
- Moraes TB, Dalazen GR, Jacques CE, de Freitas RS, Rosa AP, Dutra-Filho CS (2014) Glutathione metabolism enzymes in brain and liver of hyperphenylalaninemic rats and the effect of lipoic acid treatment. *Metab Brain Dis* 29(3):609–615
- Mutze U, Thiele AG, Baerwald C, Ceglarek U, Kiess W, Beblo S (2016) Ten years of specialized adult care for phenylketonuria – a single-centre experience. *Orphanet J Rare Dis* 11:27
- Okano Y, Nagasaka H (2013) Optimal serum phenylalanine for adult patients with phenylketonuria. *Mol Genet Metab* 110(4):424–430
- Preissler T, Bristot IJ, Costa BM et al (2016) Phenylalanine induces oxidative stress and decreases the viability of rat astrocytes: possible relevance for the pathophysiology of neurodegeneration in phenylketonuria. *Metab Brain Dis* 31(3):529–537
- Reilly C, Barrett JE, Patterson CM, Tinggi U, Latham SL, Marrinan A (1990) Trace element nutrition status and dietary intake of children with phenylketonuria. *Am J Clin Nutr* 52(1):159–165
- Robertson LV, McStravick N, Ripley S et al (2013) Body mass index in adult patients with diet-treated phenylketonuria. *J Hum Nutr Diet* 26(Suppl 1):1–6
- Rocha JC, van Spronsen FJ, Almeida MF et al (2012) Dietary treatment in phenylketonuria does not lead to increased risk of obesity or metabolic syndrome. *Mol Genet Metab* 107(4):659–663
- Sanayama Y, Nagasaka H, Takayanagi M et al (2011) Experimental evidence that phenylalanine is strongly associated to oxidative stress in adolescents and adults with phenylketonuria. *Mol Genet Metab* 103(3):220–225
- Schmittgen TDK, Livak J (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3(6):1101–1108
- Schuck PF, Malgarin F, Cararo JH, Cardoso F, Streck EL, Ferreira GC (2015) Phenylketonuria pathophysiology: on the role of metabolic alterations. *Aging Dis* 6(5):390–399
- Sharman R, Sullivan K, Young RM, McGill J (2012) Depressive symptoms in adolescents with early and continuously treated phenylketonuria: associations with phenylalanine and tyrosine levels. *Gene* 504(2):288–291
- Sierra C, Vilaseca MA, Moyano D et al (1998) Antioxidant status in hyperphenylalaninemia. *Clin Chim Acta* 276(1):1–9
- Sirtori LR, Dutra-Filho CS, Fitarelli D et al (2005) Oxidative stress in patients with phenylketonuria. *Biochim Biophys Acta* 1740(1):68–73
- Sitta A, Barschak AG, Deon M et al (2006) Investigation of oxidative stress parameters in treated phenylketonuric patients. *Metab Brain Dis* 21(4):287–296
- Sitta A, Manfredini V, Biasi L et al (2009) Evidence that DNA damage is associated to phenylalanine blood levels in leukocytes from phenylketonuric patients. *Mutat Res* 679(1–2):13–16
- Sitta A, Vanzin CS, Biancini GB et al (2011) Evidence that L-carnitine and selenium supplementation reduces oxidative stress in phenylketonuric patients. *Cell Mol Neurobiol* 31(3):429–436
- Streck EL, Vieira PS, Wannmacher CM, Dutra-Filho CS, Wajner M, Wyse AT (2003) In vitro effect of homocysteine on some parameters of oxidative stress in rat hippocampus. *Metab Brain Dis* 18(2):147–154
- Tavana S, Amini S, Hakhamaneshi MS et al (2016) Prooxidant-antioxidant balance in patients with phenylketonuria and its correlation to biochemical and hematological parameters. *J Pediatr Endocrinol Metab* 29:675–680
- ten Hoedt AE, de Sonnevile LM, Francois B et al (2011) High phenylalanine levels directly affect mood and sustained attention in

- adults with phenylketonuria: a randomised, double-blind, placebo-controlled, crossover trial. *J Inherit Metab Dis* 34(1):165–171
- Trefz F, Maillot F, Motzfeldt K, Schwarz M (2011) Adult phenylketonuria outcome and management. *Mol Genet Metab* 104(Suppl): S26–S30
- van Vliet D, Bruinenberg VM, Mazzola PN et al (2016) Therapeutic brain modulation with targeted large neutral amino acid supplements in the Pah-enu2 phenylketonuria mouse model. *Am J Clin Nutr* 104(5):1292–1300
- Vanzin CS, Biancini GB, Sitta A et al (2011) Experimental evidence of oxidative stress in plasma of homocystinuric patients: a possible role for homocysteine. *Mol Genet Metab* 104(1–2):112–117
- Waisbren SE, Noel K, Fahrbach K et al (2007) Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis. *Mol Genet Metab* 92(1–2):63–70
- Wajner M, Latini A, Wyse AT, Dutra-Filho CS (2004) The role of oxidative damage in the neuropathology of organic acidurias: insights from animal studies. *J Inherit Metab Dis* 27(4):427–448
- Weglage J, Fromm J, van Teeffelen-Heithoff A et al (2013) Neurocognitive functioning in adults with phenylketonuria: results of a long term study. *Mol Genet Metab* 110(Suppl):S44–S48
- Xiong X, Sheng X, Liu D, Zeng T, Peng Y, Wang Y (2015) A GC/MS-based metabolomic approach for reliable diagnosis of phenylketonuria. *Anal Bioanal Chem* 407(29):8825–8833