EPG SYMPOSIUM ON ADVANCES AND CHALLENGES IN PKU 2011

OXIDATIVE STRESS IN PATIENTS WITH PHENYLKETONURIA

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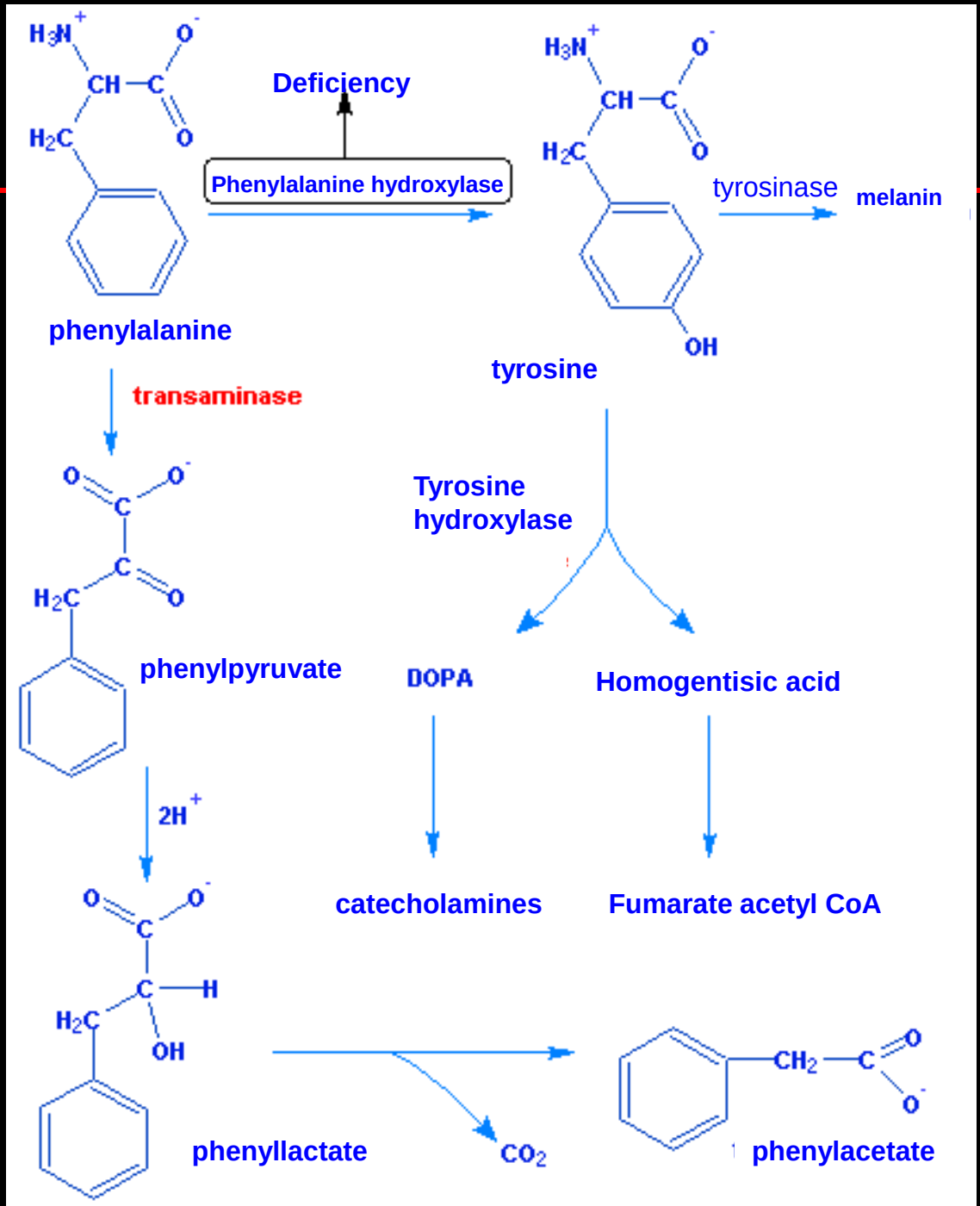
Phenylketonuria (PKU)

- IEM - 1:10.000 – 1:20.000

- 1934 – Asbjorn Folling:
  Increased urine excretion of phenylpyruvate that produced a green colour after ferric chloride reaction.


Deficiency
Phenylalanine hydroxylase
tyrosinase
melanin
phenylalanine
tyrosine
transaminase
Tyrosine hydroxylase
phenylpyruvate
DOPA
Homogentisic acid
2H+
catecholamines
Fumarate acetyl CoA
phenyllactate
CO2
phenylacetate

PKU – treatment

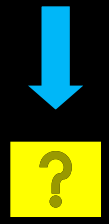
- **Semi-synthetic formulas of amino acids restricted in Phe, enriched with essential micronutrients (vitamins, minerals and trace elements).**
- **BH4**
- **Selenium and carnitine**

Patients with good adherence to treatment do not present the mental retardation characteristic of untreated PKU patients, although they can have a low IQ and neuropsychological deficits.

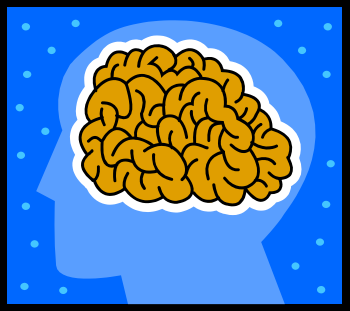
(Holtzman et al, 1986; Smith et al, 1990; Lou et al, 1985; Krause et al, 1985)

Phenylketonuria

Genetic Disease



Brain Damage



Neurologic Features

Mental Retardation

PKU – pathophysiology

❖Inhibition of transport of neutral amino acids (val, leu, ileu, tryp, thre, hist, met, tyr) in the blood-brain barrier → defective myelination. (Curtis el al, 1981;Hanley et al, 2000)

❖ ↓ Tryptophan → ↓ serotonin
Tyrosine → dopamine

❖ In vitro inhibition of pyruvate kinase by Phe → reduction of glucose uptake in the brain (Hasselbalch et al, 1996)

❖ Inhibition of creatine kinase in vivo and in vitro by Phe (Feksa et al, 2002)

❖ Decrease in the activity of succinate dehydrogenase in rat cortex with hyperphenylalaninemia and in vitro inhibition of complexes I-III of the respiratory chain by Phe (Rech et al, 2002)

❖ In cultured neurons, Phe inhibits glutamatergic synaptic transmission (Glushakov et al, 2003).

PKU Neurophysiopathology

High Phe levels

Oxidative Stress

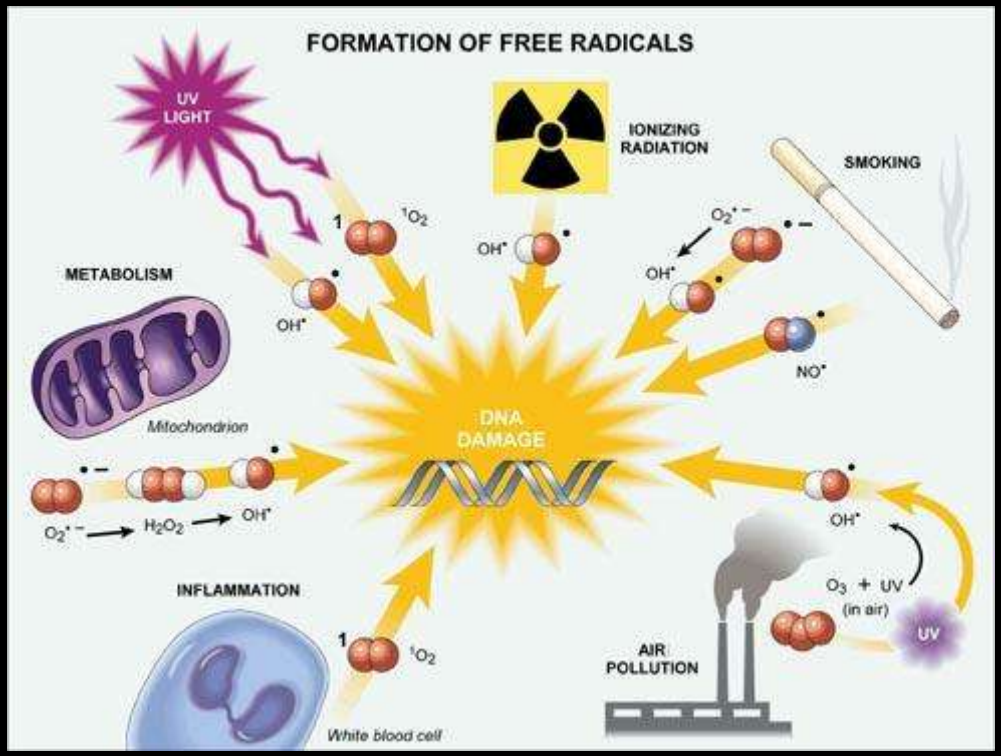
Sierra et al (1998), Hagen et al (2002), Schulpis et al (2003), Artuch et al (2004)

Free radicals

Free radical: species that contains one or more unpaired electrons.

Instability and high reactivity

Reactive oxygen species (ROS): $O2^{\bullet -}$, H_2O_2, HOCl, $ONOO^-$, $NO^{\bullet}$, $OH^{\bullet}$



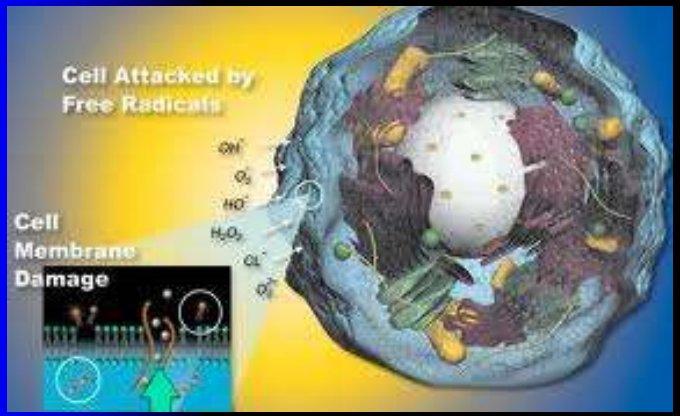
(Halliwell and Gutteridge, 2007)

Oxidative stress

Antioxidant defenses:

Enzymatic: SOD, CAT, GPx

Non-enzymatic: endogenous and exogenous substances



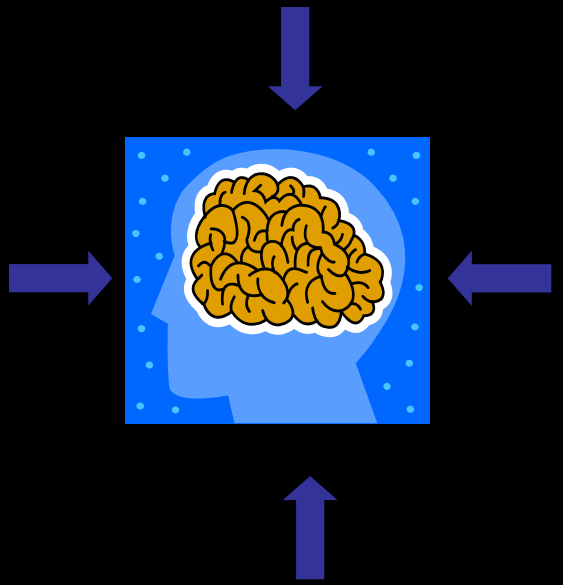
Consequences:

- Oxidative damage to lipids, proteins and DNA

(Dröge, 2002; Halliwell and Gutteridge, 2007)

CNS – High vulnerability to Oxidative Stress

- **high oxygen consumption**
- **high metabolism: ROS generation**
- **high iron and lipid contents (polyunsaturated fatty acids)**
- **low activity of antioxidant defenses**



(Halliwell and Gutteridge, 2001)

Oxidative Stress – IEM (other than PKU)

Propionic and Methylmalonic Acidemia
(Fontella et al, 2000)

Glutaric Acidemia Type I
(Latini et al, 2002)

Tyrosinemia Type I (Bird et al, 1995)

MSUD (Barschak et al, 2006)

X-ALD (Vargas et al, 2004)

Oxidative Stress – IEM

1. accumulation of toxic metabolites that lead to excessive production of free radicals
2. metabolic by-products directly or indirectly deplete the cell antioxidant capacity
3. restricted diets alter the antioxidant status

Evidence of Oxidative Stress in PKU Patients

Antioxidant Defences in PKU Patients

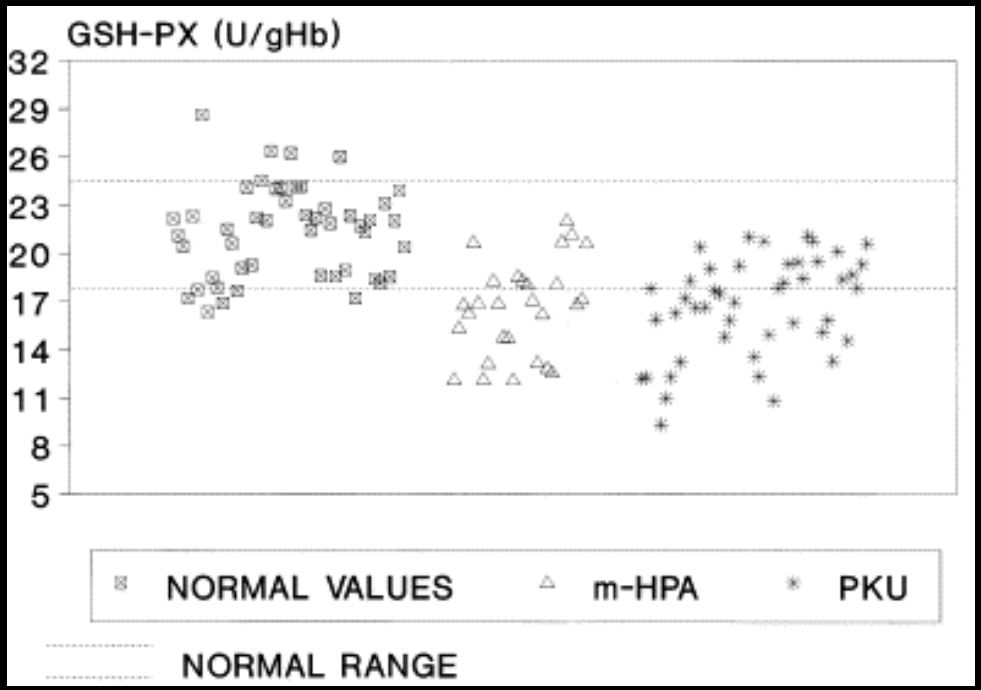
Reduction of selenium in plasma and urine of children with phenylketonuria during treatment with low protein diet:

	Chromium		Selenium	
	Plasma (n = 19, 19)	Urine (n = 14, 15)	Plasma (n = 20, 20)	Urine (n = 14, 15)
	nmol/L	*nmol/d*	*μmol/L*	*nmol/d*
PKU children				
$\bar{x} \pm$ SD	—	—	0.38 ± 0.11	58.0 ± 34.5
Median	11.5	<9.1	0.38	44.0
Range	<9.6–53.8	<3.9–11.7	0.23–0.65	25.9–153.0
Siblings				
$\bar{x} \pm$ SD	—	—	0.82 ± 0.15‡	165.2 ± 49.4§
Median	<9.6	<7.1	0.87	145.8
Range	<9.6–30.8	<2.7–15.1	0.56–1.06	98.4–260.0

(van Bakel et al, 2000; Steiner et al, 1982; Rottoli et al, 1985; Reilly et al, 1990; Darling et al, 1992; Sierra et al, 1998; Wilke et al, 1992; Gassió et al, 2008; Reilly et al, 1990)

Antioxidant Defences in PKU Patients

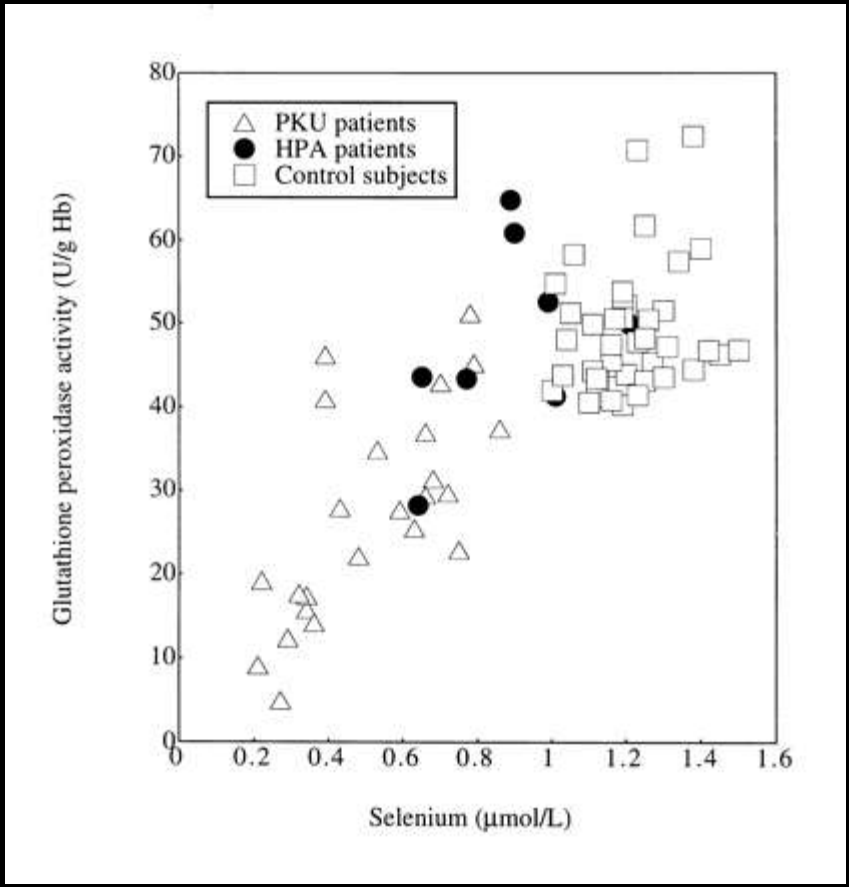
Decreased GPx activity



(Wilke et al, 1992; Reilly et al, 1990; van Bakel et al, 2000; Darling et al, 1992; Sierra et al, 1998)

Antioxidant Defences in PKU Patients

Decreased activity of GPx x selenium deficiency:



(Wilke et al, 1992; Reilly et al, 1990; van Bakel et al, 2000; Darling et al, 1992)

Antioxidant Defences in PKU Patients

Selenium supplementation does not correct the GPx activity?

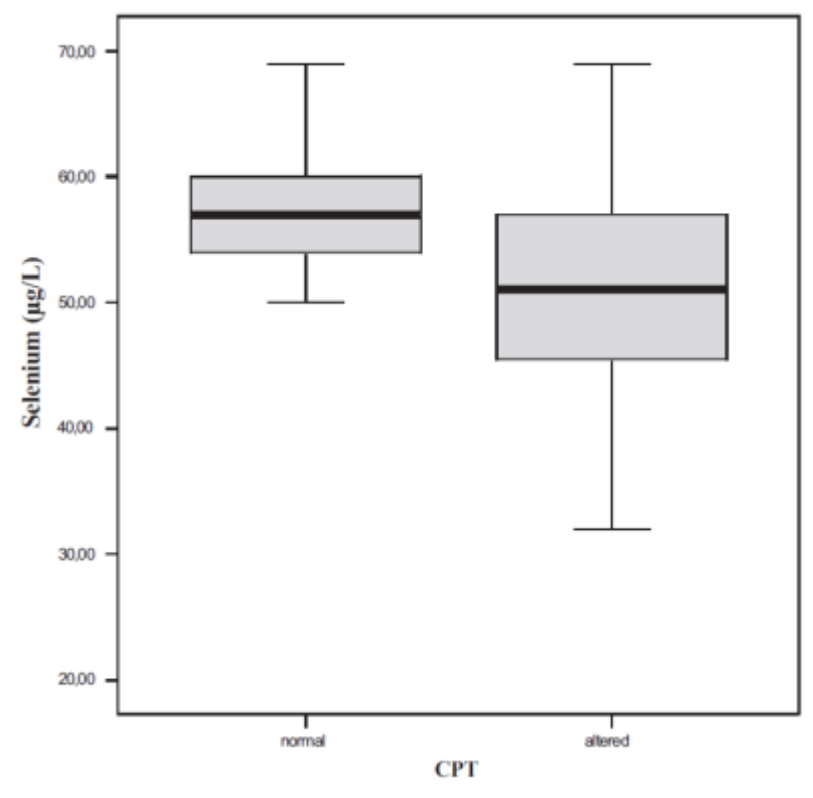
- Problems in the absorption of selenium?
- Poor dietary compliance?

(Lambruschini et al, 2005)

(Sierra et al, 1998)

Antioxidant Defences in PKU Patients

Selenium deficiency x cognitive changes:



(Castaño et al, 1997; Gassio et al, 2008)

Figure 1. Plasma selenium concentrations in PKU patients with normal and altered CPT performance. Selenium deficiency was considered for values lower than 50 µg/L. CPT = Conner's Continuous Performance Test.

Antioxidant Defences in PKU Patients

Decrease of GPx and neurological manifestations

	BP	NE Abnormal	IQ <80	EEG Abnormal
PKU normal GSH-Px	32%	27%	13%	14%
PKU low GSH-Px	65%	39%	29%	30%

Results are expressed as percent of patients with neuropsychological disturbances.

BP: behavioral problems

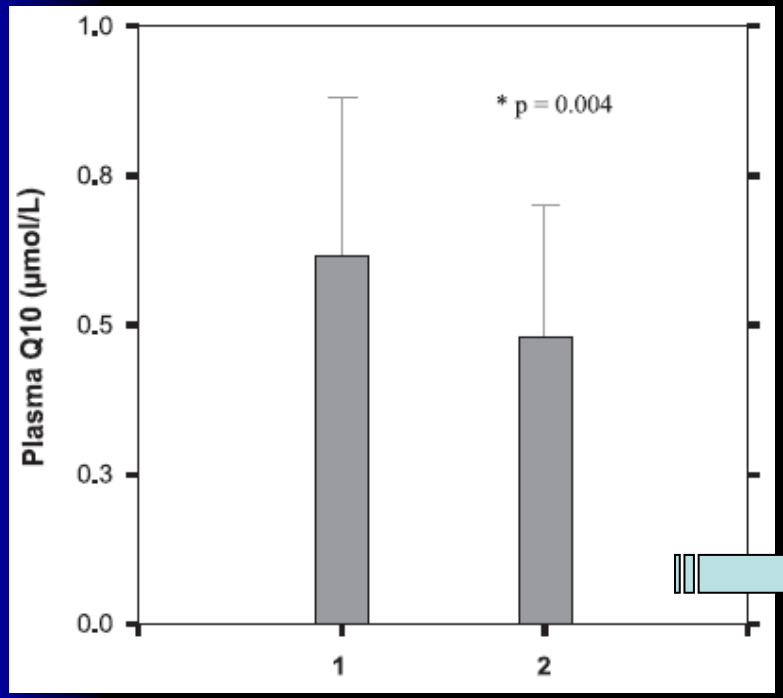
NE: neurological examination

IQ: intellectual quotient

(Sierra et al, 1998)

Antioxidant Defences in PKU Patients

Reduction of ubiquinone (coenzyme Q10) in the plasma of PKU patients:



- Q10: lipophilic antioxidant

- **Low protein diet?**

- **Inhibiting the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase by Phe, decreasing the biosynthesis of Q10?**

Control group: 0.69 µ mol / L
Group 1: well-controlled patients
Group 2: patients with poorly controlled

(Artuch et al, 1999; Gassió et al, 2008; Colomé et al, 2003; Artuch et al, 2004)

Antioxidant Defences in PKU Patients

Decreased antioxidant status (TAS and TAR):

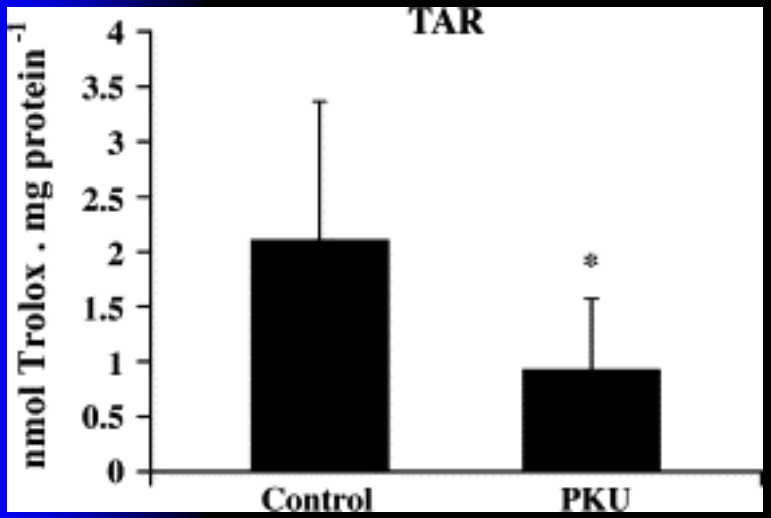


TABLE 1
Plasma antioxidant variables in patients and control subjects[1]

	PKU patients ($n = 24$)	HPA patients ($n = 10$)	Control subjects ($n = 42$)
Selenium (μmol/L)	0.52 ± 0.20[2,3]	0.92 ± 0.21[4]	1.23 ± 0.14
α-Tocopherol (μmol/L)	21.46 ± 4.06	19.25 ± 2.11	20.93 ± 6.15
Uric acid (μmol/L)	217.9 ± 50.4	235.7 ± 27.8	244.4 ± 104.8
Albumin (g/L)	39.8 ± 2.2	40.5 ± 2.3	39.5 ± 3.2
TAS (mmol/L)	1.52 ± 0.14[5]	1.52 ± 0.13[5]	1.76 ± 0.19

[1] $\bar{x}$ ± SD. PKU, phenylketonuria; HPA, hyperphenylalaninemia; TAS, total antioxidant status.

low levels of selenium

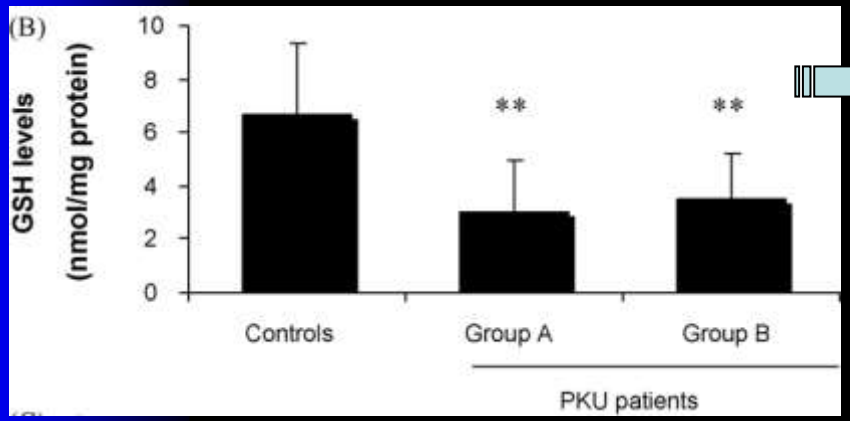
(Van Bakel et al, 2000; Sitta et al, 2006; Sitta et al, 2009; Sirtori et al, 2005)

Antioxidant Defences in PKU Patients

Decrease of GSH in erythrocytes of PKU patients

TABLE 3
Antioxidant enzyme activities of the glutathione cycle and reduced glutathione in erythrocytes of patients and control subjects[1]

	PKU patients (n = 24)	HPA patients (n = 10)	Control subjects (n = 42)
GSH (μmol/g Hb)	4.71 ± 0.64[2]	6.17 ± 1.30	7.35 ± 2.32
Glutathione peroxidase (U/g Hb)	27.75 ± 12.30[3,4]	49.59 ± 11.99	48.78 ± 7.46
SOD (U/mg Hb)	1.49 ± 0.55[2]	1.57 ± 0.36[2]	2.40 ± 0.33
Glutathione reductase (U/g Hb)	9.17 ± 1.72	9.67 ± 2.59	9.01 ± 2.01
Glutathione transferase (U/g Hb)	4.22 ± 1.69	4.11 ± 0.86	4.66 ± 1.50



Group A: Early diagnosis
Group B: late diagnosis

- Inhibition of transport system for Gly and Cys by high levels of Phe ?
- Increased formation of free radicals?

(van Bakel et al, 2000; Sitta et al, 2009)

Antioxidant Defences in PKU Patients

Correlation between selenium and GSH

	Selenium	
	r	*P*
Plasma		
α-Tocopherol (μmol/L)	−0.05	0.78
Albumin (g/L)	0.33	0.07
Uric acid (μmol/L)	0.37	<0.05
TAS (mmol/L)	0.18	0.33
FT_4 (pmol/L)	−0.46	<0.01
FT_3 (pmol/L)	0.27	0.14
FT_4:FT_3	−0.55	0.001
rT_3 (pmol/L)	−0.56	<0.001
rT_3:FT_3	−0.59	<0.0005
Erythrocyte		
Glutathione peroxidase (U/g Hb)	0.76	<0.000001
GSH (μmol/g Hb)	0.40	<0.05
SOD (U/mg Hb)	0.11	0.54
Glutathione transferase (U/g Hb)	0.05	0.79
Glutathione reductase (U/g Hb)	−0.06	0.74

(van Bakel et al, 2000; Sitta et al, 2009)

Antioxidant Defences in PKU Patients

Decrease of enzymatic antioxidant defenses (SOD and CAT):

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Table 1

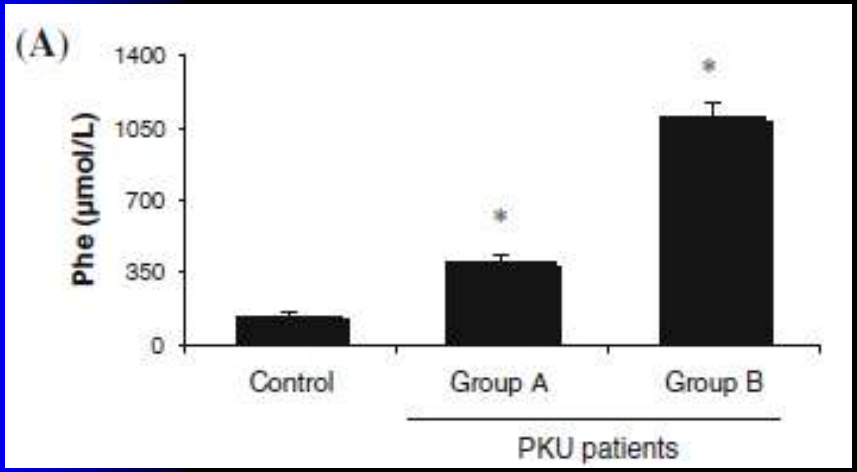
Biochemical Data of the Antioxidant System and Plasma Phenylalanine in PKU Patients and a Control Population

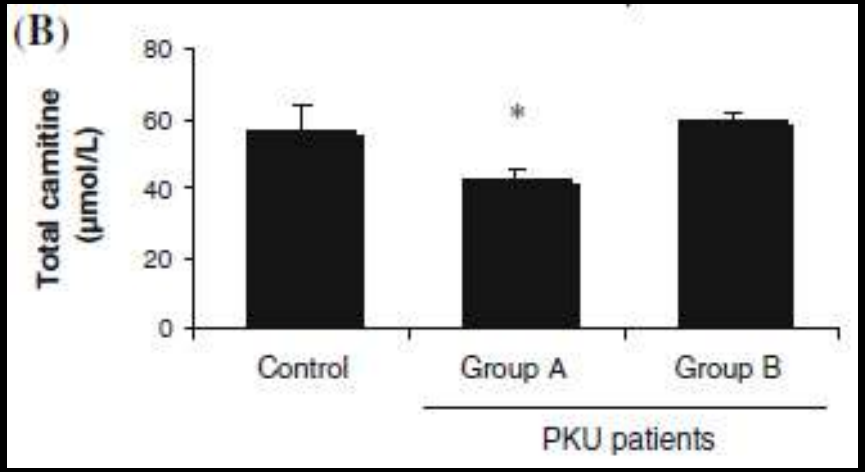
Antioxidant	PKU ($M \pm SD$)	CV ($M \pm SD$)
GPx (U/g Hb)	21 ± 5.39	22 ± 4.5
CAT (U/g Hb)	1,621 ± 224.82[a]	1,755 ± 222.84
MDA (nmol/L)	620 ± 195.74[a]	507 ± 154.06
Selenium (μg/L)	49 ± 14.33[a]	71 ± 21.48
Vit A (nmol/g prot)	1.36 ± 0.38	2.10 ± 4.12
Vit E (nmol/g prot)	20 ± 4.57	21 ± 6.74
Q10 (μmol/L)	0.52 ± 0.18[a]	0.69 ± 0.24
SOD (U/g Hb)	501 ± 53.55	485 ± 99.29
IDC 6m (μmol/L)	421 ± 188.59	65 (40–70)[b]
Tyr (μmol/L)	57.41 ± 24.84	72 (53–87)[b]

(van Bakel et al, 2000; Artuch et al, 2004; Gassió et al, 2008)

Antioxidant Defences in PKU Patients

Decreased plasma levels of L-carnitine in patients with good adherence to the PKU diet

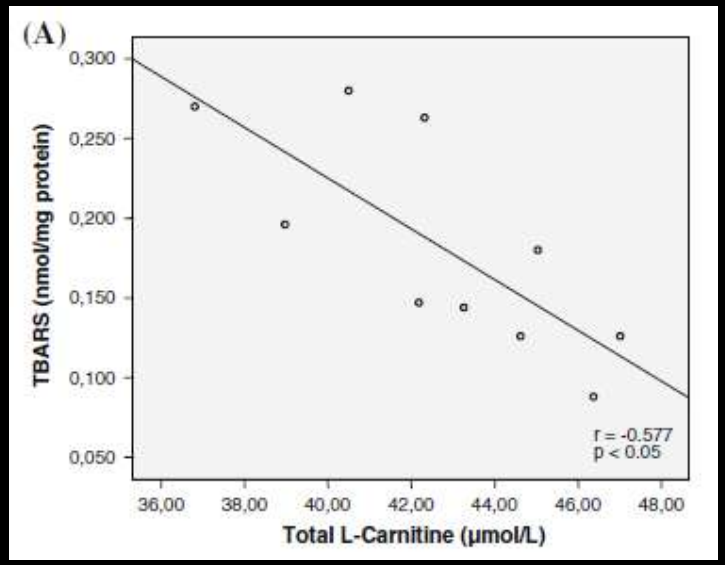


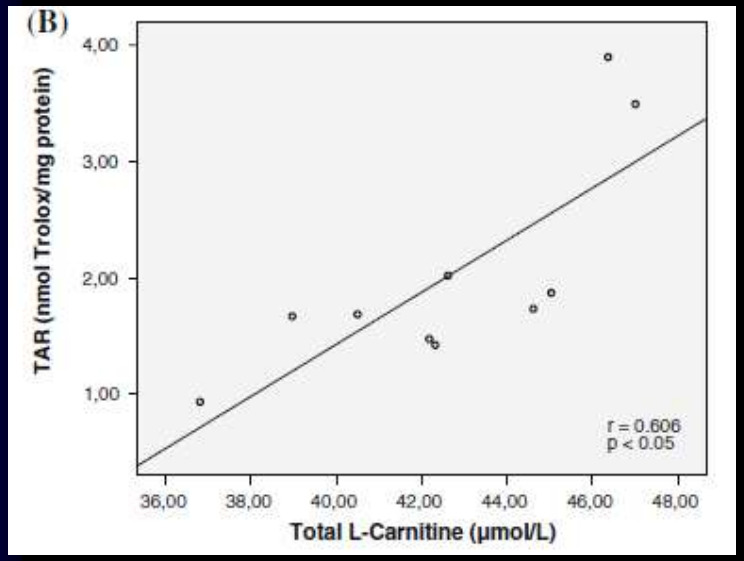


(Sitta et al, 2009)

Antioxidant Defences in PKU Patients

- Negative correlation between TBARS and carnitine.
- Positive correlation between TAR and carnitine.





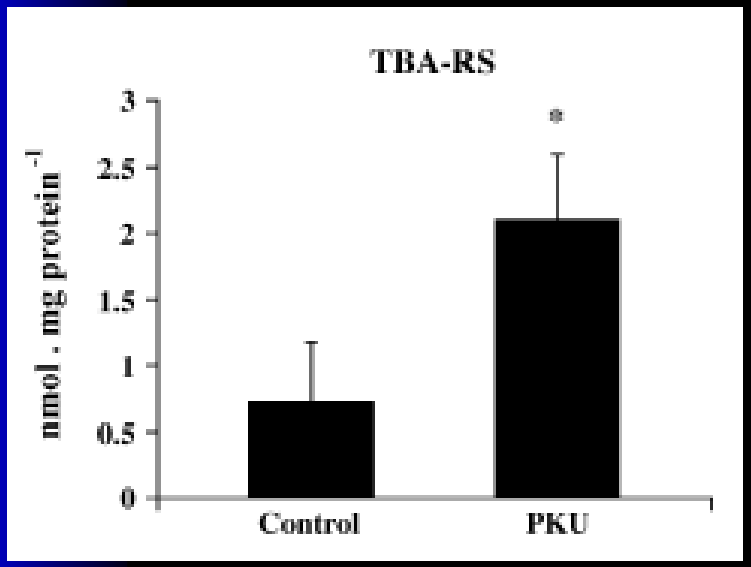
(Sitta et al, 2009)

L-carnitine:

- Main sources: red meat and dairy.
- Antioxidant and antiperoxidative

Oxidative Biomarkers in PKU Patients

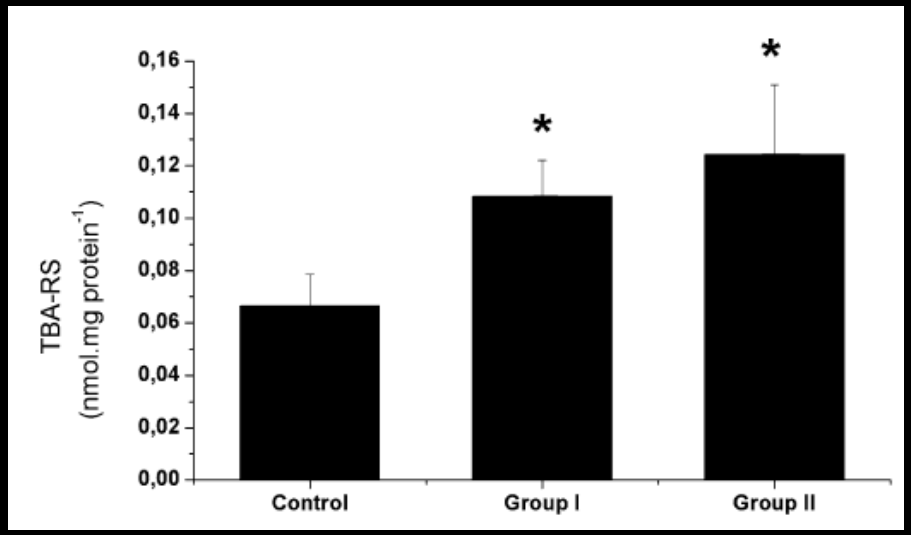
Lipid peroxidation



Diagnosis

(late diagnosis)

(Sirtori et al, 2005)



Treatment

(late diagnosis)

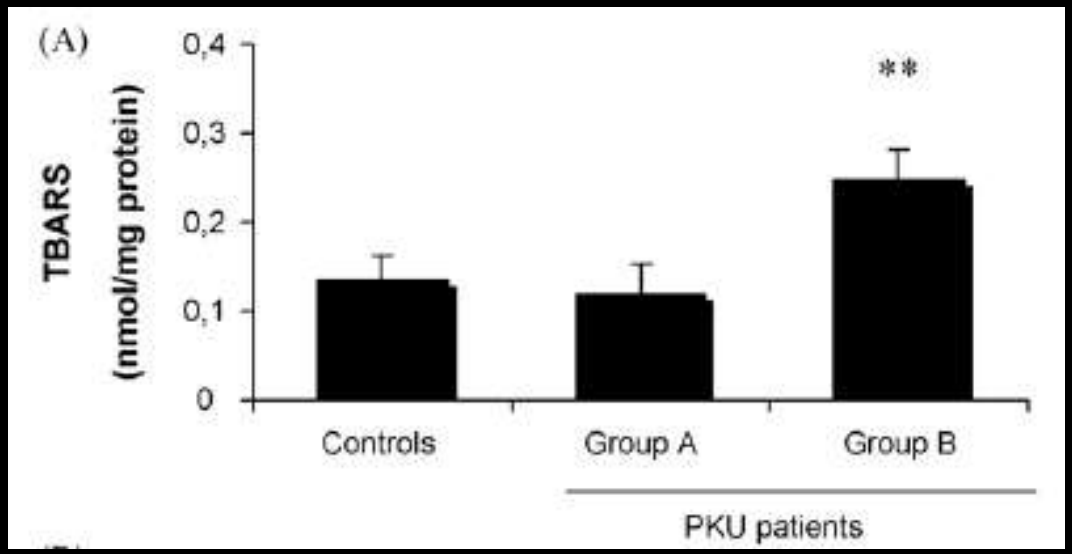
(Sitta et al, 2006)

Group I: Phe between 5.2 to 9.4 mg / dL

Group II: Phe between **17.3 to 21.1** mg / dL

Oxidative Biomarkers in PKU Patients

Lipid peroxidation



Group A: Early diagnosis
Group B: Late diagnosis

(Sitta et al, 2009)

Oxidative Biomarkers in PKU Patients

Lipid peroxidation

	PKU patients				
	All ($n = 58$)	Group 1 ($n = 25$)	Group 2 ($n = 33$)	HPA group ($n = 30$)	Control group ($n = 58$)
Phe (μmol/L)	604 (152–1407)[2,3]	607 (210–1244)[2,3]	601 (152–1407)	255 (108–607)	50 (33–76)
Q_{10} (μmol/L)	0.55 (0.2–1.67)[2,4]	0.37 (0.20–0.45)[2,3,5]	0.57 (0.46–1.67)	0.63 (0.44–1.22)	0.71 (0.36–1.10)
Tocopherol (μmol/L)	20.2 (8.5–30.1)	17.8 (8.5–25.7)[6,8]	21(13.0–30.1)	21.7 (15.1–30.0)	22.0 (12.0–36.0)
Retinol (μmol/L)	1.48 (0.6–3.6)	1.46 (0.6–3.1)	1.55 (0.8–3.6)	1.15 (0.8–1.8)	1.40 (0.6–2.3)
Ascorbate (μmol/L)	70 (36–109)	61 (44–97)	76 (36–109)	67 (38–89)	55 (8–92)
Selenium (μg/L)	49 (11.0–81.0)[2,9]	45 (11.0–68)[2,9]	49 (25–81)	60.6 (28.0–84.0)	65.4 (32.–84)
GPX (U/g Hb)	19.8 (11.4–30.1)	20 (12–30.1)	19.7 (11,4–27.8)	20.4 (14.4–26.0)	21.8 (13.9–33.6)
MDA (nmol/L)	663 (316–1404)[7]	768 (497–1341)[4,7,10]	620 (316–1404)	591 (357–946)	521 (320–914)
Tocopherol intake (mg/d)	13.1 (2.1–40.2)	12.3 (2.1–26.2)	13.8 (5.8–40.2)	NA	NA
Retinol intake (μg/d)	1622 (434–5806)	1767 (434–5806)	1523 (480–4674)	NA	NA
Ascorbate intake (mg/d)	112 (9–327)	124 (9–240)	104 (19–327)	NA	NA
Selenium intake (μg/d)	40 (6–113)	39 (6–105)	43 (8–113)	NA	NA

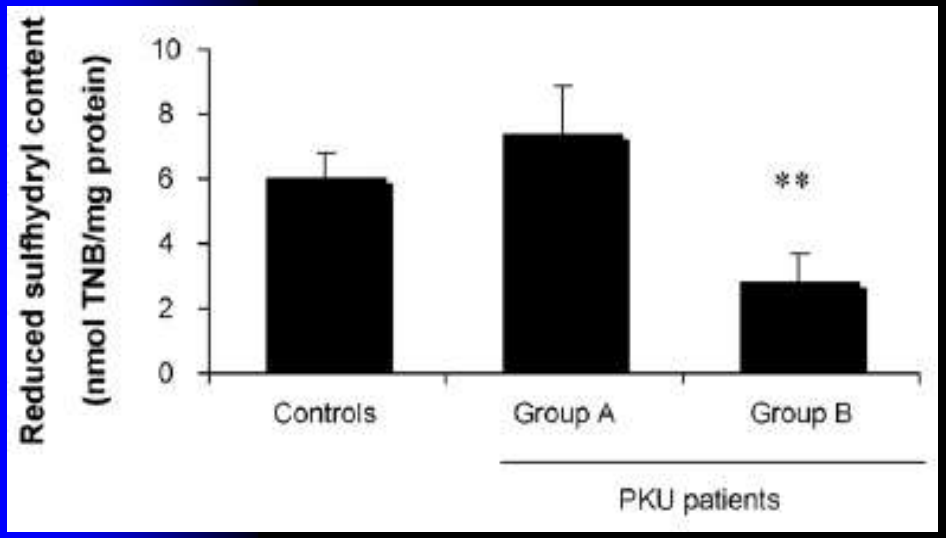
Low levels of coenzyme Q10

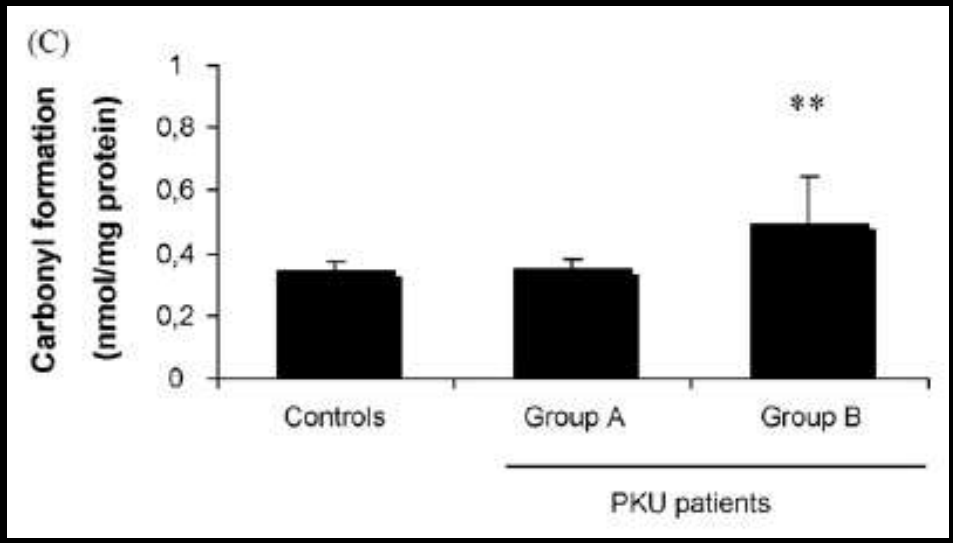
Normal levels of coenzyme Q10

(Colomé et al, 2003)

Oxidative Biomarkers in PKU Patients

Oxidative damage to proteins



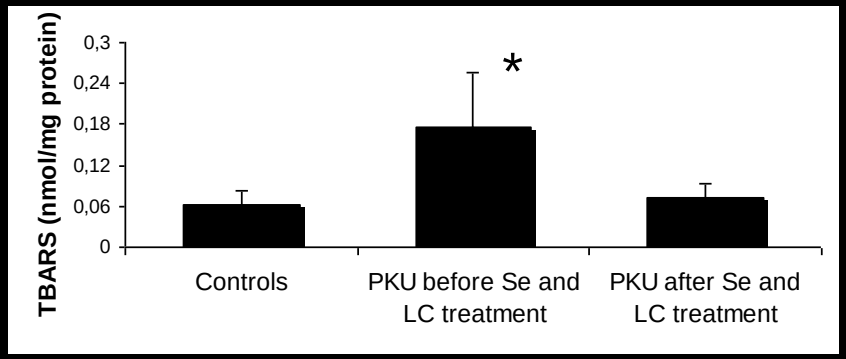


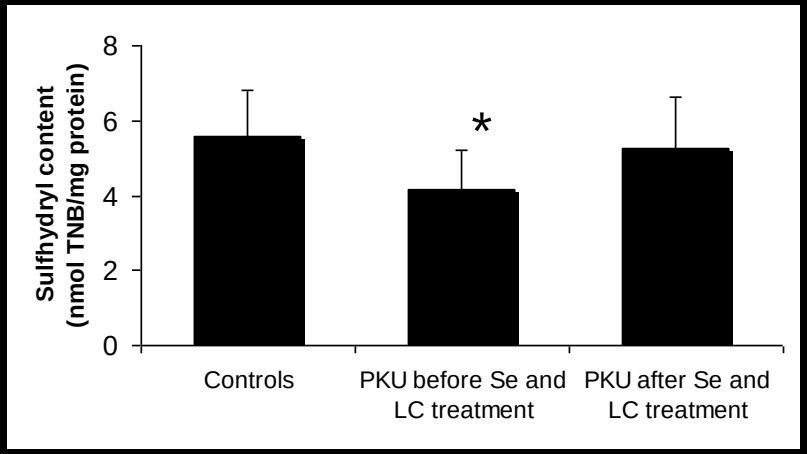
Group A: Early diagnosis

Group B: late diagnosis

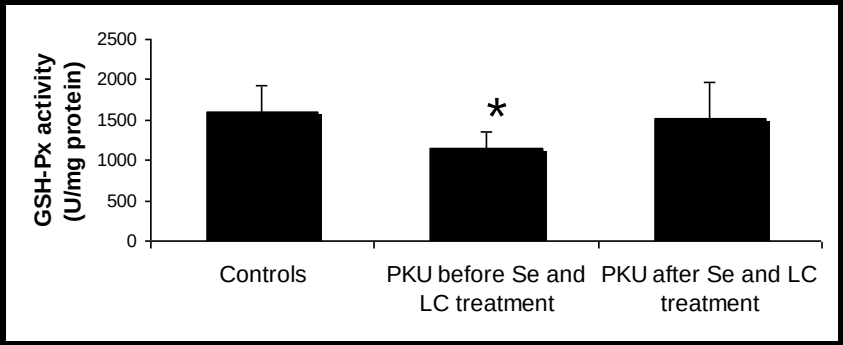
(Sitta et al, 2009)

Oxidative Biomarkers in PKU Patients



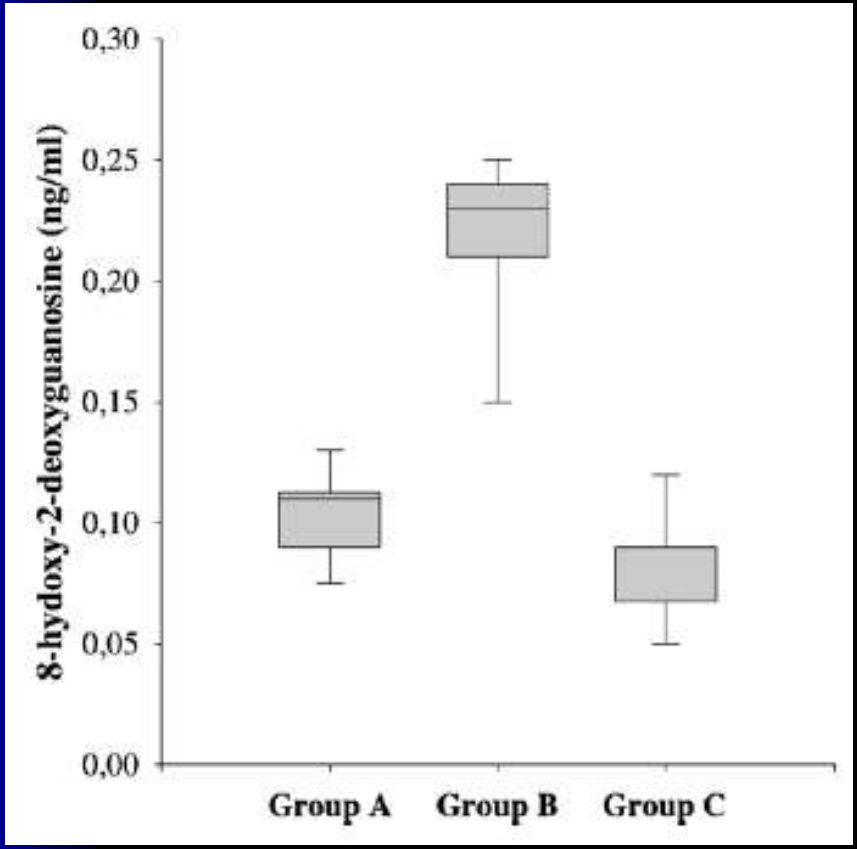


(Sitta et al, 2010)



Oxidative Biomarkers in PKU Patients

Oxidative DNA damage



Group A: patients with good adherence to treatment;

Phe = 156.6 ± 14.1 μmol/L

Group B: patients with poor adherence to treatment;

Phe = 1260.3 ± 32.8 μmol/L

Group C : Controls

(Schulpis et al, 2005)

Oxidative Biomarkers in PKU Patients

DNA damage in vitro and in vivo (comet assay)

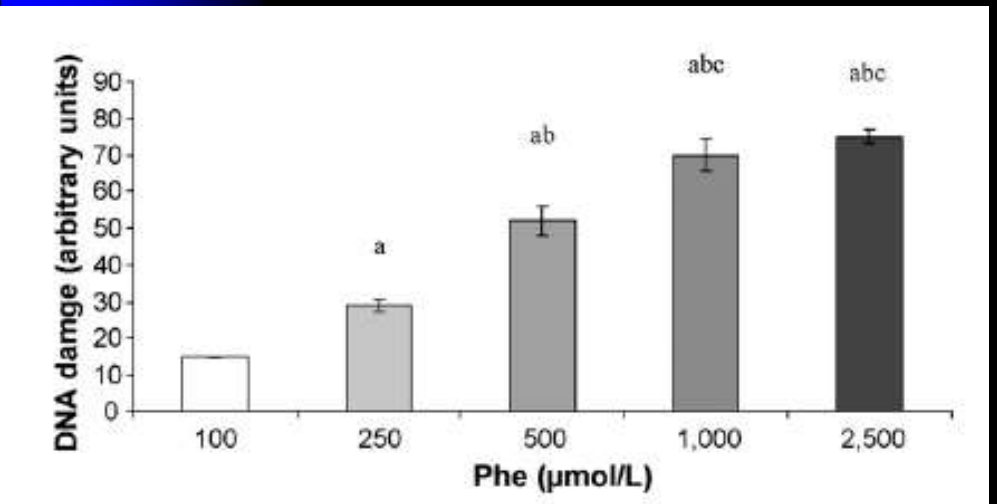


Fig. 1. *In vitro* effect of phenylalanine on DNA damage (comet assay) in leukocytes from whole blood. Data represent median ± S.E. of 3 independent experiments (individuals). (a) $p < 0.01$ compared to the Phe 100 µmol/L group; (b) $p < 0.01$ compared to the 250 µmol/L group; (c) $p < 0.01$ compared to the Phe 500 µmol/L group (Kruskal–Wallis test followed by Mann–Whitney *U*-test).

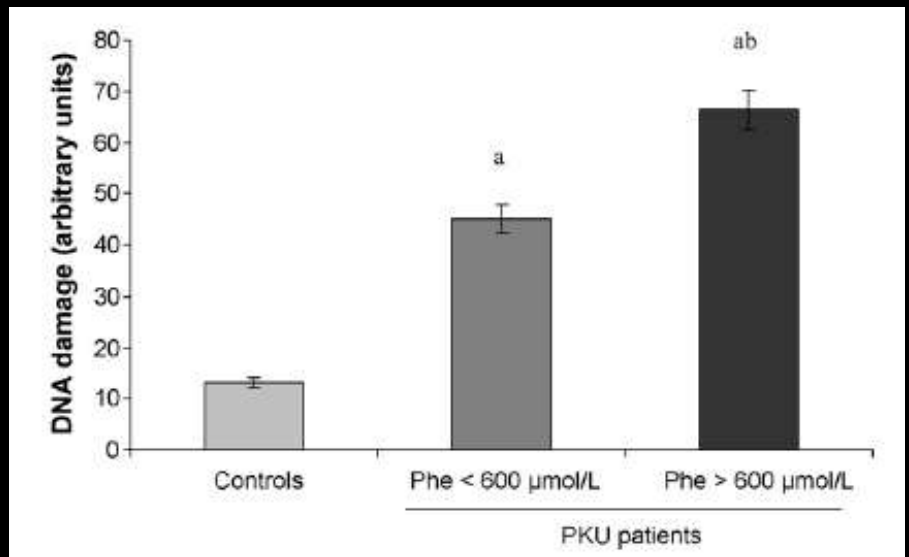
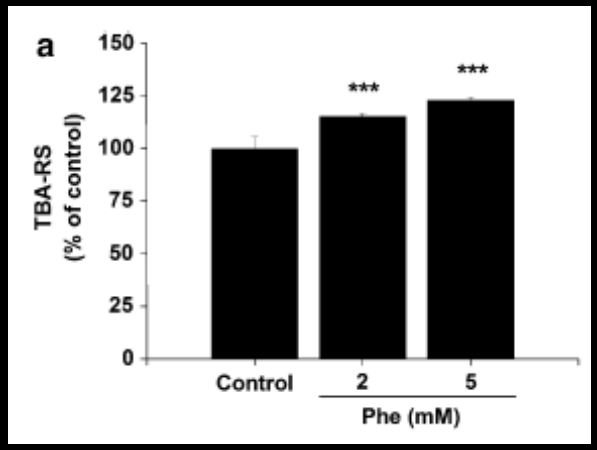


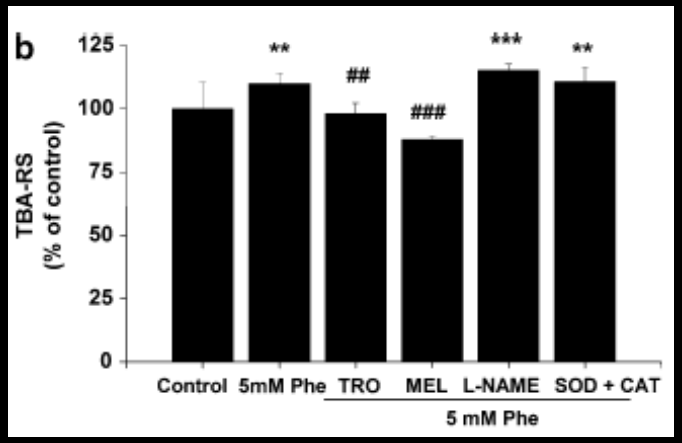
Fig. 2. DNA damage (comet assay) of peripheral blood leukocytes from two groups of PKU patients, one with Phe <600 µmol/L ($n = 8$) and the other with Phe >600 µmol/L ($n = 10$) and controls ($n = 17$). Data represent median ± S.E. (a) $p < 0.0001$ compared to the control; (b) $p < 0.001$ compared to the Phe <600 µmol/L group (Kruskal–Wallis test followed by Mann–Whitney *U*-test).

(Sitta et al, 2009)

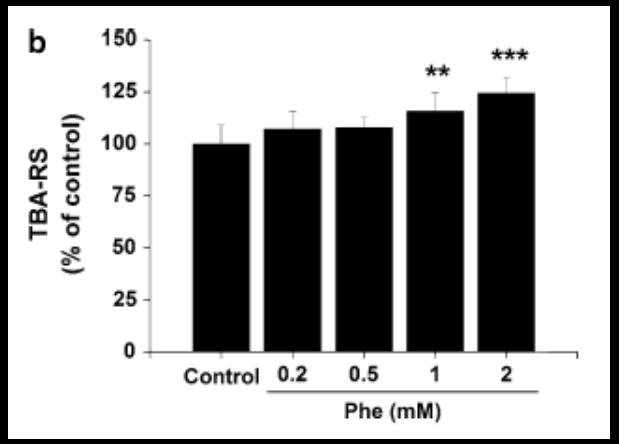
Oxidative Stress in PKU – animals studies

Fernandes et al., 2010: **in vitro effect of Phe on lipid peroxidation in rat hippocampus**

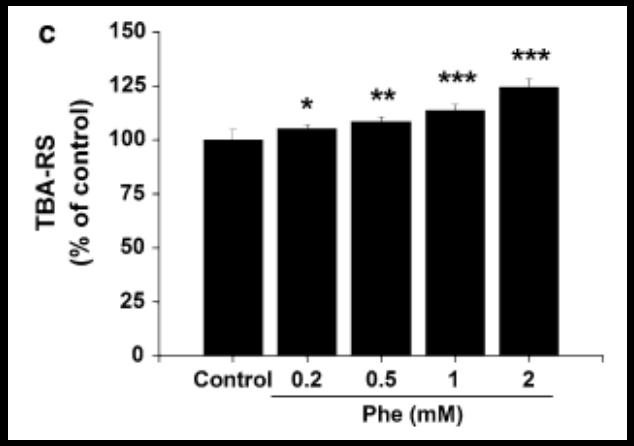




Fernandes et al., 2010: **in vitro effect of Phe on lipid peroxidation in rat cortex**



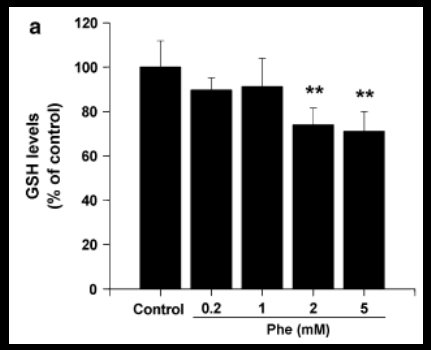
30 min



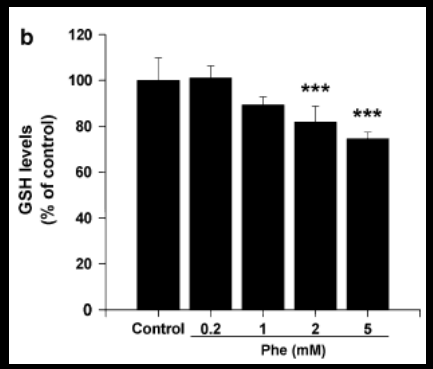
60 min

Oxidative Stress in PKU – animals studies

- **Fernandes et al., 2010: in vitro effect of Phe on glutathione levels in cortex and hippocampus of rats.**



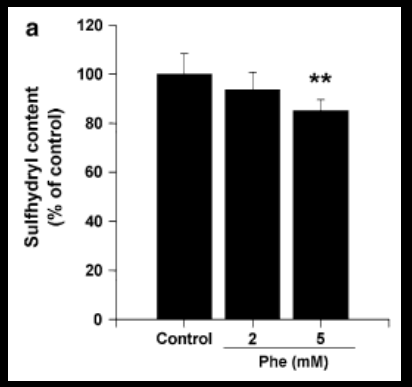
Hippocampus



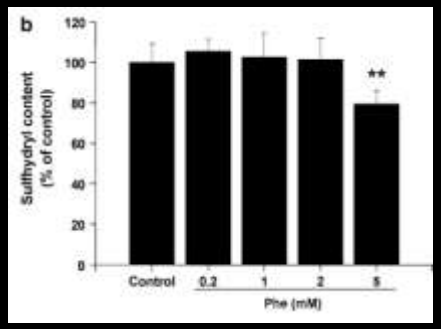
Cortex

Exposure time: 60 min

Fernandes et al., 2010: in vitro effect of Phe on oxidative damage to proteins in cortex and hippocampus of rats



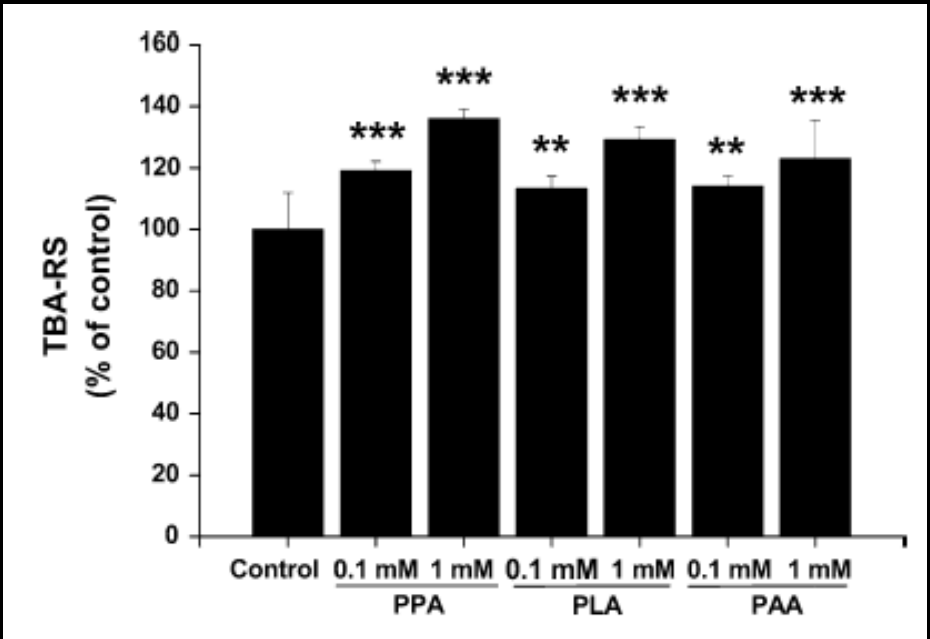
Hippocampus



Cortex

Oxidative Stress in PKU – animals studies

Fernandes et al., 2010: In vitro effects of Phe metabolites on lipid peroxidation in rat cortex



PPA: phenylpyruvate
PLA: phenyllactate
PAA: phenylacetate

Exposure time: 60 min

Final Remarks

Oxidative Stress in PKU

Oxidative damage to proteins, lipids and DNA

- **Selenium**
  GSH
- **Coenzyme Q10**
  Carnitine
- **SOD**
  CAT
  GPx
- **TAR**
  TAS

Final Conclusion

- **It can be suggested that the oxidative stress is one of the pathomechanisms of neurological damage in PKU patients**

Final Conclusion

- **Oxidative stress occurs at diagnosis and in treated PKU patients (good/bad compliance) demonstrating that:**

- **oxidative damage occurs despite the dietetic treatment**
- **dietary restrictions can contribute to depleted antioxidant status**
- **Phe is not the only metabolite involved with oxidative events**

Final Conclusion

- **Studies on new therapeutic approaches concerning oxidative stress (antioxidants) will be beneficial to PKU patients**

Thank you for your attention!

Genetic Medical Service / HCPA-Brazil