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Blood Antioxidant Status and Urinary Levels of Catecholamine Metabolites in β-Thalassemia

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It has been reported that iron overload in β-thalassemia leads to an enhanced generation of reactive oxygen species and to oxidative stress. We have studied the oxidant/antioxidant imbalance in the blood of 48 transfusion-dependent β-thalassemic patients (TLP) (17 males, 31 females, 11–22 year), under chelation therapy, and in 40 sex and age matched healthy controls (CTR). Plasma and lymphocyte levels of vitamin E (Vit E), ubiquinol ($CoQ_{10}H_2$), ubiquinone (CoQ_{10}), plasma concentrations of vitamin A (Vit A), β-carotene, lycopene, vitamin C (Vit C), total thiols, fatty acid patterns of phospholipids (PL-FA), and plasma and urinary markers of lipoperoxidation (TBA-RM, conjugated dienes, and azelaic acid (AZA), as well as the urinary levels of catecholamine and serotonin metabolites, were evaluated by gas chromatography-mass spectrometry (GC-MS), HPLC and spectrophotometry. Routine laboratory blood analyses were performed on the same samples; 39/48 TLP were HCV positive. Blood samples were collected just before transfusion, the 24 h urine samples the day before. Our results clearly showed that a severe oxidative stress occurs in the plasma of TLP in comparison with CTR. In fact, the levels of lipophilic antioxidants and ascorbate were severely depleted: $CoQ_{10}H_2$ (−62.5%), total CoQ_{10} (−35.1%), Vit E (−43.8%), β-carotene (−31.1%), lycopene (−63.7%), Vit A (−35.9%), Vit C (−23.1%). The impairment of the antioxidant status was associated with elevated plasma levels of by-products of lipoperoxidation and urinary concentrations of catecholamine metabolites and of AZA, indicating a high degree of both neurological stress and lipoperoxidation. A significant positive correlation was found between vitamin E and non-transferrin-bound iron (NTBI) ($r = -0.81$; $p < 0.001$), while no correlation was found between antioxidant depletion and ferritin serum levels, average blood consumption, or the presence of clinical complications. The administration of selective antioxidants along with an appropriate diet might represent a promising way of counteracting oxidative damage and its deleterious effects on the progression of the disease.

Keywords: β-thalassemia, oxidative stress, iron overload, ubiquinone, lipophilic antioxidants, antioxidant therapy, catecholamines

INTRODUCTION

β-thalassemia major is a homozygous inborn condition in which the rate of synthesis of β-hemoglobin chains is abnormally inhibited or absent.[1] Blood oxidative stress, mainly due to

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iron overload, has been claimed by many authors to be involved in the etiopathology of the disease.[2–6] The current therapeutic approach consists of both iron-chelating agents and transfusion of packed red blood cells (RBC).[7–10] These transfused cells contain relatively conspicuous amounts of iron, thus contributing to the iron overload in the patient. Oxidative damage is further enhanced by heme or heme-released iron, both cytoplasmic and bound to cell membranes, due to the instability of the hemoglobin moiety in endogenous thalassemic RBC.[11,12] The excess unpaired α-hemoglobin chains bind to cell membranes are readily oxidized and denatured, giving rise to typical inclusion bodies (Heinz Bodies) in erythroid precursors and, to a lesser extent, in peripheral blood erythrocytes.[13–15] Autoxidation of the globin chains, with abnormal release of superoxide anion (O_2^-),[16] induces cytoskeleton modifications, protein cross-linking with depletion of thiol residues, antioxidant depletion and lipid peroxidation of cell membranes, resulting in a shortened cell survival and massive hemolysis.[13,17] In addition, cellular enzymatic antioxidant defenses are affected, as demonstrated *in vitro* by the entrapment of purified α-chains into normal erythrocytes, leading to severe impairment of catalase (CAT) and glutathione peroxidase (GSH-Px) activity.[15,18]

The excess of iron in β-thalassemic patients (TLP) leads to the saturation of transferrin and increases levels of both ferritin, which represents an important – though debatable – parameter to monitor iron overload, and of non-transferrin-bound iron (NTBI).[19–21] This *free* form of iron is able to mediate the production of extremely reactive hydroxyl radicals ($OH^\bullet$).[22] Besides, ferritin itself is able to stimulate lipoperoxidation to an extent related to the level of iron it contains: reducing agents such as O_2^- radicals or vitamin C (Vit C) are able to cause the reductive release of Fe^{2+} from the protein, and this Fe^{2+} catalyzes the formation of $OH^\bullet$.[22–25] Iron accumulates particularly in liver, spleen, heart, pancreas and pituitary gland,[22] and many transfusion-dependent thalassemic patients suffer from liver damage, endocrinological complications and cardiac malfunctions (congestive heart failure representing the main cause of death[26]). This suggests the etiopathological involvement of an increased generation of iron-dependent reactive oxygen species (ROS), leading to oxidative damage,[22] further amplified by the depletion of antioxidants, the concentrations of which become insufficient to cope with the high levels of ROS and other radicals, affecting RBC integrity and function.

The antioxidant status in the blood of β-thalassemic patients has been so far only partially investigated: low serum levels of vitamin E (Vit E) and increased susceptibility of RBC to exogenous oxidant stress have been described.[27–30] In this connection, David *et al.*[31] have claimed that the shortened survival of normal transfused erythrocytes in thalassemic plasma may derive from the toxic effects of a pathological *plasma environment* in these patients, which is depleted of Vit E and is presumably pro-oxidant, due to the overproduction of aldehydes and lipid radicals. Only recently, both the depletion of lipophilic antioxidants and Vit C, and the increased values of some parameters of oxidative damage to polyunsaturated fatty acids (PUFA) and proteins have been clearly shown by Livrea *et al.*,[6] in the serum of 42 transfusion dependent TLP, under iron chelation therapy, aged 4–40 years.

In the present study we have performed a multiparameter analysis of the antioxidant status in 48 TLP, aged 11–22 years, undergoing a transfusional program plus chelation therapy, and 40 age and sex matched healthy controls (CTR). We evaluated: (a) plasma lipophilic and hydrophilic antioxidants, such as Vit E, ubiquinol ($CoQ_{10}H_2$), ubiquinone (CoQ_{10}), vitamin A (Vit A), β-carotene, lycopene, Vit C, total thiols; (b) Vit E, $CoQ_{10}H_2$, CoQ_{10} in peripheral blood lymphocytes (PBL); (c) the fatty acid pattern of the phospholipid fraction (PL-FA) of plasma lipids, in that lipoperoxidation, which involves PUFA as its primary reaction sites, can be

considered as a hallmark of oxidative stress; (d) plasma and urine markers of oxidative damage to plasma PUFA and proteins; (e) urinary catecholamine and serotonin metabolites.

MATERIALS AND METHODS

Samples

Fresh anticoagulated (EDTA) venous blood was obtained from 48 patients affected with homozygous β-thalassemia, 17 males and 31 females, age 11–22 years, with informed consent for the study, obtained from adult subjects or from their relatives. All patients were under a transfusional program with a mean transfusional interval of 20 days (range, 10–28 days), receiving approximately 15 ml of packed RBC per kg/body weight at each transfusion, to maintain hemoglobin (Hb) levels above 9.5–10.0 g/100 ml. Chelation therapy was performed with desferrioxamine mesylate (DFX), administrated by subcutaneous infusion at an average dose of 40 mg/kg/day (range, 30–50 mg), for 10–12 h nightly. Patients were not taking vitamin supplements. TLP were affected with the following clinical complications: HCV positive (39/48), HBSAg positive (1/48), diabetes mellitus (1/48), hypogonadism (13/48), cardiac malfunctions (1/48). Within the group, 6/48 patients were splenectomized, those who had shown clinical signs of hypersplenism, i.e. enlargement of the spleen, plus a drop in mean annual Hb levels, increased blood consumption (ml/kg/year) and reduced well-being.

Blood was collected the day before transfusion, after 12 h fasting, the 24 h urine the day before. Control blood and urine were obtained, with consent, from 40 age and sex matched healthy individuals (CTR), who were not taking any medication; their hemoglobin levels fluctuated between 12.5 and 14.2 g/100 ml.

Plasma was immediately separated by centrifugation, and divided into 0.5–1.0 ml aliquots, immediately stored at −80°C under argon. The analytical assays described below were either performed immediately (ubiquinol/ubiquinone, total thiols and ascorbate), or within 48 h. Following plasma separation, lymphocytes were obtained by centrifugation on Ficoll-Paque gradient (Amersham Pharmacia Biotech, Freiburg, Germany), and stored at −80°C under argon until analysis. Urine samples were acidified (HCl 1 N, 10%) and stored at −80°C if not immediately processed for analysis.

All reagents were of analytical grade, reference standards were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Biochemical Assays

Ubiquinol ($CoQ_{10}H_2$) and Ubiquinone (CoQ_{10})

Plasma and lymphocyte $CoQ_{10}H_2$ and CoQ_{10} were quantified simultaneously by high performance liquid chromatography (HPLC) on a HP1090 liquid chromatograph (Hewlett-Packard), on an analytical Supelcosil LC-18DB column (24 cm × 4.6 mm, 5 µm, Supelco) plus its guard column, by using on line both a 1050 diode array detector (DAD) (CoQ_{10}) and an electrochemical detector 1049A with a glassy carbon electrode (ECD) ($CoQ_{10}H_2$). Mobile phase: methanol/isopropanol (55/45, v/v), flow: 1 ml/min.[32]

Vitamin A (Vit A), β-carotene and Lycopene

Plasma Vit A, β-carotene and lycopene were assayed by HPLC with ECD detection, on a analytical Supelcosil LC-18DB column (24 cm × 4.6 mm, 5 µm, Supelco) plus its guard column. Mobile phase: A = 20 µm $NaClO_4$ in MeOH/H_2O (96/4, v/v), B = MeOH/2-propanol (55/45, v/v). Gradient program: %B = 5 for 5 min, %B = 20 in 15 min and then %B = 90 in 25 min, flow: 1 ml/min.[33]

Vitamin E (Vit E)

Determination of plasma and lymphocyte Vit E was performed by gas chromatography-mass spectrometry (GC-MS) on a HP5990 gas

chromatogragh coupled with a HP5989B mass spectrometer (Hewlett-Packard), equipped with a capillary Ultra 1 column (30 m × 0.2 mm × 0.33 μm, Hewlett-Packard), according to Passi *et al.*[34]

Fatty Acid Patterns of Plasma Phospholipids (PL-FA)

Quantitation of plasma PL-FA was performed by GC-MS on a crosslinked-FFAP capillary column (50 m × 0.32 mm × 0.52 μm, Hewlett-Packard), following purification of lipid fractions by thin layer chromatography (TLC), according to Passi *et al.*[34]

Cholesterol

Total cholesterol (CH) was assayed spectrophotometrically by using a commercial analytical kit from Sigma Chemical Co. (St. Louis, MO, USA). Free cholesterol (FC) was analyzed by GC-MS on an Ultra 1 capillary column as described by Passi *et al.*[34] The values of cholesterol in cholesterol esters (CE) were calculated by the difference between CH and FC (CE = CH − FC).

Total Thiols

Determination of plasma total thiol groups was performed by 2,2-dithionitrobenzoic acid (Ellman's Reagent), according to Hu.[35]

Ascorbic (Vit C) and Uric Acids

Plasma Vit C and uric acid were measured by HPLC, on an analytical Supelcosil LC-18DB column (24 cm × 4.6 mm, 5 μm, Supelco) plus its guard column, according to the method of Motchnik *et al.*[33]

Urinary Catecholamine and Serotonine Metabolites, and Azelaic Acid (AZA)

Urinary catecholamine and serotonin metabolites, namely homovanillic acid (HVA, deriving from dopamine), vanilmandelic acid (VMA, from epinephrine plus norepinephrine), and hydroxyindole acetic acid (HIAA, from serotonin), and azelaic acid (AZA, a marker of lipoperoxidation[36]), were assayed as trimethylsilyl ether (TMS) derivatives by GC-MS on capillary Ultra 1 column (30 m × 0.2 mm × 0.33 μm, Hewlett-Packard), according to Morrone *et al.*[37]

Plasma Lipoperoxides

Plasma lipoperoxidation levels were evaluated by both thiobarbituric acid (TBA) test[38] and 9,11 conjugated diene test. The values of TBA-reactive material (TBA-RM) were expressed as malondialdehyde (nmol/ml). Conjugated dienes were estimated spectrophotometrically at 234 nm, using a molar absorption coefficient of 27.000.[39]

Protein Carbonyls

Plasma protein carbonyls were evaluated by the 2,4-dinitrophenylhydrazine method according to Faure and Lafond.[40] Protein content was determined by the Bio-Rad Protein Essay, Bio-Rad (München, Germany).

Other Assays

NTBI was quantified colorimetrically by a bathophenanthroline-based method according to Zhang *et al.*[41] Triglycerides, free fatty acids, bilirubin, albumin, alanine transaminase, were assayed spectrophotometrically by using commercial analytical kits from Sigma Chemical Co. (St. Louis, MO, USA). Total phospholipids were quantified spectrophotometrically by a commercial analytical kit from SGM Italia (Rome, Italy). Total lipids were quantified by TLC, as the sum of phospholipid, triglyceride, free fatty acid, free cholesterol and cholesterol ester fractions. Ferritin was measured by an enzyme immunoassay (Abbott Labs, North Chicago, IL, USA).

Statistical Analysis

Each result was expressed as mean ± standard deviation (SD). Statistical significance of data was estimated using the unpaired Student's

t-test. The association of the variables was assessed using correlation analysis.

RESULTS

Thalassemic patients showed, as compared to controls, a dramatic plasma increase ($p < 0.001$) of ferritin (2887 ± 1550 vs. 91 ± 14 ng/ml) and NTBI (14.6 ± 10.5 μg/100 ml vs. non-detectable levels) (Table I), in association with a significant depletion ($p < 0.001$) of lipophilic antioxidants: $CoQ_{10}H_2$ (0.12 ± 0.11 vs. 0.32 ± 0.10 μg/ml), Vit E (5.79 ± 3.06 vs. 10.30 ± 1.10 μg/ml), total Q_{10} ($CoQ_{10}H_2 + CoQ_{10}H_2$) ($0.50 \pm 0.13$ vs. 0.77 ± 0.09 μg/ml), Vit A (0.270 ± 0.086 vs. 0.421 ± 0.048 μg/ml), β-carotene (0.164 ± 0.067 vs. 0.238 ± 0.047 μg/ml), lycopene (0.105 ± 0.087 vs. 0.289 ± 0.071 μg/ml) (Table II).

With the exception of Vit C (5.91 ± 2.17 vs. 7.68 ± 1.15 μg/ml, $p < 0.05$), the hydrophilic antioxidants, i.e., total thiols (including GSH), urate, albumin and bilirubin were not depleted (Tables I and II) and, indeed, bilirubin, a bile pigment produced by heme catabolism, was significantly increased ($p < 0.001$) due to the abnormal erythrocyte hemolysis.

TABLE I Important hematological parameters of TLP and healthy CTR

	TLP ($n = 48$)	CTR ($n = 40$)
NTBI (μg/dl)	14.6 ± 10.5 *	0
Ferritin (ng/ml)	2887 ± 1550*	91 ± 14
Albumin (g/dl)	4.87 ± 0.41	4.21 ± 0.48
Alanine transaminase (U/l)	66.4 ± 20.5*	29.1 ± 7.0
Cholesterol, total (CH, mmol/l)	3.16 ± 0.75*	4.64 ± 0.35
Cholesterol esters (CE, mg/dl)[a]	154.2 ± 31.0*	220.5 ± 25.1
Triglycerides (mg/dl)	95.9 ± 21.4	91.4 ± 15.6
Phospholipids, total (mg/dl)	157.5 ± 61.3	171.8 ± 13.7
Fatty acids, free (mg/dl)	17.3 ± 4.5	14.5 ± 2.3
Lipids, total (mg/dl)	456.3 ± 129.1	539.9 ± 71.5
Bilirubin (μg/ml)	10.7 ± 4.2*	5.9 ± 0.4

n = number of individuals in the two groups under study; results are expressed as the mean ± SD of n determinations.
[a]Cholesterol represents nearly 59% of the whole weight of CE.
*$p < 0.001$ vs. CTR.

Concerning lipid metabolism, CH and CE were significantly depleted ($p < 0.001$) (Table I), while triglycerides, phospholipids, and phospholipid PUFA (PL-PUFA) levels, in particular C18 : 2*n*-6, C20 : 3*n*-6, C20 : 4*n*-6, and C22 : 6*n*-3 were not significantly different from control values (Tables I and III).

Liver function was moderately impaired, as evidenced by the increased alanine transaminase levels ($p < 0.001$) and by the decrease of plasma CH and CE levels as compared to control (Table I).

TABLE II Antioxidant levels in plasma and lymphocytes of TLP and healthy CTR

	TLP ($n = 48$)	CTR ($n = 40$)
Plasma (μmol/l)		
Vit E	13.44 ± 7.10***	23.91 ± 2.55
$CoQ_{10}H_2$	0.14 ± 0.13***	0.40 ± 0.12
CoQ_{10}	0.44 ± 0.21	0.52 ± 0.09
$CoQ_{10}H_2 + CoQ_{10}$	0.58 ± 0.34***	0.92 ± 0.21
Vit A	0.94 ± 0.30***	1.47 ± 0.17
β-carotene	0.305 ± 0.125**	0.443 ± 0.087
Lycopene	0.197 ± 0.162***	0.538 ± 0.132
Ascorbate	33.56 ± 12.32*	43.61 ± 6.53
Urate	283.16 ± 86.85	299.23 ± 71.38
Total thiols	218 ± 65	232 ± 52
Antioxidant/total lipids ($\mu g\,ml^{-1}/mg\,ml^{-1}$)$10^{-3}$		
Vit E	1.268 ± 0.671**	1.907 ± 0.203
$CoQ_{10}H_2$	0.026 ± 0.024***	0.059 ± 0.019
CoQ_{10}	0.083 ± 0.039	0.083 ± 0.015
$CoQ_{10}H_2 + CoQ_{10}$	0.109 ± 0.028***	0.143 ± 0.017
Vit A	0.059 ± 0.019***	0.078 ± 0.009
β-carotene	0.036 ± 0.015	0.044 ± 0.009
Lycopene	0.023 ± 0.019**	0.053 ± 0.013
Lymphocytes (ng/10^6 cells)		
Vit E	61.9 ± 19.4	65.3 ± 11.1
$CoQ_{10}H_2$	12.4 ± 7.1***	28.5 ± 5.9
CoQ_{10}	51.3 ± 17.6***	36.4 ± 6.1
$CoQ_{10}H_2 + CoQ_{10}$	63.7 ± 11.4	64.9 ± 6.0

n = number of individuals in the two groups under study; results are expressed as the mean ± SD of n determinations. The relatively higher SD in β-thalassemic patients are indicating the higher fluctuation of data in this group, affecting statistical significance. Lipophilic antioxidant levels are reported as absolute values (μmol/l) and also normalized per unit plasma total lipids. With the exception of β carotene, normalization does not alter substantially the significance of data.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. CTR.

TABLE III Percent of fatty acid composition of plasma phospholipids in TLP and healthy CTR

Fatty acid (%)	TLP ($n=48$)	CTR ($n=40$)
C16:0	23.00 ± 1.86	22.29 ± 2.39
C18:0	19.44 ± 1.47	20.55 ± 1.97
C18:1	12.46 ± 0.67	12.95 ± 0.82
C18:2	19.61 ± 1.30	20.22 ± 1.80
C20:3*n*-6	4.15 ± 0.88	3.93 ± 0.98
C20:4*n*-6	17.61 ± 1.86	16.52 ± 2.06
C22:6*n*-3	3.03 ± 1.15	2.96 ± 2.20
Others	0.71	0.58
% Saturated FA	42.30 ± 3.53	42.0 ± 2.52
% PUFA	45.29 ± 4.34	39.6 ± 4.51
C18:2/(C20:3*n*-6 + C20:4*n*-6)[a]	0.91 ± 0.23	1.16 ± 0.10

n = number of individuals in the two groups under study; results are expressed as the mean ± SD of n determinations. *Others*: other minor fatty acids.
[a]This ratio is an indicator of the microsomal Δ-6 desaturase activity.

TABLE IV Indices of oxidative damage to lipids and proteins in the plasma of TLP and healthy CTR

	TLP ($n=48$)	CTR ($n=40$)
TBA-RM (nmol/ml)	3.37 ± 0.83*	2.05 ± 0.69
Conjugated dienes (nmol/ml)	11.5 ± 4.3*	5.8 ± 2.6
Protein carbonyls (nmol/ml proteins)	0.81 ± 0.13*	0.36 ± 0.06

n = number of individuals in the two groups under study; results are expressed as the mean ± SD of n determinations.
*$p < 0.001$ vs. CTR.

TABLE V Urinary levels of catecholamine and serotonin metabolites and azelaic acid in TLP and healthy CTR

	TLP ($n=48$)	CTR ($n=40$)
HVA (mg/24 h)	3.23 ± 2.02*	1.27 ± 0.41
VMA (mg/24 h)	1.68 ± 0.64*	0.86 ± 0.28
HIAA (mg/24 h)	1.93 ± 0.98*	0.62 ± 0.33
AZA (mg/24 h)	3.10 ± 1.09**	0.83 ± 0.35

n = number of individuals in the two groups under study; results are expressed as the mean ± SD of n determinations.
*$p < 0.01$, **$p < 0.001$ vs. CTR.

$CoQ_{10}H_2$ was significantly decreased also in the PBL of thalassemics (12.4 ± 7.1 vs. 28.5 ± 5.9 ng/10^6 cells, $p < 0.001$), while Vit E results were normal, and CoQ_{10} levels increased ($p < 0.001$) in respect to controls. In any case, total ubiquinone, i.e. $CoQ_{10}H_2 + CoQ_{10}$, was not significantly different from control levels (Table II).

The stability of PL-PUFA and of hydrophilic antioxidants (with the exception of ascorbate) is in apparent contradiction with the significant increase ($p < 0.001$) of plasma TBA-RM (3.37 ± 0.83 vs. 2.05 ± 0.69 nmol/ml), conjugated dienes (11.5 ± 4.3 vs. 5.8 ± 2.6 nmol/ml), protein carbonyls (0.81 ± 0.13 vs. 0.36 ± 0.06 and nmol/mg proteins), and urinary AZA (3.10 ± 1.09 vs. 0.83 ± 0.35 mg/24 h) (Tables IV and V). This latter parameter is a reliable marker of lipoperoxidation, being a specific and stable end-product of both free and esterified oxidized PUFA, as previously shown.[36]

The impairment of the antioxidant status in the plasma of patients was consistent with the elevated levels of urinary catecholamine and serotonin metabolites (HVA, VMA, HIAA, $p < 0.01$), indicating an elevated neurological involvement (Table V), while no correlation was found with ferritin, NTBI, or with the score of clinical complications. The only significant correlation was found between Vit E and NTBI ($r = -0.81$; $p < 0.001$).

DISCUSSION

Our results clearly show that a severe oxidative stress occurs in the plasma of TLP in comparison with controls. In fact, the levels of lipophilic antioxidants and Vit C are dramatically depleted (Table II): ubiquinol (−62.5%), total CoQ_{10} (−35.1%), Vit E (−43.8%), β-carotene (−31.1%), lycopene (−63.7%), Vit A (−35.9%), Vit C (−23.1%). However, it is important to underline that the standard deviations reported in Tables I–IV, higher in patients than in controls, are indicating an elevated degree of fluctuation of data, affecting statistical significance and consequently the degree of oxidative stress among TLP.

In particular, the marked drop of $CoQ_{10}H_2$, not counterbalanced by a concomitant increase of CoQ_{10} (Table II), indicates that the biosynthesis of total ubiquinone, i.e., $CoQ_{10}H_2 + CoQ_{10}$, is decreased, as shown by its low plasma levels (−35.1% vs. CTR). It is worth mentioning that $CoQ_{10}H_2$ is the only known lipophilic antioxidant that mammalian cells can synthesize *de novo* and for which there are enzymic NAD(P)H dependent mechanisms able to (re)generate it from CoQ_{10}.[42,43] It is well known that CoQ_{10}, in addition to its function as an electron and proton carrier in mitochondria, acts as a powerful antioxidant in its reduced form $CoQ_{10}H_2$, by preventing both the initiation and the propagation of lipoperoxidation in biological membranes.[44,45] $CoQ_{10}H_2$ is able to sustain efficiently the chain breaking antioxidant capacity of Vit E, by generating it from the α-tocopheryl radical[46] which, otherwise, would need the cooperation of hydrophilic antioxidants such as vitamin C, and/or GSH. Moreover, as $CoQ_{10}H_2$ is essential to maintain Vit E status and function, depletion of $CoQ_{10}H_2$, in turn, contributes to further exacerbate the deficiency of Vit E.

Ubiquinol is significantly reduced also in PBL of thalassemic patients, but this depletion is counterbalanced by a concomitant increase of CoQ_{10}, while Vit E levels lie within normal ranges (Table II). Although, according to literature,[1–31,52–61] lymphocytes do not appear to be oxidatively involved in the disease, the observed unbalanced ratio $CoQ_{10}H_2/CoQ_{10}$ may argue for an oxidative damage affecting these cells. In this connection, Yamashita and Yamamoto[47] have recently proposed that the imbalance of the plasma ratio $CoQ_{10}H_2/CoQ_{10}$ can be considered a marker of oxidative stress. Nevertheless, in the group of TLP under study (age 11–22 year), we have observed no increase in morbidity for infections as compared to age matched CTR, though some authors have reported that infections represent the second cause of death, among the younger patients.[26]

With the exception of ascorbate, the hydrophilic antioxidants, i.e., total thiols, urate, albumin and bilirubin are not decreased (Tables I and II) and, indeed, bilirubin, a bile pigment produced by heme catabolism, is significantly increased, likely due to the enhanced erythrocyte hemolysis. Bilirubin may inhibit peroxidation of PUFA bound to albumin, this latter being a *sacrificial antioxidant*, present at high concentrations in plasma (40–60 mg/ml).[48,49] Albumin binds nonceruloplasmin copper tightly and iron weakly, and is able to scavenge any $OH^{\bullet}$ or other reactive species generated on its surface, without relevant damage to the protein due to its rapid turnover.[48,50]

The deficiency of all measured lipophilic antioxidants is associated with an elevated increase of the markers of oxidative injury to PUFA and proteins, i.e., TBA-RM, conjugated dienes, protein carbonyls and urinary AZA (Tables IV and V). Our findings, which are in full accordance with the results of Livrea *et al.*,[6] fit well with the findings of Podda *et al.*[51] They have shown that, after UV irradiation of human skin equivalents, ubiquinol is the most susceptible antioxidant to depletion, followed by ubiquinone and Vit E, while water soluble antioxidants are quite resistant to oxidative damage by UV light.

Since the main role of lipophilic antioxidants is that of inhibiting the initiation step of lipoperoxidation and/or of acting as chain breaking agents during the peroxidative process, their deficiency in TLP plasma may be ascribed to their consumption in countering lipoperoxidation induced mainly by iron overload, which is evidenced by the elevated amounts of ferritin and NTBI (Table I), both able to participate in $OH^{\bullet}$ generation. It has been reported that during iron chelation by subcutaneous infusion of desferrioxamine, NTBI levels decrease nearly to normal values, but become elevated within 2–4 h after discontinuation of the chelator.[52] The use of continuous 24-h chelation is desirable in order to maintain NTBI at low levels. Nevertheless, desferrioxamine becomes toxic when iron levels in the body are low, possibly due to its prooxidant action, exacerbated by the presence of

ascorbate.[53,54] It should therefore be advisable to associate chelation therapy with the administration of lipophilic antioxidants.

However, the observed net drop of lipophilic antioxidants in TLP might partially be due also to lipid malabsorption. In this connection, a relationship has been found in the serum of these patients between low levels of Vit E and reduced concentrations of total lipids,[2] particularly of cholesterol.[55–57] As a matter of fact, we have found that cholesterol deficiency concerns mainly CE, likely due to liver dysfunction, while other lipid fractions such as triglycerides, phospholipids and their PUFA patterns, are in the normal range (Tables I and III).

Surprisingly, the elevated markers of lipoperoxidation are in sharp contrast with the percentages of PUFA, such as C18 : 2*n*-6, C20 : 3*n*-6, C20 : 4*n*-6, and C22 : 6*n*-3, in the plasma phospholipids of the patients, which are not significantly different from those of CTR (Table III). It could be speculated that plasma PL-PUFA protection is afforded by albumin, bilirubin and other hydrophilic antioxidants, and that the remarkable amounts of TBA-RM, conjugated dienes, and AZA may be ascribed to the selective oxidation of erythrocyte PL-PUFA, more unsaturated than plasma ones,[58] released in the plasma following hemolysis. In our opinion, however, also plasma PL-PUFA undergo oxidation, and the apparent stability of their concentration depends on the continuous contribution of PL-PUFA deriving from erythrocyte hemolysis.

The abnormal systemic oxidative stress in thalassemics is consistent with the elevated excretion of urinary catecholamine and serotonin metabolites such as HVA, VMA and HIAA (Table V), deriving respectively from dopamine, epinephrine plus norepinephrine, and serotonin. These urinary acids are clear markers of an active neurological stress, probably due to an abnormal emotional state of the patients, secondary to unsatisfactory clinical and physical conditions. An elevated release of the neurological mediators from pre-synaptic cells into the blood stream is able to induce an increased vasoconstriction and/or vasodilatation in the different body districts.[59,60] This leads to increased ROS production by ischemia-hypoxia-reoxygenation mechanisms, thus contributing to the alteration of the antioxidant pool homeostasis in the patients.

To date, trials have been attempted with the administration of Vit E at high dosage, without clear beneficial effects on RBC survival and transfusional requirements,[27,61–63] which can be summarized quoting Rachmilewitz *et al.*:[27] *"while there seem to be practical and theoretical indications to treat thalassemic patients with vit E, this by itself is incapable of correcting the variety of changes in red blood cell membrane components"*.

However, it must be pointed out that:

(a) Vit E is generally administered as d,l-α-tocopherol acetate, which contains only 9–11% natural α-tocopherol (RRR-α-tocopherol), the remaining aliquot being represented by other seven isomers which are not utilized by the organism and are excreted with the bile;

(b) The chain-breaking antioxidant activity of RRR-α-tocopherol on membranes and plasma lipoproteins is highly potentiated by the synergistic association with suitable amounts of ubiquinol/ubiquinone and other natural lipophilic antioxidants, such as β-carotene, Vit A and lycopene. This antioxidant treatment in the β-thalassemic patients, associated with an appropriate diet, is presently under scrutiny in our Institutes.

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