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# Modulation of apoptosis and improved redox metabolism with the use of a new antioxidant formula

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**Abstract**

Oxidative stress is involved in the pathogenesis of a wide spectrum of diseases, implicating that strategies directed at counterbalancing oxidative processes could have a role in clinical medicine. There is also an evidence that oxidative stress acts as a major determinant of apoptotic cell death. Many studies have reported favourable effects of antioxidant formulas on several parameters of the oxidant–antioxidant balance, but none of them has focused whether antioxidant formulas could modulate apoptosis. We investigated in 20 healthy individuals the effect of supplementation with a formula containing α-tocopherol, α-lipoic acid, coenzyme $Q_{10}$, carnitines, and selenomethionine, on plasma oxidant status and peroxide levels, erythrocyte antioxidant enzymes, lymphocyte apoptosis, and generation of ROS at the mitochondrial level. Control subjects received only carnitines or an incomplete formula with α-tocopherol, α-lipoic acid, coenzyme $Q_{10}$, and selenomethionine. Supplementation with the complete formula resulted in a significant increase in the plasma antioxidant status that was mirrored by a decrease in blood peroxide levels and a reduced generation of ROS at the mitochondrial level. This was associated with a significant decrease in the frequency of peripheral blood lymphocytes, with either CD4 or CD8 phenotype, undergoing apoptosis. Less consistent results were found when either incomplete formula was used. Our study suggests that supplementation with antioxidant formulas can modulate the process of apoptosis under *in vivo* conditions. The clinical potential of this strategy in the treatment of diseases with an elevated commitment to apoptosis should be explored. 

*Keywords:* Antioxidants; Apoptosis; Carnitine; Coenzyme $Q_{10}$; Lymphocytes; Vitamin E

## 1. Introduction

Antioxidants are believed to be important in health maintenance through the modulation of oxidative processes in the body [1]. Oxidative damage with the unregulated production of ROS has been implicated in a growing list of clinical disorders such as atherosclerosis, ischaemia–reperfusion injury, rheumatoid arthritis, cancer, stroke, cataract, Parkinson's disease, and Alzheimer's disease [1,2]. Mechanisms responsible for the ROS-mediated injury to cells and tissues mainly include lipid peroxidation, oxidative DNA damage, and protein oxidation [3], but there is also evidence that ROS can induce the process of

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*Abbreviations:* 7-AAD, 7-amino-actinomycin D; ABAP, 2,2′-azobis(2-amidinopropane); CAT, catalase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DPPH, 1,1-diphenyl-2-picryl-hydrazil; Eth, ethidium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FSC, forward scatter; GSH, glutathione; GSHPX, glutathione peroxidase; HE, hydroethidine; MBB, monobromobimane; *m*ClCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PE, phycoerythrin; PI, propidium iodide; $Q_{10}$, coenzyme $Q_{10}$ oxidized form (ubiquinone); $QH_2$, coenzyme $Q_{10}$ reduced form (ubiquinol); R-PE, R-phycoerythrin; ROS, reactive oxygen species; SOD, superoxide dismutase; SSC, side scatter; $T_0$, base-line; $T_1$, after treatment; TRAP, total radical-trapping antioxidant parameter.

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cell death [4,5]. Indeed, unbalance in the endogenous antioxidant system can modulate cellular proliferation, either in a positive or a negative way, respectively leading to a stimulation in cell proliferation at low levels of peroxides or to apoptotic/necrotic cell death at higher concentrations [6]. Based on this background, it is clear that investigating compounds able to counteract this oxidative damage may have a relevant clinical impact. Balanced human diets contain multiple antioxidants and there is a strong evidence that additive and synergistic interactions intervene among those antioxidant substances [1]. Under a clinical perspective, this suggests that the use of formulas containing multiple substances with antioxidant properties has the potential to provide a significantly better protection against oxidative stress than the use of each single antioxidant alone.

In this study, we investigated in a group of healthy individuals the *in vivo* effects of feeding a formula containing six antioxidant substances on several parameters of oxidative stress, cellular apoptosis, and mitochondrial function. The formula components α-tocopherol, coenzyme $Q_{10}$, carnitines, selenomethionine, and α-lipoic acid were chosen on the basis of their known antioxidant and/or antiapoptotic activity and even on the basis of their synergistic interactions which would ultimately result in the increase of the global antioxidant activity of the formula [7–11]. Even though selenomethionine and carnitines are weak antioxidants *in vitro*, clinical investigations have demonstrated a significant stronger activity *in vivo* [11,12]. Furthermore, carnitines have been recently shown to act as important anti-apoptotic mediators [12,13].

## 2. Materials and methods

### 2.1. Materials

Coenzyme $Q_{10}$ ($Q_{10}$, ubiquinone), L-carnitine, *N*-acetyl-L-carnitine, α-tocopherol, α-lipoic acid and selenomethionine were kindly provided by SigmaTau. Ubiquinol ($QH_2$) was prepared by dithionite reduction of $Q_{10}$ according to Lang *et al.* [14]. R-PE, linoleic acid, cytochrome *c*, trolox, DPPH, SOD from bovine liver, 7-AAD, menadione and *m*ClCCP were from Sigma-Aldrich. ABAP was from Polyscience. MBB, HE and DCFH-DA were from Molecular Probes. RPMI culture medium, FCS, antibiotics and glutamine were from Life Technologies. Anti-hCD4 or -hCD8 antibodies were from Becton Dickinson. Gradient grade HPLC solvents were from Fluka. All other reagents were analytical grade products from Merck.

### 2.2. In vitro assessment of the antioxidant potential of the formula

Prior to *in vivo* experiments, the formula was subjected to an *in vitro* assessment of its antioxidant capacity. The formula was dissolved in ethanol at a final concentration of 10 mg/mL and then the following experiments were performed.

The ability of the formula to scavenge the DPPH free radical was performed as described in [15]. Superoxide anion scavenging activity was measured by the cytochrome *c* reduction method at 550 nm [16]. The ability of the mixture to scavenge hydrophilic peroxyl radicals was evaluated by the method of DeLange and Glazer [17]. Hydroxyl radical scavenging activity was determined as in [18]. The inhibition of lipid peroxidation was measured according to Pryor *et al.* [19].

All the buffers and solutions used in the analyzes were prepared by the use of ultrapure water of the highest electrical resistance (>18.2 M Ω $cm^2$, obtained through a Milli-Q Millipore filter system) to minimise the presence of metals that could catalyze autooxidation of the test compounds. All the *in vitro* experiments were made against suitable blanks with the vehicle alone to evaluate the effect of solvent on the various parameters tested. Solvent interference in the assay, if present, was subtracted from the experimental value of the analyzes. Spectrophotometric determinations were performed with a Kontron spectrophotometer, Model Uvikon 930. Spectrofluoromeric determinations were performed with a Jasco spectrofluorometer, Model FP-770.

### 2.3. In vivo assessment of the antioxidant potential of the formula

#### 2.3.1. Study design

This trial was a 16-week randomized trial. A total of 20 healthy subjects (12 men, 8 women) were included in the study if they fulfilled the following inclusion criteria: non-smokers, not taking vitamin/antioxidant or oestrogen supplements, thyroxin, or lipid-lowering drugs; normal plasma glucose, hepatic and renal function tests; no acute medical conditions at least 3 months prior to entry into the study. Participants were advised not to adopt any particular lifestyle, to adhere to their usual diet and physical activity during the course of the experiments and not to vary their consumption of vitamin-rich foods; none were taking any additional supplements or medications during the study period. Participants gave written informed consent and were requested to report on compliance on a weekly basis.

Participants were assigned to receive antioxidant supplements in the form of one sachet per day over a 3-week period. The formula was taken daily in the morning after meals. The supplements contained the following per sachet: *N*-acetyl-L-carnitine 100 mg, L-carnitine 100 mg, selenomethionine 0.05 mg, α-tocopherol 10 mg, coenzyme $Q_{10}$ 100 mg and α-lipoic acid 100 mg. Control group A (5 subjects) received only *N*-acetyl-L-carnitine and L-carnitine and control group B (6 subjects) was given

only selenomethionine, α-tocopherol, α-lipoic acid and coenzyme $Q_{10}$.

Subjects enrolled in the study group and subjects in both control groups were similar with respect to age (29–40 years), body mass-index, and lipid profile at entry into the study (data not shown). Safety assessments included the evaluation of adverse events and vital signs, hematological tests, biochemical tests, urine analysis, and physical examination. These assessments were done at base-line before treatment was started and, then, at weekly intervals during the treatment period and 2 weeks after completion of the study.

### 2.3.2. Assay of antioxidant enzymes

Enzyme activities were determined in fresh erythrocytes. After centrifugation of 1 mL blood, the erythrocytes were washed three times with isotonic solution and then lysed in bidistilled water (final volume 5 mL). SOD activity was determined according to Flohé and Otting [20], by monitoring the inhibition of cytochrome *c* reduction induced by superoxide anion generated by the xanthine/xanthine oxidase system. A calibration curve for the calculation of enzyme units was obtained by the use of purified SOD from bovine liver. CAT activity was determined according to Pippenger *et al.* [21], by evaluating the rate of the decomposition of $H_2O_2$ at 240 nm. GSHPX activity was determined according to Flohé and Gunzler [22] by monitoring the continuous reduction of oxidized GSH produced by GSHPX in the presence of an excess of glutathione reductase. The concomitant oxidation of NADPH was monitored spectrophotometrically. Results are expressed as enzyme units/mg hemoglobin.

### 2.3.3. Plasma vitamin E, coenzymes $Q_{10}$ and $QH_2$ measurement

Vitamin E and coenzyme $Q_{10}$ determinations were performed by the procedure of Lang *et al.* [14]. Plasma was obtained from freshly drawn heparinized venous blood by brief centrifugation (800 *g* for 5 min at 4°). One milliliter plasma was mixed with 1 mL ethanol containing 0.1 mM butylated hydroxytoluene and extracted with 3 mL hexane. The hexane phase was then evaporated to dryness under nitrogen stream and re-dissolved in ethanol. Four hundred microliters was filtered onto 0.45 μm filters and then an aliquot of 100 μL was analyzed by HPLC. The HPLC system consisted of a Waters apparatus, equipped with two 510 pumps, a Rheodyne injection valve with a 100 μL loop, a Symmetry 300 column (C18 reverse phase, 4.6 cm × 25 cm, 5 μm particle size), thermostated at 27° with a guard column (10 mm) of the same material matrix, a Waters 996 Diode array detector and a Waters 474 spectrofluorometer detector. The two detectors were set up in line, the column effluent first passing through the UV detector. The elution was performed at a flow rate of 1 mL/min with a gradient consisting of a mixture of A (80/20 v/v methanol/$H_2O$) and B (95/5 v/v ethanol/isopropanol). The initial conditions were 39% A and 61% B. After 16 min the mobile phase was changed linearly over 2 min to 100% B; which continued for 10 min, after which the system reversed linearly over 2 min to the initial conditions. Peak identification was performed on the basis of the retention time and of the absorption spectrum for ubiquinol (Rt = 25.1 min; $\lambda_{max}$ = 290 nm) and ubiquinone (Rt = 27.1 min; $\lambda_{max}$ = 275 nm), and retention time, absorption spectrum and fluorescence for Vitamin E (Rt = 18.2 min; $\lambda_{max}$ = 292 nm; $\lambda_{ex}$ = 220 nm, $\lambda_{em}$ = 335 nm). Peak quantitation was performed by automatic peak area integration using a dedicated software (Millennium[32]). Results are expressed as nmoles/mL plasma.

### 2.3.4. TRAP assay

TRAP was measured by a fluorescent method using a L-3 Luminescence Spectrometer from Perkin-Elmer, equipped with a thermostatically controlled cell-holder, according to the method described by Ghiselli *et al.* [23]. The TRAP was evaluated by measuring the total protection time of the plasma as regards to a fluorescent protein, the R-PE, that was subject to constant oxidative stress produced by ABAP, a generator of peroxyl radicals. The decay in the fluorescence was measured at 37° and pH 7.0, after the kinetic reaction. Trolox (a hydrosoluble analog of Vitamin E) was inserted as internal standard after a 30% decay in fluorescence. In this way, after having extrapolated the lag phase (*T*) of the plasma and of the Trolox, the TRAP value was calculated according to the following proportions:

$$\text{Trolox concentration} : T\,\text{Trolox} = \text{TRAP plasma} : T\,\text{plasma}.$$

Lastly, the value was multiplied by the stoichiometric factor of Trolox (2) and by the dilution factor of plasma (50). Results are expressed as trolox eq./L.

### 2.3.5. Plasma hydroperoxides quantitation

The assessment of the oxidising capacity in blood serum was carried out using the D-ROMs kit test produced by DIACRON s.r.l., in accordance with the protocol supplied by the manufacturer. The method is based upon the capacity of transition metals to catalyze the formation of hydroxyl radicals in the presence of hydroperoxides by Fenton-type reactions. The hydroxyl radicals produced, whose quantity is directly proportional to the amount of peroxides present in plasma, were trapped by molecules of *N*,*N*-diethyl-*p*-phenylene diamine, with the formation of a chromogen with a $\lambda_{max}$ at 505 nm.

### 2.3.6. Lymphocyte isolation

PBMCs were separated from heparinized peripheral blood by Lymphoprep gradient centrifugation, washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 IU/mL

penicillin/streptomycin, 10 mM HEPES, and 1 mM L-glutamine. In the apoptosis assay, PBMCs ($5 \times 10^5$/mL) were cultured in complete medium for 12 hr at 37° in a 5% $CO_2$-humidified atmosphere. In addition, for the analysis of mitochondrial functions, aliquots of cells were isolated and maintained in a complete culture medium at 4° until labeling.

*2.3.7. Expression of surface and intracellular antigens*

The absolute counts of cells bearing either the CD4 or the CD8 phenotype were determined by flow cytometry. PBMCs were stained with the following antibodies: PE-labeled anti-hCD4 or anti-hCD8. For staining of surface antigens $5 \times 10^5$ PBMCs were washed in PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS–BSA–$NaN_3$) followed by incubation for 20 min at 4° with the monoclonal antibodies previously described. For determination of background staining, cells were incubated with 20 μL of mouse IgG1-PE. Then, after two washing with PBS–BSA–$NaN_3$ containing 2% FCS, the labeled cells were analyzed by flow cytometry using a FACScan flow cytometer equipped with a 15 mW air-cooled 488 nm argon-ion laser. For each sample 10,000 viable lymphocytes were gated, following size (forward scatter, FSC) and granularity (side scatter, SSC) parameters.

*2.3.8. Staining of apoptotic nuclei with PI*

Lymphocyte apoptosis was quantified as the percentage of cells with hypodiploid DNA using the technique of Nicoletti *et al.* [24]. Briefly, following a short term culture, cell suspensions were centrifuged at 200 *g* for 10 min. For staining of surface antigens, aliquots of $1 \times 10^6$ cells were incubated with FITC-conjugated anti-hCD4 or anti-hCD8 monoclonal antibodies as previously described and, after washing, the pellet was gently re-suspended in 1 mL of hypotonic fluorochrome solution (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100, 0.05 mg/mL RNase A). Cells were kept overnight at 4°, then analyzed in their staining solution on a FACScan flow cytometer. Apoptotic nuclei appeared as a broad hypodiploid DNA peak which was easily discriminable from the narrow peak of nuclei with normal (diploid) DNA content in the red fluorescence channel. Orange PI fluorescence was collected after a 585/42 nm band pass filter and was displayed on a four-decade log scale. Acquisition on the flow cytometer was done in the low sample flow rate setting (12 μL/min) to improve the coefficient of variation on the DNA histograms. Lymphocytes, including live, early and late apoptotic cells, were gated on the basis of their FSC and SSC parameters, and fluorescence data were gated on FSC vs. PI fluorescence dual-parameter contour plots for exclusion of monocytes, debris and clumps. This method of gating allowed ready discrimination of debris (very low FSC and decreased PI fluorescence) from dead cells (low FSC and high PI fluorescence). A minimum of 10,000 events were collected on each sample.

*2.3.9. Phenotypic analysis of apoptotic T cells*

Quantification and phenotypic analysis of apoptotic cells from the short-term cultured lymphocytes was performed by staining apoptotic cells with 7-AAD as reported by Schmid *et al.* [25]. This method was shown to discriminate between early and late apoptotic cells due to their increased membrane permeability. Cultured lymphocytes were first incubated with FITC-conjugated monoclonal antibodies to surface antigens as described above, and washed cells were then incubated with 20 μg/mL of 7-AAD for 20 min at 4° protected from light. Stained cells were further fixed with 1% paraformaldehyde in PBS in the presence of 20 μg/mL of non-fluorescent actinomycin D to block 7-AAD staining within apoptotic cells and avoid non-specific labeling of living cells. Finally, the double-stained cells were incubated overnight at 4° in the dark and were then analyzed in their staining solution by a FACScan flow cytometer. The green fluorescence was collected after a 530/30 nm band pass filter, the red fluorescence from 7-AAD was filtered through a 650 long pass filter. Scattergrams were generated by combining FSC with 7-AAD fluorescence, and regions were drawn around clear-cut populations having negative (live cells), dim (early apoptotic cells), and bright fluorescence (late apoptotic cells). A minimum of 10,000 events were collected on each sample.

*2.3.10. Analysis of mitochondrial functions*

For the simultaneous assessment of surface markers and ROS generation, such as superoxide anion and hydroperoxides, cells were first stained with PE-labeled anti-hCD4 or anti-hCD8 antibodies and then exposed for 15 min at 37° to 2 mmol/L HE and for 1 hr at 37° to 5 mM DCFH-DA, respectively [26]. In control experiments, cells were labeled after pre-incubation with the uncoupling agent *m*ClCCP (50 mmol/L, 37°, 30 min), or the ROS-generating agent menadione (1 mmol/L, 37°, 1 hr). For DCFH-DA, a positive control (cells kept 2 min in 15 mM $H_2O_2$ and washed three times) was inserted. MBB stains GSH. In the presence of glutathione-S-transferase, MBB does not combines enzymatically with GSH at low concentrations, resulting in GSH-specific fluorescence [27]. Briefly, T cells were pelleted and resuspended in 1 mL medium containing 40 μM MBB for 10 min at room temperature in the dark. Cells were placed on ice before analysis performed on a FACScan cytofluorometer. FSC and SSC parameters were gated on the major population of normal-sized lymphoid cells. After suitable compensation, fluorescence was recorded at different wavelengths: FITC, DCFH-DA and MBB at 525 nm, PE at 575 nm and HE at 600 nm.

*2.4. Statistical analysis*

All the results are expressed as means ± SD. Statistical comparison between groups was made using Student's *t*-test. *P*-values $<0.05$ were regarded as significant.

## 3. Results

### 3.1. In vitro antioxidant activity of the formula

Prior to the *in vivo* study, we tested the antioxidant properties of the complete formula under *in vitro* conditions, and it revealed to be an efficient antioxidant preparation. The complete mixture was a good scavenger of the hydroxyl radical, as it inhibited lipid and protein peroxidation and was a good quencher of the DPPH radical, even at low concentrations of the individual compounds. The formula was not a scavenger for the superoxide anion (data not shown).

### 3.2. Plasma antioxidant status and peroxide levels

Supplementation with the complete antioxidant formula for 21 days resulted in a significant increase in the total antioxidant status (Table 1). Supportive evidence for this improved antioxidant status was obtained by measurement of TRAP values ($1631 \pm 306$ at the end of the treatment vs. $1510 \pm 270$ at base-line; $P < 0.05$) and lipid peroxidation products ($272 \pm 51$ vs. $301 \pm 73$; $P < 0.05$) in blood plasma. We observed a slight increase in TRAP values also in control group A subjects who were given only carnitines and a negligible trend towards reduction in control group B subjects who were given only selenomethionine, α-tocopherol, α-lipoic acid and coenzyme $Q_{10}$. However, differences between TRAP values measured at the end of the study period and those measured at base-line did not reach the statistical significance for either control group. Likewise, we found a trend toward a decrease in plasma peroxide levels also in control groups A and B but the difference between post- and pre-treatment did not reach a statistical significance.

Base-line ($T_0$) plasma levels for Vitamin E, total coenzymes $Q_{10}$ and $QH_2$ were $25.6 \pm 5.38$, $0.97 \pm 0.27$, and $0.46 \pm 0.12$ nmol/mL, respectively, which are comparable to data reported in the literature [14,28,29]. Supplementation with the complete antioxidant formula for 3 weeks resulted into an increase in mean plasma levels of α-tocopherol and total coenzyme $Q_{10}$ ($28.8 \pm 6.9$ and $1.4 \pm 0.59$, respectively; $P < 0.05$ for both parameters) (Table 1). It is of interest that complete formula supplementation resulted in a 1.5-fold increment of the plasma coenzyme concentration, mainly in its reduced form. Actually, a modification of these parameters was also observed in both control groups, but the results were less striking and again the differences observed did not reach the statistical significance, except for total $Q_{10}$ in control B.

### 3.3. Activity of antioxidant enzymes in red blood cells

In the treatment group, we observed that feeding the complete formula resulted in a significant increase in the specific activity of the antioxidant enzyme GSHPX at the end of the treatment period ($T_1$) compared with $T_0$ values ($0.036 \pm 0.011$ at base-line, $0.046 \pm 0.007$ after treatment; $P < 0.01$) (Table 2). We found a comparable increase in GSHPX activity also in control group B ($0.032 \pm 0.018$ at

Table 1
Plasma antioxidant status

| | Complete formula[a] | | Control A[b] | | Control B[c] | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_1$ | $T_0$ | $T_1$ | $T_0$ | $T_1$ |
| Vitamin E (nmol/mL) | 25.61 ± 5.38 | 28.81 ± 6.89* | 25.82 ± 6.25 | 26.78 ± 6.86 | 22.56 ± 3.71 | 23.37 ± 3.37 |
| $QH_2$ (nmol/mL) | 0.462 ± 0.117 | 0.784 ± 0.259 | 0.338 ± 0.116 | 0.229 ± 0.148 | 0.428 ± 0.058 | 0.441 ± 0.124 |
| $Q_{10}$ tot (nmol/mL) | 0.969 ± 0.266 | 1.414 ± 0.591* | 1.017 ± 0.220 | 1.353 ± 0.492 | 0.910 ± 0.160 | 1.042 ± 0.335* |
| TRAP (trolox eq./L) | 1510 ± 270 | 1631 ± 306* | 1602 ± 200 | 1654 ± 145 | 1582 ± 141 | 1536 ± 272 |
| LOOH (U. Carr.) | 301 ± 73 | 272 ± 51* | 327 ± 38 | 292 ± 51 | 263 ± 41 | 259 ± 55 |

The values shown are means ± SD. *$P < 0.05$.
[a] $N = 9$.
[b] $N = 5$.
[c] $N = 6$.

Table 2
Antioxidant enzymes activity

| | Complete formula[a] | | Control A[b] | | Control B[c] | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_1$ | $T_0$ | $T_1$ | $T_0$ | $T_1$ |
| CAT (U/mg Hb) | 22.54 ± 3.19 | 15.60 ± 1.63** | 25.34 ± 1.91 | 24.53 ± 3.04 | 22.50 ± 3.87 | 20.87 ± 1.80 |
| SOD (U/mg Hb) | 5.54 ± 1.36 | 7.63 ± 3.10 | 5.78 ± 2.61 | 6.43 ± 2.11 | 5.75 ± 1.89 | 7.37 ± 1.55 |
| GSHPX (U/mg Hb) | 0.036 ± 0.011 | 0.046 ± 0.007** | 0.033 ± 0.02 | 0.037 ± 0.014 | 0.032 ± 0.018 | 0.038 ± 0.017** |

The values shown are means ± SD. **$P < 0.01$.
[a] $N = 9$.
[b] $N = 5$.
[c] $N = 6$.

base-line, $0.038 \pm 0.017$ after treatment, $P < 0.01$). No significant change in SOD activity was found after supplementation either with the complete antioxidant formula or the incomplete formulas. CAT activity was significantly decreased at the end of the study period in the group given the complete formula as compared to base-line ($22.54 \pm 3.19$ at base-line, $15.60 \pm 1.63$ after treatment; $P < 0.05$); a trend towards a reduction in CAT activity was also observed in both control groups but the difference with values measured before feeding either the incomplete formula did not reach the statistical significance.

### 3.4. Lymphocyte apoptosis

Supplementation with the antioxidant formula was associated with a reduced susceptibility of lymphocytes to apoptosis. In fact, a lower number of lymphocytes were undergoing apoptosis in treated patients after 3 weeks of supplementation as compared to base-line. This was established by staining apoptotic nuclei with PI [24], which detects late events of apoptosis such as chromatin condensation and DNA fragmentation. Following 12 hr of incubation in complete medium, the rate of spontaneous apoptosis was significantly decreased in CD4 and CD8 lymphocytes taken after 3 weeks of treatment as compared with pre-treatment levels ($6.1 \pm 2.59$ and $6.8 \pm 3.03$ at base-line, $2.8 \pm 1.52$ and $3.9 \pm 2.09$ at the end of the treatment for CD4 and CD8 cells, respectively; $P < 0.001$ for CD4 and $P < 0.01$ for CD8) (Table 3). Supplementation with carnitines only had a comparable, and even greater, impact on the frequency of CD4 and CD8 lymphocytes undergoing apoptosis ($7.2 \pm 1.19$ and $12.2 \pm 3.24$ at $T_0$, $3.9 \pm 1.19$ and $5.4 \pm 1.94$ at $T_1$, respectively for CD4 and CD8 lymphocytes; $P < 0.01$ for both CD4 and CD8 cells). Even subjects who were given selenomethionine, α-tocopherol, α-lipoic acid, and coenzyme $Q_{10}$ had a decrease in the frequency of apoptotic lymphocytes compared to pre-treatment levels ($6.7 \pm 1.58$ and $8.6 \pm 1.91$ at base-line, $3.7 \pm 1.59$ and $5.9 \pm 1.12$ at the end of the treatment for CD4 and CD8 cells, respectively, $P < 0.05$ for both parameters).

We confirmed these results measuring apoptosis also with 7-AAD, a fluorescent DNA-intercalating agent which only penetrates the membrane of cells undergoing apoptosis and thus exhibit a shrunken phenotype (reduced FSC) (Table 3). This staining discriminates between early and late apoptotic cells [25], and the extent of lymphocyte apoptosis using both methods was closely comparable in samples taken from both the treatment and control groups (data not shown). The FSC and SSC characteristics of cells stained with PI and 7-AAD confirmed that those cells were indeed undergoing apoptosis (data not shown).

### 3.5. Generation of ROS

As shown in Table 4, circulating lymphocytes from the healthy volunteers enrolled in this study contained a fraction of cells which were able to oxidize the nonfluorescent lipophilic (i.e. membrane-permeable) dye HE into the hydrophilic fluorescent product Ethidium (Eth). Since HE is particularly sensitive to superoxide anion, this change is thought to reflect the generation of superoxide anion [26]. Moreover, lymphocytes were labeled using DCFH-DA, a fluorochrome that detects hydroperoxide generation [26]. We found that supplementation with the complete antioxidant formula was associated with a strong decrease in the percentage of such cells, which bear an $Eth^{high}$ and DCFH-DA-positive phenotype, as compared to pre-treatment levels (Table 4). Statistical analysis revealed a highly significant difference between pre- and post-treatment levels with respect to CD4 and CD8 cells stained with either HE or DCFH-DA ($P < 0.001$ for both parameters). A significant reduction in the frequency of $Eth^{high}$ and DCFH-DA-positive CD4 and CD8 subset was also found in the control groups A and B even though the impact of supplementation on those parameters, although statistically significant, was less striking as compared to the treatment with the complete formula (Table 4).

Table 3
Staining for apoptotic lymphocytes

| | Complete formula[a] | | Control A[b] | | Control B[c] | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_1$ | $T_0$ | $T_1$ | $T_0$ | $T_1$ |
| PI staining | | | | | | |
| CD4 | $6.11 \pm 2.59$ | $2.81 \pm 1.52^{***}$ | $7.23 \pm 1.19$ | $3.89 \pm 1.19^{**}$ | $6.69 \pm 1.58$ | $3.73 \pm 1.59^{*}$ |
| CD8 | $6.81 \pm 3.03$ | $3.94 \pm 2.09^{**}$ | $12.19 \pm 3.24$ | $5.35 \pm 1.94^{**}$ | $8.60 \pm 1.91$ | $5.86 \pm 1.12^{*}$ |
| 7-AAD staining | | | | | | |
| CD4 | $6.14 \pm 2.60$ | $2.78 \pm 1.51^{***}$ | $7.23 \pm 1.13$ | $3.89 \pm 1.14^{**}$ | $6.73 \pm 1.65$ | $3.75 \pm 1.55^{*}$ |
| CD8 | $6.81 \pm 3.05$ | $3.92 \pm 2.06^{**}$ | $12.18 \pm 3.23$ | $5.40 \pm 1.88^{**}$ | $8.61 \pm 1.97$ | $5.87 \pm 0.98^{*}$ |

The values shown are means ± SD. Numbers refer to the percentage of apoptotic cells among the CD4 and CD8 lymphocytes, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

[a] $N = 9$.

[b] $N = 5$.

[c] $N = 6$.

Table 4
Staining for ROS and GSH at the mitochondrial level

| | Complete formula[a] | | Control A[b] | | Control B[c] | |
|---|---|---|---|---|---|---|
| | $T_0$ | $T_1$ | $T_0$ | $T_1$ | $T_0$ | $T_1$ |
| HE staining | | | | | | |
| CD4 | 9.98 ± 3.22 | 3.06 ± 0.95*** | 14.19 ± 5.35 | 5.06 ± 1.21** | 10.36 ± 4.80 | 5.14 ± 1.43 |
| CD8 | 17.20 ± 4.82 | 4.48 ± 1.72*** | 19.88 ± 6.11 | 9.76 ± 4.88* | 13.37 ± 3.88 | 6.43 ± 1.07** |
| DCFH-DA staining | | | | | | |
| CD4 | 13.42 ± 3.02 | 8.34 ± 2.28*** | 13.22 ± 2.34 | 9.89 ± 2.30 | 14.19 ± 2.85 | 9.20 ± 1.37** |
| CD8 | 14.59 ± 6.57 | 8.78 ± 3.63** | 19.22 ± 5.81 | 13.18 ± 4.34 | 21.15 ± 6.09 | 14.23 ± 3.56 |
| MBB staining | | | | | | |
| CD4 | 55.13 ± 7.71 | 59.18 ± 4.58* | 56.92 ± 4.58 | 61.07 ± 3.49 | 51.61 ± 3.72 | 60.28 ± 1.91* |
| CD8 | 69.37 ± 4.98 | 74.91 ± 6.29** | 64.87 ± 7.22 | 76.30 ± 6.77* | 63.70 ± 5.87 | 72.51 ± 4.30* |

The values shown are means ± SD. Numbers refer to the percentage of cells which bear an $Eth^{high}$ or DCFH-DA positive phenotype, or GSH positive cells among the CD4 and CD8 lymphocytes, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

[a] $N = 9$.

[b] $N = 5$.

[c] $N = 6$.

The treatment with the complete formula was also associated with an increased frequency of circulating lymphocytes with either CD4 or CD8 surface phenotype that stained positive for glutathione (CD4: 55.1 ± 7.71 and 59.2 ± 4.58; CD8: 69.4 ± 4.98 and 74.9 ± 6.29, at $T_0$ and $T_1$, respectively, $P < 0.05$ and <0.01, respectively). We observed a similar trend even in subjects given only acetyl-L-carnitine and L-carnitine (CD4: 56.9 ± 4.58 and 61.1 ± 3.49; CD8: 64.9 ± 7.22 and 76.3 ± 6.77, at $T_0$ and $T_1$, respectively, $P < 0.18$ and <0.05, respectively), or the incomplete formula containing selenomethionine, α-lipoic acid, α-tocopherol, and coenzyme $Q_{10}$ (CD4: 51.6 ± 3.72 and 60.3 ± 1.91; CD8: 63.7 ± 5.87 and 72.5 ± 4.30, at $T_0$ and $T_1$, respectively, $P < 0.05$ for both parameters).

### 3.6. Safety profile

Treatment with the antioxidant formulas was well tolerated and none of the subjects enrolled experienced adverse effects. No abnormality was disclosed with hematological and biochemical tests (data not shown).

## 4. Discussion

The role of free radical oxidative damage in the pathophysiology of human diseases is currently a topic of considerable interest as free radical activity has been implicated in a wide spectrum of clinical conditions, ranging from cancer to atherosclerosis, stroke and neurodegenerative diseases [1–3]. The putative role of free radicals has been based mainly on the detection of products of oxidative damage to biologic molecules including proteins, lipids, and DNA [1–3]. Natural antioxidant mechanisms exist to prevent or delay oxidation but they have been found to be defective in many of those diseases [1–3]. Furthermore, protective effects of a wide spectrum of putative antioxidant compounds, including Vitamin E, selenium, α-lipoic acid, coenzymes $Q_{10}$, and carnitines, have been shown to counteract oxidative damage in numerous studies [1,7–11]. This background has stimulated interest in the possibility of supplementing antioxidant formulas as a tool to prevent or slow the progression of such diseases even under *in vivo* conditions, and several investigations have indeed confirmed the potential benefit of this strategy [1,7,8]. However, there is some evidence that low doses of antioxidants (similar to those available from dietary sources) may be beneficial while high doses may paradoxically act as pro-oxidants [30,31]. This has raised concern about the optimal composition and dosage of antioxidant formulas to be used *in vivo* for attaining the maximum benefit with no risk of accelerating the disease process and a minimum risk of adverse effect.

In this study, we investigated the impact of supplementing a complete antioxidant formula containing selenomethionine, α-lipoic acid, coenzyme $Q_{10}$, α-tocopherol, and carnitines on several measures of oxidant activity. The significance of our investigation is 3-fold. First, protection afforded by each individual antioxidant compound present in the formula has previously been demonstrated either *in vitro* or by clinical studies [7–11]. Second, we compared the efficacy of the complete formula with two incomplete formulas containing, only carnitines (group A) or only selenomethionine, α-tocopherol, α-lipoic acid and coenzyme $Q_{10}$ (group B), respectively. Third, we investigated the impact of these antioxidant formulas on both cell apoptosis and the generation of ROS at the mitochondrial level.

We found that supplementation with the complete antioxidant formula led to a significant increase in the levels of TRAP activity in the peripheral blood, whereas supplementation with the incomplete formulas had a strikingly lesser impact on this parameter. These results are further

strengthened by measurement of plasma peroxide levels as only subjects given the complete formula had a statistically significant reduction in LOOH levels. Supplementation with either incomplete formula, in contrast, had a marginal effect on peroxide levels and differences between pre- and post-treatment values did not reach the statistical significance. It is important to note that the effect of treatment on functional parameters, such as TRAP, and plasma peroxide levels was not associated with clear-cut and consistent variations in plasma concentrations of antioxidant compounds, such as Vitamin E and coenzymes $Q_{10}$ and $QH_2$. This discrepancy is difficult to be explained. It is possible that plasma concentrations of Vitamin E or $Q_{10}$ and $QH_2$ are less reliable indicators than TRAP values or peroxide levels of oxidant–antioxidant balance and redox metabolism. However, further studies are required to better understand this issue. Several previous studies lend support to the usefulness of TRAP in evaluating the antioxidant capability of blood and other extracellular fluids [32]. There is evidence in favor of the view that the overall antioxidant status, as measured by TRAP values, reflects the efficacy of antioxidant therapy more efficiently than the measurement of any single antioxidant in the peripheral blood or in any other biological fluid. TRAP mainly measures the total peroxyl radical-trapping capacity, and practically all chain-breaking antioxidants are included in the measurement, but excludes other kinds of antioxidants, such as transition metal binding proteins and antioxidant enzymes [23,32]. However, when we looked specifically at this latter parameter we found that GSHPX activity was significantly increased in the group given a three-week supplementation with the complete antioxidant formula. We found a comparable increase in GSHPX activity also in subjects who were given the incomplete formula containing selenomethionine, α-tocopherol, α-lipoic acid and coenzyme $Q_{10}$ (control group B), whereas no change was detected in the group given only carnitines (control group A). In contrast, we detected a significant reduction in CAT activity at the end of the treatment period in the group given the complete formula and a similar trend was also observed in subjects supplemented with either control formula. In the latter, however, the difference compared to base-line did not reach the cut-off level for statistical significance. These findings are somewhat unclear, but a likely explanation is that the decrease in CAT activity during supplementation with antioxidant formulas is an indirect measurement of the switch in overall redox balance towards a more antioxidant cellular milieu. Taking all data together, our results are in agreement with the view that the complete antioxidant formula provides a greater antioxidant effect compared to the two incomplete formulas.

Under a clinical perspective, it appears reasonable to conceive that combining several different antioxidant compounds in the composition of formulas would offer a greater benefit compared to that provided by the use of each single compound or even formulas containing a limited spectrum of antioxidant compounds. In recent years, many studies of antioxidant therapy have been performed focusing on a single compound, both *in vitro* and *in vivo* [1–3]. However, this approach may not accurately reflect the interactions intervening between and among the various antioxidants as they occur in the intact cell, especially when using formulas containing compounds with different pharmacokinetics, mechanisms of action, and molecular targets. Furthermore, the cellular and biochemical pathways ultimately responsible for the antioxidant activity of most of those compounds are still poorly understood. Even though experimental evidence for an *in vivo* interaction is still inconclusive, several studies have proposed that interactions and synergism may occur among putative antioxidant compounds and this could significantly enhance the efficacy of treatment [1–3]. This suggests that, at least in terms of biological and antioxidant activity, the impact on the oxidant–antioxidant balance should be expected to be greater if using formulas containing a wide spectrum of antioxidant compounds compared with the use of formulas containing a limited number of antioxidant compounds. In other words, the protection provided by antioxidant therapy is likely to be substantially dependent on the number and diversity of antioxidants in the formulas rather than on the antioxidant efficacy of each single compound. The results we provide in this study support this hypothesis. A potential drawback of this strategy is that the complex mixture of antioxidant compounds does not allow to precisely identify which constituent(s) were responsible for the observed effect. However, this should not be regarded as a true limitation. In a clinical setting, indeed, two major issues should be targeted when testing the efficacy of a new strategy of antioxidant therapy, i.e. the ultimate impact on the antioxidant activity at the level of cellular microenvironment and the safety of treatment. Both these parameters have been fulfilled by supplementation with the experimental complete formula we have assayed.

An innovative and important matter we have examined, that has not been previously investigated in similar studies, is the effect of antioxidant treatment on the process of apoptosis. No report to the best of our knowledge has so far studied the energy metabolism and oxidant stress at the mitochondrial level in lymphocytes taken from patients undergoing supplementation with antioxidant formulas. Oxidative stress and ROS are involved in the process of apoptosis, in particular through mediating the expression and/or activity of pro-apoptotic endogenous mediators, including Fas-FasL, tumor necrosis factor, caspases, and ceramide [33–36]. ROS are indeed generated in response to a wide spectrum of apoptotic stimuli and can in turn induce apoptosis in a variety of cells [4,5]. Furthermore, the generation of these metabolites with the accompanying intracellular depletion of antioxidants, in particular GSH, invariably precedes and is associated with apoptosis [4,5].

The finding that apoptosis can be blocked by inhibiting or neutralizing ROS further confirms their pro-apoptotic role [4,5]. Among the intracellular sources of ROS, mitochondria are the most important and recent evidence has emerged that these organelles also participate in the decision of cells to undergo apoptosis and in the executioner phase of the process [37]. Interestingly, the change in mitochondrial permeability transition that precedes and triggers nuclear apoptosis results in production of ROS [37], and also the redox state in mitochondria is a determinant of apoptosis and may regulate it [38].

In conclusion, we have found a strict association between the effect of antioxidant treatment on the pro-oxidant intracellular milieu, as characterized by mitochondrial generation of ROS, and the commitment to apoptosis [25–27]. Indeed, the supplementation with the complete antioxidant formula resulted into a significant decrease in the frequency of apoptotic CD4 and CD8 lymphocytes and this effect was closely paralleled by the reduction in the frequency of lymphocytes stained positive for the HE and DCFH-DA phenotype. In turn, this was associated with a significant increase in the frequency of lymphocytes that stained positive for GSH. Our data support the view that oxidant stress and the generation of ROS could be an important target for antiapoptotic therapies. This would be of relevance in clinical disorders associated with a high rate of apoptosis. For example, the progression of HIV infection towards more advanced stages of immune deficiency appears to be paralleled by an enhanced rate of CD4 cell loss through an apoptotic mechanism [39]. Furthermore, it should also be considered that antioxidant compounds, such as carnitines that are included in the antioxidant formula we tested in this study, have been recently shown to down-modulate apoptosis in pilot clinical trials in HIV-infected subjects [12,13]. The potential of this antioxidant formula as an antiapoptotic tool should be formally investigated in these clinical settings.

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