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Original article

Effects of 1-month R-α-lipoic acid supplementation on humans oxidative status: a pilot study

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Summary. Alpha-lipoic acid (α-LA), an endogenous antioxidant co-factor of important enzymatic complexes, is rapidly catabolised in dihydrolipoic acid becoming an even stronger antioxidant for strengthening the activity of endogenous antioxidants (vitamin C, E, glutathione). *AIM.* To evaluate the effects of an oral use liquid formulation containing the natural and more active α-lipoic acid (R-αLA) enantiomer on 20 subjects oxidative status: 10 apparently healthy subjects and 10 affected by risk factors (such as excess weight, hypertension) and/or diseases (such as diabetes, polyneuropathy). The subjects' oxidative status was assessed by measuring blood concentrations of: reactive oxygen species (ROS), total antioxidant capacity (TAC), glutathione, Vitamin C and Vitamin E at baseline (T0) and after 1-month' supplementation (T1) with R-αLA. Moreover, cysteine and homocysteine levels, well-known cardiovascular risk factors, were measured. *Results.* Interesting findings on the cellular antioxidant regeneration cycle: a significant decrease in reduced glutathione (GSH) levels and GSH/GSSG ratio, a slight downward trend in oxidised glutathione (GSSG) and vitamin C and E concentrations. Due to the brief treatment period, the study did not however show any significant differences in the oxidative "status" balancing parameters (ROS and TAC) and on homocysteine and cysteine levels. Remarkable were the findings regarding patients who reported a lessening, or even the disappearance, of pain symptoms. *Comment.* After 1-month' treatment, the R-αLA oral use liquid formulation seemed to reinforce endogenous antioxidant activity and, notably, helped mitigate the pain symptoms typical of several stress oxidative-dependent diseases. This effect could probably depend on greater bioavailability of the liquid formulation.

Key words: α-lipoic acid, oxidative status, glutathione, inflammatory diseases

Introduction

All human cells are continuously exposed to multiple oxidising and reducing factors, both as a result of natural physiological processes (i.e. mitochondrial respiration and other metabolic pathways), and pathophysiological conditions (i.e. inflammation, hypoxic, ischemic or reperfusion injuries, radiation and metabolism of exogenous substances) (1). This exposure leads to the formation of "reactive species" including both free radicals (highly reactive molecules with an unpaired electron, which, reacting with other molecules, tend to complete their orbital octet) and non-radical species (e.g. hydrogen peroxide) (1).

Approx. 90% of Reactive Oxygen Species (ROS) are produced at a mitochondrial level. In physiological conditions, the body defends itself from these substances by an extended system of antioxidant enzymes

and scavenger molecules, preventing and stopping the chain propagation of these radical reactions (2). In healthy conditions, there is a balance between endogenous and exogenous ROS and their neutralisation by endogenous and exogenous antioxidant defence mechanisms (total antioxidant capacity, TAC) giving rise to a physiological oxidative *status*. The right balance between oxidants and antioxidants is essential for all the physiological functions, as numerous proteins involved in the intracellular signal transduction pathways (receptors, kinase and phosphatase, transcription factors) are sensitive to even slight alterations of this balance (1, 3).

Table 1 summarises the functions of the antioxidants, which, even if in low concentrations, inhibit or significantly reduce oxidation of their specific substrate (4, 5).

In Table 2, the antioxidants are classified as Primary and Secondary according to their mechanism of action (6).

When ROS concentration is higher than physiological, and TAC is insufficient to neutralise ROS, a pathological condition (known as "oxidative stress") is produced giving rise to alterations (both functional and structural of the cells and tissues) potentially responsible for, or promoting the onset and/or permanence of various chronic diseases (7,8).

Table 1. Functions of antioxidants

• to prevent the formation of new free radicals;
• to react with the reactive species before they can attack important native biomolecules;
• to interrupt the radical propagation chain;
• divert the radical species

α-lipoic acid

α-lipoic acid, or α-thioctic acid (1, 2-dithiolane-3-pentanoic acid) (αLA) is an amphiphilic molecule of small dimensions, with high permeability through the plasma membranes, and high versatility, since it has the capacity to exert its numerous functions in both lipophilic and hydrophilic environments (9,10). Ubiquitous molecule, with extensive tissue diffusion, it has been identified for the first time as a co-factor of enzymatic complexes involved in the oxidative decarboxylation reactions of α-ketoacids; thus it takes part in Krebs cycle contributing to the cellular energy requirement (11). Numerous *in vitro* studies on animal models and subsequent clinical studies on humans allowed to attribute many and different functions to αLA. It has been reported that αLA affects the glucose and lipid metabolism (12, 13), regulates the appetite (14), combats complications of diabetic origin (15) and myocardial and cerebral reperfusion injuries (16), helps the treatment of neurodegenerative diseases (17), and activates apoptosis in tumour cell lines (18). Therefore, rather than defining a single functional property, it would be more correct to state that αLA triggers pleiotropic effects due to its different cellular mechanisms of action.

Indeed, thanks to an amidic bond between its carboxylic group and the lysine residues of various proteins, αLA is sequestered by various protein complexes (e.g. pyruvate dehydrogenase); on the one hand, this limits cell availability of αLA, while on the other it brings about targeted compartmentalisation.

Another important αLA functional group is the dithiolane ring which is subjected to redox reactions by the cellular redox systems (NAD^+/NADH; $NADP^+$/NADPH) allowing the α-lipoic acid to assume an oxidised form (αLA) or a reduced form (DHLA) (Fig.1A)

Table 2. Classification of antioxidants according to their mechanism of action

Primary *"Preventive antioxidants"*	**Secondary** *"Chain-breaking antioxidants"*
Main action They tend to remove newly-formed free radicals or to prevent their formation	**Main action** They sequester or neutralise free radicals, preventing them from creating damage and from spreading
Enzymes and/or Molecules Superoxide dismutase, glutathione peroxidase, catalase, ferritin, transferrin, albumin	**Enzymes and/or Molecules** Thiols, uric acid, vitamin C, E, B9, β-carotene, bilirubin

(9, 10). The αLA/DHLA constitutes a powerful endogenous antioxidant system, acting at various levels in order to contrast ROS, protecting the cell from oxidative damage. Additionally, the single forms demonstrate scavenger properties towards different radical species, so that the αLA/DHLA system amplifies its chemical actions through the transfer of electrons (9) both regenerating some endogenous antioxidant systems (glutathione, vitamin C, vitamin E) (19) and chelating heavy metals (zinc, copper, lead, arsenic), so as to limit their toxicity and facilitate their elimination (20). Due to all these potentialities, the use of αLA was suggested as supplementation in clinical settings (i.e aging or oxidative stress-dependent diseases, characterised by a drastic decrease in endogenous defences, including the *de novo* synthesis of αLA itself).

Moreover, beneficial effects have been reported, but only after αLA intravenous administration, in the treatment of neuropathic pain associated with diabetic neuropathy (21). In the case of formulations for oral use, the daily αLA dose must be increased leading to the risk of undesirable effects and/or prolonging the treatment period.

The main problems of αLA oral treatment are the reduction of inter-individual variability due to gastrointestinal absorption, the increase in plasma and tissue bioavailability and half-life time (22); all these characteristics limit αLA use in therapeutic protocols. Although this antioxidant is used as a drug in some countries, it is normally registered as a dietary supplement thus overcoming the intrinsic limitations due to its pharmacokinetic properties (22, 23).

For some years, the Italian market has been selling a liquid formulation for oral use containing only the natural and biologically more active form of αLA, i.e. the enantiomer R-αLA (Fig. 1B), which improves the αLA solubility and preserves its stability in the gastric environment (24). The increased R-αLA bioavailability and its prolonged presence in plasma compared to the analogous solid formulation correlated with an increase in biological efficacy, as shown in the experimental model of Type 2 diabetes (24). Moreover, in recent preliminary clinical studies, this formulation proved effective in increasing the quality of life in patients with peripheral neuropathy by reducing their pain symptoms (25) and nocturnal cramps (26).

Glutathione

Glutathione (GSH), one of the most powerful endogenous antioxidants present in all tissues, is synthesised inside the cell by three amino acids: cysteine and glycine which are linked together by a normal peptide bond, and glutamate, bound to cysteine by an atypical peptide bond (gamma linkage) formed between the glutamate side chain carboxylic group and the amine group of cysteine. By the gamma linkage, glutathione resists the degradations of intracellular peptidase and

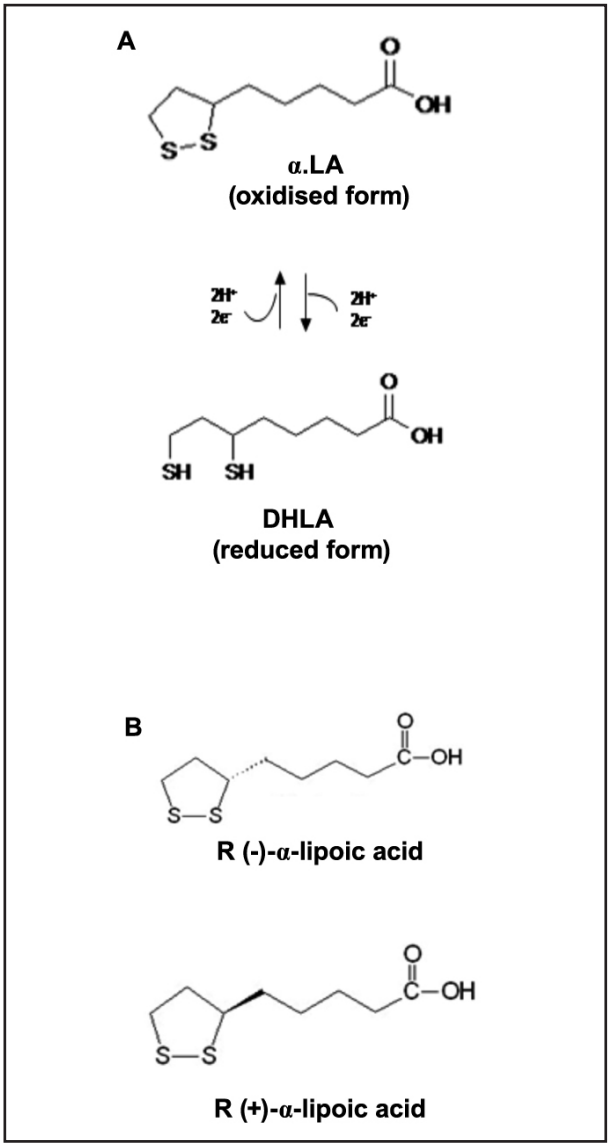

Figure 1. A) Oxydised form (αLA) and reduced form (αDHLA) of α-lipoic acid; B) Enantiomers (R) and (S) of α-lipoic acid

acts in enzymatic and non-enzymatic reactions alike. The metabolised GSH is *de novo* regenerated from the three amino acids.

Glutathione, together with selenium, forms the glutathione peroxidase enzyme (GPx), a powerful intracellular antioxidant that reduces hydrogen peroxide (H_2O_2) to two molecules of water, using as substrate the reduced GSH form that, bound to another GSH, forms the oxidised glutathione (GSSG). The oxidised glutathione can be retransformed into two molecules of GSH by GSSG reductase (GSr) through the NADH or NADPH as the redox co-factor of GSr.

Glutathione exerts its fundamental antioxidant action in the red blood cell, where it is present both in reduced and oxidised form, at a concentration ranging from 1-10 mM, preventing haemolysis. The GSH action in the mitochondria is very important to contrast pathophysiological conditions generating oxidative stress (27); moreover, GSH has a wider action compared to catalase reducing hydrogen peroxide levels but only in peroxisomes.

The liver is the organ most heavily involved in the GSH metabolism which, synthesised, is transported both into the intracellular compartments and into the extracellular spaces (bile and plasma) (27-29). Table 3 shows the multiple functions performed by GSH in the various cellular processes.

Since GSH is involved in many cellular processes, the alteration of its homeostasis is involved in the etiology and progression of different pathological conditions including neurodegeneratives (Alzheimer, Parkinson, Multiple Sclerosis), age-related diseases (cataract, glaucoma), osteoporosis, cancer, cardiovascular and lung diseases, diabetes mellitus, inflammatory and diseases of the immune system, viral and microbial infections (28-30).

Glutathione acts through two different mechanisms: either reacting with free radicals (e.g. superoxide or hydroxyl radicals) in non-enzymatic reactions or as electrons donor for the peroxides reduction catalysed by glutathione peroxidase (27).

The GSH/GSSG ratio is the intracellular redox potential useful to prevent the shift in balance when the oxidative stress exceeds cellular ability to transform GSSG into GSH. In quiescent cells, GSH amounts are 100 times more than its oxidised form GSSG.

During oxidative stress, the transient alteration of the GSH/GSSG ratio changes from 100 to 10 or even to 1. A shift of this balance towards GSSG indicates a greater formation of free radicals causing an increase in oxidative stress if short of antioxidant defences. The GSSG can be transported actively out of the cells or react with proteins having a sulfhydryl group, leading to the formation of a mixed disulphide. Severe oxidative stress is, therefore, also accompanied by an intracellular GSH depletion (27).

All other amino acid residues are subject to post-transductional modifications such as oxidation or formation of disulphides. Cysteine, an amino thiol present in many human proteins, is the most reactive nucleophilic residue. Oxidative/nitrosative damage can alter the reduced state of the cell by reacting with thiol residue of proteins sensitive to the reduction. In any case, GSH is able to maintain the redox homeostasis at an intracellular level. In a healthy body, more than 90% of blood glutathione is in its free form. Thus, the GSH/GSSG ratio is normally greater than 10/1 (27, 28).

Table 3. Functions of glutathione

• Defence against free radicals
• Maintenance and regulation of the reduced state of thiols in the cells where it exerts a crucial role in cell differentiation, proliferation and apoptosis
• Detoxification from xenobiotics and their metabolites
• Cysteine reserve
• Modulation of the immune functions
• Protection against infections caused by exogenous microbial organisms
• Chelation of toxic metals, contributing to their elimination from the body

Vitamin C

Vitamin C (L-ascorbic acid), belonging to the group of water-soluble vitamins, acts as an antioxidant against free radicals and ROS, preventing lipid peroxidation. Moreover, it almost eliminates hydrosoluble radicals and acts in synergy with vitamin E, which is fat-soluble. In fact, vitamin E radical, formed as intermediate product after the ROS attack, regenerates immediately by vitamin C, because of more Vitamin C than Vitamin E present in the body.

Vitamin E

The term Vitamin E covers a group of chemical compounds with antioxidant properties. The most important compound for humans is α-Tocopherol, which protects from the oxidation the polyunsaturated fats, some vitamins, hormones and enzymes. It acts as a scavenger of free radicals, preventing the radical propagation reactions leading to ROS generation.

The aim of this study was to evaluate in volunteers the possible effects, on the oxidative and/or inflammatory state, of a R-α-lipoic acid dietary supplement (commercially available in a liquid formulation for oral use). The study particularly sets out to verify whether the greater bioavailability of this R-αLA formulation could affect some parameters of the oxidative status in humans.

Materials and methods

Study population and methods

Twenty outpatients (9M/11F, aged 55.6±14) were recruited from Obesity and Work Center "L. Devoto" of Fondazione Ca' Granda Ospedale Maggiore, Policlinico of Milan, Italy.

Upon entering the study, each participant signed an informed consent form and gave detailed information about his or her general health, usual dietary intake, life-style. Pregnancy or lactation, oral contraceptives, gastrointestinal disorders, unstable psychiatric function, alcohol abuse, currently smoking or use of dietary supplements were considered exclusion criteria.

Ten apparently healthy participants (5 F) were chosen as controls (CTRL) and 10 (6 F), classified as Patients, were affected by risk factors of oxidative stress, such as overweight/obesity (3 patients), arterial hypertension (1 patient) and/or by diseases, such as multiple sclerosis (1 patient), diabetic polyneuropathy (2 patients), fibromyalgia (2 patients), carpal tunnel syndrome (1 patient).

Moreover, routine blood count examination confirmed his/her suitability for enrolment in the study (data not shown).

Each participant in order to evaluate baseline nutritional *status* had to fill in the Questionario Osservatorio Grana Padano (OGP) (31); his/her information was then analysed by the OGP software according to the consumption of micro and macronutrients recommended by LARN (National Recommended Energy and Nutrient Intake Levels) (32). At the same time, blood nutritional state (vitamin B12, vitamin E, vitamin C, folates and zinc levels), oxidative status (ROS, TAC, glutathione, vitamin C and vitamin E levels) and cardiovascular risk factors (cysteine and homocysteine levels) of each subject were measured. Finally, the treatment started with R-α-lipoic acid dietary supplement, in liquid form for oral use (once/day for one month, taken on empty stomach, before breakfast).

After 1-month's supplementation (T1), only oxidative status parameters were re-tested as the treatment period was too short to modify the subjects' nutritional conditions.

Most participants reported good compliance (85%).

The research was carried out in accordance with the principles stated in the Declaration of Helsinki for Research on Human Subjects and was approved by the local Ethics Committees (Study registration number: 1370).

Product description

Liponax (I.B.N. Savio srl, Italy) was the name of the nutraceutic product used in the study: a liquid formulation for oral use containing 300 mg of R–αLA, the natural and biologically more active enantiomer of α-lipoic acid (24).

Biochemical parameters

Peripheral blood samples were drawn after an over-night fast. Blood specimens from each subject were collected in tubes, either (2 tubes) without additive to assess ROS, TAC, vitamin C and E levels or (3 tubes) containing ethylenediaminetetraacetic acid (EDTA), to prevent coagulation, for complete blood count (CBC), GSH, cysteine and Hcy concentration assays. The two tubes for serum assays, left for 30 minutes at room temperature to facilitate the spontaneous coagulation, were divided into aliquots for ROS, TAC, vitamin C and E assays after centrifugation.

Two EDTA samples were immediately placed on ice: one for GSH measurement was immediately put on ice and, within 10 minutes, put at -80°C until analysed; the other for plasma cysteine and Hcy concentrations assay was immediately put on ice and centrifuged within 30 minutes at 2500 g, 4°C for 10 min, frozen and stored at -80°C. In the third EDTA sample, CBC was assessed by routine laboratory analyser SysmexXT (Dasit, Cornaredo, Milan, Italy).

ROS and TAC concentrations were measured using commercial kits (dROMs test and OXY-Adsorbent test, respectively, Diacron International, Grosseto, Italy) on a dedicated spectrophotometer, Diacron F.R.E.E. CARPE DIEM, as previously reported (33). Serum vitamin C and E and whole blood GSH were assessed by kits (Chromsystems Instruments and Chemicals GmbH-Grafelfing, Germany). Homocysteine and cysteine were measured by an HPLC system in isocratic conditions, equipped with an automatic refrigerated injection system (Varian, Palo Alto, CA, USA) and with a fluorometer (Jasco Europe, Cremella, LC, Italy) and a UV/VIS detector (Shimadzu Corporation, Kyoto, Japan) (34).

Cysteine and homocysteine levels were assessed by enzymatic routine methods on Modular P automated analyser (Hitachi-Roche, Basel, Switzerland). All serum and plasma parameters were evaluated according to the relevant reference intervals or cut off currently used in our routine laboratory.

Statistical Analysis

As the distribution of the data analysed with the Kolmogorov-Smirnov test did not present a normal distribution, Mann Whitney's non-parametric statistical analysis and Wilcoxon's test for paired data were applied (software Graph Pad Prism v6.02), making it possible to compare the levels of analytes/metabolites in the groups of patients. The cut-off value for statistical significance was set at $p<0.05$.

Results

Baseline nutritional parameters.

Based on the OGP questionnaire (31), Patients showed a lower B12, folates and vitamin E intake than the healthy volunteers (CTRL), even if not significantly so (Figure 2). On the other hand, Patients showed a significantly lower vitamin C ($p=0.05$) and zinc ($p <0.05$) intake than Controls (Figure 3).

Oxidative status and cardiovascular risk parameters evaluated at T0 and T1

Table 4 shows the data regarding oxidative status and cardiovascular risk parameters of the 20 volunteers, evaluated all together due to their small number.

The present pilot study showed interesting findings on the cellular antioxidant regeneration cycle after only 1-month supplementation (Figure 4). In fact, the participants had a significant decrease in reduced glutathione (GSH) levels ($p<0.001$) and in GSH/GSSG ratio ($p<0.001$); a downward trend, even if slight, was observed in oxidised glutathione (GSSG) and in vitamin C and E concentrations, more evident for vitamin C than for vitamin E. On the contrary, due to the brief treatment period, the study did not show any significant differences in the balancing parameters of the oxidative *status* (ROS and TAC) and homocysteine and cysteine levels.

At the end of one month's treatment, the patients reported a decrease in, or even the disappearance of their pain symptoms, which was definitively remarkable.

Discussion

The interesting results of the present pilot study have shown that 1-month's supplementation with R-α-lipoic acid, well known for its antioxidant properties, had a beneficial effect on the general health of the compliant participants.

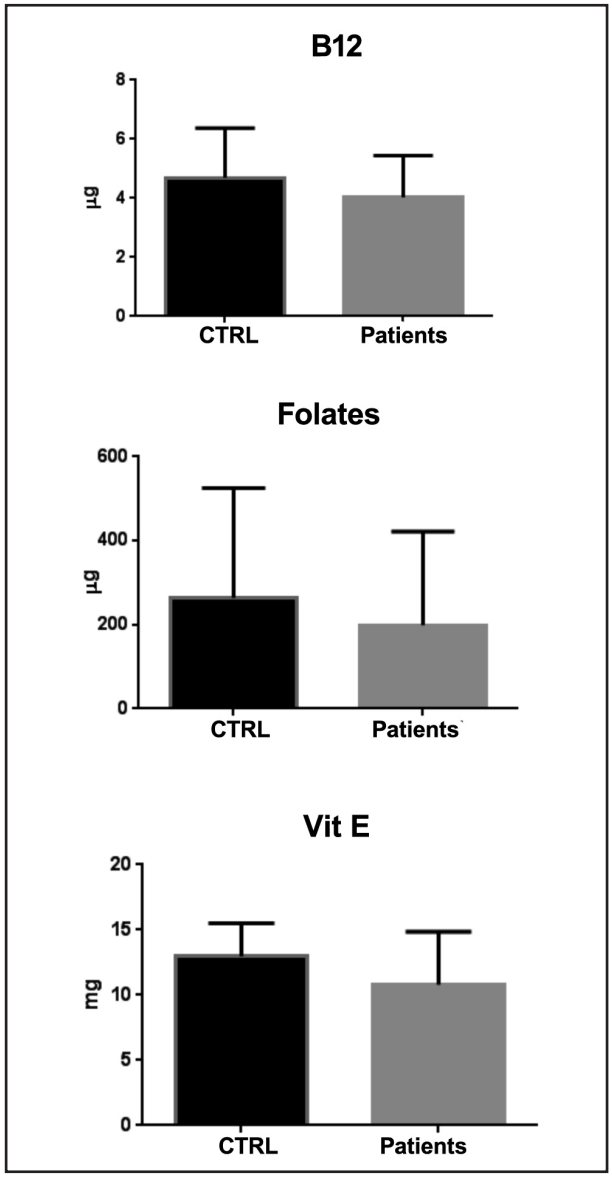

Figure 2. Estimate of the intake of vitamin B12, folates and vitamin E in healthy volunteers (CTRL) and Patients

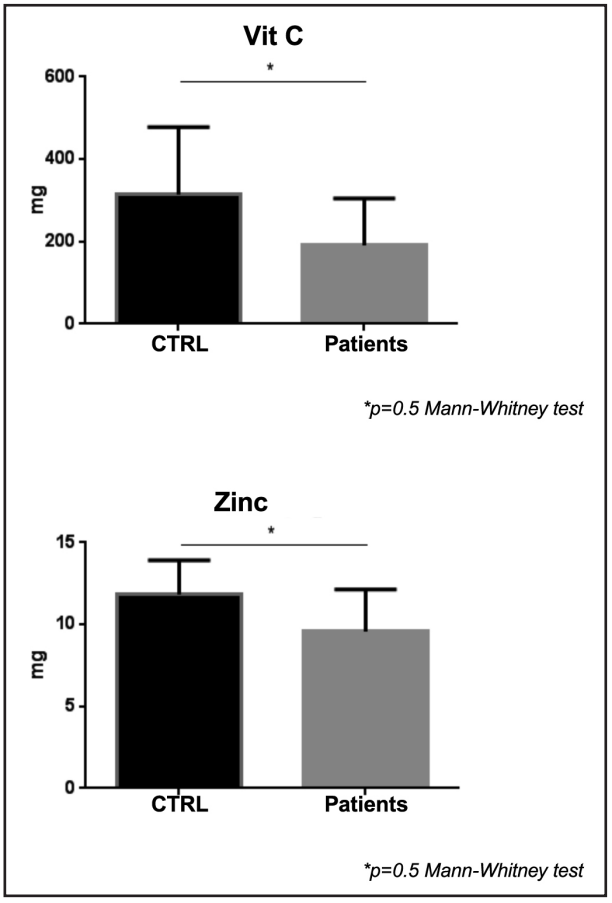

Figure 3. Estimate of the intake of vitamin C and zinc in healthy volunteers (CTRL) and Patients

α-Lipoic acid is present in nature in two enantiomeric forms, R-αLA being the more biologically active. αLA acts as an endogenous co-factor of enzymatic complexes important for the production of energy. The small molecular structure and its solubility allow αLA to spread to all districts of the body. Moreover, its amphiphilic nature makes it more effective than other antioxidants such as vitamin C (water-soluble) and vitamin E (lipid-soluble).

Inside the cells, α-lipoic acid is rapidly catabolised into α-dyhydrolipoic acid, with an even stronger antioxidant effect, since it has both the power to inactivate free radicals and reactive oxygen species, and strengthen the activity of endogenous antioxidants (vitamin C, E, GSH), regenerating them and/or increasing their intracellular quantity (9, 10, 35-37).

In the present study, thanks to OGP questionnaire (31), self-compiled by the study participants at T0, it was possible to distinguish between subjects with general clinical conditions more compromised (presence of major diseases) from those "apparently" healthy or with a slightly altered baseline condition (CTRL). Then, any baseline differences in individual nutritional status and antioxidant activity were compared.

Interestingly (Figg. 2, 3), lack of vitamin and low zinc levels were observed only in the subjects with dis-

Table 4. Concentrations of all the analytes determined in all of the volunteers at the two time intervals T_0 and T_1

Analyte (reference interval or cut-off)	T_0	T_1
ROS (< 300 U CARR)	438,6 ± 82.5	480.2 ± 92.8
TAC (> 350 mmol HCL/mL)	774.9 ± 197.5	793.7 ± 204.4
GSH (500-1500 μmol/L)	820.9 ± 169.9	450.3 ± 205.4*
GSSG (25-150 μmol/L)	172.4 ± 44	155.7 ± 45.4
GSH/GSSG (10-15)	5.1 ± 2.1	2.9 ± 0.8*
Vitamin C (460-1490 μg/dL)	2414.5 ± 1124	2146.5 ± 681
Vitamin E (500-2000 μg/dL)	1633.8 ± 570.3	1537.4 ± 576.6
CYS P TOT (172-296 μmol/L)	184.1 ± 19	186.1 ± 21.1
HCY P TOT (3-20 μmol/L)	12.6 ± 4.3	13 ± 3.8

**p<0.0001*

eases, according to *in vitro* studies on the protective functions of zinc (38). In addition, it is important to point out that most Patients' diseases, in general, are also treated with drugs, and, hypothetically, the alteration of their oxidative status could be due both to the disease and to the interfering effect of drugs.

As previously reported, when evaluating oxidative status at T0 and T1, for a correct statistical analysis of the various biochemical parameters, Patients and control group were considered all together. Also "apparently" healthy volunteers could have greater or lesser alterations in the oxidation/antioxidation system.

The present pilot study, although carried out on a small number of volunteers and without comparing it to a placebo group, showed some interesting beneficial effects of the R-αLA innovative liquid formulation for oral use, which could be useful in a therapeutic protocol for diseases. Even though the trial period was short, the subjects reported that the supplementation had had anti-inflammatory analgesic (at a physical level) and antioxidant (at a biochemical level) effects. Interestingly, 14 subjects reported an improvement in their wellbeing and physical performance after one month of the R-αLA oral treatment. Only one volunteer reported an undesired effect (the onset of slight tachycardia during supplementation) but nonetheless continued the treatment. Moreover, 5 patients, who had complained of pain symptoms at the time of enrolment (one affected by carpal tunnel, two with diabetic polyneuropathy, two with fibromyalgia), observed a marked improvement in their chronic symptoms and were able to reduce or stop taking analgesics (NSAIDs) during treatment.

According to previous studies on animals (10, 39, 40), these results show the importance of using α-lipoic acid as a dietary supplement in a clinical context; moreover, the oral formulation seems to increase the bioavailability of this antioxidant.

As shown in Table 4, other analytes assessed in this study (i.e. ROS and TAC and the well known cardiovascular risk factors, homocysteine and cysteine) did not produce any significant difference after one month's nutraceutical treatment.

This could depend on a number of the inter-individual factors (age, gender, dietary habits, lifestyle, diseases, drugs, smoking habits), conditioning the epigenetics and, consequently, the *status* of each subject. All these parameters remained more or less unvaried since they generally need a longer treatment (at least three months, i.e. the mean life of the red blood cell) in order to change significantly (7, 8, 41). Moreover, as recently reported (42), baseline ROS levels – and particularly TAC – only change in the presence of a marked increase in oxidative stress while, in the present study, participants showed a condition of oxidative balance rather than stress.

The actual body's oxidative status is determined in a precise and accurate manner by measuring both forms of GSH and, even more important, its GSH/GSSG ratio (27, 43). Notably, in the present study, by

evaluating "as a whole" the regeneration cycle of cellular antioxidants (GSH, Vitamin C and E), we observed a significant drop in both GSH levels and in the GSH/GSSG ratio and a slight downward trend in both GSSG and vitamin C at T1 (Table 4 and Fig 4). This could imply the antioxidant action of R-αLA oral supplementation.

GSH plays an important role in cellular resistance against oxidative damage, by supplying enzymes involved in the ROS metabolism, eliminating the potentially toxic oxidation products and reducing thiols groups of oxidised proteins. The GSH capacity, "upregulated" in the presence of oxidative stress, was measured by the biosynthetic and recycling pathways of GSH. The GSH/GSSG ratio determines the "intracellular redox potential" preventing the shift in balance when the oxidative stress exceeds cellular ability to transform GSSG into GSH. In humans, blood assessment of GSH and GSSG and their ratio was considered an index of the entire body's oxidative status, a useful indicator of the risk of diseases.

Figure 5 summarises the cycle and clarifies the most important results of the present pilot study.

Vitamin E, transformed into its radical after ROS neutralisation, was regenerated by vitamin C, which is oxidised and, in turn, regenerated by enzymatic reactions by GSH (43-46).

This could partially explain the reduction in GSH and the decrease in vitamin C required to restore vitamin E, but it does not seem to consider the vitamin C concentration normally much greater than vitamin E concentration. A hypothetical explanation might be that water-soluble vitamin C is actively carried by specific transporters (e.g. Na^+- and GSH-dependent) necessary both for maintaining reserves and for recycling the powerful antioxidant vitamin E (localised in areas rich in lipids, such as cellular membranes, adipose tissue, liver). Therefore, large quantities of vitamin C promote the important functions of vitamin E, protecting it from over-rapid oxidation (44-46).

The results of this study agree with the results of other authors' studies *in vitro* and *in vivo* in which

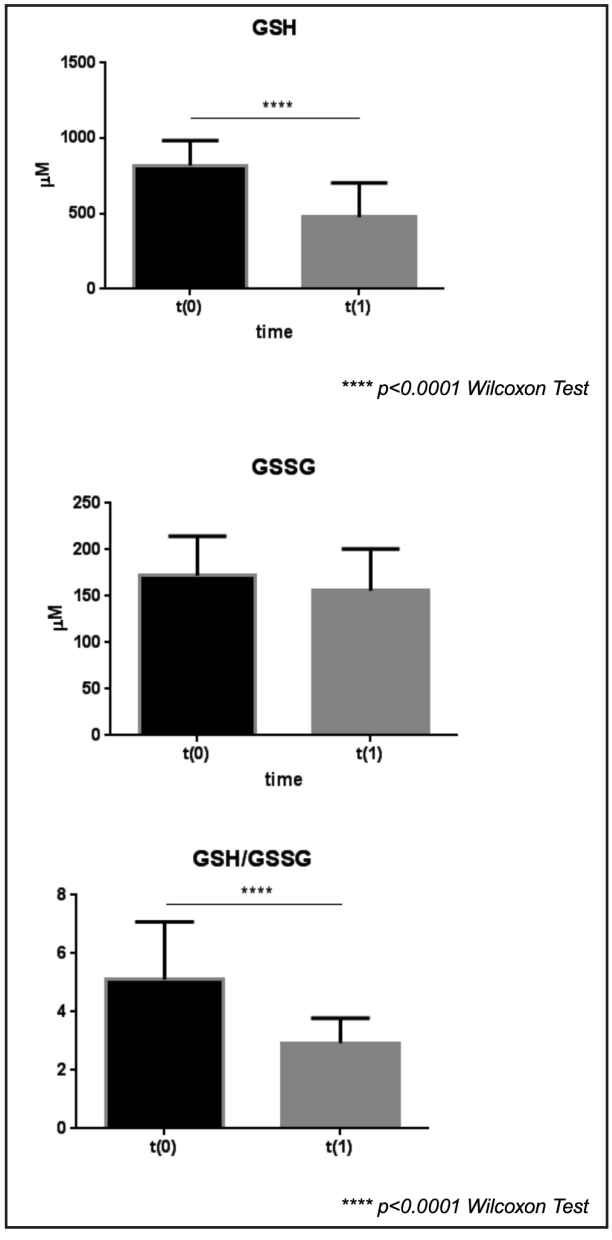

Figure 4. Concentrations of GSH, GSSG and GSH/GSSG at T_0 and T_1

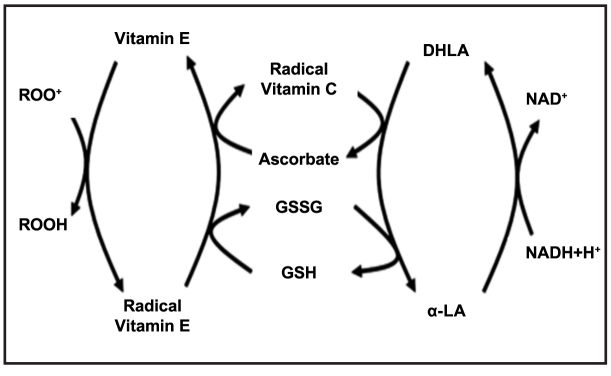

Figure 5. The role of α-lipoic acid, in both forms, oxidised and reduced, in the regeneration cycle of cellular antioxidants

α-lipoic acid promoted an increase in intracellular glutathione, inducing its absorption by the plasma (44-46), while the decrease in the GSH/GSSG ratio could indicate an antioxidant response following supplementation. Additionally, these results agree with studies conducted on other dietary supplements (41) measuring the same antioxidant panel.

Moreover, the findings of the present study suggest that supplementation with R-αLA can promote the absorption of vitamin C and also partly of vitamin E from plasma, to the benefit of their intracellular increase. Both vitamins could be used in that dense network of antioxidants to which glutathione belongs, according to other authors (44-46).

The significant decrease in blood GSH does not produce an increase in GSSG, and therefore might not correlate to its oxidation. Moreover, after R-αLA supplementation, the GSH total amounts dropped suggesting an increase in its metabolism and/or its cellular internalisation. This hypothesis will have to be confirmed by more in-depth studies even if R-αLA seems to promote an "internalisation" of circulating glutathione in cells, which need it for greater antioxidant activity requirements.

The present pilot study is perfectly in line with the activity of the "antioxidant regeneration cycle", which, after the intake of R-α lipoic acid, showed a correct sequence of the various substrates involved.

Lastly, speculative hypotheses as regards α-LA could be increased by bioavailability of the active R-α lipoic acid liquid formulation. In fact, the pharmacokinetic parameters (Cmax, AUC, Tmax) of αLA are better than those achieved with an analogous solid formulation (e.g. tablets) (22).

Last but not least, absorption of vitamins varies on the basis of subjective factors connected with physical conditions, lifestyle and drug intake, if any.

In conclusion, the present pilot study, the first carried out on humans, evaluated the possible changes in oxidative status after only 1-month supplementation with highly bioavailable R-α lipoic acid, and, in agreement with previous studies, interestingly showed subject's metabolic modifications related to oxidative status.

Although many aspects of the mechanism of action *in vivo* are still to be clarified, it is clear that dietary supplements of R-αLA (even for oral use) can increase plasma bioavailability and improve disorders connected with oxidative stress. Consequently, this pilot study also lays the foundations for future clinical studies with more in-depth investigations on the close relationship between the exogenous supplementation of a powerful antioxidant, such as R-αLA, and the complex network of endogenous substances designated protecting the cells.

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All authors read and approved the final manuscript.

Conflict of interest

The authors confirm that there are no conflicts of interest. FR contributed to the description of the Liponax sol used in this study and kindly provided by I.B.N. Savio srl - Pomezia (RM).

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