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The Impact of Different Antioxidant Agents alone or in Combination on Reactive Oxygen Species, Antioxidant Enzymes and Cytokines in a Series of Advanced Cancer Patients at Different Sites: Correlation with Disease Progression

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In the present study we tested the ability of different antioxidant agents, used alone or in combination, to reduce the reactive oxygen species (ROS) levels and to increase the glutathione peroxidase (GPx) activity. Moreover, we tested the ability of such antioxidant agents to reduce the serum levels of proinflammatory cytokines IL-6 and TNFα. Fifty-six advanced stage cancer patients with tumors at different sites were included in the study: they were mainly stage III (12.5%) and stage IV (82.1%). The study was divided into two phases. In the 1st phase 28 patients were divided into five groups and a single different antioxidant agent was administered to each group. The selected antioxidant agents were: alpha lipoic acid or carboxycysteine-lysine salt, amifostine, reduced glutathione, vitamin A plus vitamin E plus Vitamin C. In the 2nd phase of the study 28 patients were divided into five groups and a combination of two different antioxidant agents was administered to each group. The antioxidant treatment was administered for 10 consecutive days. The patients were studied at baseline and after antioxidant treatment. Our results show that all single antioxidants tested were effective in reducing the ROS levels and three of them in increasing GPx activity, too. Among the combinations of antioxidant agents, three were effective in reducing ROS, while three were effective in increasing GPx activity (arm 4 was effective in both instances). Comprehensively, the "antioxidant treatment" was found to be effective both on ROS levels and GPx activity. Moreover, the antioxidant treatment was able to reduce serum levels of IL-6 and TNFα. Furthermore, a correlation was shown between the Eastern Cooperative Oncology Group Performance Status of patients and blood levels of ROS, GPx activity, serum levels of proinflammatory cytokines.

Keywords: Antioxidant agents; Reactive oxygen species; Glutathione peroxidase; Cytokines; Disease progression; Cancer patients

Abbreviations: ROS, reactive oxygen species; OS, oxidative stress; GPx, glutathione peroxidase; IL, interleukin; TNF, tumor necrosis factor; ALA, alpha lipoic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; ECOG PS, eastern cooperative oncology group performance status; CARR U, carratelli units; GR, glutathione reductase; SD, standard deviation

INTRODUCTION

Oxidation is the transfer of electrons from one atom to another and represents an essential part of aerobic life and normal metabolism, since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP.[1] However, problems may arise when the electron flow becomes uncoupled (transfer of unpaired single electrons), generating free radicals: the oxygen-centered free radicals are known as ROS. In addition to the ROS

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radicals, in living organisms, there are also other ROS non-radicals. It is accepted that ROS play different roles *in vivo*. Some are positive and are related to their involvement in energy production, phagocytosis, regulation of cell growth and intercellular signalling and synthesis of biologically important compounds.[2] However, ROS may be very damaging, since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, to induce oxidations, which cause membrane damage, protein modification including enzymes and DNA damage. This oxidative damage is considered to play a causative role in aging and several degenerative diseases, such as heart diseases, cataracts, cognitive dysfunction and cancer.[3] Humans have evolved with antioxidant systems to protect against free radicals. These systems include some antioxidants produced in the body, namely endogenous, and others supplied from the diet, namely exogenous. Endogenous antioxidants include enzymatic defences, such as Se-glutathione peroxidase (GPx), catalase and superoxide dismutase, which metabolize superoxide, hydrogen peroxide and lipid peroxides, hence preventing the formation of the toxic $OH^{\bullet}$, as well as non-enzymatic defences, such as glutathione, histidine-peptides, the iron-binding proteins transferrin and ferritin, lipoic acid, reduced CoQ_{10}, melatonin, urate and plasma protein thiols, with the last two accounting for the major contribution to the radical-trapping capacity of plasma.

Several mechanisms may lead to OS in cancer patients. The first one is the altered energy metabolism which may be attributable to symptoms such as anorexia/cachexia, nausea and vomiting which prevent a normal nutrition and thereby a normal supply of nutrients such as glucose, proteins and vitamins, leading eventually to accumulation of ROS such as hydroxyl radicals, superoxide radicals and others. The second mechanism is a non-specific chronic activation of the immune system with an excessive production of proinflammatory cytokines, which in turn may increase the ROS production.[4] A third mechanism may be the result of the use of antineoplastic drugs: many of them, particularly alkylating agents and cisplatin, are able to produce an excess of ROS and therefore lead to OS.[5] Thus, we hypothesize that the body redox systems which include antioxidant enzymes and low molecular weight antioxidants may be dysregulated in cancer patients and that this disorder is potentiated as a function of disease progression.

In our previous preliminary study we have demonstrated that the blood levels of ROS of advanced stage cancer patients were significantly higher, while the GPx activity was significantly lower than controls. Moreover, the values of proinflammatory cytokines IL-6 and TNFα were significantly higher in cancer patients than controls.

Those results warranted to carry out an active therapeutic intervention such as the administration of antioxidant agents aimed at preventing and/or correcting OS in cancer patients.

To counteract ROS and OS several approaches have been tried both in experimental systems and in humans. Among the most used antioxidant agents there are alpha lipoic acid (ALA), cysteine-containing compounds, amifostine, GSH and vitamins. ALA is present in human cells in a bound lipoillysine form, in mitochondrial proteins that play a central role in oxidative metabolism: it has recently gained considerable attention as an antioxidant.[6] It has been reported to have beneficial effects in disorders associated with OS, inducing a substantial increase in cellular reduced glutathione and restoring severely glutathione deficient cells.[7] Within drug-related antioxidant pharmacology ALA is a model compound that enhances understanding of the mode of action of antioxidants in drug therapy.

Among the cysteine-containing compounds, the carboxycysteine-lysine salt appears to be one of the most interesting: the cysteine is a known precursor for glutathione synthesis that has been shown to act on redox balance and to be capable of significantly improving the antioxidant potential by elevating reduced glutathione levels.[8] Carboxycysteine-lysine salt protects alpha 1 antitripsin from inactivation by hypochlorous acid: in fact, having a chemical structure similar to methionine, it competes with the latter against the oxidative activity of ROS. The carboxycysteine-lysine salt is able to protect DNA from the ROS activity by concentrations of 2.5 mM.

Amifostine, an analogue of cysteamine, is a phosphorilated aminothiol prodrug that is dephosphorilated at the tissue site by membrane-bound alkaline phosphatase to its active metabolite, the free thiol, WR-1065. WR-1065 is the form of the drug that is rapidly taken up into cells and it is the major cytoprotective metabolite. Oxidation of WR-1065 forms the symmetrical disulfide, WR-33278, which is structurally similar to the naturally occurring polyamine, spermine, and indeed it shares certain biochemical properties with the polyamines that may contribute to some of the pharmacologic and clinical properties associated with amifostine.

GSH is a key molecule in the redox body homeostasis. OS induces the transformation of GSH into oxidized glutathione (GSSG) by the action of GPx: GSSG may in turn be transformed into glutathione protein mixed disulfide or reduced back to GSH by glutathione reductase (GR). During cancer growth, the glutathione redox status (GSH/GSSG) decreases in blood of tumor-bearing animals and humans, too. This effect is mainly due to

an increase in GSSG levels. Two reasons may explain this increase: (1) the increase in peroxide production by the tumor that, in addiction to changes affecting the glutathione-related and the antioxidant enzyme activities, can lead to GSH oxidation within the red blood cells and (2) an increase of GSSG release from different tissues into the blood. The GSH/GSSG ratio in blood also decreases in patients bearing breast or colon cancers and this change associates with higher GSSG levels, especially in advanced stage of cancer progression.[9]

Antioxidant vitamins, which include vitamin A, C and E, are hypothesized to decrease cancer risk and prevent tissue damage by trapping organic free radicals and/or deactivating reactive oxygen molecules.[10] Many studies have been carried out attempting at demonstrating a preventive role for vitamins as antioxidant agents against cancer and other diseases. The discrepancies between the results of these studies may be explained by the type of population studied (general or high risk subjects), the different doses of supplementation (nutritional levels or higher), the number of antioxidant tested (one, two or more) and the type of administration (alone or in balanced association). So, it appears that their preventive effect may be related to multiple nutrients consumed at nutritional doses and in combination, and optimal effect may be expected with a combination of nutrients at levels similar to those found in a healthy diet.[11]

Aim of the Study

The aims of the present study were to demonstrate that the blood levels of ROS of advanced stage cancer patients were pathologically high and the antioxidant enzyme GPx was low. We tested the ability of different antioxidant agents, used alone or in combination, to reduce the ROS levels and to increase the antioxidant enzyme activity. Moreover, we tested the ability of such antioxidant agents to reduce the serum levels of proinflammatory cytokines IL-6 and TNFα. The results were correlated with the relevant clinical indices of patients, the most important of which is the performance status, with the aim of finding their predictive, i.e. prognostic, role for disease outcome.

PATIENTS AND METHODS

Patients

The protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects: last amendment adopted by the 52nd WMA General Assembly, Edinburgh, October 2000). The study was approved by the Ethical Committee of the University of Cagliari Medical School. Written informed consent was obtained from all patients. Fifty-six advanced stage cancer patients with tumors at different (10) sites were included in the study (mean age 59.5 years, range 34–75; M/F ratio: 29/27; mainly stage III, 12.5% and stage IV, 82.1%). The majority of patients had head and neck cancer: they were habitual smokers and often heavy alcohol drinkers and for these reasons they exhibit high levels of ROS. However, the most frequent tumors such as lung, breast and colon cancer are adequately represented. We have selected this wide range of tumor sites to getting information on as much as possible number of tumors in a relatively small sample of patients. All patients were referred to Medical Oncology Department, Policlinico Universitario, University of Cagliari Medical School, Cagliari, Italy. Their clinical characteristics are reported in Table I. Performance status was quantified using the WHO-approved ECOG PS scale.[12] The great majority of patients included in the study was chemotherapy-naive and only five patients were studied during a chemotherapy regimen, but no patient was treated with antioxidant agents contemporaneously to chemotherapy.

Twenty age–sex-matched healthy individuals were studied as controls.

TABLE I Characteristics of all patients studied

	Number of patients	(%)
Patients	56	
Age (years)		
Mean	59.5	
Range	34–75	
Sex		
Male	29	51.8
Female	27	48.2
Performance status (ECOG)		
0	17	30.4
1	22	39.2
2	14	25.0
3	3	5.4
Stage		
I	2	3.6
II	1	1.8
III	7	12.5
IV	46	82.1
Cancer site		
Head and neck	26	46.3
Lung	6	10.6
Breast	7	12.5
Pancreas	2	3.6
Colorectal	3	5.4
Ovary	3	5.4
Endometrium	3	5.4
Melanoma	2	3.6
Kidney	2	3.6
Myeloma	2	3.6
Weight: kg-mean (range)	63.4 (40–96)	
Height: m-mean (range)	1.61 (1.49–1.75)	

TABLE II Characteristics of patients included in the first phase of the study

	Number of patients				
	Arm 1	Arm 2	Arm 3	Arm 4	Arm 5
Patients (28)	6	5	7	6	4
Sex					
Male	3	2	3	2	
Female	3	3	4	4	4
Performance status (ECOG)					
0	1	2	1		
1	3	3	2	3	4
2	2		4	2	
3				1	
Stage					
II					
III		1		1	1
IV	6	4	7	5	3
Cancer site					
Head and neck	3	3	4	1	
Lung				2	1
Breast	2	1		1	1
Colorectal	1	1			
Ovary			2		
Endometrium			1	1	
Melanoma				1	1
Myeloma					1

Arm 1: Alpha lipoic acid 200 mg/day orally. Arm 2: *N*-acetylcysteine 1800 mg/day i.v or carboxycysteine-lysine salt sachets 2.7 g/day orally. Arm 3: Amifostine 375 mg/day i.v. Arm 4: Reduced glutathione 600 mg/day i.v. Arm 5: Vitamin A 30,000 IU + Vitamin E 70 mg + Vitamin C 500 mg/day orally. All treatments were administered during 10 days continuously.

TABLE III Characteristics of patients included in the second phase of the study

	Number of patients				
	Arm 1	Arm 2	Arm 3	Arm 4	Arm 5
Patients (28)	12	5	4	3	4
Sex					
Male	9	4	3	2	1
Female	3	1	1	1	3
Performance status (ECOG)					
0	10			1	2
1	2	2	1		2
2		3	2	1	
3			1	1	
Stage					
I	2				
II	1				
III	2	1			1
IV	7	4	4	3	3
Cancer site					
Head and neck	8	1	3	2	1
Lung		3			
Breast	1				1
Pancreas				1	1
Colorectal		1			
Ovary	1				
Endometrium			1		
Kidney	2				
Myeloma					1

Arm 1: Alpha lipoic acid 200 mg/day + carboxycysteine lysine-salt sachets 2.7 g/day. Arm 2: Alpha lipoic acid 200 mg/day + Amifostine 375 mg/day i.v. Arm 3: Carboxycysteine lysine salt sachets 2.7 g/day orally + Amifostine 375 mg/day i.v. Arm 4: Reduced glutathione 600 mg/day i.v + Amifostine 375 mg/day i.v. Arm 5: Alpha lipoic acid 200 mg/day + reduced glutathione 600 mg/day i.v. All treatment were administered during 10 days continuously.

The study was divided into two phases: in the first phase 28 patients were divided into five groups and a single different antioxidant agent was administered to each group (Table II). The selected antioxidant agents were: ALA capsules (Tiobec, Laborest, Nerviano, Milan, Italy) 200 mg/day orally (arm 1), *N*-acetylcysteine vials 1800 mg/day i.v. or carboxycysteine-lysine salt sachets (Fluifort, Dompè, Milan, Italy) 2.7 g/day orally (arm 2), amifostine vials (Ethyol, Schering Plough, Milan, Italy) 375 mg/day i.v. (arm 3), GSH vials 600 mg/day i.v. (arm 4), vitamin A tablets 30,000 IU/day orally plus vitamin E tablets 70 mg/day orally plus vitamin C tablets 500 mg/day orally (arm 5). The antioxidant treatment was administered for 10 consecutive days. The patients were studied at baseline and after antioxidant treatment.

In the second phase of the study 28 patients were divided into five groups and a combination of two different antioxidant agents was administered to each group (Table III). The selected combinations of antioxidant agents were: ALA 200 mg/day orally + carboxycysteine-lysine salt sachets 2.7 g/day orally (arm 1), ALA 200 mg/day orally + amifostine 375 mg/day i.v. (arm 2), carboxycysteine-lysine salt sachets 2.7 g/day orally + amifostine 375 mg/day i.v. (arm 3); GSH 600 mg/day i.v. + amifostine 375 mg/day i.v. (arm 4), ALA 200 mg/day orally + GSH 600 mg/day i.v. (arm 5). The antioxidant treatment was administered for 10 consecutive days. The patients were studied at baseline and after antioxidant treatment.

Assessment of Blood Levels of Reactive Oxygen Species (ROS) and Antioxidant Enzyme Glutathione Peroxidase (GPx)

The ROS were determined by the D-Roms test (Callegari, Parma, Italy). This method is based on the ability of transition metals to catalyse, in the presence of peroxides, the formation of hydroperoxides which are then trapped by an alchylamine. The alchylamine reacts with a specific chromogen reagent and develops a colored complex measurable through a kinetic reaction at 505 nm using a spectrophotometer (Form CR 2000, Callegari, Parma, Italy). Results are expressed in CARR U, where one CARR U corresponds to 0.08 mg/dl of hydrogen peroxide.[13] The method is considered specific and sensitive: within-run variations were less than 2.6% and between-run variations less than 4.6%.[14]

Erythrocyte GPx activity was measured using a commercially available kit (Ransod; Randox Lab, Crumlin, UK). Heparinized whole blood samples were diluted with diluting agent to convert the GPx to the reduced form; incubated for 5 min and then diluted with Drabkin's reagent to avoid falsely elevated results due to the presence of peroxidases in

human blood. The diluted sample was mixed with reagent (constituted by glutathione, GR and NADPH) and Cumene Hydroperoxide. GPx catalyses the oxidation of GSH by Cumene Hydroperoxide. In the presence of GR and NADPH the GSSG is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance after 1 and 2 min at 340 nm is measured. The result obtained was expressed in units/l of haemolysate and was multiplied by the appropriate dilution factor (41) to obtain the result in U/l of whole blood.

Serum Levels of Proinflammatory Cytokines and IL-2

Proinflammatory cytokines (IL-6, TNFα and IL-1β) and IL-2 were detected by a "sandwich" ELISA test (Biosource Europe SA, Belgium for IL-6 and TNFα; Immunotech SA, Marseille, France for IL-1β and IL-2) using monoclonal antibodies (mAbs) for two different epitopes of the cytokine molecule. The absorbance of the sample at 450 nm for IL-6, TNFα and IL-1β, and at 405 nm for IL-2, was measured with a spectrophotometer (Sirio, Seac, Florence, Italy). A standard curve was prepared by plotting the absorbance value of the standards versus corresponding concentrations. The concentration of the cytokine in the sample was determined by extrapolating from the standard curve. Ranges of the assay were: 5–1000 pg/ml for IL-1β and IL-2; 2–1000 pg/ml for IL-6; 10–1200 pg/ml for TNFα. Intra-assay variations were: 5% for IL-1β; 3% for IL-6, TNFα and IL-2. Inter-assay variations were 7% for IL-1β, IL-2 and TNFα; 8% for IL-6. The results are expressed in pg/ml. More details of the techniques used are described in our previous reports.[15,16]

Statistical Analysis

The results were expressed as mean ± standard deviation. Student's *t*-test for the difference of the means was used. Paired Student's *t*-test was used to compare the values of parameters at baseline and after antioxidant treatment. Significance was determined at the 5, 1 and 0.1% level, two-sided.

RESULTS

Assessment of Blood Levels of ROS and Antioxidant Enzyme GPx

The blood levels of ROS at baseline were significantly higher in cancer patients than in controls, as well as GPx activity values at baseline were significantly lower in cancer patients than in controls (Table IV).

TABLE IV Assessment of blood levels of ROS, GPx activity, serum proinflammatory cytokines and IL-2 of 56 cancer patients and 20 controls

	Controls	Patients	*p* Value
ROS (Carr U)	172 ± 32.2	403.4 ± 78.1	0.000
GPx (U/l)	10813 ± 2134.7	6770.6 ± 2355.2	0.000
IL-6 (pg/ml)	1 ± 2.5	29.1 ± 20.5	0.000
TNFα (pg/ml)	19 ± 6.7	41.0 ± 27.0	0.000
IL-1β (pg/ml)	11.5 ± 5.6	20.0 ± 13.3	0.007
IL-2 (pg/ml)	37.2 ± 23	18.4 ± 12.9	0.000

Results are expressed as mean ± standard deviation. Significance was calculated by Student's *t*-test for comparison to controls.

The comparison of the blood levels of ROS and GPx activity at baseline and after antioxidant treatment of the pooled patients (putting together the two phases of the study and all arms) showed a significant decrease of blood levels of ROS after antioxidant treatment compared to baseline and a significant increase of GPx activity (Table V). Both the patients enrolled in the first phase of the study and those enrolled in the second phase of the study (putting together the different arms) showed a significant decrease of ROS levels and a significant increase of GPx activity after antioxidant treatment compared to baseline (Table V). This comparison was made with the aim to provide evidence of the effectiveness of an "antioxidant treatment" on blood levels of ROS and GPx activity because the single arms of the study included a small number of patients not enough possibly to reach a statistical significance.

The first phase of the study showed that the blood levels of ROS decreased significantly after antioxidant treatment compared to baseline in all arms with different levels of significance ($p = 0.051$, 0.018, 0.007, 0.05, 0.047, respectively). The GPx activity increased significantly after antioxidant treatment compared to baseline in three arms (arm 2, $p = 0.005$; arm 3, $p = 0.006$; arm 4, $p = 0.052$) (Table VI).

The second phase of the study showed that the blood levels of ROS decreased significantly after antioxidant treatment compared to baseline in three arms (arm 1, $p = 0.008$; arm 4, $p = 0.014$; arm 5, $p = 0.013$). The GPx activity increased significantly after antioxidant treatment in three arms (arm 2, $p = 0.010$; arm 3, $p = 0.027$; arm 4, $p = 0.027$) (Table VII).

The comparison of the relative effectiveness of the different antioxidant treatments is reported in Tables VI and VII: the difference between the arms is never statistically significant due to the fact that the statistical power of this comparison is substantially influenced by the small number of patients included in each arm.

TABLE V Assessment of blood levels of ROS and GPx in cancer patients at baseline and after antioxidant treatment

	Number of patients	ROS			GPx		
		Baseline	After	*p* Value	Baseline	After	*p* Value
Pooled patients	56	403.4 ± 78.1	345.9 ± 71.4	0.000	6770.6 ± 2355.2	9263.7 ± 2318.4	0.000
Patients enrolled in the first phase of the study	28	414.4 ± 86.0	352.1 ± 80.1	0.007	6793.0 ± 2310.5	9032.0 ± 2289.0	0.000
Patients enrolled in the second phase of the study	28	392.4 ± 69.1	339.7 ± 62.4	0.004	6748.3 ± 2441.2	9558.6 ± 2375.5	0.000

Significance between values at baseline and after antioxidant treatment was calculated by paired Student's *t*-test.

Assessment of Serum Levels of Proinflammatory Cytokines and IL-2

As demonstrated in our previous studies, the values of serum proinflammatory cytokines IL-6 and TNFα were significantly higher in cancer patients than controls. The values of serum IL-2 were lower as compared to controls (Table IV).

The comparison of the serum values of proinflammatory cytokines at baseline and after antioxidant treatment of the pooled patients (putting together the two phases of the study and all arms) showed a significant decrease of IL-6 ($p = 0.043$) and TNFα ($p = 0.023$) after antioxidant treatment compared to baseline. The phases 1 and 2 of the study (putting together all arms) showed a significant decrease of TNFα ($p = 0.014$) and IL-6 ($p = 0.037$), respectively, after antioxidant treatment compared to baseline (Table VIII).

The first phase of the study showed a significant decrease of serum values of IL-1β in the arm 3 ($p = 0.037$), TNFα in the arm 4 ($p = 0.057$) and a significant increase of IL-2 in the arm 1 ($p = 0.043$) after antioxidant treatment compared to baseline (data not shown). The second phase of the study showed a significant decrease of serum values of TNFα in the arm 2 ($p = 0.009$) after treatment compared to baseline (data not shown).

Stratification of Patients According to the ECOG PS Status (0–1 versus 2–3)

The baseline blood levels of ROS and IL-6 of patients with ECOG PS 0–1 were significantly lower than those of patients with ECOG PS 2–3 (Table IX). The stratification of patients according to ECOG PS status showed that the blood levels of ROS decreased significantly after antioxidant treatment compared to baseline both in ECOG PS 0–1 and in ECOG PS 2–3 patients. Conversely, the GPx activity increased significantly after antioxidant treatment in both groups. IL-6 decreased significantly after treatment in ECOG PS 0–1 patients, while TNFα decreased significantly after treatment in ECOG PS 2–3 patients (Table X).

Safety

The administration of antioxidant agents has been proven to be safe: no adverse events were recorded except only one patient who had a short episode of orthostatic hypotension after amifostine administration, spontaneously cleared up in few minutes.

DISCUSSION

The OS is considered to play a key role in cancer. Despite our increasing understanding of the possible

TABLE VI Assessment of blood levels of ROS and GPx activity in cancer patients at baseline and after antioxidant treatment with single agents (first phase of the study)

		ROS			GPx		
	Number of patients	Baseline	After	*p* Value	Baseline	After	*p* Value
Arm 1	6	445.2 ± 99.8	347.3 ± 98.3	0.051	6033.8 ± 1040.9	8146.8 ± 2741.1	0.082
Arm 2	5	330.8 ± 83.4	257.8 ± 46.2	0.018	6412.4 ± 1340.7	9823.6 ± 2363.9	0.005
Arm 3	7	440.6 ± 87.0	396.3 ± 68.0	0.007	6625.0 ± 1797.2	9160.6 ± 1277.6	0.006
Arm 4	6	434.8 ± 64.4	382.4 ± 57.7	0.050	6765.0 ± 2288.7	8511.6 ± 2295.6	0.052
Arm 5	4	401 ± 63.4	348.3 ± 56.0	0.047	9009.8 ± 4689.2	10414 ± 3034.2	0.340

Arm 1: Alpha lipoic acid 200 mg/day orally. Arm 2: *N*-acetilcysteine 1800 mg/day i.v or carboxycysteine-lysine salt oral solution 2.7 g/day. Arm 3: Amifostine 375 mg/day i.v. Arm 4: Reduced glutathione 600 mg/day i.v. Arm 5: Vitamin A 30,000 IU + Vitamin E 70 mg + Vitamin C 500 mg/day orally. All treatments were administered during 10 days continuously. Significance between values at baseline and after antioxidant treatment was calculated by paired Student's *t*-test. Comparison of the relactive effectiveness of the different antioxidant treatments: Arm 2 vs. 1: ROS $p = 0.615$; GPx $p = 0.508$, Arm 2 vs. 3: ROS $p = 0.192$; GPx $p = 0.348$, Arm 2 vs. 4: ROS $p = 0.303$; GPx $p = 0.153$, Arm 2 vs 5: ROS $p = 0.458$; GPx $p = 0.164$. Arm 2, which showed the highest mean difference between baseline and after-treatment values, was selected as the reference arm.

mechanisms through which OS exerts a regulatory role in tumor growth and progression (including genomic instability,[17] oncogene activation[18] and angiogenesis),[19] several important questions remain unanswered. It is unclear whether OS in tumor results from an increased oxidant production or from a failure of antioxidant systems.[20] While important changes in cellular redox homeostasis during tumor growth have been documented in experimental models,[21,9] such variations have not been demonstrated in humans. Most of the difficulties encountered in these studies are related to the complexity of the biochemical pathways that regulate the cellular redox balance. A wide variety of oxidizing molecules such as ROS and/or depleting agents can alter the glutathione redox state, a key compound in the regulation of body redox homeostasis, which is normally maintained by the activity of GSH-depleting (GPx) and -replenishing enzymes (GR). The importance of glutathione and related enzymes and their variation in tumors has been so far poorly investigated.[9,22]

In the present study, we provide evidence that ROS production of advanced stage cancer patients was significantly higher than that of normal individuals and it was somehow related to disease progression and particularly to patient general status such as PS: indeed, highest values were found in ECOG PS 2–3 patients. The GPx activity (which is one of the physiologically most important antioxidant defence systems) shows an inverse relationship such as cancer patients exhibit significantly lower values than controls[23–26] and, moreover, a good correlation exists with patient PS, too: indeed, the lowest GPx values were seen in patients with PS 2–3. Among the antioxidant enzymes present in body fluids the GPx was found to be in our hands the most relevant and reliable of a series of antioxidant activities such as GR, superoxide dismutase and total antioxidant status.[23] We acknowledge, however, that "the data reported in the literature on antioxidant enzymes in different human cancer types are controversial"[23] and that other studies report different results. Taking together the results of ROS and GPx, cancer patients show a typical pattern of overt OS, in which the *reduced* antioxidant defence systems are insufficient to cope with the *increased* oxidant production.[27]

TABLE VII Assessment of blood levels of ROS and GPx activity in cancer patients at baseline and after antioxidant treatment with combination of different agents (second phase of the study)

		ROS			GPx		
	Number of patients	Baseline	After	*p* Value	Baseline	After	*p* Value
Arm 1	12	394.8 ± 61.8	345.1 ± 50.7	0.008	7641.7 ± 2548.9	10614.4 ± 2064.1	0.223
Arm 2	5	379.2 ± 57.3	350.4 ± 41.9	0.094	4239.4 ± 821.8	7220.8 ± 1126.2	0.010
Arm 3	4	412.5 ± 67.7	361.5 ± 42.8	0.140	7503.0 ± 1783.7	9850.3 ± 2373.6	0.027
Arm 4	3	348.7 ± 77.3	258.0 ± 59.0	0.014	6314.0 ± 859.0	10755.7 ± 1569.5	0.027
Arm 5	4	414.3 ± 112.1	349.5 ± 106.5	0.013	6775.3 ± 3256.9	9711.5 ± 3456.4	0.087

Arm 1: Alpha lipoic acid 200 mg/day + carboxycysteine lysine salt sachets 2.7 g/day. Arm 2: Alpha lipoic acid 200 mg/day + Amifostine 375 mg/day i.v. Arm 3: Carboxycysteine lysine salt sachets 2.7 g/day orally + Amifostine 375 mg/day i.v. Arm 4: Reduced glutathione 600 mg/day i.v. + Amifostine 375 mg/day i.v. Arm 5: Alpha lipoic acid 200 mg/day + reduced glutathione 600 mg/day i.v. All treatments were administered for 10 days continuously. Significance between values at baseline and after antioxidant treatment was calculated by paired Student's *t*-test. Comparison of the relactive effectiveness of the different antioxidant treatments: Arm 4 vs. 1: ROS $p = 0.224$; GPx $p = 0.268$, Arm 4 vs. 2: ROS $p = 0.108$; GPx $p = 0.206$, Arm 4 vs. 3: ROS $p = 0.265$; GPx $p = 0.278$, Arm 4 vs. 5: ROS $p = 0.191$; GPx $p = 0.367$. Arm 4, which showed the highest mean difference between baseline and after treatment values, was selected as the reference arm.

TABLE VIII Assessment of blood levels of proinflammatory cytokines in cancer patients at baseline and after antioxidant treatment

	Number of patients	IL-6			TNFα			IL-1β			IL-2		
		Baseline	After	*p* Value	Baseline	After	*p* Value	Baseline	After	*p* Value	Baseline	After	*p* Value
Pooled patients	56	29.3 ± 20.6	22.0 ± 15.6	0.043	41.3 ± 27.2	31.4 ± 18.0	0.023	20.2 ± 13.4	21.2 ± 14.3	0.544	18.6 ± 13.0	19.2 ± 9.3	0.673
Patients enrolled in the first phase of the study	28	28.0 ± 20.0	22.3 ± 17.2	0.293	46.5 ± 28.1	30.6 ± 15.9	0.014	21.3 ± 15.0	24.0 ± 15.4	0.376	21.8 ± 14.3	20.9 ± 10.0	0.903
Patients enrolled in the second phase of the study	28	31.2 ± 21.9	21.6 ± 13.5	0.037	33.9 ± 24.8	32.5 ± 21.0	0.821	18.7 ± 11.0	16.8 ± 11.8	0.536	13.9 ± 9.4	16.5 ± 7.6	0.260

Significance between values at baseline and after antioxidant treatment was calculated by paired Student's *t*-test.

The above reported data prompted us to carry out the present study to verify whether the administration of different antioxidant agents, administered either orally or i.v. to cancer patients, was feasible and effective, namely able to reduce the blood levels of ROS and to increase antioxidant enzymes, as well as to reduce the proinflammatory cytokines IL-6 and TNFα, which are known to be involved in cancer cachexia.

Indeed, multiple relationships betweeen OS and cancer cachexia have been found and a key role as mediators of both events is played by proinflammatory cytokines.[4,28]

Among the antioxidants, we have selected for the present study first ALA and cysteine-containing compounds, which showed, alongside their antioxidant efficacy, to be able to restore important immunological functional defects in peripheral blood mononuclear cells isolated from cancer patients,[29] as well as other well known and of wide clinical use such as vitamins A, C, E, GSH and amifostine.

The reasons for this choice were based on different considerations: (1) they include both orally and i.v. administered compounds which address the different personal preferences and/or compliance of patients; (2) they have been shown to be effective in our and in other hands; (3) they have different mechanisms of action. Indeed, numerous recent data demonstrated that antioxidant agents alone or in combination are effective in reducing the OS and even on cancer progression. In fact, supplementation with vitamin C or an antioxidant mixture containing vitamin C, ALA and vitamin E increases plasma F(2)-isoprostane levels, an index of OS in humans with high body mass index.[30] A recent paper provides evidence that *N*-acetylcysteine has a strong anti-angiogenic potential that could be exploited for preventing cancer progression.[31] Moreover, in a population-based study estimating the consumption of the antioxidant vitamins A, C, D, E and various carotenoids, the dietary intake of these compounds has been found to reduce the risk of ovarian cancer.[32]

The present study shows that all antioxidants tested were effective in reducing ROS levels and three of them, namely cysteine-containing compounds, amifostine and GSH, in increasing GPx activity, too. Among the combinations of antioxidant agents, ALA + carboxycysteine, GSH + amifostine and ALA + GSH were effective in reducing ROS, while GSH + amifostine, ALA + amifostine and carboxycysteine + amifostine were effective in increasing GPx activity. As reported in the "Results" section, the "antioxidant treatment" was found to be effective both on ROS levels and GPx activity. Considering the results, it is to be taken into account that the duration of treatment was short (10 days)

TABLE IX Comparison between baseline levels of ROS, GPx activity, serum proinflammatory cytokines and IL-2 of ECOG PS 0–1 with ECOG PS 0–2 patients

	Patients ECOG PS 0–1	Patients ECOG PS 2–3	*p* Value
ROS (Carr U)	386.6 ± 73.6	436.2 ± 78.0	0.023
GPx (U/l)	7108.4 ± 2605.1	6112.8 ± 1638.3	0.136
IL-6 (pg/ml)	21.6 ± 10.3	42.3 ± 26.9	0.000
TNFα (pg/ml)	38.6 ± 26.2	45.1 ± 28.6	0.398
IL-1β (pg/ml)	18.5 ± 11.4	22.6 ± 16.0	0.273
IL-2 (pg/ml)	19.6 ± 14.3	16.4 ± 10.1	0.389

Results are expressed as mean ± standard deviation. Significance was calculated by Student's *t*-test.

and perhaps not all its potential benefit could have been exploited: certainly in the clinical use this treatment must be planned over a much longer period of time. What it is to be considered the best antioxidant treatment, it is not yet established: in the present study the comparison of the relative effectiveness of the antioxidant treatments showed no difference between the different arms due to the fact that the statistical power of this comparison is substantially influenced by the small number of patients included in each arm. Several factors must be taken into consideration for the choice: the effectiveness, the safety, the compliance of the patients, the treatment feasibility and the costs or cost/effectiveness of treatment. For instance, considering the results of the present study, we can suggest the combination of ALA and carboxycysteine for outpatients as this treatment can achieve the best compliance of the patients, while for inpatients a treatment with GSH + ALA may be suggested addressing both the patient compliance and the effectiveness. Regarding amifostine, alongside its effectiveness, it is to be considered the high cost of the treatment, the need of intravenous injection and, although rare and not severe, the incidence of adverse effects (hypotension).

In the present study we confirm, as reported in several our previous papers,[4,33,34] that the levels of proinflammatory cytokines, and particularly IL-6 and TNFα, were higher in cancer patients as compared to controls and that antioxidant treatment in pooled patients was able to reduce serum levels of IL-6 and TNFα. Interestingly, antioxidant agents such as *N*-acetylcysteine, precursor of the synthesis of GSH and the same GSH, were reported to inhibit the production of TNFα[35] and, moreover, glutathione prodrugs were found able to decrease the production of TNFα, IL-6 and IL-8.[36] The short duration of the antioxidant treatment must be considered in the evaluation of the results.

In the present study, we show that a correlation exists between ECOG PS and baseline blood levels of ROS, GPx activity, serum levels of proinflammatory cytokines, whereas the effectiveness of treatment was not significantly influenced by ECOG PS status. We believe that this correlation between the patient biological parameters relevant to OS and the clinical most important index of disease progression, such as ECOG PS, represents the most significant novelty of our research.

In summary, our results warrant further investigation with an adequate clinical trial to test the hypothesis that supplementation of antioxidant agents may prevent/protect cancer patients from OS, either spontaneously occurring or enhanced by the treatment with cisplatin or other oxidative damage-inducing drugs.[37,38]

A phase III clinical trial based on the reported results is soon to be activated in our Institution.

TABLE X Stratification of patients according to the ECOG PS status (0–1 versus 2–3)

	ECOG PS 0–1 patients			ECOG PS 2–3 patients		
	Baseline	After	*p* Value	Baseline	After	*p* Value
ROS levels (Carr U)	386.6 ± 73.6	327.3 ± 69.4	0.000	436.2 ± 78.0	382.1 ± 61.9	0.023
GPx activity (U/l)	7108.4 ± 2605.1	9712.7 ± 2388.6	0.000	6112.8 ± 1638.3	8465.6 ± 2008.7	0.000
IL-6 (pg/ml)	21.6 ± 10.3	16.7 ± 9.5	0.037	42.3 ± 26.9	31.7 ± 19.2	0.171
TNFα (pg/ml)	38.6 ± 26.2	33.0 ± 19.2	0.298	45.1 ± 28.6	27.5 ± 15.4	0.024
IL-1β (pg/ml)	18.5 ± 11.4	20.8 ± 14.5	0.451	22.6 ± 16.0	22.8 ± 14.9	0.968
IL-2 (pg/ml)	19.6 ± 14.3	21.5 ± 10.0	0.510	16.4 ± 10.1	15.7 ± 6.5	0.801

Significance between values at baseline and after antioxidant treatment was calculated by paired Student's *t*-test.

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