Concurrent decline of several antioxidants and markers of oxidative stress during combination chemotherapy for small cell lung cancer

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Abstract

Objectives: To investigate the oxidant effects of adriamycin-containing chemotherapy (CT), we evaluated various antioxidants, total antioxidant capacity (TRAP) and different parameters of oxidative and nitrosative stress during combination CT.

Design and methods: Blood samples were obtained from 16 small cell lung cancer patients at baseline and several times during the first, second and sixth CT cycles.

Results: There were significant decreases in serum urate and serum proteins during all cycles, serum TRAP during the first two cycles, plasma ascorbic acid and serum TBARS during the first cycle, and serum conjugated dienes and plasma alphatocopherol during the last cycle. The baseline levels of tocopherols increased significantly between the first and sixth CT cycles. Higher levels of baseline plasma thiols were associated with better overall survival ($p = 0.008$).

Conclusions: Adriamycin-containing CT causes significant oxidative stress as implied by reduced levels of protective antioxidants. Long-term CT treatment seems to enhance lipid peroxidation.

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Keywords: Antioxidants; Oxidative stress; Nitrosative stress; Small cell lung cancer; Chemotherapy

Introduction

Lung cancer is the leading cause of cancer deaths in the world. It is divided into two groups: non-small cell lung cancer and small cell lung cancer (SCLC), the latter comprising approximately 20% of all lung cancers [1]. Most SCLC patients are not candidates for surgery, since SCLC tends to metastasize early, and therefore combination chemotherapy (CT) is the treatment of choice [2,3]. SCLC is chemosensitive and combinations of etoposide with cisplatin or adriamycin with vincristin and cyclophosphamide (CAV) have been standard treatment for a long time [4,5]. However, several new treatment strategies are being investigated to improve treatment results [2].

Many chemotherapeutic regimens exert their effects, at least partly, through the oxidative pathway. These drugs include the anthracyclins (e.g. adriamycin, epiadriamycin and daunorubicin),

Abbreviations: CT, chemotherapy; SCLC, small cell lung cancer; CAV, cyclophosphamide + adriamycin + vincristin; TRAP, total peroxyl radical trapping antioxidant potential; TBARS, thiobarbituric acid reactive substances; NOx, nitrate+nitrite; HPLC, high-performance liquid chromatography.

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procarbazine, bleomycin, vincristin, cyclophosphamide, etoposide and mitomycin [6]. The anticancer action of Adriamycin is related to its interactions with topoisomerase II, which leads to DNA fragmentation and cell death [7]. Adriamycin also enhances protein oxidation [8]. The human body has developed a complex antioxidant defense strategy to counteract these deleterious effects of external and internal oxidants [9].

In a previous study, we showed that anthracyclin-based chemotherapy produces oxidative stress, as evidenced by a decreased plasma total antioxidant capacity (TRAP) after CT treatment in SCLC patients [10]. Reduced levels of various antioxidants have been reported during combination chemotherapy of patients with hematological malignancies [11,12]. However, this study concentrated on a solid tumor (lung cancer), and to our knowledge, only a couple of studies have examined the oxidative status during CT of SCLC patients [10,13]. As the adverse events caused by Adriamycin might also be related to free radical formation and induction of lipid peroxidation [14,15], it would be interesting to explore whether oxidative stress marker levels could predict the occurrence of adverse events. This hypothesis has been tested in SCLC patients in only one previous study, which showed that increased lipid peroxidation markers are associated with better overall survival; however, that study examined only TBARS and Schiff’s bases [13].

Only TRAP and its components were investigated in our previous study [10]. To assess oxidative stress in a more comprehensive way, we measured levels of ascorbic acid, alpha- and gammacopherol, urate, thiols, total antioxidant capacity (TRAP) and markers of oxidative and nitrosative stress (oxidized proteins, proteins, TBARS, conjugated dienes, nitrite, nitrite + nitrate) at baseline before CT treatment and at several time points during CAV chemotherapy administered to SCLC patients. We also examined whether these markers predict adverse events, response to treatment and overall patient survival.

Materials and methods

Study group and procedures

Sixteen untreated patients with histologically or cytologically verified SCLC participated. The main inclusion criteria were: Karnofsky performance status ≥ 70%, no major cardiac, hepatic or metabolic disease, and planned CAV treatment. Patients with unstable angina pectoris, goit, a history of cancer (except basalioma or cervical carcinoma in situ) or regular allopurinol or acetylcysteine medication were excluded. No participants had taken vitamins or herbal supplements for three months prior to the study. Data on smoking habits, other diseases, symptoms and medication were recorded on a standardized questionnaire modified from the ATBC (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study) [16]. Smokers were defined either as current smokers or as smokers who had stopped smoking less than 6 months previously, and ex-smokers as subjects who had stopped smoking more than 6 months previously. The lifetime cigarette consumption was expressed as pack-years (cigarette packs smoked/day × years smoked). Patient characteristics are shown in Table 1.

Before treatment, all patients underwent a complete physical examination and chest radiography, ECG, chest and upper abdominal computed tomography, urinalysis and blood tests (see below). Bronchoscopy and bone scintigraphy were performed as clinically indicated.

All patients were treated and followed up at the Department of Oncology at Tampere University Hospital or at the Department of Pulmonary Diseases at Turku University Hospital, Finland.

Ethics

The ethics committees of Tampere and Turku University Hospitals approved the study protocol. Written informed consent was obtained from all the patients before any study procedures.

Blood tests

All patients underwent laboratory testing at baseline. The tests were: full blood count, serum C-reactive protein, sodium, potassium, creatinine, alanine transferase, aspartate transferase, alkaline phosphatase, albumin and neuron-specific enolase (NSE). Blood cell count and serum chemistry analysis were repeated prior to each cycle of CT.

The blood samples for oxidative stress markers were taken as follows: 20 mL of venous blood was drawn from an antecubital vein into two tubes, 10 mL into a Vacutainer tube containing ethylenediaminetetraacetic acid (EDTA) and 10 mL into a sterile tube. The samples for ascorbic acid and alphatocopherol analysis were immediately protected from light. Samples were centrifuged at 2800 g for 10 min and the plasma for ascorbic acid analysis was mixed (1:10) with 5% metaphosphoric acid and isosorbate. Samples were stored at −70 °C until analysis.

The first oxidative stress marker blood sample was taken 10–15 min before the start of CT, usually at 12 A.M. Additional bloods samples were taken 3, 5, and 19 h after the start of CT.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics.</th>
<th>Lung cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65.3 (8.7; 50–78)</td>
<td>Males</td>
</tr>
<tr>
<td>Males</td>
<td>14 (87.5)</td>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 (5.7; 19.8–43.6)</td>
<td>FEV1 (% of predicted)</td>
</tr>
<tr>
<td>FEV1 (% of predicted)</td>
<td>65.8 (14.7; 41–85)</td>
<td>Smoking</td>
</tr>
<tr>
<td>Smoking</td>
<td>Ex-smokers 6 (37.5)</td>
<td>Current smokers 10 (62.5)</td>
</tr>
<tr>
<td>Current smokers 10 (62.5)</td>
<td>10 (62.5)</td>
<td>Pack-years 44.1 (24.2; 12.5–100)</td>
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<tr>
<td>Pack-years 44.1 (24.2; 12.5–100)</td>
<td>44.1 (24.2; 12.5–100)</td>
<td>Stage of lung cancer</td>
</tr>
<tr>
<td>Stage of lung cancer</td>
<td>III 6 (37.5)</td>
<td>IV 10 (62.5)</td>
</tr>
<tr>
<td>III 6 (37.5)</td>
<td>6 (37.5)</td>
<td>Karnofsky performance status</td>
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<tr>
<td>Karnofsky performance status</td>
<td>70 2 (12.5)</td>
<td>80 3 (18.8)</td>
</tr>
<tr>
<td>70 2 (12.5)</td>
<td>2 (12.5)</td>
<td>90 10 (62.5)</td>
</tr>
<tr>
<td>80 3 (18.8)</td>
<td>3 (18.8)</td>
<td>100 1 (6.3)</td>
</tr>
<tr>
<td>90 10 (62.5)</td>
<td>10 (62.5)</td>
<td>100 1 (6.3)</td>
</tr>
</tbody>
</table>

Values are means (SD; range) or absolute values (percentages).
infusion (at 3 P.M., 5 P.M. and 7 A.M. the following day). The samples were collected during the first, second and last (usually sixth) CT cycles.

Chemotherapy treatment

CT treatment consisted of adriamycin 50 mg/m², vincristine 1.5 mg/m² (maximum dose 2 mg each time) and cyclophosphamide 750 mg/m². All regimens were administered through a peripheral vein. Adriamycin was always infused first over 30 min, after that vincristine as a bolus and finally cyclophosphamide over 30 min. Antiemetic medication, usually a 5-HT3 antagonist, was given 1/2 hour before the first CT infusion, and 3 liters of isotonic fluids were given during the first and second CT infusions to prevent the effects of any tumorlysis syndrome. Ten patients (63%) also received 300 mg allopurinol and 6–12 mg sodium bicarbonate before each CT treatment. The chemotherapy treatment was administered during the first day of the cycle. The chemotherapy cycle was repeated at 21-day intervals (second to sixth) CT cycles.

Assessment of treatment response and adverse events

The adverse events during the treatment were recorded three weeks after each CT cycle. The adverse events were evaluated according to World Health Organization (WHO) criteria [17]. Response to treatment was assessed by physical examination, laboratory tests and chest radiography after every two cycles of CT and classified according to WHO criteria [17,18]. Complete response (CR) was defined as the disappearance of all known disease determined by two observations not less than four weeks apart; partial response (PR) was defined as a 50% or more decrease in the size of one or more measurable lesions measured during two observations not less than four weeks apart; no change (NC) was defined as a less than 50% decrease but not more than 25% increase in the size of one or more measurable lesions; progressive disease (PD) was defined as a 25% or greater increase in the size of one or more measurable lesions or the appearance of new lesions [17,18]. Patients with complete or partial response were classified as responders and those with no change or progressive disease were classified as non-responders.

Analyses

Plasma thiols

Total plasma thiols were determined from 100 μL of plasma using a spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (Ellmann’s reagent) according to the method described in [19]. The coefficient of variation between the series was 6.6%.

Plasma ascorbic acid

Plasma ascorbic acid concentration was determined by ion-paired reversed-phase HPLC coupled with an electrochemical (EC) detector. The chromatography system comprised a Hewlett-Packard 1090 HPLC (Hewlett-Packard, Palo Alto, CA, USA), an EC detector with an ESA Coulometric Cell Model 5011A, a Hewlett-Packard LL0014 integrator, a reversed-phase fully end-capped Supelcosil LC18DB HPLC column (250×4.6 mm, 5 μm particle) and a Supelguard LC-18-DB guard column (20×4.6 mm, 5 μm particle; Sigma-Aldrich Co., St. Louis, MO, USA). An ECD potential of +70 mV was used for oxidizing extra components and +280 mV for ascorbic acid [20]. The coefficient of variation between the series was 5.5%.

Serum urate

Serum urate was determined by an enzymatic method (Konelab, Thermo Fisher Scientific Oy, Vantaa, Finland) using uricase, peroxidase and ascorbate oxidase. The coefficient of variation between the series varied from 1.4 to 2.3% and the accuracy (bias) was +1.4% in an external quality assessment program (Labquality Ltd, Helsinki, Finland).

Plasma tocopherols

To 50 μL plasma, 0.4 mL of a 50% ethanolic solution containing ascorbic acid and BHT and 50 μL of the internal standard tocol was added. After mixing, the analytes were extracted with 1 mL hexane. A 0.8 mL hexane aliquot was evaporated under nitrogen and the residue dissolved in 100 μL of methanol. Gamma- and alphatocopherol were separated with an Inertsil ODS-3 column (2.1×100 mm, 3 μm, GL Sciences, Tokyo, Japan). The mobile phase was methanol, 0.3 mL/min; 5 μL was injected into the column and the tocopherols were detected by their fluorescence at 292/324 nm. Peak height/internal standard ratios were compared to the ratios of a reference plasma with values traceable to NIST certified serum standards, 968b (National Institute of Standardization and Technology, Gaithersburg, MD, USA) [21,22]. The coefficient of variation between the series was 5.2%. The results are expressed as mg/L.

Plasma NOx and nitrite

To measure nitrite+nitrate (NOx) concentrations, vanadium (III) chloride (VCl₃) in hydrochloric acid was used to convert nitrite and nitrate to nitric oxide (NO), which was then quantified by the ozone-chemiluminescence method [23,24]. The samples were first treated with two volumes of ethanol at −20 °C for 2 h to precipitate proteins. A sample of 20 μL was injected into a cylinder containing a saturated solution of VCl₃ in 1 M HCl at 95 °C, and the nitric oxide formed under these reducing conditions was measured using an NOA 280 nitric oxide analyzer (Sievers Instruments Inc., Boulder, CO, USA) with sodium nitrate as standard. In the measurement of nitrite concentration, the deproteinized samples were injected into a cylinder containing sodium iodide (1% wt/vol) in acetic acid at room temperature to convert nitrite to nitric oxide, which was measured as before. The detection limit was 0.2 μmol/L for nitrite and 1.5 μmol/L for NOx.

Serum protein oxidation, diene conjugation, TBARS, TRAP

Chemicals. 1,1,3,3-Tetraethoxypropane was purchased from Sigma Chemical Co. (St.Louis, MO, USA). 2,4-dinitrophenylhydrazine and ethyl acetate were obtained from Merck (Darmstadt,
carried out as described by Reznick and Packer [25]. The protein was washed in ethyl acetate and dissolved in guanidine hydrochloride. Protein carbonyl content was quantified by scanning the samples from 320 to 535 nm [27]. The standard used was 1,1,3,3-tetraethoxypropane (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Aldrich Chem. Co. (Milwaukee, WIS, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich Chem. Co. (Milwaukee, WIS, USA).

**Assay procedures.** Protein carbonyl determinations were carried out as described by Reznick and Packer [25]. The sample was treated with 2,4-dinitrophenylhydrazine and TCA. The protein was washed in ethyl acetate–ethanol (1:1 vol/vol) and dissolved in guanidine hydrochloride. Protein carbonyl content was quantified by scanning the samples from 320 to 410 nm in a spectrophotometer. The peak absorbance was used to calculate protein carbonyl content (extinction coefficient 22,000 L mol⁻¹ cm⁻¹). Two different methods (diene conjugation and TBA-reactive substances) were used to estimate serum levels of lipid peroxides. For the measurement of diene conjugation, lipids extracted from serum samples (100 μL) by chloroform–methanol (1:1 vol/vol) were dried under nitrogen atmosphere and then redissolved in cyclohexane, were analyzed spectrophotometrically (at 234 nm) as described in [26]. For the analyses of TBA-reactive substances serum samples (100 μL) were diluted in phosphate buffer and heated together with TBA solution (375 mg/mL) in a boiling water bath for 15 min. The tubes were then cooled, and the absorbances measured at 535 nm [27]. The standard used was 1,1,3,3-tetraethoxypropane. The antioxidant potential of serum samples (total peroxyl radical trapping antioxidant potential, TRAP) was estimated by their potency in resisting ABAP-induced peroxidation [28,29]. In brief, 0.45 mL of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.02 mL of 120 mM linoleic acid, 0.05 mL of luminol (0.5 mg/mL) and 20 μL of serum sample were mixed in the cuvette and the assay was initiated with 0.05 mL of ABAP (83 mg/mL). Chemiluminescence was measured in duplicate cuvettes at 37 °C until a peak value for each sample was detected. Peroxyl radical trapping capacity was defined by the half-peak time point. Trolox served as a standard radical scavenger. The concentrations of plasma thiols, plasma ascorbic acid, serum urate, plasma nitrite and NOx, serum protein oxidation, serum diene conjugation, serum TBARS and serum TRAP are expressed as μmol/L.

**Serum proteins**

**Instrumentation.** Cobas Integra, F.Hoffman-La Roche Ltd, Basel, Switzerland. **Assay procedure.** Serum proteins were analyzed colorimetrically by the Biuret method, in which divergent copper react with the peptide bonds of proteins under alkaline conditions to form the characteristic pink to purple biuret complex [30,31]. Sodium potassium tartrate prevents copper hydroxide precipitation and potassium iodide prevents the auto-reduction of copper. The color intensity is proportional to the protein concentration. It is measured by measuring the increase in absorbance at 552 nm. The intra-assay coefficient of variation was 0.88% and the interassay one 1.80%. The results are expressed as g/L.

**Statistics**

Oxidative stress markers are the primary variables of the study. The results are given as medians and interquartile ranges (IQR). The repeated baseline (at 0 h, before CT) measurements during the 1st, 2nd and 6th cycles were analyzed using Friedman’s non-parametric analysis of variance. In the case of a significant result, the Wilcoxon signed rank test was used for paired comparisons (1st vs. 2nd, 1st vs. 6th and 2nd vs. 6th). The same method was applied to the within-cycle results at 0, 3, 5 and 19 h. First, Friedman’s analysis of variance was used for the 1st, 2nd and 6th cycles separately, after which the Wilcoxon signed ranks test was used for paired comparisons (baseline vs. 3 h, baseline vs. 5 h, baseline vs. 19 h), if the global test was significant. Spearman’s rank correlation and Mann–Whitney’s U test were used to study the association between patient characteristics and the oxidative stress markers. Spearman’s rank correlation was used to study the associations between baseline oxidative stress markers and other laboratory measurements. The oxidative stress markers at baseline during the 1st cycle were divided into two groups (values below median and values above median). Kaplan–Meier’s method was used to plot the survival curves. The log–rank test was used to compare the survival distributions for higher vs. lower levels for oxidative stress markers. The survival times are given as the median and the 95% confidence interval. Fisher’s exact test was used to study the association between the baseline level of oxidative stress markers, response to treatment and adverse events. p values below 0.05 were considered statistically significant. Statistical analyses were performed using SPSS (release 15.0) software (SPSS Inc. Chicago, IL, USA).

**Results**

**Study groups, adverse events and response to treatment**

Sixteen SCLC patients (14 males and two females) were entered in the study. Ten (63%) of the patients were current smokers and six (37%) had stopped smoking. The median pack-years of the patients were 44.1 (range 12.5–100). In addition to cancer, two (12.5%) of the patients had cardiac disease, one (6%) had chronic arrhythmia, four (25%) had hypertension and one (6%) had chronic obstructive pulmonary disease (COPD). None of the patients had rheumatic disorders, diabetes, asthma, chronic bronchitis, tuberculosis or asbestosis.

The majority (n = 12, 75%) of the patients completed the planned CT treatment. There were no significant treatment delays. The mean total doses delivered at each cycle were as follows: adriamycin 91 mg (range 80–110 mg), cyclophosphamide 1360 mg (range 1050–1650 mg) and vincristin 2 mg. Twelve (75%) patients experienced adverse events during treatment. Overall toxicity during CT was mild and there were no serious adverse events; however, three patients (19%) experienced transient grade III hematological toxicity. Nine
patients (56%) had grade I/II hematological toxicity. Other adverse events consisted of total alopecia (n = 7), nausea (n = 4), fatigue (n = 4) and dyspnea (n = 4). No colony-stimulating factors were used during the CT treatment.

After six cycles of treatment, one patient (6.3%) achieved complete response, 11 patients (68.8%) a partial response and two patients (12.5%) had stable disease. Two patients (12.5%) had progressive disease during the treatment, and the CAV chemotherapy was discontinued after the first CT cycle (one patient) or after two cycles of CT (one patient). The overall response rate was 75%. Two of the patients received carboplatin-etoposide (CAR-E) chemotherapy treatment after CAV treatment, one patient received pulmonary irradiation and four patients received cranial irradiation after CAV chemotherapy. No samples were obtained during these subsequent treatments.

Table 2
Baseline levels (measured at 12 A.M. before chemotherapy infusion) of antioxidants and oxidative stress markers during 1st, 2nd and 6th cycles.

<table>
<thead>
<tr>
<th></th>
<th>1st cycle</th>
<th>2nd cycle</th>
<th>6th cycle</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma thiols (μmol/L)</td>
<td>0.31 (0.28–0.36)</td>
<td>0.315 (0.28–0.35)</td>
<td>0.33 (0.29–0.35)</td>
<td>0.199</td>
</tr>
<tr>
<td>Plasma alphatocopherol (mg/L)</td>
<td>7.86 (7.03–8.88)</td>
<td>8.07 (7.18–9.02)</td>
<td>10.10 (8.16–11.07)</td>
<td>0.045</td>
</tr>
<tr>
<td>Plasma gammatomatocopherol (mg/L)</td>
<td>0.765 (0.51–1.22)</td>
<td>0.76 (0.58–1.36)</td>
<td>0.97 (0.57–1.82)</td>
<td>0.041</td>
</tr>
<tr>
<td>Plasma ascorbic acid (μmol/L)</td>
<td>57.7 (27.0–75.7)</td>
<td>45.4 (31.2–66.8)</td>
<td>39.8 (19.8–82.1)</td>
<td>0.717</td>
</tr>
<tr>
<td>Serum urate (μmol/L)</td>
<td>318 (294–422)</td>
<td>313 (269–403)</td>
<td>307 (279–363)</td>
<td>0.741</td>
</tr>
<tr>
<td>Serum oxidized proteins (μmol/L)</td>
<td>3.32 (2.57–3.87)</td>
<td>2.53 (2.20–3.11)</td>
<td>2.97 (2.18–3.30)</td>
<td>0.273</td>
</tr>
<tr>
<td>Serum conjugated dienes (μmol/L)</td>
<td>57.8 (44.0–75.2)</td>
<td>60.7 (46.8–81.4)</td>
<td>70.9 (60.7–77.2)</td>
<td>0.092</td>
</tr>
<tr>
<td>Serum TRAP (μmol/L)</td>
<td>925 (840–1059)</td>
<td>925 (730–1072)</td>
<td>876 (779–1035)</td>
<td>0.704</td>
</tr>
<tr>
<td>Serum TBARS (μmol/L)</td>
<td>5.65 (4.82–7.79)</td>
<td>5.46 (4.22–6.34)</td>
<td>4.85 (4.40–6.27)</td>
<td>0.209</td>
</tr>
<tr>
<td>Serum proteins (g/L)</td>
<td>69.5 (67.0–71.8)</td>
<td>69.0 (65.0–73.0)</td>
<td>70.0 (65.5–73.3)</td>
<td>0.581</td>
</tr>
<tr>
<td>Plasma nitrite (μmol/L)</td>
<td>0.48 (0.38–0.63)</td>
<td>0.61 (0.46–0.65)</td>
<td>0.50 (0.34–0.60)</td>
<td>0.150</td>
</tr>
<tr>
<td>Plasma NOx (μmol/L)</td>
<td>35.3 (28.5–43.0)</td>
<td>28.9 (24.4–43.1)</td>
<td>28.4 (23.6–44.1)</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Results are given as medians (IQR). Number of patients: n = 16 at 1st cycle, n = 13 at 2nd cycle and n = 10 at 6th cycle.

* Friedman’s test; patients with all three baseline measurements are included (n = 10).
Baseline levels of oxidative stress markers

The baseline levels of oxidative stress markers were obtained at 12 A.M. before the start of CT infusion. These measurements were performed during the 1st, 2nd and 6th CT cycles.

The baseline levels of alphatocopherol and gammatocopherol increased \( (p = 0.045 \text{ and } p = 0.041, \text{ respectively}) \) and NOx decreased \( (p = 0.020) \) significantly from the 1st to the 6th cycle (Fig. 1). The change in these markers was not significant when the 1st cycle was compared with the 2nd cycle. Instead, the significant increase/decrease was caused by the 6th cycle. The changes in baseline levels were not significantly different for the other antioxidants or oxidative stress markers, although the levels of conjugated dienes tended to increase from the 1st to the 2nd and 6th cycles \( (p = 0.092) \) (Table 2).

Oxidative stress markers during 1st chemotherapy cycle

Most prominent changes in antioxidants and oxidative stress markers occurred during the first chemotherapy cycle. There were significant decreases in the levels of urate \( (< 0.001) \), ascorbic acid \( (p = 0.021) \), proteins \( (p = 0.004) \), TBARS \( (p = 0.012) \) and TRAP \( (p = 0.001) \). Significant reductions were seen in the levels of urate and TRAP at all timepoints during the cycle. The median changes after 3, 5 and 19 h were \(-41, -50 \text{ and } -65 \mu\text{mol/L for urate, } -5.7, -2.3 \text{ and } -4.3 \mu\text{mol/L for ascorbic acid, } -3.0, -2.0 \text{ and } -5.0 \text{ g/L for proteins, } -0.53, -0.42 \text{ and } -0.14 \mu\text{mol/L for TBARS and } -49, -97 \text{ and } -49 \mu\text{mol/L for TRAP compared with the baseline value (0 h) of the cycle (Fig. 2).}

Oxidative stress markers during 2nd chemotherapy cycle

There was another significant decrease in the levels of urate and TRAP also during the second CT cycle at all measured timepoints at 3 P.M., 5 P.M. and 7 A.M. the following day \( (p = 0.002, p = 0.038, \text{ respectively}) \). Proteins decreased at 3 and 19 h after the start of CT \( (p = 0.009, p = 0.017) \), whereas oxidized proteins increased significantly during the CT cycle measured 19 h after administration of the first chemotherapeutic drug \( (p = 0.009) \). The median changes after 3, 5 and 19 h were \(-18, -26 \text{ and } -29 \mu\text{mol/L for urate, } -49, -49 \text{ and } -49 \mu\text{mol/L for TRAP and } -5.0, -1.0 \text{ and } -4.5 \text{ g/L for proteins compared with the baseline values (Fig. 2).}

Oxidative stress markers during the final chemotherapy cycle

During the last chemotherapy cycle, significant changes from baseline at 12 A.M. were recorded in the levels of urate \( (p = 0.005) \), alphatocopherol \( (p = 0.008) \), proteins \( (p = 0.004) \) and conjugated dienes \( (p = 0.022) \). The median changes after 3, 5 and 19 h were \(-22, -20 \text{ and } -8.5 \mu\text{mol/L for urate, } -0.6, -0.2 \text{ and } -0.8 \text{ mg/L for alphatocopherol, } -4.0, -3.0 \text{ and } -5.0 \text{ g/L for proteins and } -0.8, -5.6 \text{ and } -6.4 \mu\text{mol/L for conjugated dienes (Fig. 2).}

Baseline serum protein levels correlated negatively with patients’ age \( (R = -0.522, p = 0.038) \). There were no other correlations between patients’ age and antioxidants or markers of oxidative stress at baseline.

There were positive correlations between serum TRAP and serum urate \( (R = 0.804, p = 0.000) \), plasma nitrite and serum conjugated dienes \( (R = 0.511, p = 0.043) \) and serum TBARS \( (R = 0.621, p = 0.010) \), and between plasma NOx and plasma alphatocopherol \( (R = 0.529, p = 0.043) \). Negative correlations were noted between plasma ascorbic acid and serum oxidized proteins \( (R = -0.521, p = 0.039) \), serum TBARS and plasma.
The occurrence of adverse events during treatment was not significantly associated with any of the baseline levels of oxidative stress markers measured at 12 A.M. during the 1st CT cycle.

Both baseline ascorbic acid ($p = 0.077$) and oxidized proteins ($p = 0.077$) had a weak association with response to treatment. If baseline levels of ascorbic acid were above the median (>57.7 μmol/L), patients had a better response to treatment than if the levels were below the median (partial/complete response to the treatment was 100% vs. 50%, respectively). Baseline levels of oxidized proteins above the median (>3.32 μmol/L) indicated a poorer response to treatment than values below the median (partial/complete response 50% vs. 100%).

The overall median progression-free survival time was 5.9 months (95% CI 5.0 to 6.8 months) and the survival 5.9 months (95% CI 0.1 to 11.7 months). At the end of the planned follow-up period of 60 months all patients had died, and only two patients survived more than 24 months (26 and 59 months). There was a statistically significant association between baseline plasma thiol levels and overall survival ($p = 0.008$). If the thiol levels were below the median (<0.306 μmol/L), median survival was 5.5 months; otherwise median survival was 10.0 months (Fig. 3). There was also a tendency towards better overall survival (8.8 months vs. 5.7 months, $p = 0.068$) when baseline serum NOx levels were above the median (>35.3 μmol/L).

**Discussion**

To our knowledge, this is the largest study so far to evaluate different antioxidants and parameters of oxidative and nitrosative stress in SCLC patients during free radical-generating CT. In this study, most of the antioxidants or oxidative stress markers examined tended to decrease during CT treatment as measured from baseline to 3, 5 and 19 h after the start of CT treatment. In serum urate, plasma alphatocopherol, plasma ascorbic acid, serum proteins, serum conjugated dienes, serum TBARS and serum TRAP, the change was significant during at least one cycle during the whole treatment period (Fig. 2), however, the most prominent changes occurred during the first CT cycle.

There was a significant reduction in plasma TRAP three h after the very first adriamycin infusion ($p = 0.016$) and the biggest reduction occurred 5 h after the start of CT ($p = 0.001$). TRAP levels returned to baseline 19 h after the start of CT ($p = 0.018$), a finding we made in a previous study showing that adriamycin-containing chemotherapy increases free radical production as evidenced by decreased levels of TRAP [10]. The reduction of total TRAP ($p = 0.001$) during the first CT cycle is due to a reduction of the components of TRAP, namely urate ($p < 0.001$), thiols ($p = 0.082$), ascorbic acid ($p = 0.021$) and alphatocopherol ($p = 0.093$). This is also in accordance with our previous report [10]. Significant decreases were also seen in TBARS ($p = 0.012$) and proteins ($p = 0.004$) during the first CT cycle. The decline in the levels of various antioxidants during the first CT cycle may reflect a failure in antioxidant defense mechanisms against CT-induced oxidative damage.

Significant decreases in many of the antioxidants and markers of oxidative stress were also recorded during the second and last CT cycles. The most significant changes took place in the levels of urate and TRAP, which corresponds to the changes during the first CT cycle (Fig. 2). Our finding is in accordance with a previous study, which showed that urate decreases during high-dose CT [11]. This study also noted significant decreases in serum proteins during all CT cycles examined; interestingly, the protein levels returned to baseline prior to the next cycle. Thus, no notable changes were seen in serum proteins when examining the baseline levels during the whole treatment period (Table 2).

Other demographics, such as smoking, concomitant diseases or the patients’ age, did not significantly influence the antioxidant or oxidative stress marker levels during any of the CT cycles. A notable exception was serum protein levels, which correlated negatively with the patients’ age. This is in agreement with a previous study showing that albumin concentration decreases with age [32]. As only two of the patients were women, no reliable analysis could be performed on the effect of gender on antioxidant and oxidative stress marker levels.

Adriamycin generates free radicals by two distinct mechanisms: redox cycling and a Haber–Weiss type of reaction [7]. The
other two drugs administered in this study also generate free radicals [6]. Studies show that cyclophosphamide treatment decreases antioxidant levels and reduces antioxidant enzyme levels in breast cancer patients [33]. Cyclophosphamide initiates peroxidation in membrane lipids, while the antitumoral action of vincristine also includes peroxidative damage [34,35]. Thus, the reductions in the levels of antioxidants may be due to increased free radical production caused by the chemotherapeutic drugs. These drugs may also lower antioxidant enzyme concentrations in plasma during chemotherapy [33], but this was not addressed in the present study.

Urate is the end product of purine metabolism and one of the major antioxidants in human plasma. Urate may play an essential role in protecting cells against free radical-induced damage [36]. Urate inhibits lipid peroxidation and may act by preserving ascorbic acid [37,38]. The water-soluble vitamin C (ascorbic acid) has a broad antioxidant activity [9,39]. A previous study has shown that ascorbic acid has a critical protective role against oxidative stress and that lipid peroxidation occurs only after ascorbic acid is completely depleted [40]. Since the concentration of urate in the plasma is 5–10 times higher than that of ascorbic acid [36], urate and ascorbic acid may be the first antioxidants to be depleted after exposure to oxidants [41]. Also, protein oxidation may be inhibited by chain-breaking antioxidants [42].

This study supports the view that urate acts as one of the most important free radical scavenging agents, as significant reductions in the serum concentration of urate took place during each examined CT treatment during the 1st, 2nd and 6th CT cycles. On the other hand, the plasma urate level may also decrease as a consequence of increased renal excretion after the high fluid load needed during CT [11]. Allopurinol is a known inhibitor of xanthine oxidase and was administered to the majority of patients before CT treatment to prevent tumorlysis syndrome. However, administration of allopurinol might partly account for the decreased levels of urate during CT treatment as CT is known to induce oxidative DNA damage, which in turn increases uric acid levels through the action of xanthine oxidase [43]. Therefore, factors other than the protective role of urate may also contribute to the decrease in urate levels during each CT cycle. However, no significant changes were noted in the baseline levels of urate measured at 12 A.M. during each cycle between the first and last chemotherapy cycle. On the contrary, during the same 4 1/2-month period, a marked, although statistically non-significant, decrease occurred in the levels of ascorbic acid (Table 2), which supports the view that ascorbic acid plays an essential role in protecting against oxidants.

Alphatocopherol is a lipid-soluble compound present in lipid membranes and plasma lipoproteins [44]. It protects cells against lipid peroxidation and blocks nitrosamine formation [45,46]. A study suggests that alphatocopherol is the final main antioxidant consumed after exposure to oxidants [10]. The present study supports this, since alphatocopherol levels decreased significantly during the last CT cycle (10.10 mg/L at 12 A.M., 8.80 mg/L at 3 P.M., 9.20 mg/L at 5 P.M. and 8.76 mg/L at 7 A.M. the following day, \( p = 0.008 \)); this is consistent with a previous study [47]. It is also possible that the decrease in alphatocopherol is a result of its enhanced breakdown during CT treatment [48]. The present study adds to the current knowledge by showing that the baseline levels of both alphatocopherol and gammatocopherol increased significantly from first to last CT cycle (\( p = 0.045 \) and \( p = 0.041 \), respectively), while the levels of conjugated dienes rose (\( p = 0.092 \)). It is known that TBARS is a rather unspecific marker of lipid peroxidation [9,49] and no significant changes in the levels of TBARS were noted between the baseline levels measured during the first and last CT cycles. Conjugated dienes reflect the early onset of lipid peroxidation and are less sensitive to compensatory antioxidant mechanisms than the other lipid peroxidation markers [49]. The increase in conjugated dienes reflects increased lipid peroxidation caused by CT, which is best evidenced after several CT cycles at the end of the treatment period. The main antioxidants seem to have protected against peroxidative damage at the beginning of the CT treatment and are consumed by the last CT cycle. It has been shown that lipid peroxidation induces apoptosis, as both free radicals and lipid peroxides enhance pro-apoptotic p53 and suppress anti-apoptotic bcl-2 expression [50,51]. Previous in-vitro studies have also shown that derivatives of alphatocopherol increase apoptosis and decrease the proliferation of tumor cells [52]. In the present study, samples were obtained of eleven patients during the sixth CT cycle. Of these patients one (9%) had stable disease and ten (91%) achieved a partial or complete response to treatment, which indicates that the increase in baseline levels of lipid peroxidation markers and tocopherols at the end of the treatment may be associated with inhibition of cancer growth and better response to CT [13].

Nitric oxide (\( \text{NO} \)) is a multifunctional molecule involved in a variety of physiological and pathological processes [53,54]. It has an important role in the initiation of apoptosis in various cell types [55]. Nitrite and nitrate in plasma reflect the levels of \( \text{NO} \) [54]. Elevated levels of \( \text{NO} \) have been found in cancerous colon tissue [56]. In the present study, the levels of NOx decreased during the course of CT treatment. An interesting hypothesis is that this may be related to the decrease of tumor mass, as most of the patients who received the sixth CT cycle were classified as responders [56].

No previous clinical follow-up studies have been performed to record associations between antioxidants and oxidative stress marker levels at baseline and response to chemotherapy in SCLC patients. The present study revealed an association, albeit a non-significant one, between treatment response and baseline ascorbic acid (\( p = 0.077 \)) and oxidized proteins (\( p = 0.077 \)). Interestingly, an inverse correlation was seen between baseline levels of plasma ascorbic acid and serum oxidized proteins (\( R = -0.521, \ p = 0.039 \)). Previous studies have speculated that ascorbic acid might reduce the growth of tumor xenografts in mice [57]. Naturally, larger studies are needed to explore the associations between antioxidants and response to treatment.

To our knowledge, the present study is the first to show an association between plasma thiols and overall survival (\( p = 0.008 \)). We noted that the patients’ overall survival was longer if baseline plasma thiols were above the median. A recent study showed that head and neck cancer patients with a higher than median post-
radiotherapy glutathione value survive longer than those whose value is below the median [58]. An in-vitro study has shown that different human lung cancer cell lines have different redox properties and that cells with higher expressions of thioredoxin were more susceptible to the anticancer actions of chemotherapeutic drugs [59]. Measuring the ratio of oxidized glutathione to reduced glutathione in the present study would have provided useful information for the hypothesis that increased glutathione is associated with overall survival; this is one limitation of our findings. Again, larger studies are clearly needed to explore the associations between antioxidants and overall survival.

Several studies have recommended the use of antioxidant supplementation during combination CT [6,8,60]. However, as free radical formation is desirable during adriamycin-containing CT, routine antioxidant supplementation might counteract the beneficial effects of the treatment [61]. On the other hand, our finding that higher thiol levels are associated with better survival indicates that further studies are warranted to find out whether antioxidant supplementation could produce less augmentation of the adverse events or improve response and survival after CT for SCLC.

Conclusions

The concentrations of the most important antioxidants are reduced in the blood during CT, probably because antioxidant defense mechanisms are activated to combat the free radical storm produced by the CT. Different oxidative stress markers seem to behave differently in this respect. Repetitive polychemotherapy with radical-generating compounds may exceed the antioxidant capacity of cancer patients and lead to high oxidative stress. This study also suggests that higher baseline thiols may predict better overall survival.

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