MARIKA CROHNS

Antioxidants, Cytokines and Markers of Oxidative Stress in Lung Cancer

Associations with adverse events, response and survival

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ACADEMIC DISSERTATION
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To my family

"Everything that we see is a shadow cast by that which we do not see"
Martin Luther King, Jr.

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LIST OF ORIGINAL COMMUNICATIONS

This doctoral dissertation is based on the following original publications which are later referred to in the text by their Roman numerals (I–V):

I Marika Crohns, Seppo Saarelainen, Marina Erhola, Hannu Alho, Pirkko Kellokumpu-Lehtinen. Impact of radiotherapy and chemotherapy on biomarkers of oxidative DNA damage in lung cancer patients. Clin Biochem 2009;42:1082–1090. *E-pub 6 March, 2009*

II Marika Crohns, Seppo Saarelainen, Hannu Kankaanranta, Eeva Moilanen, Hannu Alho, Pirkko Kellokumpu-Lehtinen. Local and systemic oxidant/antioxidant status before and during lung cancer radiotherapy. Free Radic Res 2009;43:646–657. *E-pub 12 May, 2009*

III Marika Crohns, Kari Liippo, Marina Erhola, Hannu Kankaanranta, Eeva Moilanen, Hannu Alho, Pirkko Kellokumpu-Lehtinen. Concurrent decline of several antioxidants and markers of oxidative stress during combination chemotherapy for small cell lung cancer. Clin Biochem 2009;42:1236–1245. *E-pub 13 May, 2009*

IV Marika Crohns, Seppo Saarelainen, Jukka Laitinen, Kimmo Peltonen, Hannu Alho, Pirkko Kellokumpu-Lehtinen. Exhaled pentane as a possible marker for survival and lipid peroxidation during radiotherapy for lung cancer – a pilot study. Free Radic Res 2009;43:965–974. *E-pub 8 August, 2009*

V Marika Crohns, Seppo Saarelainen, Seppo Laine, Tuija Poussa, Hannu Alho, Pirkko Kellokumpu-Lehtinen. Cytokines in bronchoalveolar lavage fluid and serum of lung cancer patients during radiotherapy – association of interleukin-8 and VEGF with survival. Cytokine 2010;50:30–36. *E-pub 30 December, 2009*

ABBREVIATIONS

8-OHdG	8-hydroxy-2´-deoxyguanosine
8-oxoGua	8-oxoguanine
8-oxodG	8-oxo-2´-deoxyguanosine
BER	base excision repair
BMI	body mass index
CAT	catalase
CD	conjugated dienes
COPD	chronic obstructive pulmonary disease
CT	chemotherapy
DNA	deoxyribonucleic acid
ECD	electrochemical detector
ECOG	Eastern Cooperative Oncology Group
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EORTC	European Organization for Research and Treatment of Cancer
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GC	gas chromatography
GPx	glutathione peroxidase
GSH	glutathione
GSR	glutathione reductase
GSSG	reduced glutathione
Gy	Gray
HPLC	high-performance liquid chromatography
IL	interleukin
LC	lung cancer
LDL	low-density lipoprotein
LOOH	lipid hydroperoxide
MDA	malondialdehyde
MS	mass spectrometry
NER	nucleotide excision repair
NO	nitric oxide
NOx	nitrite+nitrate
NO_2	nitrite
NO_3	nitrate
NSCLC	non-small cell lung cancer
PUFA	polyunsaturated fatty acids

ROS	reactive oxygen species
RT	radiotherapy
RTLF	respiratory tract lining fluid
RTOG	Radiation Therapy Oncology Group
SCLC	small cell lung cancer
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
TNM	Tumor Nodus Metastasis (cancer staging system)
TRAP	total peroxyl radical trapping antioxidant potential
UICC	International Union Against Cancer
WHO	World Health Organization

ABSTRACT

Lung cancer represents a major health concern with over 1.3 million cases diagnosed worldwide each year. A growing body of evidence has indicated that lung cancer, among other cancers, is associated with increased production of reactive oxygen species (ROS). Also ionizing radiation induces the formation of ROS, which can damage lipids, proteins and DNA. In addition, many chemotherapeutic agents seem to be associated with the generation of free radicals and increased lipid peroxidation. Despite accumulating data on ROS in cancer, there are few studies examining antioxidants, cytokines and markers of oxidative stress during cancer treatments.

To evaluate oxidative stress in lung cancer, we examined the levels of a number of antioxidants, various cytokines, total peroxyl radical trapping antioxidant potential (TRAP) and a number of parameters of oxidative and nitrosative stress in 65 lung cancer patients and 66 non-cancer controls. Our aim was to assess the local and systemic effects of lung cancer and its treatment by radiotherapy and chemotherapy on these markers. The markers were also evaluated in relation to various clinicopathological parameters, and possible associations between these markers and adverse events, response to treatment and overall survival of the patients were investigated.

The findings here would indicate that lung cancer is associated with increased oxidative stress and production of certain cytokines. It was also demonstrated that bronchoalveolar lavage (BAL) fluid can be a useful medium for analyzing local cytokine and oxidative stress marker status in lung cancer patients. The involvement of radiotherapy and chemotherapy produces free radicals, as evidenced by a decrease in the levels of various antioxidants and elevation of certain markers of oxidative stress during these treatments. However, it appears that the body´s own antioxidant defense system is fairly sufficient to counteract the increase in ROS during lung cancer treatments. Higher exhaled pentane levels, as a marker of lipid peroxidation, seemed to predict better survival. In addition, these data imply that serum and BAL fluid IL-8 and serum VEGF may be useful prognostic factors in lung cancer patients. A further interesting future research topic is the association between plasma and BAL thiols and survival of patients. However, additional studies are called for to explore the possible associations between oxidative stress markers and adverse events and response to treatment.

TIIVISTELMÄ

Keuhkosyöpä on merkittävä maailmanlaajuinen terveysongelma. Yli 1,3 miljoonaa ihmistä sairastuu siihen vuosittain. Suomessa keuhkosyöpä on miesten toiseksi yleisin ja naisten neljänneksi yleisin syöpämuoto. Aiemmat tutkimukset ovat osoittaneet, että keuhkosyövässä, kuten monissa muissakin syövissä, vapaiden radikaalien tuotanto on lisääntynyt. Myös sädehoidossa syntyy syöpäsoluja tappavia vapaita radikaaleja, jotka voivat vaurioittaa lipidejä, proteiineja ja DNA:ta. Lisäksi useat solunsalpaajat aiheuttavat vapaiden radikaalien muodostumista ja lipidiperoksidaatiota. Vapaiden radikaalien osuudesta syövässä on saatu paljon tutkimustietoa, mutta antioksidantteja, sytokiineja ja oksidatiivisen kuormituksen merkkiaineita keuhkosyövän hoitojen aikana on tutkittu vähän.

Väitöskirjatutkimukseen osallistui 65 keuhkosyöpäpotilasta ja 66 kontrollihenkilöä, joilla ei ollut syöpää. Osallistujilta määritettiin useita antioksidantteja, sytokiineja, kokonaisantioksidanttikapasiteetti sekä oksidatiivisen ja nitrosatiivisen kuormituksen merkkiaineita. Tutkimustyön tavoitteena oli arvioida keuhkosyövän sekä säde- ja solunsalpaajahoidon paikallisia ja systeemisiä vaikutuksia mainittuihin merkkiaineisiin. Lisäksi työn tavoitteena oli tutkia oksidatiivisen kuormituksen merkkiaineiden yhteyttä muihin taustatekijöihin sekä niiden soveltuvuutta syövän hoitojen yhteydessä ilmenevien sivuvaikutusten, hoitovasteen ja elossaolon ennustamiseen.

Väitöskirjatutkimuksessa todettiin, että keuhkosyöpään liittyy lisääntynyt vapaiden radikaalien ja tiettyjen sytokiinien tuotanto. Tutkimus osoittaa myös keuhkojen tähystyksessä saatavan bronkoalveolaarinesteen tarkoituksenmukaisuuden mitattaessa paikallisesti sytokiineja ja oksidatiivisen kuormituksen merkkiaineita keuhkosyöpäpotilailla. Tutkimuksen mukaan sekä sädehoito että solunsalpaajahoito lisäävät vapaiden radikaalien tuotantoa, sillä tiettyjen antioksidanttien tasot laskivat hoidon aikana samalla kun oksidatiivisen kuormituksen merkkiaineiden tasot nousivat. Vaikuttaa kuitenkin siltä, että elimistön omat puolustusmekanismit pystyvät melko hyvin torjumaan keuhkosyövän hoitojen aikaista oksidatiivista kuormitusta. Tutkimuksen mukaan korkeammat lipidiperoksidaation merkkiaineen, uloshengitetyn pentaanin, tasot ennustivat pitempää elossaoloaikaa. Tulosten perusteella myös seerumin ja bronkoalveolaarinesteen interleukiini-8 ja seerumin verisuonikasvutekijä (VEGF) saattavat olla hyödyllisiä merkkiaineita ennustettaessa keuhkosyöpäpotilaiden elossaoloaikaa. Mielenkiintoinen tutkimusaihe tulevaisuudessa olisi myös plasman ja bronkoalveolaarinesteen tiolitasojen yhteys elossaoloon. Laajempia tutkimuksia tarvitaan kuitenkin selvittämään oksidatiivisen vaurion merkkiaineiden mahdollisia yhteyksiä hoitojen aikaisiin sivuvaikutuksiin sekä hoitovasteeseen.

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the Western world (Jassem, 2007). In Finland it is the second most common cancer among men, with 1511 new cases in 2007, and the fourth most common cancer in women, with 675 new cases in 2007 (Finnish Cancer Registry, 2009). Cigarette smoke is the main risk factor for the development of lung cancer (Pryor et al., 1985). Lung cancer is divided into two main entities: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Treatment is based on the tumor type, the extent of disease and performance status of the patient (Pirozynski et al., 2006).

In NSCLC surgery is the therapy of choice in stage I–IIIA tumors. In addition to surgery, both radiotherapy and chemotherapy are used. For unresectable stage III patients, concurrent chemoradiotherapy is the standard. In advanced stage IV tumors, only palliative treatment can be offered. A significant proportion of all lung cancer patients require active intervention and receive radiotherapy (Jassem, 2007). However, as most lung cancer cases are inoperable, the overall 5-year survival of lung cancer patients is poor, approximately 8–15% (Giaccone, 2002, Jassem, 2007, Finnish Cancer Registry, 2009).

SCLC is significantly more chemosensitive than NSCLC. SCLC has a tendency to disseminate early and chemotherapy is therefore the mainstay of treatment (Stupp et al., 2004, Murray and Turrisi, 2006). Despite active treatment, most SCLC patients eventually experience a relapse, which is often refractory to further treatments. The challenge thus remains to explore new, better treatment options for SCLC (Ohe, 2004).

Free radicals are highly reactive molecules or atoms which contain one or more unpaired electrons in their outer orbitals (Singal et al., 2000). They are constantly formed during cellular processes, e.g. during energy production and activation of phagocytosing cells, and by auto-oxidation of different molecules (Halliwell and Gutteridge, 2007, p. 19–23). Oxidative stress is thus an inevitable consequence of aerobic life. The implication of free radical reactions in the pathogenesis of various diseases is nowadays generally accepted (Halliwell and Cross, 1994). To control the influence of reactive oxygen species (ROS), aerobic cells have developed their own antioxidant defense system, which includes both enzymatic and non-enzymatic components (Bakan et al., 2003). However, oxidative stress may occur if the production of ROS exceeds the antioxidant capacity of the cell (Powell et al., 2005).

There has been increasing interest in the role of free radicals and antioxidants in cancer during recent years. Oxidative stress has been suggested to play a key role in carcinogenesis (Cerutti, 1994, Lu, 2007). Nonetheless the exact role of free radicals especially during cancer treatments is still largely unknown. Free radicals have been shown to mediate the anti-cancer actions of many chemotherapeutic regimens (Faber et al., 1995, Dűrken et al., 2000). Also radiotherapy exerts its cytotoxic effects through

free radicals, either by direct action on DNA with damage as a consequence or indirectly by producing ROS (Riley, 1994). Despite active investigation, knowledge is lacking concerning the local and systemic effects of free radical-generating treatments in lung cancer.

These series of studies were undertaken to elucidate the role of different antioxidants, cytokines and markers of oxidative and nitrosative stress in lung cancer patients before and during radiotherapy and chemotherapy for lung cancer. Results were also compared to those in healthy controls. We also aimed to determine the associations between these markers and adverse events, response to treatment and patient survival.

2. REVIEW OF THE LITERATURE

2.1 Free radicals and oxidative stress

All aerobic cells and plants require oxygen (O_2) for their energy production. The percentage of oxygen in the atmosphere is 21% in dry air (Halliwell and Gutteridge, 2007, p. 1). However, it is known that even this 21% of oxygen has harmful effects, most of them due to the formation of free oxygen radicals. Cells continuously produce free radicals as a part of their normal metabolic processes. Under normal metabolic conditions 2–5% of the O_2 consumed by mitochondria is converted to reactive oxygen species (Lopaczynski and Zeisel, 2001). Hence, the prevention of excess free radical formation is a vital step for cell survival (Valko et al., 2006).

Electrons in atoms are located in orbitals. Each orbital can hold at maximum two electrons. An unpaired electron is defined as any electron which is alone in an orbital. A free radical is a molecule which contains one or more unpaired electrons in its outer orbital (Halliwell et al., 1992).

Most common free radicals are superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), nitric oxide ($NO^{\bullet}$), thiyl ($RS^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) and trichloromethyl ($CCl^{\bullet}$) radicals. The hydrogen atom ($H^{\bullet}$) also has a single electron and is highly reactive. It diffuses only a couple of molecular diameters before reacting with the closest cellular component and can thus act as a free radical (Powell et al., 2005). The reactivity of a superoxide radical is much lower than that of a hydroxyl radical. Superoxide radicals may also serve as precursors for other reactive oxygen species. Reactive oxygen species (ROS) is a term used of highly reactive compounds which can also act as free radicals. ROS include e.g. hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) (Halliwell et al., 1992, Powell et al., 2005). Free radicals can act by three different mechanisms, namely addition, donation and removal of electrons.

a) addition $x^{\bullet}+y \rightarrow [x–y]^{\bullet}$
b) electron donation $x^{\bullet}+y \rightarrow y^{\bullet-}+x^{+}$
c) electron removal $x^{\bullet}+y \rightarrow x^{-}+y^{\bullet+}$

The main characteristic of free radicals is their high reactivity. As their half-life is relatively short, most free radicals cause damage locally, close to their sites of production (Singal et al., 2000). The reactions of free radicals normally proceed in chain reactions: one radical begets another (Halliwell and Cross, 1994). When two radicals meet, the reaction can be terminated. Free radicals are able to bind most cellular components; they can react with unsaturated fatty acids in membrane lipids, damage DNA and denaturate proteins (Huang et al., 1999). Most of the toxicity of free radicals in vivo is thought to arise from reactions catalyzed by metal ions, such as Cu(II) and Fe(III), as first observed by Fenton in 1876 (Halliwell and Gutteridge, 2007, p. 40–42). Reactions where a metal ion acts as catalyst can be summarized as:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

ROS can be produced endogenously or exogenously. In vivo free radicals are formed during normal aerobic respiration, by activation of phagocytosing cells, in peroxisomes where fatty acids are degraded, and by auto-oxidation of various molecules (Frei et al., 1989, Halliwell, 2007). These endogenous radical production ways account for most of the oxidants produced by cells. Additional endogenous sources of cellular ROS are neutrophils, eosinophils and macrophages (Valko et al., 2006). Exogenously free radicals are formed e.g. by cigarette smoke, ionizing radiation, pollutants, organic solvents, anesthetic gases, hyperoxic environments and pesticides (Church and Pryor, 1985, Riley, 1994). It is estimated that in a given cell, 10^5 oxidative DNA lesions are formed each day (Powell et al., 2005).

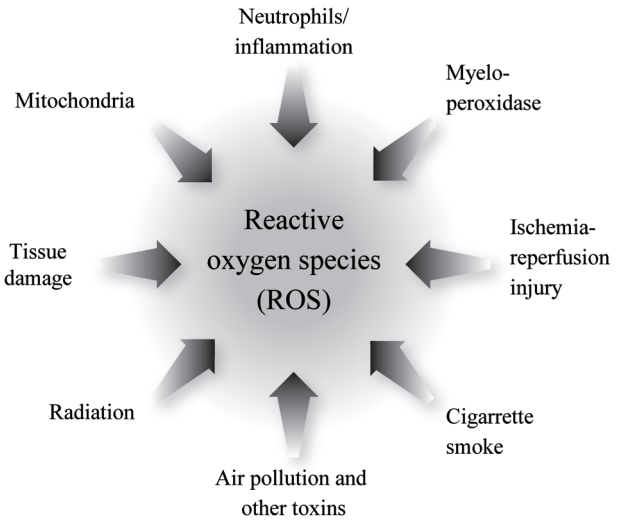

Figure 1. *Endogenous and exogenous sources of reactive oxygen species (ROS)*

Oxidative stress has been proved to play a significant role in carcinogenesis. ROS can act both anticarcinogenically e.g. by promoting cell-cycle stasis, apoptosis and necrosis, and inhibiting angiogenesis or, conversely, procarcinogenically, due to their ability to promote proliferation, invasiveness, angiogenesis and metastasis, and suppress apoptosis (Halliwell, 2007). Oxidative stress is defined as "the biomolecular damage caused by attack of reactive oxygen species upon the constituents of living organisms" (Halliwell and Gutteridge, 2007, p. 187).

Free radicals have, as mentioned, very short half-life and are therefore difficult to measure. Direct means of measuring free radicals include electron spin resonance and spin trapping methods (Baum et al., 2003). Free radicals can also be labeled chemically and analyzed by spectroscopic methods. However, the most common approach is to measure the derivates or end-products of oxidation processes such as lipid peroxidation products (Holley and Cheeseman, 1993). Indirect methods include

measurement of antioxidant levels or the total antioxidant status. Total antioxidant capacity (TAC) has been used to assess oxidative stress in the whole body (Wayner et al, 1985, Gönenç et al., 2001). There are numerous assays available, the results of which, do not however, correlate well with each other (Prior and Cao, 1999). In principle, a tissue or blood sample is added to a free radical-generating system, and the ability of the tissue or blood to resist oxidation is measured. Wayner and associates (1985) originally developed a test to measure the (secondary) antioxidant activity of plasma and introduced the abbreviation "TRAP", referring to "total peroxyl radical trapping antioxidant potential". This test is based on the amount of oxygen consumption during lipid peroxidation reaction, induced by thermal decomposition of an azo-compound, 2,2'-azobis (2-amidinopropane hydrochloride) (ABAP). The results are expressed as μmol of peroxyl radicals trapped by one liter of plasma. The separate contribution of ascorbic acid, uric acid and alpha-tocopherol to TRAP is assessed by measuring their stoichiometric factors.

2.2 Antioxidant defense system

According to the commonly accepted definition by Halliwell and Gutteridge (2007, p. 81), an antioxidant is "any substance that delays, prevents or removes oxidative damage to a target molecule". The human body contains a complex antioxidant defense system which depends on the dietary intake of antioxidants as well as the endogenous production of antioxidative compounds such as glutathione (Clarkson and Thompson, 2000). Antioxidants can act at different levels and by diverse mechanisms in the oxidative sequence (Halliwell and Gutteridge, 2007, p. 79–80).

Antioxidants can be classified into a number of different groups:

1. Antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR)
2. Antioxidative proteins: hemoglobin, ceruloplasmine, transferrin, albumin, lactoferrin
3. Small-molecular-weight compounds: ascorbic acid (vitamin C), tocopherols (vitamin E), glutathione (GSH), uric acid, selenium, bilirubin, glucose
4. Ubiquinone (coenzyme Q-10)
5. Flavonoids
6. Protein sulfhydryl (SH) groups (thiols)

(Duarte and Lunec, 2005, Halliwell and Gutteridge, 2007, p. 150–151)

The three major antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx or GSH-Px). SOD and CAT are proteins which act primarily in the cell cytoplasm and form the first line defense against oxidants, e.g. superoxide anion and hydrogen peroxide (Gago-Dominguez and Castelao, 2006). SOD exists in several isoforms with different active metals in the center and different amino acid constituency. In humans, three different forms of SOD are cytosolic-CuZn-SOD, mitochondrial Mn-SOD, and extracellular SOD. Fe-SOD exists in animals but not in humans. Though CuZn-SOD is located in most parts of the cell, Mn-SOD is the most important scavenger of $O_2^{\bullet -}$, converting it to hydrogen peroxide and oxygen (Halliwell, 2007). Thus CuZn-SOD and Mn-SOD catalyse the following reaction:

$$2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

The generated H_2O_2 is removed mainly by peroxiredoxins, thioredoxin-dependent peroxidase enzymes (Halliwell, 2007). CAT neutralizes hydrogen peroxide. SOD and CAT also function as anticarcinogenes by inhibiting the initiation and promotion phases in carcinogenesis (Kumaraguruparan et al., 2005, Halliwell, 2007).

Glutathione peroxidase (GPx) is a selenoprotein, and essential for the conversion of glutathione to oxidized glutathione. There are four different forms of GPx enzymes. Glutathione peroxidase reduces lipidic or non-lipidic hydroperoxides as well as H_2O_2 while oxidizing glutathione (Michiels et al., 1994).

$$H_2O_2 + 2\ GSH \rightarrow GSSG + 2\ H_2O$$

Glutathione reductase, also known as GSR or GR, reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, where NADPH is used as the reducing agent (Meister, 1988). Glutathione reductase is a flavoenzyme and an important predictor of general oxidant/antioxidant status (Halliwell and Gutteridge, 2007, p. 113–114).

MnSOD, CAT and GPx are all necessary for cell survival. In addition to SOD, CAT and GPx, there are numerous antioxidant enzymes reacting with and detoxifying compounds produced by oxidative insult (Sies, 1993). For example, glutathione S-transferase catalyses the conjugation of GSH to various endogenous and exogenous electrophilic compounds (Halliwell and Gutteridge, 2007, p. 118). The levels of different antioxidative enzymes are reportedly lower in the serum of cancer patients compared to healthy controls (Bakan et al., 2003).

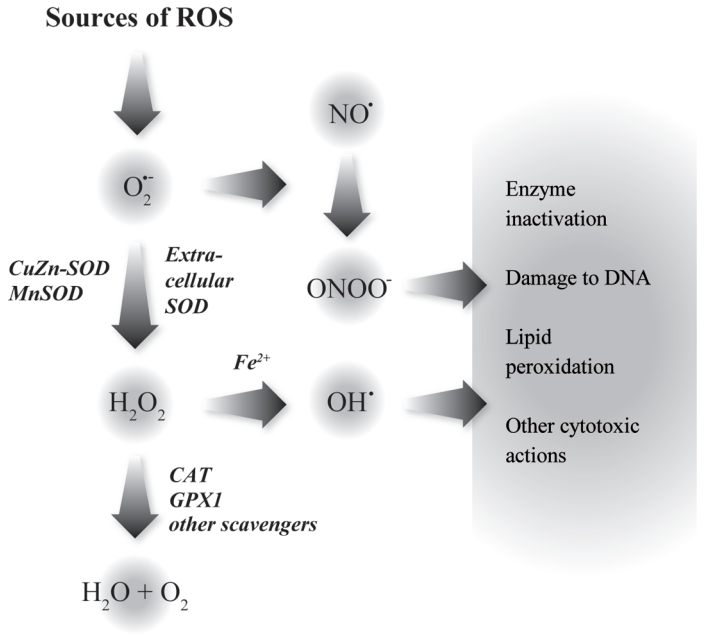

Figure 2. *Action of various antioxidative enzymes (SOD=superoxide dismutase, CAT=catalase, GPX1=glutathione peroxidase1)*

Transition metals include 38 different elements, among them iron, nickel, chromium, copper and lead. Essential micronutrients include cobalt, copper, chromium, fluorine, iron, iodine, manganese, molybdenum, selenium and zinc. Transition metals, except for copper, contain one electron in their outermost shell and can therefore act as free radicals. Also copper, due to its ability to gain and lose electrons, can act as a free radical. Iron and copper are common catalysts of oxidation reactions. Iron is essential to aerobic life and is the fourth most abundant element on Earth (Halliwell and Gutteridge, 2007, p. 38–39). Many transition metals play a key role in various biological processes by activating or inhibiting enzymatic reactions or competing with metalloproteins or other elements for binding sites. The transition metals may thus have indirect or direct effects on the carcinogenic process (Huang et al., 1999). Transition metal binding proteins act extracellularly (e.g. transferrin, haptoglobin, lactoferrin and hemopexin). In addition, ceruloplasmine and albumin bind free copper ions (Nappi and Vass, 2000). For instance, lactoferrin can bind two molecules of Fe^{3+} per molecule of protein and may thus prevent iron from undergoing free radical-mediated reactions. In the normal situation iron and copper are bound to these carrier proteins, but presentation of metal ions from cigarette smoke or air pollutants may circumvent this (Frei et al., 1989).

Of the antioxidants vitamin C, tocopherols, urate, glutathione and protein thiols were the primary variables in these studies. A more detailed description of these is therefore given in the following.

2.2.1 Vitamin C (ascorbic acid)

Vitamin C, ascorbic acid, is a water-soluble chain-breaking antioxidant, known to be the most effective aqueous phase antioxidant in human plasma (Frei et al., 1989). It plays a vital role in various biological processes (Duarte and Lunec, 2005). Ascorbic acid also inhibits lipid peroxidation, oxidation of low-density lipoproteins and protein oxidation (Frei, 1991 & 2004). The concentrations of ascorbic acid are much lower in plasma than in human tissues. Studies suggest that ascorbic acid is the first plasma antioxidant to be consumed after exposure to oxidants. Ascorbic acid is easily oxidized to dehydroascorbic acid, which is known to have effects similar to those of ascorbic acid in vivo (Frei et al., 1989). Ascorbic acid can react directly with superoxide, hydroxyl radicals and singlet oxygen. Vitamin C and E can also spare glutathione and prevent its oxidation (Duarte and Lunec, 2005). Vitamin C is important in recycling the tocopherol radical of vitamin E to an active reduced state (Niki, 1987). Of note, ascorbic acid may display pro-oxidant effects in the presence of free transition metal catalysts (Frei et al., 1989).

2.2.2 Vitamin E (tocopherol)

Vitamin E is the major lipid-soluble antioxidant present in lipid membranes and human plasma lipoproteins (Abiaka et al., 2001). It exists in 8 different isoforms, of which alpha-tocopherol is biologically the most important. Vitamin E is a strong inhibitor of apoptosis and a stabilizer of biological membranes and is known to act on all steps of membrane oxidative damage (Kolanjiappan et al., 2002, Gago-Dominquez and Castelao, 2006). Alpha-tocopherol functions in vivo as a strong protector against lipid peroxidation and also blocks nitrosamine formation (Slater, 1984, Niki et al., 1991).

Our previous findings would indicate that it is the final main antioxidant consumed after exposure to oxidants (Erhola et al., 1996). Even though alpha-tocopherol is the major or even the only lipid-soluble chain-breaking antioxidant in human plasma, it is responsible for only 2–3% of the total peroxyl radical trapping capacity (TRAP) of plasma (Burton et al., 1983, Uotila et al., 1994). Depending on the circumstances, alpha-tocopherols can either initiate or inhibit apoptosis (Bartsch et al., 2000). In the presence of iron, alpha-tocopherol may exert pro-oxidant effects, this, however, not being likely to occur in the human body since most transition metals are bound to proteins (Frei et al., 1989). Other functions of vitamin E include inhibition of cell adhesion, proliferation and protein kinase activity as well as enhancement of immunity and modulation of gene expression (Brigelius-Flohé et al., 2002).

2.2.3 Urate (uric acid)

Urate (uric acid) is present in high concentrations intra- and extracellularly. It is an important hydrophilic radical scavenger (Gil et al., 2006). Urate is the excreted end product of purine metabolism where xanthine oxidase catalyses the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both of these steps, molecular oxygen is reduced and superoxide anion and hydrogen peroxide, respectively, are formed. Urate is the principal constituent of adenosine triphosphate, DNA and RNA (Kumar et al., 2008).

Urate is a powerful scavenger of singlet oxygen, peroxyl radicals and $OH^{\bullet}$ radicals (Ames et al., 1981). It is the main contributor to human total antioxidant capacity (TRAP) (Wayner et al., 1987, Uotila et al., 1994). It protects unsaturated fatty acids from oxidation and may act by preserving ascorbic acid (Davies et al., 1986). Since the concentration of urate in the plasma is 5–10 times higher than that of ascorbic acid, urate and ascorbic acid may be the first antioxidants to be depleted after exposure to oxidants (Ames et al., 1981, Cross et al., 1992). Urate is also an iron chelator and may thus protect ascorbic acid from iron-catalysed generation of reduced iron (Halliwell and Gutteridge, 2007, p. 157).

2.2.4 Thiols and glutathione (GSH)

Thiols, molecules which contain -SH groups, include for example thioredoxins, cysteine and reduced glutathione. Albumin is the major component of total -SH groups in the plasma. Thiols are essential for overall protein function (Halliwell and Gutteridge, 2007, p. 120, Cengiz et al., 2008). They are nucleophilic and react avidly with free radicals. They may protect molecules from injury caused by radiation by donating hydrogen (Navarro et al., 1997). It has been suggested that although plasma protein thiols easily become oxidized after exposure to oxidants, they are insufficient radical scavengers and are mainly consumed by auto-oxidation (Frei et al., 1989).

Reduced glutathione (GSH) is a tripeptide with a free thiol group (-SH group) which consists of three amino acids joined together (glutamic acid, cysteine and glycine). It is highly abundant in the cytosol, nuclei and mitochondria (Valko et al., 2006). Oxidized glutathione (GSSG) is formed by joining two reduced glutathione molecules by their -SH groups. Glutathione peroxidase catalyses the reduced form of glutathione (GSH) to

the oxidized form (GSSG). It is thought that only less than 0.2% of the total GSH exists as GSSG, as some GSH is bound to low-molecular-weight compounds and proteins (Meister, 1988). The synthesis of glutathione takes place mainly in the cytoplasm. The rate-limiting enzyme for GSH synthesis is glutamate-cysteine ligase, which is found to be highly expressed in non-small cell lung cancer (Soini et al., 2001).

Glutathione has multiple functions: it modulates cell proliferation and plays a key role in protecting cells against oxidants (Navarro et al., 1999, Kumaraguruparan et al., 2005). GSH has functions in catalysis, metabolism and transport, and is involved in protein synthesis (Meister, 1988). Conjugation with GSH has been suggested as a primary mechanism for the detoxification of lipid peroxidation products (Gago-Dominquez and Castelao, 2006). The redox system of GSH consists of primary and secondary antioxidants (glutathione peroxidase, glutathione reductase, glutathione-S-transferase, glucose-6-phosphate dehydrogenase). GSH is also held to protect against radiation-induced cell damage and adverse events caused by a number of chemotherapeutic regimens including cisplatin (Navarro et al., 1999, Pelicano et al., 2004). Higher levels of GSH have been found in NSCLC tumor specimens compared with lung tissue (Blair et al., 1997). Interestingly, the drug resistance of tumors and increased thiol levels have been noted to have an association (Meister, 1988).

Table I. *Major antioxidant defenses of human blood*

Antioxidant	Solubility	Concentration	Deficiency	Function
Vitamin C (ascorbic acid)	water-soluble	30-90 μM in human plasma	scurvy	- inhibits lipid peroxidation - a powerful scavenger of $^1O_2$, HOCl, O_3, $NO_2^{\bullet}$ - scavenges $O_2^{\bullet}$, $HO_2^{\bullet}$, $OH^{\bullet}$, $RO_2^{\bullet}$, thiyl and oxysulphur radicals - co-operates with vitamin E - protects against phagocytic adhesion to endothelium induced by oxidized LDL *(Frei et al, 1989, Frei 1991, Yoshida et al., 2003, Frei 2004, Duarte and Lunec, 2005, Halliwell and Gutteridge, 2007)*
Vitamin E (exists in 8 isoforms: α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols)	lipid-soluble	20–35 μM in plasma in adults, located in the interior of membranes and in lipoproteins	short-term shortage in humans causes no symptoms although increases susceptibility of membranes to peroxidation	- powerful inhibitor of lipid peroxidation - quenches and reacts with singlet oxygen - interacts with vitamin C - γ-tocopherol induces apoptosis in prostate cancer cells - enhances immunity, inhibits cell adhesion and proliferation (*Slater, 1984, Mirvish 1986, Niki et al., 1991, Yoshida et al.,* 2003, *Halliwell and Gutteridge, 2007*)
GSH (glutathione)	water-soluble	0.1–10 μM in human plasma (99% as GSH and 1% in reduced form, GSSG)	defects in GSH metabolism cause hemolysis and neurological disorders, GSH synthetase deficiency causes acidosis and possibly brain damage, glutathione reductase deficiency exhibits cataract formation	- major intracellular antioxidant - mediator of several physiological reactions including cellular signaling - involved in the metabolism of ascorbic acid - prevents protein SH groups from oxidizing and cross-linking - reacts with $OH^{\bullet}$, HOCl, $ONOO^-$, $RO^{\bullet}$, $RO_2^{\bullet}$, $NO_2^{\bullet}$ - can protect cells against heavy metals, irradiation, oxidation and toxic compounds (*Meister, 1988, Kumaraguruparan et al., 2005, Halliwell and Gutteridge, 2007, Forman et al., 2009*)
Coenzyme Q 10 (ubiquinone)	lipid-soluble	0.4–1 μM in human plasma	primary coenzyme Q 10 deficiency may cause neurological and muscular symptoms, renal disease	- present primarily in mitochondria where plays an important role in electron transport - may act as antioxidant - protects against lipid peroxidation *(Diomedi-Camassei et al., 2007, Halliwell and Gutteridge, 2007)*
Urate (uric acid)	limited solubility to water	0.2–0.4 mM in plasma, also present in many body fluids	decreased levels seen in liver diseases and some cancers such as breast and gastrointestinal cancer	- powerful scavenger of ROS - reacts avidly with peroxynitrite - can chelate metal ions *(Ames et al., 1981, Abiaka et al., 2001, Halliwell and Gutteridge, 2007)*
β-carotene	lipid-soluble	0.2–0.6 μM in human plasma (levels vary with diet)	dry skin, susceptibility to infection and night blindness as deficiency of vitamin A	- precursor of vitamin A - inhibits lipid peroxidation *(Halliwell and Gutteridge, 2007)*

2.3 Antioxidants in the respiratory tract

The lung is a multifunctional organ which has a role in the exchange of gases while at the same time the mucosal surface interacts with external oxidants. Bronchoscopy and bronchoalveolar lavation (BAL) are the only tools to examine local conditions of the alveolar level of the lungs. Nowadays, BAL is widely accepted as a means to diagnose various pathological conditions. However, in clinical studies, bronchoscopy and BAL are rarely performed due to their laboriousness. BAL provides information of the cellular and non-cellular components and oxidative status on the first line defense system of the lungs, the respiratory epithelium (Schock et al., 2003).

The alveolar space of the lungs includes type I and II pneumocytes and endothelial cells. These cells lie on the basement membrane and the intestinal tissue, which consists of elastic fibers and mesenchymal and inflammatory cells, which are in contact with capillary vessels. Type II pneumocytes secrete the protein components in the surfactant (Abratt and Morgan, 2002, Halliwell and Gutteridge, 2007, p. 17). The epithelial cells of the trachea are the first cells of the lung to encounter toxic products, e.g. air pollution or tobacco smoke (Shull et al., 1991). Macrophages and lymphocytes are the major cell populations within the BAL fluid (Sutinen et al., 1995). The kinetics of the antioxidants in the respiratory tract lining fluid (RTLF) has remained markly understudied (Cross et al., 2002). For example, it is still not known how nutritional antioxidants or antioxidants delivered directly by the airway reach RTLF.

RTLF contains a variety of antioxidative enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. It also contains vitamins C, E and A and other antioxidative compounds such as albumin, ceruloplasmine and transferrin (Cross et al., 1994). The concentration of glutathione is over 100-fold higher in the epithelial lining fluid than in the blood (Cantin and Bégin, 1991). It is known that the antioxidants in RTLF are not evenly distributed in the various areas of the respiratory tract. Proximally, where thicker airways exist, RTLF contains large quantities of uric acid, whereas distal RTLFs are thinner and are rich in ascorbic acid and glutathione (Cross et al., 1998, Van der Vliet and Cross, 2000). It has been speculated that ambient pollutant gases such as ozone or cigarette smoke react with RTLF components and do not actually reach the underlying respiratory tract epithelial cells (Cross et al., 2002). It has been shown that ozone reacts rapidly with ascorbic acid and uric acid, and to a lesser extent with glutathione. Ascorbic acid and uric acid may thus constitute the first line defense system in RTLF against external oxidants (Mudway et al., 2001). It has also been proved that the epithelial cells of the respiratory tract respond to different oxidative attacks by selective induction of antioxidant enzymes (Shull et al, 1991).

A balance has to be maintained between the normal encounter with oxidants (through respiration) and antioxidant defense mechanisms. The term “respiratory burst” is used when invading neutrophils, macrophages and eosinophils induce the production of singlet oxygen and hydrogen peroxide, which damage the surrounding tissues and enhance production of ROS (Cooke et al., 2003).

2.4 Lipid peroxidation

Lipid peroxidation is a form of oxidative damage in cell membranes determined as free radicals reacting with polyunsaturated fatty acids (Lopaczynski and Zeisel, 2001). Polyunsaturated fatty acids (PUFAs) are abundant in cellular membranes and also in low-density lipoproteins (LDL). They comprise mainly n-3 and n-6 PUFAs, and are especially sensitive to free radicals (Feurgard et al., 1998, De Zwart et al., 1999). The interaction of ROS and lipids consists of three different steps: initiation, propagation and termination. In the initiation phase, conjugated dienes are formed as hydrogen atom is abstracted from a lipid methylene group. Conjugated dienes absorb ultraviolet light at 230–235 nm, and their measurement is regarded as an accurate and repeatable marker of lipid peroxidation (Clarkson and Thompson, 2000). The molecular oxygen reacts with carbon-centered free radicals and thus lipid hydroperoxides (LOOH) are formed (Zieba et al., 2000). LOOH may cause alterations in membrane structure and function which, however, may be reversible (Lopaczynski and Zeisel, 2001). LOOH are formed earlier in the pathway leading to the formation of malondialdehyde (MDA) and are the source of highly reactive aldehydes (Urso and Clarkson, 2003). The aldehydes, for example MDA and 4-hydroxynonenal (HNE), have the potential to modify DNA and proteins. They are also capable of inducing apoptosis or necrosis in various cells (Lopaczynski and Zeisel, 2001). Measuring MDA levels in the plasma or serum provides a suitable in vivo index of lipid peroxidation (Morabito et al., 2004). TBA (thiobarbituric acid) reacts with numerous chemical species (including nucleic acids, amino acids, proteins, phospholipids and aldehydes) when heated under acidic conditions and produces a typical pink chromophore which can be measured by UV or fluorescence detection (Seljeskog et al., 2006).

Lipid peroxidation markers can also be examined non-invasively. Peroxidation of n-3 and n-6 polyunsaturated fatty acids in cellular membranes results in the formation of ethane and pentane, respectively (Kneepkens et al., 1994, Feurgard et al., 1998). Both are volatile hydrocarbons readily excreted in the breath (Kneepkens et al., 1994, Mohler et al., 1996). Quantitation of exhaled pentane is a particularly accurate means to measure in vivo lipid peroxidation, as the fatty acids from which exhaled pentane is derived are abundant in cell membranes (Clarkson and Thompson, 2000). However, there are large variations between exhaled pentane results reported in different studies. Technical differences and ambient air composition may partly explain this (Clemens et al., 1983). One study suggests that exhaled ethane and pentane correlate with other markers of lipid peroxidation, for example conjugated dienes, malondialdehyde and TBARS (Riely et al., 1974).

Numerous in vivo studies have supported the view that lipid peroxidation has a key role in carcinogenesis (Gago-Dominguez and Castelao, 2006). Also the production of ROS by circulating inflammatory cells in tumor tissues might promote lipid peroxidation, which also additionally gives rise to active oxygen species (Seven et al., 1999).

2.5 Protein oxidation

Different markers of protein oxidation include protein carbonyl derivates, oxidized amino acid side chains, protein fragments, and formation of advanced glycation end products (Lopaczynski and Zeisel, 2001). Protein carbonyl derivates are formed early in the oxidation process and are generated as the peptide main chain is cleaved or as some amino acid side chains (arginine, lysine, proline, or threonine) are oxidized (Berlett and Stadtman, 1997, Dean et al., 1997, Lopaczynski and Zeisel, 2001, Morabito et al., 2004). The carbonyl groups are relatively stable (Morabito et al., 2004).

Protein oxidation may result in loss of protein function as well as increased degradation of soluble proteins catalysed by the proteasome pathway (Berlett and Stadtman, 1997, Orlowski, 1999). Protein oxidation has also been shown to be a chain reaction and may be inhibited by chain-breaking antioxidants (Neuzil et al., 1993, Benderitter et al., 2003). It is thought, however, that protein damage can be repaired and is a non-lethal event for the cell (Valko et al., 2006).

Despite active research on protein oxidation in other fields of medicine, knowledge of the role of protein oxidation in carcinogenesis remains scant. Relatively few studies, moreover, have examined protein oxidation among cancer patients. One such, investigating Hodgkin´s lymphoma patients, revealed that cancer patients have higher circulating protein carbonyl groups compared to healthy controls (Morabito et al., 2004). It has been suggested that membrane damage during radiotherapy may be partly due to protein oxidation (Lenz et al., 1999).

The most frequently employed mode of analysis of oxidative protein damage is measurement of the formation of protein carbonyl groups as a reaction between free radicals, e.g. hydroxyl radical with amino acid side groups. Protein carbonyls are most accurately measured by HPLC or ELISA, although also other methods have been applied (Dean et al., 1997, Urso and Clarkson, 2003). However, there has been criticism of the analytical methods applied. Elevated levels of protein carbonyls are considered to reflect the amount of oxidative stress, but may also be a sign of protein dysfunction caused by the disease itself (Dalle-Donne et al., 2003).

2.6 Oxidative damage to DNA

DNA is especially sensitive to damage due to its potential to create cumulative mutations which may disrupt cellular homeostasis (Powell et al., 2005). ROS have the ability to cause permanent structural changes in DNA, as base-pair mutations, deletions, insertions, rearrangements and sequence amplification (Cerutti, 1994). The most common site for point mutations is the guanine-cytosine (G-C) pair (Halliwell and Gutteridge, 2007, p. 222). Continuous oxidative damage to DNA may lead to alterations in signaling cascades or gene expression, may induce or arrest transcription, and may cause replication errors and genomic instability (Powell et al., 2005). Free radical reactions with DNA may eventually lead to transformation of the epithelium from normal to malignant (Burcham, 1998).

DNA can be damaged in numerous ways, e.g. as a consequence of radiation or chemotherapy. Following damage to DNA, a number of purine- and pyrimidine-derived compounds are formed, and the repair products of these compounds are excreted

in the urine. Over 100 oxidative DNA adducts and more than 20 oxidized purine and pyrimidine bases have hitherto been identified (Powell et al., 2005). 8-oxo-7,8-dihydroguanine (8-oxoGua) and its 2´-deoxynucleoside equivalent, 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodG) are the most abundant and frequently studied oxidative DNA lesions (Rozalski et al., 2004). Measurement of urinary 8-oxoGua and 8-oxodG are considered reliable markers of oxidative damage to DNA available in clinical use (Shigenaga et al., 1994).

2.6.1 Repair of oxidative damage to DNA

The majority of oxidatively damaged DNA bases are repaired by the base excision repair (BER) pathway. BER involves four steps: 1) removal of the oxidized base by DNA glycosylase 2) removal of the deoxyribose phosphate group from the AP site by AP endonuclease resulting in a single nucleotide gap 3) filling of this gap with a DNA polymerase and 4) sealing of the strand break and completion of the repair process by DNA ligase (Seeberg et al., 1995, Elliott et al., 2000). There are short-patch and long-patch BER repairs, which differ by the enzymes involved and the number of nucleotides removed (Elliott et al., 2000, Powell et al., 2005). Radiation-induced base damage and single-strand breaks are repaired by the BER pathway (Meyn et a1., 2009). Other DNA repair mechanisms include nucleotide excision repair (NER) and mismatch repair; e.g. some forms of radiation-induced base damage are repaired by the mismatch repair pathways. Non-homologous end-joining (NHEJ) and homologous recombination (HRR) are responsible for the repair of double-strand breaks (Meyn et al., 2009). There is evidence that the induction of DNA repair enzymes occurs as a response to oxidative stress (Powell et al., 2005).

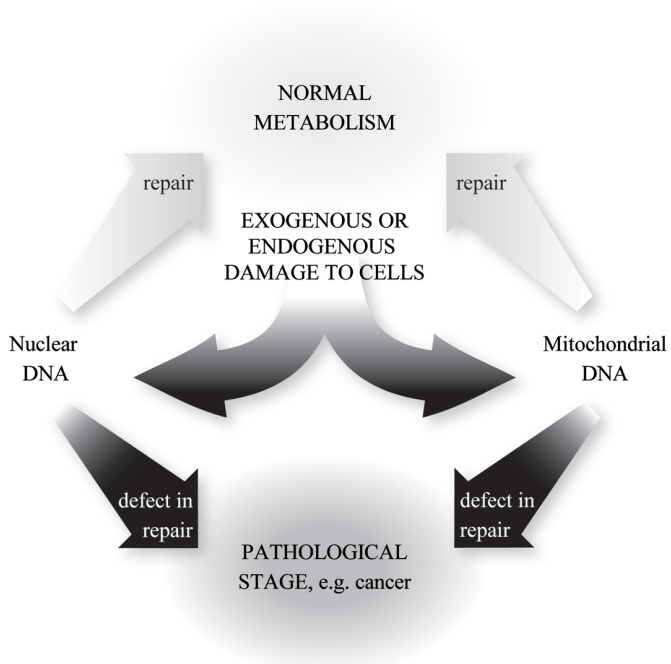

Figure 3. *Schematic representation of normal cellular metabolism, oxidative damage to DNA and repair of damage*

2.7 Nitrosative stress

Nitric oxide ($NO^{\bullet}$) is a free radical formed in various cell types by nitric oxide synthases (NOS). It is involved in many physiological and pathological processes: it has a role in neurotransmission, blood pressure regulation, various defence mechanisms, relaxation of smooth muscle cells and immune regulation (Valko et al., 2006). $NO^{\bullet}$ is soluble in both aqueous and lipid media. It has an extremely short half-life, only a few seconds in an aqueous environment, and is thus a reactive molecule which can either be oxidized or form complexes with other molecules (Metzger et al., 2006). In plasma and other physiological fluids $NO^{\bullet}$ is oxidized to nitrite (NO_2), whereas in whole blood $NO^{\bullet}$ and nitrite are oxidized to nitrate (NO_3) (Bryan and Grisham, 2007). The blood $NO^{\bullet}$ level does not necessarily reflect the $NO^{\bullet}$ status of the tissues. However, measuring plasma nitrite has been considered an index of endogenous production of $NO^{\bullet}$ (Metzger et al., 2006).

Endogenous nitric oxide is a double-edged molecule, acting as an important physiological signaling molecule mediating various cellular functions; on the other hand it induces cytotoxic and mutagenic effects when present in excess (Kaynar et al., 2005). Nitric oxide has an important role in the initiation of apoptosis in various cell types (Ellis et al., 1998). It has been shown that increased production of nitric oxide may protect cells from oxidative stress (Gönenç et al., 2006). In contrast, tumor-associated nitric oxide has pleiotrophic effects of the formation of cancer, tumor growth and metastasis (Xie, 2001).

Nitric oxide can react with the superoxide anion ($O_2^{\bullet-}$), which produces the peroxynitrite anion ($ONOO^-$). This reaction is one of the fastest in human biology (Forman et al., 2009). Peroxynitrite is itself cytotoxic and can decompose to a hydroxyl radical or nitrogen dioxide (Taysi et al., 2003). Peroxynitrite is a powerful oxidant and may trigger lipid peroxidation, inhibit mitochondrial electron transport, oxidize thiol compounds and oxidize and nitrate DNA (Taysi et al., 2003, Powell et al., 2005, Valko et al., 2006). The reactive nitrogen species (RNS) play a significant role in DNA damage, especially in inflammation. The reaction of peroxynitrite with guanine results in the formation of 8-nitroguanine (8-NG) (Halliwell, 2007). Interactions of proteins with peroxynitrite result in nitration of tyrosine and subsequently nitrotyrosine synthesis. Overproduction of reactive nitrogen species is referred as nitrosative stress (Klatt and Lamas, 2000), although the term “oxidative stress” is widely used to cover also nitrosative stress (Halliwell and Gutteridge, 2007, p. 32).

2.8 Free radicals and formation of cancer

In recent years the relationship between cancer and oxidative stress has been extensively studied. The pathogenesis of cancer is a multistage process which involves mutations in critical genes required for maintenance of the cellular homeostasis (Powell et al., 2005). Oxidative mechanisms have a a role in the initiation, promotion and progression of carcinogenesis (Toyokuni et al., 1995, Cooke et al., 2006). Oxidative DNA modifications are more common in cancerous tissues than in surrounding cancer-free tissues, which reinforces the conception that ROS play a role in the development of cancer (Jaruga et al., 1994). Persistent oxidative damage to DNA or impairment of antioxidant defense

systems have been linked to mutation, activated transcription factors, modification of gene expression and chromosomal aberrations, i.e. genomic instability, processes which have been described in the progressions of cancer (Toyokuni et al., 1995, Morabito et al., 2004).

Inflammation is known to cause DNA alterations (Cooke et al., 2003). Chronic inflammation due to infection or injury is estimated to contribute to 25% of all cancers in the world (Coussens and Werb, 2002). Various chemical carcinogens such as chlorinated compounds, metal ions, barbiturates, phorbol esters, aromatic hydrocarbons and some peroxisome proliferators have been shown to induce oxidative stress and damage to DNA. They may therefore partly account for the development especially of work-related cancers. Very rare hereditary diseases such as Xeroderma pigmentosum, Fanconi´s anemia and Cocknay´s syndrome are also associated with an increased cancer risk due to deficiencies in nucleotide excision repair (NER) (Powell et al., 2005).

Many cancers are associated with increased production of ROS (Toyokuni et al., 1995, Cooke et al., 2006). The increased oxidative stress in cancer may be attributable to a variety of factors:

1) increased formation of ROS when the antioxidative defense mechanism works normally
2) unchanged status of exposure to ROS while the antioxidant defense mechanisms are decreased
3) failure in repair of oxidative damage, which leads to increased presence of ROS
4) combination of the above (Halliwell, 2007).

2.9 Cytokines

Cytokines are soluble proteins, peptides or glycoproteins produced by the mononuclear cells of the immune system (usually lymphocytes or monocytes). They have low molecular mass, usually <80 kDa, and many of them are glycated (Whicher and Evans, 1990). Cytokines belong to the signaling molecules and have an important task in cellular communication. They are also involved in several processes during embryogenesis and are critical for the development and function of innate and adaptive immune responses. Certain cytokines, for example interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNFα), have a central role in myeloid cell proliferation and differentiation. Many cytokines are multifunctional and have diverse actions and target cells. They also interact with each other at cellular level (Whicher and Evans, 1990, Halliwell and Gutteridge, 2007, p. 218–219) Among others, IL-1, IL-2, IL-6, IL-8 and TNFα are cytokines which have a role in the regulation of inflammation (Whicher and Evans, 1990). Tumors often induce inflammation and thus secrete cytokines which recruit phagocytes and lymphocytes. As known, inflammation can also promote cancer development (Halliwell, 2007). Each cytokine has a cell-surface receptor through which they exert their actions (Whicher and Evans, 1990).

Cytokines are important in several biological processes in malignant tumors. They are produced by both normal and tumor cells, and can act as paracrine and autocrine tumor cell growth factors (Whicher and Evans, 1990, Whicher and Banks, 1995). There are numerous ways by which cytokines act. They facilitate tumor growth, invasion and metastasis by inducing DNA damage or inhibition of DNA repair through ROS, by

inactivation of tumor suppressor genes, modulation of cell:cell adhesion molecules or by influencing tissue remodeling via the action of matrix metalloproteinases. They may also have direct or indirect control over tumor cell migration (Balkwill and Mantovani, 2001). On the other hand, cytokines may also possess growth-inhibitory qualities (Whicher and Banks, 1995).

TNFα is synthesized by monocyte/macrophage cell lines and lymphocytes. It interacts with other cytokines and hormones, and participates in immune stimulation, acute-phase response and inflammatory events. TNFα activates cellular functions and produces various cell-proliferative effects, and also exerts cytolytic and cytostatic effects (Whicher and Evans, 1990, Whicher and Banks, 1995). However, TNFα also evinces protumor effects (Whicher and Banks, 1995).

A large body of evidence has been gathered on cytokines in various cancers (Whicher and Banks, 1995). In this study, we decided to concentrate on some of the most common cytokines related to tumor biology, namely, vascular endothelial growth factor (VEGF), TNFα, IL-1β, IL-6, IL-8, IL-12 and IL-18.

2.9.1 Interleukins

Immunomodulating agents such as interferons and interleukins (IL), belong to the cytokines. New interleukins are continually being discovered: to date more than 30 have been identified. The latest cytokine is IL-35, whose receptor is as yet unknown (Collison et al., 2007). Mostly interleukins are synthesized, in addition to monocytes, macrophages and endothelial cells, by helper CD4+ T lymphocytes. Also chemokines belong to the cytokine group. These mediate chemoattraction (chemotaxis) between cells.

IL-1β is a monocyte-derived T-cell-promoting factor. It promotes antigen-specific immune responses and inflammation (Whicher and Evans, 1990).

IL-6 is secreted by monocytes in a wide variety of molecular forms with molecular masses between 21.5–28 kDA. In addition, it can be synthesized by many cell types, for example T-cells, macrophages and stromal cells (Heikkilä et al., 2008). It is a pleiotropic cytokine which acts in inflammation and antigen-specific immune response. The primary function of IL-6 is to induce acute-phase protein production in the liver as well as the final maturation of B-cells (Whicher and Evans, 1990). IL-6 exhibits synergism with other cytokines, e.g. IL-1 and TNFα, and may be also induced by other cytokines. IL-6 is known to be produced by several types of cancer cells, e.g. renal carcinoma and melanoma cells, and might have a potential as a prognostic marker in these cancers (Whicher and Evans, 1990, Giri et al., 2001). IL-6 is also synthesized by a number of cells in the lung parenchyma, among them alveolar macrophages, T lymphocytes, type II pneumocytes and lung fibroblasts (Chen et al., 2002). According to one review, elevated levels of circulating IL-6 concentrations are seen in various tumor types when compared to healthy controls. However, the relevance of IL-6 in the etiology of cancer is still unknown (Heikkilä et al., 2008).

IL-8 is the only chemokine originally named interleukin. It also belongs to the inflammatory cytokines. It is a potent neutrophil chemotactic factor, promoting neutrophil adhesion, migration and degranulation (Xie, 2001). IL-8 has been thought to have a role in enhancing tumor cell proliferation, tumor cell motility and adhesion as well as neoangiogenesis (Nacinović-Duletić et al., 2008). IL-8 may be produced

by various normal and tumor cells and might be involved in tumor angiogenesis (Xie, 2001).

IL-12 belongs to the family of heterodimeric cytokines produced by phagocytic cells, dendritic cells and B cells. It has a wide range of immunoreactive activities such as activation of NK cells and induction of T-helper cells enhancing the activities of cytotoxic lymphocytes (Colombo and Trinchieri, 2002).

IL-18 is a proinflammatory cytokine with an important role in autoimmune and inflammatory diseases. Among other functions, IL-18 induces the production of Th1 cytokines and is involved in the cell-mediated cytotoxicity of T- and NK- cells. IL-18 is also related to IL-1β. It induces innate defense against tumors in mice (Dinarello, 1999).

Two different sources of enhanced production of interleukins have been proposed as possible mechanisms following irradiation: 1) if the tumor does not respond to the treatment, the stromal cells of the tumor will continue to produce various cytokines; 2) the cell injury caused by irradiation may trigger the production of different cytokines (Ganellin and Triggle, 1997, p. 192). It has further been shown that the number of macrophages increases shortly after irradiation and these have been evidenced to produce various cytokines (Rabbani et al., 2005).

2.9.2 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a sub-family of growth factors which plays an important role as a signaling protein related to angiogenesis. Angiogenesis is critical for tumor development and growth and also necessary for metastasis. Even though platelet-derived growth factor and fibroblast growth factor are involved in angiogenesis, VEGF plays a central role in this process (Zhan et al., 2009). The term VEGF covers a number of proteins from two different families: the human VEGF family consists of VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF). The VEGF receptor family includes three protein-tyrosine kinases and two non-protein kinase receptors (neuropilin-1 and 2) (Roskoski, 2007). All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (Valko et al., 2006). Increased expression of VEGF has been found in most human tumors, including NSCLC (Beinert et al., 2000).

VEGF-A, commonly known as VEGF, is a 45 kDa homodimeric glycoprotein with diverse angiogenic activities. It is an endothelial cell-specific mitogen and has two receptors. VEGF is one of the most potent and specific angiogenic molecules, implicated in endothelial cell proliferation and migration as well as in increased vascular permeability (Dvorak et al., 1995, Ferrara and Davis-Smyth, 1997).

2.10 Previous studies on the effect of cytokines and oxidative stress in cancer

In recent years there has been growing interest in the role of free radicals in cancer. The role of oxidative stress in relation to the initiation and progression of a large variety of cancers has been investigated and, based on the findings, it has been suggested that oxidative stress plays an important role in carcinogenesis by means of induction of mutagenesis (Akbulut et al., 2003).

Lungs from cancer patients contain 25–75 8-oxodG/10^5 deoxyguanosine in the apparently normal tissue and two- to three-fold higher values in the lung tumor, in addition to several other oxidative DNA modifications (Olinski et al., 1992). Some tumor lines have been shown to produce significant amounts of hydrogen peroxide, which might be responsible for the increased oxidative damage to DNA seen in cancer (Cooke et al., 2003).

It has previously been shown that lung and breast cancer patients have approximately 50% higher levels of modified DNA base (8-oxoGua) excreted in the urine compared to controls, though no changes were seen in the nucleoside form (8-oxodG) (Olinski et al., 2002). A number of studies of lung, colorectal, gynecological, bladder, prostate, breast cancer and lymphoma patients provide evidence for elevated urinary marker levels of oxidative damage to DNA (Tagesson et al., 1995, Yamamoto et al., 1996, Erhola et al., 1997b, Rozalski et al., 2002, Chiou et al., 2003, Miyake et al., 2004, Mei et al., 2005).

A growing body of evidence suggests that cancer causes disturbances in oxidant/antioxidant balance and induces lipid peroxidation. Elevated levels of TBARS with concomitant decreases in several antioxidative enzymes are found in cervical, oral squamous cell, breast and prostate cancer and Hodgkin´s lymphoma patients compared to controls (Kolanjiappan et al., 2002, Manju et al., 2002, Subapriya et al., 2002, Morabito et al., 2004, Gonçalves et al., 2005, Yeh et al., 2005, Aydin et al., 2006). As far as lung cancer is concerned, a number of studies have shown that cancer patients present with increased serum lipid peroxidation marker levels compared to healthy controls (Look and Musch, 1994, De Cavanagh et al., 2002, Taysi et al., 2003, Kaynar et al., 2005, Mahdavi et al., 2009).

Our previous findings suggested that lung cancer is associated with reduced levels of TRAP and its components, except for uric acid (Erhola et al., 1997a). Other studies have likewise found reduced levels of various antioxidants and antioxidative enzymes in lung cancer patients (Polat et al., 2002, Bakan et al., 2003, Kaynar et al., 2005). However, measurements of the levels of antioxidative enzymes in tumors have yielded inconclusive results (Cerutti, 1994).

Several studies have investigated bronchoalveolar lavage (BAL) fluid to assess the oxidant/antioxidant balance in the lungs in various benign pulmonary diseases (Schock et al., 2003, Drost et al., 2005, Starosta et al., 2006, Emad A and Emad V, 2007). However, only a few studies have examined the local RTLF status of lung cancer patients. Increased glutathione and reduced superoxide dismutase levels are reported in the BAL fluid of lung cancer patients compared to non-cancer controls (Melloni et al., 1996). The levels of vascular endothelial growth factor (VEGF) are also elevated in the BAL fluid of lung cancer patients compared to healthy controls (Beinert et al., 1999). Arias-Diaz and colleagues (1994) detected elevated levels of TNFα, IL-6 and NO_3 in the BAL fluid of lung cancer patients compared to healthy controls. Another study identified higher IL-6 and IL-1β levels in the BAL fluid of lung cancer patients compared to patients with benign lung disease (Matanić et al., 2003). Also elevated serum levels of various cytokines are reported in several lung cancer studies (Yanagawa et al., 1995, Martín et al., 1999, Mantovani et al., 2000). The major studies concerning antioxidants, cytokines and markers of oxidative stress in cancer are listed in Table II.

Table II. *Main studies on antioxidants, cytokines, oxidative and nitrosative stress markers in cancer patients (ca=cancer, pts=patients, BAL fluid=bronchoalveolar lavage fluid, TAC=total antioxidant capacity, IL=interleukin, TNFα=tumor necrosis factor alpha, sIL-2R=serum interleukin-2 receptor)*

Reference	Number of pts	Tumor type	Parameters measured	Observed significant differences (p values not given)
Abiaka C et al. (2001)	90 pts, 110 sex- and age-matched controls	36 gastrointestinal, 28 breast, 26 other cancer	Plasma alpha-tocopherol, urate, total cholesterol	Alpha-tocopherol, urate and alpha-tocopherol/cholesterol ratios ↓ in ca pts vs. controls
Arias-Díaz J et al. (1994)	22 pts, 8 subjects without cancer	lung cancer	BAL fluid TNFα, IL-1 and IL-6, NO, phosphatidylcholine levels	BAL fluid TNFα levels, IL-6 and NO_2^-/NO_3^- ↑ in ca pts vs. controls, phosphatidylcholine levels ↓ in ca pts vs. controls
Bakan N et al. (2003)	21 pts, 20 healthy controls	chronic lymphocytic leukemia	Serum glutathione peroxidase (GSH-Px), glutathione reductase, CuZn superoxide dismutase (CuZn-SOD), GSH, NO, MDA	GSH-Px, CuZn-SOD activities, GSH ↓ in ca pts vs. controls, NO and MDA ↑ in ca pts vs. controls
Battisti V et al. (2008)	80 pts, 50 healthy controls	acute lymphoblastic leukemia	Plasma TBARS, thiols, serum vitamin E, protein carbonyls, blood CAT, SOD	TBARS and protein carbonyls ↑ in ca pts vs. controls
Burgaz S et al. (1996)	94 pts, 92 age-, sex- and smoking-matched controls	13 lung, 21 breast, 26 head and neck, 17 genitourinary, 17 other cancers	Serum total carotenoids, uric acid	Carotenoids ↓ in ca pts vs. controls, uric acid ↑ in ca pts vs. controls
Calişkan-Can E et al. (2008)	39 pts, 31 healthy controls	lung cancer	Serum MDA, β-carotene, retinol, vitamin C and E, urine 8-OHdG	Urine 8-OHdG, serum MDA ↑ in ca pts vs. controls, β-carotene, retinol, vitamin C and E ↓ in ca pts vs. controls
Dalaveris E et al. (2009)	30 pts, 15 age- and gender-matched healthy controls	lung cancer	Serum and exhaled breath concentrate (EBC) TNFα, VEGF, 8-isoprostane	Serum and EBC TNFα ↑ in ca pts vs. controls, serum VEGF and serum 8-isoprostane ↑ in ca pts vs. controls
Diakowska D et al. (2008)	75 pts, 79 healthy controls	esophageal cancer	Serum TBARS, total antioxidant status (TAS), 8-OHdG	TBARS and 8-OHdG levels ↑ in ca pts vs. controls, TAS ↓ in ca pts vs. controls
Dowlati A et al. (1999)	32 pts, 24 COPD pts	lung cancer	Serum and BAL fluid IL-6, BAL fluid lactate dehydrogenase	BAL fluid IL-6 ↑ in ca pts vs. controls
Erhola M et al. (1998)	57 pts, 76 age-, sex- and smoking-matched controls	lung cancer	Plasma TRAP, vitamin E, C, A, uric acid	TRAP, vitamin C and E ↓ in ca pts vs. controls
Gönenç A et al. (2001)	36 pts, 41 healthy controls	26 breast, 12 lung cancer	Plasma MDA, serum uric acid, albumin, cholesterol, triglycerides	MDA and uric acid ↑ in ca pts vs. controls
Gönenç A et al. (2006)	15 pts, 15 controls with benign breast disease	breast cancer	Serum and tissue MDA, TAC, nitrite, nitrate, lipid hydroperoxides, total cholesterol, triglycerides, HDL- and LDL-cholesterol	Serum and tissue MDA ↓ in ca pts vs. controls, serum and tissue nitrate and nitrite ↑ in ca pts vs. controls, tissue TAC ↑ in ca pts vs. controls, serum total cholesterol and HDL-cholesterol ↓ in ca pts vs. controls

Kaynar H et al. (2005)	32 pts, 16 healthy controls	lung cancer	Erythrocyte GSH-Px, glutathione-S-transferase (GST), CAT, xanthine oxidase (XO), CuZn-SOD, GSH, NO, MDA	MDA, NO, GSH levels and SOD, CAT, XO activities ↑ in ca pts vs. controls
Kumaraguruparan R et al. (2002)	30 pts, 10 pts with benign fibroadenoma + 40 healthy controls	breast cancer	Plasma and erythrocyte TBARS, plasma ascorbic acid, vitamin E, SOD, CAT, GSH, GPx, GST	Plasma and erythrocyte TBARS ↑ in ca pts vs. fibroadenoma pts and in pts vs. healthy controls, plasma GSH, ascorbic acid, vitamin E, activities of SOD, CAT, GPx and GST ↓ in ca pts vs. fibroadenoma pts and controls
Lee HL et al. (2008)	77 pts, 131 healthy controls	non-Hodgkin lymphoma	Serum and urine IL-8	Urine IL-8/creatinine ↑ in ca pts vs. controls
Mahdavi R et al. (2009)	57 pts, 22 healthy controls	16 lung, 22 head and neck, 19 gastrointestinal cancer	Serum MDA, total antioxidant status (TAS), vitamin C	MDA concentrations ↑ in ca pts vs. controls, except in lung cancer. Vitamin C ↓ in ca pts vs. controls
Mantovani G et al. (2002)	82 pts, 36 healthy controls	different advanced stage cancers	Erythrocyte SOD, GPx, serum GR, TAS, IL-6, TNFα, IL-2, leptin	SOD, IL-6 and TNFα ↑ in ca pts vs. controls, GPx and leptin ↓ in ca pts vs. controls
Matanić D et al. (2003)	26 pts, 15 pts with benign lung disease	lung cancer	BAL fluid and blood TNFα, IL-1β, IL-6	BAL fluid IL-6, IL-1β ↑ in ca pts vs. controls
Oltra AM et al. (2001)	35 pts, 30 age-matched healthy controls	chronic lymphocytic leukemia	Lymphocyte SOD, CAT, GPx, GSSG/GSH, 8-oxodG	SOD, CAT ↓ in ca pts vs. controls, GPx and 8-oxodG ↑ in ca pts vs. controls
Riedel F et al. (2004)	24 pts, 28 healthy controls	head and neck squamous cell carcinoma	Serum and tissue IL-18, serum IL-8, IL-10, IL-12, interferon (IFN)-gamma, plasma endotoxin	Serum IL-18 ↑ in ca pts vs. controls
Rutkowski P et al. (2002)	156 pts, 50 healthy controls	soft tissue sarcoma	Serum TNFα, IL-1ra, IL-6, IL-8, IL-10, VEGF, basic fibroblast growth factor (bFGF), sIL-2R alpha, sIL-6R, TNFRI, TNFRII	TNFα, IL-1ra, IL-6, IL-8, IL-10, VEGF, bFGF, sIL-2R alpha, TNFRI, TNFRII ↑ in ca pts vs. controls
Saygili EI et al. (2003)	20 pts, 20 age-matched healthy controls	colorectal cancer	Plasma TBARS, cholesterol, vitamin E, vitamin C	TBARS ↑ in ca pts vs. controls, vitamin C ↓ in ca pts vs. controls
Taysi S et al. (2003)	25 pts, 15 healthy controls	advanced laryngeal cancer	Plasma MDA, NO, NO_3	MDA, NO, NO_3 ↑ in ca pts vs. controls
Yeh CC et al. (2005)	117 pts, 40 healthy controls	breast cancer	Whole blood GPx, SOD, glutathione reductase (GRX), plasma MDA, GSH, vitamin A, E, C	MDA ↑ in ca pts vs. controls, activities of GPx, SOD, GRX ↑ in ca pts vs. controls, GSH, vitamin C ↓ in ca pts vs. controls

2.11 Studies during radiotherapy

The changes in the local and systemic oxidant/antioxidant status during cancer treatments are largely unstudied and so far the data are conflicting. Studies show that BAL fluid transforming growth factor (TGF)-1β, IL-6, and vascular endothelial growth factor (VEGF) levels rise during radiotherapy for lung cancer (Beinert et al., 2000, Barthelemy-Brichant et al., 2004). In contrast, serum levels of fibroblast growth factor-2 and VEGF concentrations decrease significantly after radiotherapy for various cancers (Ria et al., 2004).

Elevated levels of malondialdehyde, glutathione and glutathione-related enzymes are noted after radioiodine treatment in patients with thyroid cancer (Konukoğlu et al., 1998). In another study, a decrease in GSH/GSSG levels, mainly due to an increase in GSSG concentration, has been detected after exposure to ionizing radiation (Navarro et al., 1997). In irradiated mice, the levels of glutathione reductase, glucose-6-phosphate dehydrogenase, glutathione peroxidase and glutathione-S-transferase have also been shown to decrease two hours after irradiation (Navarro et al., 1997).

In a previous study of ours we showed that the levels of urinary 8-OHdG increased after radiotherapy and chemotherapy for lung cancer (Erhola et al., 1997b). A few other studies support this finding, as elevated levels of urinary or lymphocyte 8-oxodG are found after radiotherapy treatment (Tagesson et al., 1995, Bialkowski et al., 1996, Bergman et al., 2004). In the last mentioned study, the excretion of 8-OHdG showed a biphasic pattern, whereas excretion of MDA produced a single peak appearing 11–19 days after initiation of chemoradiotherapy (Bergman et al., 2004).

Table III. *Main studies on antioxidants, cytokines and oxidative stress markers during radiotherapy for cancer (RT=radiotherapy, pts=patients, CT=chemotherapy, BAL fluid=bronchoalveolar lavage fluid, IL=interleukin, sig=significant, ns=non-significant, TBI=total body irradiation, BMT=bone marrow transplantation)*

Reference	Number of pts	Treatment type	Parameters measured	Observed changes during radiotherapy (p values not given)
Akmansu M et al. (2005)	34 head and neck cancer pts	external beam irradiation	Serum TNFα, IL-6 before RT and after fifth week of RT	TNFα ↑ in all pts (sig), IL-6 ↑ in pts treated with postoperative adjuvant RT (sig)
Barthelemy-Brichant N et al. (2004)	11 lung cancer pts	external beam irradiation	BAL fluid transforming growth factor-beta$_1$ (TGF-beta$_1$), IL-6 before, during and 1, 3, and 6 months after RT	BAL fluid TGF-beta$_1$, IL-6 from the irradiated areas ↑ after RT (sig), the increase in TGF-beta$_1$ levels tended to be greater in patients who developed severe pneumonitis
Beinert T et al. (2000)	94 lung cancer pts (82 NSCLC, 12 SCLC)	external beam irradiation followed by CT or CT alone	BAL fluid VEGF, oxidized methionine in proteins before, during and after treatment	BAL fluid VEGF, oxidized methionine ↑ in ca pts at baseline. BAL fluid VEGF ↑ in pts undergoing CT administered after RT (sig)
Bergman V et al. (2004)	11 pts with hematological malignancies	TBI and high-dose CT preceding BMT	Urinary 8-OHdG and MDA before and during RT	9/11 pts had ↑ in urinary 8-OHdG (8–25 times the initial baseline level) on days 0–7 after RT (ns); the excretion ↓ during the aplastic period and ↑ at engraftment (ns)
Bialkowski K et al. (1996)	3 lung, 1 colorectal cancer pts	external beam irradiation	Lymphocytes 8-oxodG before and after RT	8-oxodG in lymphocytes ↑ after RT (p not tested)
Bøhn SK et al. (2006)	29 head and neck cancer pts, 51 healthy controls	external beam irradiation	Plasma GSH, cysteine, homocysteine, cysteinyl-glycine	GSH ↓ in pts compared to controls at baseline (sig), pts with lowest post-radiotherapy GSH levels had the lowest 36 months survival
Erhola M et al. (1998)	16 lung cancer pts	external beam irradiation	Plasma TRAP, vitamin E, C, A and uric acid measured nine times during RT	Vitamin E ↓ after 6 Gy (sig). Uric acid ↑ towards the end of the RT at 50 Gy (sig)
Gisterek I et al. (2007)	37 pharyngeal and laryngeal cancer pts	external beam irradiation	Serum VEGF	VEGF ↓ after RT (sig)
Konukoğlu D et al. (1998)	30 thyroid cancer pts, 20 age-matched healthy controls	radioiodine treatment	Erythrocyte MDA, GSH levels and activities of GSH-peroxidase and GSH-reductase	MDA ↑ and GSH levels and activities of GSH-related enzymes ↓ in pts after surgery vs. healthy controls (sig). MDA and GSH levels and GSH-related enzyme activities ↑ after trt (sig)
Navarro J et al. (1997)	12 breast, 7 lung cancer pts	external beam irradiation	Plasma GSH, GSSG, GPx, GR	GSH/GSSG ↑ (due to GSSG ↑) at 24 Gy and 50 Gy during RT (in breast cancer) and at 50 Gy in lung cancer pts (sig)
Ria R et al. (2004)	33 pts (12 breast, 3 rectum, 2 lung, 16 other cancers)	external beam irradiation	Serum fibroblast growth factor-2 (FGF-2), VEGF, hepatocyte growth factor (HGF)	FGF-2 and VEGF ↓ after RT (sig). The extent of their diminution is related to the radiation dose and response
Roszkowski K et al. (2008)	27 head and neck cancer pts	external beam irradiation	Urinary 8-oxodG, 8-oxoGua, leukocyte 8-oxodG before and one day after RT	Urinary 8-oxodG ↑ 24 hr after the last fraction (ns). No changes in urinary 8-oxoGua and 8-oxodG (ns)
Sabitha KE and Shyamaladevi CS (1999)	12 oral cancer pts, 12 age- and sex-matched healthy controls	external beam irradiation	Red blood cell superoxide dismutase (SOD), catalase, GPx, GR, glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH), serum MDA	MDA ↑ in untreated and RT-treated oral cancer patients compared to normal subjects. SOD, catalase, GPx, GSR, GST and G6PDH ↓ during RT (sig)

2.12 Studies during chemotherapy

Our previous study investigated the plasma total peroxyl radical trapping antioxidant parameter (TRAP) and its main components (uric acid, vitamin E, thiols) during CAV (cyclophosphamide, adriamycin, vincristine) chemotherapy for small cell cancer. A significant decrease in the levels of TRAP was evidenced during the first chemotherapy cycle, the levels recovering to baseline 20 hours after the start of chemotherapy. During the second chemotherapy cycle, a decrease in the unidentified antioxidant components occurred (Erhola et al., 1996). In a study by Dűrken and co-workers (2000) TRAP and different antioxidants were also examined during high-dose chemo- and chemoradiotherapy preceding bone marrow transplantation. The levels of TRAP and uric acid were seen to decrease significantly during treatments. An increase in the levels of TBARS was also seen after conditioning therapy. Interestingly, the decreased levels of different antioxidants recovered partly two weeks after bone marrow transplantation. Another study detected elevated levels of TBARS, as a lipid peroxidation marker, and Schiff's bases, a short-lived species formed by carbonyl groups reacting with amines, during combination chemotherapy (carboplatin, vincristine and etoposide) for SCLC patients (Nowak and Janczak, 2006). A study conducted in Hodgkin´s lymphoma patients showed decreased levels of various antioxidants and increased levels of nitric oxide and MDA after adriamycin-containing combination chemotherapy (Kaya et al., 2005).

Several studies have investigated plasma antioxidants during high-dose chemotherapy for hematological malignancies and revealed that the levels of plasma glutathione, alpha- and gamma-tocopherol and β-carotene decreased after conditioning chemotherapy (Ladner et al., 1989, Jonas et al., 2000). On the other hand, plasma vitamin C, zinc and glutathione peroxidase concentrations increased during two weeks after bone marrow transplantation (Jonas et al., 2000).

Another group explored the effects of adriamycin-containing chemotherapy on urinary excretion of 5-hydroxymethyluracil (HMUra), serum TBARS, plasma retinol and plasma alpha-tocopherol. The levels of urine HMUra and serum TBARS were found to be increased during chemotherapy, suggesting that adriamycin induces nucleic acid alterations. Retinol and alpha-tocopherol levels decreased during chemotherapy (Faure et al., 1996). (Table IV)

Table IV. *Studies on antioxidants, oxidative and nitrosative stress markers during chemotherapy or chemoradiotherapy for cancer (CT=chemotherapy, pts=patients, GSH-Px=glutathione peroxidase, TBI=total body irradiation)*

Reference	Number of patients, tumor type	Treatment	Parameters measured and time points	Observed significant differences (p values not given)
Dürken M et al. (2000)	16 pts with hematological malignancies	busulfan, VP-16, cyclo-phosphamide (n=8), VP-16, cyclophosphamide + TBI (n=8)	Plasma TRAP, alpha-tocopherol, uric acid, protein SH groups, ascorbate, bilirubin, ubiquinone-10, TBARS, polyunsaturated fatty acids (PUFA), allantoin	TRAP ↓ after conditioning CT and CT+TBI, TRAP ↑ during 2nd day of TBI, PUFA ↓, and TBARS ↑ during conditioning therapy, allantoin and ubiquinone-10 ↑ after CT
Erhola M et al. (1996)	12 SCLC pts	adriamycin, vincristine, cyclophosphamide	Plasma TRAP, vitamin E, urate, protein SH groups, ascorbate	TRAP, urate and ascorbate ↓ during first CT cycle
Faber M et al. (1995)	15 various cancer pts, 20 healthy controls	adriamycin containing CT	Plasma thiobarbituric acid-reactive material (TBARM), GSH, GSH-Px, zinc, selenium, vitamin E, β-carotene, vitamin A, copper	TBARM ↑ in ca pts vs. controls. Selenium ↓ in ca pts vs. controls. TBARM ↑ after CT
Faure H et al. (1996)	14 pts (breast, testis, lung, uterus, hematological cancers)	adriamycin containing CT	Urine thymine oxidative lesion-5-hydroxymethyluracil (HMUra), serum TBARS, retinol, alpha-tocopherol	Urine HMUra, TBARS ↑ by CT, retinol and alpha-tocopherol ↓ at the end of CT
Jonas C et al. (2000)	24 hematological cancer pts receiving bone marrow transplantation (BMT)	conditioning CT	Plasma GSH, GSH-Px, alpha- and gamma-tocopherol, vitamin C, zinc, cysteine-cystine before treatment and at days 1, 3, 7, 10, and 14 after BMT	GSH and alpha- and gamma-tocopherol ↓, the GSH redox state became more oxidized after conditioning CT. Cystine, vitamin C, zinc concentrations, GSH-Px activity ↑ over time
Kaya E et al. (2005)	34 Hodgkin's lymphoma pts	adriamycin, bleomycin, vincristine and dexamethasone treatment	Serum superoxide dismutase (SOD), GSH-Px, catalase (CAT), MDA, NO and enzyme activities before CT, on days 1 and 7 during CT	SOD, CAT, GSH-Px ↓ on days 1 and 7, NO and MDA ↑ compared to baseline
Ladner C et al. (1989)	13 hematological cancer pts receiving bone marrow transplantation (BMT)	12 Gy total body irradiation + cyclophosphamide CT. In addition etoposide for high-risk pts	Plasma alpha-tocopherol and beta-carotene, serum lipid hydroperoxides 8 days before BMT, on the day of BMT, 12 days after BMT	Alpha-tocopherol and beta-carotene ↓ following conditioning therapy. Lipid hydroperoxides ↑ during etoposide treatment
Look MP and Musch E (1994)	44 pts, 52 healthy controls	polychemotherapy	Plasma TBARS at baseline (n=44) and after CT (n=12)	TBARS ↑ in ca pts vs. controls. After CT, TBARS ↑ vs. pre-treatment value
Nowak D and Janczak M (2006)	37 SCLC pts	carboplatin, vincristine, etoposide	Serum TBARS and Schiff's bases (SB) before and 6, 24 h after onset of the 1st, 3rd and 6th cycles	TBARS and SB levels ↑ 24 h after 1st CT cycle in the whole group. In pts with early progression TBARS and SB ↓ 6 and 24 h after the 1st cycle

2.13 Exhaled hydrocarbon studies

Elevated levels of exhaled ethane or pentane are reported in several pulmonary diseases, e.g. in asthma, obstructive sleep apnea, chronic obstructive pulmonary disease (COPD), cystic fibrosis and interstitial lung diseases (Olopade et al., 1997a+b, Paredi et al., 2000a+b, Kanoh et al., 2005). Also other inflammatory conditions such as Crohn's disease, as well as ischemic heart disease, are associated with increased production of exhaled hydrocarbons (Mendis et al., 1995, Wendland et al., 2001). Studies including critically ill patients and preterm infants have also been made and show elevated levels of exhaled hydrocarbons in these conditions (Drury et al., 1997, Schubert et al., 1998, Pitkänen et al., 2004).

Only a few studies have explored exhaled hydrocarbons in cancer patients. According to Hietanen and co-workers (1994), breast cancer patients show increased exhaled pentane production compared to healthy controls. Exhaled hydrocarbons during radiotherapy have been addressed in only one case report, that by Arterbery and co-workers (1994). In that study exhaled ethane levels were measured in a unique radiotherapy situation, namely under total body irradiation for chronic myeloid leukemia. The measurements were performed daily over four consecutive days over a total dose of 12 Gy. The investigators noted an elevation of exhaled ethane levels on the second day of radiotherapy, when the clinical manifestations of adverse events were also most prominent. The pretreatment levels of exhaled ethane on day 3 and 4 decreased below pretreatment exhaled ethane levels on day 1, which might imply to enhanced antioxidative defense mechanisms during radiotherapy. However, measurement of exhaled hydrocarbons involves a difficult technique with possible confounding factors, which may partly explain its infrequent use.

Some studies have been performed using exhaled air analysis in the diagnosis of lung cancer (Phillips et al., 2003, Horváth et al., 2009). Interestingly, exhaled pentane has been one of the gases identified in a predictive model to diagnose lung cancer (Phillips et al., 2003). However, none of these techniques has proved sufficiently specific, and further research is still needed on the topic.

2.14 Previous studies on associations with adverse events and response

There appears to be a paucity of data on antioxidants and markers of oxidative stress in relation to adverse events and clinical response. One study has reported that the degree of acute radiosensitivity correlated with urinary levels of 8-oxodG at a radiotherapy dose of 12 Gy, as lower urinary 8-oxodG levels were found in breast cancer patients with pronounced skin reactions compared to patients with minor skin reactions during radiotherapy. However, only 17 patients participated in this study and at a later point during radiotherapy, no such correlations were seen (Haghdoost et al., 2001). Another report involving 13 patients with oral carcinoma showed that patients with higher pretreatment plasma glutathione levels developed less severe acute radiation mucositis (Bhattathiri et al., 1994). In contrast, an examination of plasma glutathione, uric acid and ascorbate values in head and neck cancer patients who received continuous hyperfractionated accelerated radiotherapy (CHART) revealed no associations between

pretreatment plasma GSH, uric acid or ascorbic acid levels and the grade of mucositis (Wardman et al., 2001).

Accumulating evidence suggests that overexpression of cytokines may be associated with the occurrence of adverse events during radiotherapy, mainly radiation pneumonitis (Chen et al., 2002, Novakova-Jiresova et al., 2004, Arpin et al., 2005, Hill, 2005). In addition, one study has observed that higher baseline erythrocyte SOD and lower GPx activities are associated with more frequent occurrence of radiation pneumonitis. In the study in question, involving 15 NSCLC patients, the levels of catalase were not associated with risk of pneumonitis (Park et al., 2007).

Our previous study with 37 SCLC patients showed that subjects evincing complete or partial response to chemotherapy showed a significant decrease in the levels of urinary 8-OHdG/creatinine, whereas those with no change or progressive disease showed an increase (Erhola et al., 1997b). Another study examining 20 SCLC patients showed that 82% of patients with a higher relative levels of DNA mutations evinced complete response after chemotherapy treatment compared to 18% of patients with lower relative levels of mutations (Silva et al., 2000).

2.15 Previous studies on associations with survival

Several lines of evidence suggest that overexpression of cytokines, especially VEGF, is a poor prognostic factor for survival. Overexpression of VEGF is linked to poor prognosis in many tumor types, for example prostate, gastric, cervical, pancreatic and colorectal cancer (Green et al., 2007). Numerous other studies involving lung cancer patients have also gathered information on this aspect; in all of them, however, the number of patients has been relatively small (Salven et al., 1998, Chen et al., 2003, Hasegawa et al., 2005). In a review by Bremnes and co-workers (2006), 10 out of 16 studies showed that elevated blood VEGF levels had a negative prognostic impact on survival in NSCLC; the data concerning the prognostic role of basic fibroblast growth factor (bFGF) was inconclusive. In another study investigating serum IL-1, TNFα, IL-2, IL-6 and interferon (IFN)-γ in 66 lung cancer patients, elevated serum IL-6 levels were associated with shorter survival during an approximately two-year follow-up period (Martín et al., 1999). Also serum IL-8 has been suggested to correlate with survival in lung cancer according to univariate analysis, without however, independent significance in multivariate analysis (Orditura et al., 2002).

One study involving 37 SCLC patients showed that better survival correlated with an increase in lipid peroxidation marker levels, namely TBARS, after first chemotherapy cycle consisting of carboplatin, etoposide and vincristine. In the study in question, patients with negative Schiff´s bases and increments of TBARS had significantly shorter survival than those with positive values over a follow-up time of almost five years (Nowak and Janczak, 2006). Another study involving 29 head and neck cancer patients reported a better outcome after 36 months´ follow-up in those whose post-radiotherapy GSH values were above median (Bøhn et al., 2006). Based on results on 44 patients, it has been suggested that urinary 8-oxodG might be a reliable prognostic marker of overall survival in lymphoma patients, although no real follow-up was included to evaluate this (Honda et al., 2000). Still, only limited evidence is provided on associations between antioxidants and oxidative stress markers and survival.

3. OXIDANT EFFECTS OF RADIOTHERAPY

Radiotherapy was introduced in the field of cancer more than one hundred years ago when X-rays were discovered. Linear accelators have been in use since the 1940s. Though the mechanism of action of ionizing radiation has long been clear, the local effects of radiotherapy at cellular level are still partly obscure (Riley, 1994, DeVita et al., 2005, p. 268).

Ionizing radiation interacts with water and this generates highly reactive free radicals, which mediate the antitumor effect of radiotherapy (Riley, 1994). Irradiation might damage DNA directly as energy is absorbed by DNA or indirectly as free radicals are formed (Halliwell, 2007). The most important free radical generated in irradiation is the hydroxyl radical ($OH^{\bullet}$). The hydroxyl radical is highly reactive and has the capability to damage DNA bases, proteins and lipids (Riley, 1994, Halliwell and Gutteridge, 2007, p. 484). Radiation induces damage to cells and may possibly induce cell necrosis, apoptosis, accelerated senescence and terminal differentiation (Halliwell, 2007, DeVita et al., 2005, p. 269–270). As a consequence of radiation, cells may sustain lethal damage; otherwise the damage may be repaired and not lead to cell death. The main types of DNA damage caused by irradiation include base damage, single-strand breaks and double-strand breaks. Repair of damage may be divided into potentially lethal damage and sublethal damage repair. Due to sophisticated DNA repair mechanisms, base and single-strand damage are usually not lethal although they are more abundant than double-strand breaks (Meyn et al., 2009). The most important response of cells to irradiation is apoptosis (Dewey et al., 1995). In fact, in vitro work has shown that ionizing radiation cause cells to release signal enhancing apoptosis (Lyng et al., 2001). It has been shown that cells with a high capacity for potentially lethal damage repair show little apoptotic response to radiation (Schmidberger et al., 2003). In general, cells respond to radiation in a number of ways:

1) error-free (base excision repair, nucleotide excision repair) or error-prone mutagenic repair of damaged DNA
2) gene expression modulation
3) cell death

DNA repair mechanisms are usually found in all types of normal healthy cells (Riklis et al., 1996). Irradiation is also known to induce inflammatory-type responses, e.g. infiltration of neutrophils and activation of macrophages (Lorimore et al., 2001, Bergman et al. 2004).

Radiotherapy is applied in fractions. Fractionation involves the size and number of radiation increments. An in vitro study has shown that small fractionation schemes produce less lipid peroxidation than single higher radiotherapy fractions (Haidenberger et al., 2003). Another in vitro work has suggested that fractionated radiation induces

apoptosis through caspase-3 and reactive oxygen species-mediated apoptosis (Bucci et al., 2006).

However, ionizing radiation also causes damage to constituents other than DNA (Riley, 1994). Lipid peroxidation is thought to be one of the main causes of damage during irradiation, and previous studies have noted amplified lipid peroxidation markers during radiotherapy (Konukoğlu et al., 1998, Sabitha and Shyamaladevi, 1999). Irradiation also causes oxidation of membrane protein sulfhydryl groups (Edwards et al., 1984), and it is possible that the levels of glutathione-related enzymes (glutathione peroxidase and glutathione reductase) decrease during radiotherapy (Konukoğlu et al., 1998). Interestingly, resistance to radiotherapy has been suggested to be due to high intracellular levels of glutathione (Navarro et al., 1997).

4. LUNG CANCER

4.1 Epidemiology of lung cancer

Lung cancer is one of the most common cancers in the world, with approximately 1.3 million new cases diagnosed every year. Its incidence and mortality are lower in developing countries than in the non-developing countries (Parkin et al., 2005). Whereas in the USA the incidence among men has undergone a significant reduction in the last 5 years, in women and in some other parts of the world it continues to increase (Ferlay et al., 2007). In Finland lung cancer is the second most common cancer in men after prostate cancer; in the year 2007, 1511 new cases were diagnosed. For women, it is the fourth most common after breast, bowel and endometrial cancer, accounting for 5.2% of all new cases. In 2007, 675 new lung cancer cases were diagnosed among women (Finnish Cancer Registry, September 2009). The incidence in men in Finland has been decreasing from the mid-1970s as the prevalence of male smokers has also declined. In contrast, the lung cancer incidence among women has been increasing, and there have been estimates to suggest that the trend will continue (Finnish Cancer Registry, September 2009). The prognosis for lung cancer is generally poor, the median overall survival in NSCLC being 8–11 months with a 5-year survival rate of 8–15% (Giaccone et al., 2002, Jassem, 2007, Finnish Cancer Registry, 2009).

4.2 Etiology of lung cancer

Cigarette smoke is the main cause of lung cancer; it has been estimated that lung cancer mortality is attributable to cigarette smoking in approximately 90% of men and in 80% of women (D´Addario et al., 2009). However, genetically determined variations in the metabolism of tobacco-derived carcinogens influence the individual risk (Bartsch et al., 2000). The health risks caused by cigarette smoking are not limited to smokers. Exposure to "secondhand" smoke, or environmental tobacco smoke, significantly increases the risk of lung cancer in nonsmokers. There is a dose-response relation between exposure and lung cancer risk. Cessation of smoking is the only effective way to reduce the cancer risk (Bartsch et al., 2000).

Cigarette smoke has been shown to contain about 4,000 chemical agents, including over 60 carcinogens (Hecht, 1999). One of most widely studied carcinogens is the polycyclic aromatic hydrocarbon (PAH) carcinogen benzo[*a*]pyrene (BaP), in addition to which cigarette smoke also contains carcinogenic nitrosamines, aromatic amines, aldehydes, volatile organic compounds, oxidants, and metals (Hecht, 2006). Cigarette smoke oxidizes thiols to disulfides, inactivates the alpha1-proteinase inhibitor and activates phagocytes to release more radicals. It contains two different populations of free radicals, one in the tar phase and one in the gas phase. The tar phase contains mostly

quinone/hydroquinone complex, held to be capable of reducing molecular oxygen to produce superoxide radical, and furthermore hydrogen peroxide and hydroxyl radicals. The gas phase contains small oxygen- and carbon-centered radicals which are more reactive than the radicals produced by tar phase (Church and Pryor, 1985). Cigarette smoke also has direct carcinogenic effects on the epithelial cells of the respiratory tract (Subapriya et al., 2002). Long-term cigarette smoke exposure may lead to decreased plasma levels of various antioxidants such as vitamin C and A (Yanbaeva et al., 2007). Cigarette smoke also causes lipid peroxidation and nicks the DNA of the adjacent tissues. It produces high concentrations of oxidizing radicals in the microloculi in the lung, leading to microhemorrages in the airways, and subsequent release of iron (Pryor and Stone, 1993).

The polycyclic aromatic hydrocarbons (PAH) in cigarette smoke initiate the formation of squamous cell carcinoma in the major bronchi of the lungs. It has been hypothesized that the alkylating carcinogens in cigarette smoke are responsible for the increased incidence of adenocarcinoma. The mechanism of cigarette smoke-induced lung cancer may in fact vary depending on the histology and anatomical location of the tumor (Godschalk et al., 2002). Also the reduction in the risk of lung cancer after cessation of smoking is faster in small cell lung and squamous cell cancer and less evident in adenocarcinoma (Devesa et al., 2005). Other risk factors for lung cancer include radon and various occupational exposures for example asbestos, silica, arsenic and chromium. Their role is, however, much less significant than exposure to cigarette smoke (Alberg and Samet, 2003).

Knowledge of the molecular and cellular basis of lung cancer is rapidly expanding. The condition is initiated by activation of oncogenes and inactivation of tumor suppressor genes (Fong et al., 2003). It is estimated that mutation in the K-ras proto-oncogene is responsible for 10–30% of all lung adenocarcinomas (Herbst et al., 2008). Other gene mutations involved in the pathogenesis of lung cancer include molecular alterations in EGFR, Ras and p53. There are also data to indicate that different molecular mechanisms are responsible for the development of lung cancer in smokers and non-smokers (Planchard et al., 2009). Recent findings suggest that some cytochrome-P, glutathione S-transferase, NADPH:quinone oxidoreductase and N-acetyltransferase genotypes are associated with an increased risk of lung cancer. Lung cancer has also been shown to be associated with impaired DNA repair capacity (Hansen, 2008, p. 7).

4.3 Classification of lung cancer

Lung cancer can be divided into two main entities based on the size and appearance of the malignant cells: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). The three subgroups of NSCLC are squamous cell lung carcinoma (45%), adenocarcinoma (40%), and large cell lung carcinoma (5%) (Brambilla et al., 2001). NSCLC accounts for approximately 75% of lung cancers, whereas 20% of lung tumors are classified as SCLC (Gridelli et al., 2004).

World Health Organization/ International Association for the Study of Lung Cancer: Histological Classification of Lung Tumors

Malignant epithelial tumors

1. Squamous cell carcinoma
   - Papillary
   - Clear cell
   - Small cell
   - Basaloid
2. Small cell carcinoma
   - Combined small cell carcinoma
3. Adenocarcinoma
   - Acinar
   - Papillary
   - Bronchioloalveolar carcinoma (Nonmucinous/ Mucinous / Mixed mucinous and nonmucinous or intermediate cell type)
   - Solid adenocarcinoma with mucin
   - Adenocarcinoma with mixed subtypes
   - Variants (Well-differentiated fetal adenocarcinoma/ Mucinous (colloid) adenocarcinoma/ Mucinous cystadenocarcinoma/ Signet ring adenocarcinoma/ Clear cell adenocarcinoma)
3. Large cell carcinoma
   - Large-cell neuroendocrine carcinoma (Combined large-cell neuroendocrine carcinoma)
   - Basaloid carcinoma
   - Lymphoepithelioma-like carcinoma
   - Clear cell carcinoma
   - Large cell carcinoma with rhabdoid phenotype
4. Adenosquamous carcinoma
5. Carcinomas with pleomorphic, sarcomatoid or sarcomatous elements
   - Carcinomas with spindle and/or giant cells (Pleomorphic carcinoma/ Spindle cell carcinoma/ Giant cell carcinoma)
   - Carcinosarcoma
   - Pulmonary blastoma
   - Others
6. Carcinoid tumor
   - Typical carcinoid
   - Atypical carcinoid
7. Carcinomas of salivary-gland type
   - Mucoepidermoid carcinoma
   - Adenoid cystic carcinoma
   - Others
8. Unclassified carcinoma

(Travis et al., 1999, Brambilla et al., 2001)

4.4 Staging of lung cancer

TNM staging is based on the size of the tumor (T), regional lymph nodes (N) and metastasis (M) characteristics as established by the International System for Staging Lung Cancer adopted by the American Joint Committee on Cancer (Posther and Harpole, 2006). By providing information on the extent of disease TNM staging aids in predicting survival and is thus essential when making treatment decisions. Clinical TNM staging is

often associated with poorer outcomes than the pathological TNM staging performed after surgery (Tanoue, 2008).

The TNM staging is mostly used in NSCLC, whereas SCLC is often categorized as limited-stage and extensive-stage disease according to a staging system developed by the Veteran´s Administration Lung Cancer Study Group. Patients with limited disease have tumors restricted to the ipsilateral hemithorax, i.e. tumor masses do not extend beyond the primary tumor, mediastinal and bilateral hilar lymph nodes and bilateral supraclavicular nodes. Superior vena cava syndrome, Pancoast syndrome and ipsilateral pleural effusion are also classified as limited disease. Extensive-stage disease characterizes metastasized SCLC, including also widespread thoracic disease such as pericardial effusion (Simon and Turrisi, 2007).

The TNM staging used in the present studies was published in 1997 (Sobin and Wittekind, 1997). However, in 2009 an updated version of the classification was released and will be implemented one year after publication (Rami-Porta et al., 2009). It should nonetheless be remembered that the TNM staging system does not take into account the biological features of lung tumors (Tanoue, 2008).

The TNM classification of lung cancer (UICC)

Primary tumor (T)

TX	Primary tumor cannot be assessed, or proven by the presence of malignant cells in sputum or bronchial wash but not visualized by imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus
T2	Tumor with any of the following features of size or extent: More than 3 cm in greatest dimension Involving main bronchus, 2 cm or more distal to the carina Invading the visceral pleura Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung
T3	Tumor of any size that directly invades the following: chest wall (including superior sulcus tumors), diaphragm, mediastinal pleura, or parietal pericardium; or tumor in the main bronchus less than 2 cm distal to the carina but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung
T4	Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina, separate tumor nodule(s) in the same lobe; or tumor with a malignant pleural effusion

Regional lymph node (N)

The regional lymph nodes are intrathoracic, scalenic and supraclavicular nodes

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes, including direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral, or contralateral scalenic or supraclavicular lymph node(s)

Distant metastasis (M)

MX	Presence of distance metastasis cannot be assessed
MO	No distant metastasis
M1	Distant metastasis present, includes separate tumor nodule(s) in a different lobe (ipsilateral or contralateral)

(Sobin and Wittekind, 1997)

The International Staging system for Lung Cancer			
Stage	***T***	***N***	***M***
Occult	TX	N0	M0
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
	T2	N0	M0
Stage II	T1	N1	M0
	T2	N1	M0
	T3	N0	M0
Stage IIIa	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIb	Any T	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1

(Mountain, 1997)

4.5 Features of lung cancer

There are four major forms of lung cancer described by the World Health Organization (WHO): adenocarcinoma, squamous cell carcinoma, large-cell carcinoma and small cell lung carcinoma; however, mixed forms (e.g. adenosquamous carcinomas) are relatively common (Brambilla et al., 2001, D´Addario et al., 2009). Sometimes small cell lung carcinomas are mixed with non-small cell lung cancer histologies (adenocarcinoma or squamous carcinoma) (Brambilla et al., 2001). During the last few decades the number of squamous cell carcinomas has decreased and that of adenocarcinomas increased. Interestingly, adenocarcinomas are the commonest form of lung cancer in women, the young and non-smokers. However, among women the rates of squamous cell carcinoma and SCLC have also recently been rising (Devesa et al., 2005). The vast majority of SCLC, carcinoids and 15% of NSCLC contain neuroendocrine cells. The major function of these cells is the elaboration, storage and secretion of small peptides and biogenic amines (Gustafsson et al., 2008).

The various histological forms of lung cancer develop in different areas of the organ; central location is characteristics of SCLC and squamous cell carcinoma, whereas adenocarcinomas and large cell carcinomas develop mainly peripherally; 40% of non-small cell cancers are centrally located (Posther and Harpole, 2006).

SCLC is substantially more chemo-and radiosensitive compared to NSCLC. Also compared to NSCLC, SCLC has a greater tendency to develop earlier and more widespread metastases. SCLC accounts for 13–20% of all lung cancers and carries a very poor prognosis- without treatment the median overall survival is 2–4 months (Simon and Turrisi, 2007). Although NSCLC develops distant metastases to a lesser degree, over 50% of patients have metastases at a late stage in the course of the disease. Patients with

N2 local metastasis and adenocarcinoma as a histological subtype are more prone to brain metastasis (Tanoue, 2008). Lung cancer can spread locally (i.e. intrathoracically), regionally via lymphatics or hematogenously forming distant metastases. Metastases may occur in most organs, but are more frequent in the liver, bones, lungs, brain and adrenal glands (DeVita et al., 2005 p. 762, Tanoue, 2008). Prognostic factors include tumor size, stage of the cancer, histological type, performance status, weight loss prior to diagnosis, mutation of K-ras oncogene and deletion of tumor suppressor genes (p53) (Chao et al., 2002, p. 307, DeVita et al., 2005, p. 747–748). Recently new indicators of poor prognosis for NSCLC have been found, for example Ki-67 and microvessel density, whereas the overexpression of Bcl-2 might predict better survival (Zhan et al., 2009).

4.6 Symptoms and diagnosis of lung cancer

The most common symptoms of lung cancer are cough, hemoptysis, pain, dyspnea, loss of appetite, loss of weight, frequent pneumonias, anorexia, malaise and weakness (Hamilton et al., 2005, Sher et al., 2008). More rare symptoms such as Horner´s syndrome may also occur. SCLC can often be associated with paraneoplastic symptoms. Ectopic production of adrenocorticotropic hormone (ACTH) may cause Cushing´s syndrome and increased antidiuretic hormone (ADH) secretion can lead to a syndrome of increased ADH secretion (SIADH) (Gustafsson et al., 2008). Paraneoplastic symptoms may also include neuropathy or other neurological deficiencies (Sher et al., 2008).

The diagnosis of lung cancer is based on physical examination, radiological findings (including chest X-ray and chest and upper abdominal computerized tomography and/or magnetic resonance imaging) as well as pathological report. Fiberoptic bronchoscopy allows endoscopic acquisition of brushing, lavage and transesophageal biopsies for pathological analysis. Bronchoscopy is also a valuable tool to evaluate the bronchial tree and to plan surgical resection (Posther and Harpole, 2006, Sher et al., 2008). Endoscopic ultrasound may also be performed to aid in assessment of lymph node involvement (Krasnik et al., 2006). Computerized tomography is essential for the evaluation of staging and therapeutic planning in lung cancer. The addition of positron-emission-tomography (PET) to the diagnostics of lung cancer, especially in evaluating mediastinal lymph nodes, has greatly improved the sensitivity of diagnosis. In addition, mediastinoscopy may be performed to explore possible metastasis to the mediastinum. Tumor markers are not routinely used or recommended in the diagnosis of lung cancer (Posther and Harpole, 2006, D'Addario et al., 2009).

4.7 Evaluation of performance status

Karnofsky performance status was developed as far back as the 1940s to measure the patient's performance and ability to cope in everyday life objectively (Karnofsky and Burchenal, 1949, p. 196). Since then, it has been commonly used in daily patient practice as well as in clinical studies. The Karnofsky scale runs from 100 to 0, 100 indicating uncompromised health and 0 death.

Karnofsky performance status scale	
100	Normal, no complaints, no evidence of disease
90	Able to carry on normal activity, minor signs and symptoms of the disease
80	Normal activity with effort, some signs and symptoms of disease
70	Cares for self. Unable to carry on normal activity or to do active work
60	Requires occasional assistance, but is able to care most of his needs
50	Requires considerable assistance and frequent medical care
40	Disabled, requires special care and assistance
30	Severely disabled, hospitalization is indicated although death is not imminent
20	Very sick, hospitalization necessary, active support treatment necessary
10	Moribund, fatal processes progress rapidly
0	Dead

(Karnofsky and Burchenal, 1949)

In the 1980s, ECOG (Eastern Cooperative Oncology Group) developed a similar kind of classification known as the World Health Organization (WHO) or Zubrod´s performance status classification. The ECOG scale runs from 0 to 5, with 0 referring to perfect health and 5 to death (Oken et al., 1982).

ECOG performance status scale	
Grade	
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair
5	Dead

(Oken et al., 1982)

4.8 Treatment of non-small cell lung cancer

Treatment of lung cancer is based on the staging of the tumor (TNM stage) and the performance status of the patient. Also patients´ other diseases, pulmonary and cardiac function as well as compliance with treatment must be taken into account when making treatment decisions (Gridelli et al., 2003).

Surgery is the most efficient curative treatment for non-small cell lung cancer. Depending on various factors, surgery may comprehend either pulmectomy or lobectomy. Also sleeve lobectomy and bilobectomy are possibilities depending on the tumor burden and location. The main complications of surgery include atrial arrhythmias, atelectasis, prolonged air leak and pneumonia. There have been reports of mortality among the elderly (1.5%) or those with compromised pulmonary function (2.1%). Other complications due to surgery may include deep venous thrombosis and other thromboembolic events, prolonged chest drainage, atelectasis, bronchopleural fistula, pneumothorax or bleeding (Posther and Harpole, 2006).

4.8.1 Stage I–II disease

In stage I and II resection should be performed whenever possible and cisplatin-based adjuvant chemotherapy added when appropriate, especially in stage II disease (Tanoue, 2008). Unfortunately, only 15–20% of lung tumors can be radically resected (Gridelli et al., 2003). In the early stages, the addition of radiotherapy after surgery has not been found to improve survival (Gridelli et al., 2003). In patients inoperable due to comorbidities or compromised pulmonary function, radical aimed radiotherapy is the treatment of choice (D´Addario et al., 2009). In such cases, only the primary tumor is treated in stage I disease, whereas the clinical target volume of stage II disease also includes the ipsilateral nodal area (Chao et al., 2002, p. 309).

4.8.2 Stage III disease

Stage III lung cancer is a particularly heterogeneous group of tumors and no exact treatment guidelines can be given. Treatment of stage III cancer is also a subject of intense clinical research due to its poor prognosis. The status of mediastinal lymph nodes (N2) often determines whether the patient is operable. In Europe and Japan patients with known N2-positive tumors are treated with surgery and post-operative chemotherapy, whereas in the USA chemotherapy or chemoradiotherapy are used (Posther and Harpole, 2006). The optimal management of patients with multifocal disease remains controversial (Battafarano et al., 2002). In inoperable cases, radiotherapy or chemoradiotherapy are used. There are two different schedules for combining chemotherapy and radiotherapy, administrating them either sequentially or concurrently (Jassem, 2007). In sequential treatment, combination chemotherapy is administered for 4–6 cycles followed by thoracic radiotherapy. The concomitant mode produces more adverse events; the long-term toxicity has nonetheless been similar in sequential and concurrent treatment studies. Failure to maintain local control after curative treatment in NSCLC is still the greatest challenge (Gridelli et al., 2003, Fietkau, 2004). The 5-year survival rate for locally advanced lung cancer is 13% and 5% for stages IIIA and IIIB, respectively (Gridelli et al., 2003). Second-line therapy (docetaxel, erlotinib or pemetrexed in non-squamous cell cancer) may be beneficial in some patients (Fossella et al., 2003, D´Addario et al., 2009).

4.8.2.1 Stage III A

In stage IIIA lung cancer surgery should be considered whenever possible. Postoperative radiotherapy is advocated for the treatment of positive or close surgical margins, positive hilar or mediastinal lymph nodes. However, postoperative radiation would appear to have no impact on survival to judge from published randomized studies, whereas it reduces the incidence of local recurrences (Johnson and Turrisi, 2000). Neoadjuvant chemotherapy or chemoradiotherapy followed by surgery are recommended options for patients with N2 disease, as surgery alone has yielded unsatisfactory results (Jassem, 2007). However, further research is warranted in this field. Otherwise concurrent chemoradiotherapy is the standard of care for stage IIIA patients (Rigas and Kelly, 2007). For patients not suitable for combination treatment, either chemotherapy or radiotherapy may be used.

Platinum-based chemotherapy has shown a survival advantage over other regimens in NSCLC (Bunn and Thatcher, 2008). According to one meta-analysis, cisplatin-based treatment produces higher response rates than combination chemotherapy with carboplatin (HR=1.36, $p<0.001$). However, no significant differences have been seen in the overall survival (HR=1.050, $p=0.515$). Cisplatin-based treatment is more often associated with grade 3 or 4 nausea and vomiting compared to carboplatin treatment, whereas carboplatin-based treatment is associated with more severe (grade 3–4) thrombocytopenia (Hotta et al., 2004). The most commonly used platinum-based doublets (platinum with gemcitabine or vinorelbine or docetaxel or paclitaxel) have however proved equally effective in relation to survival; their adverse event profile differs (Gridelli et al., 2003). Of these chemotherapeutic agents, taxanes are the most widely investigated (Rigas and Kelly, 2007). Pemetrexed, a multi-target antifolate agent, has been used in combination with cisplatin in the treatment of NSCLC (Ricciardi et al., 2009) According to another meta-analysis, four cycles of chemotherapy seem to be adequate (Socinski et al., 2003). Single chemotherapy should be used in elderly and poor performance status (WHO 2) patients (Gridelli et al., 2004). In addition to the commonly used chemotherapy regimens, a wide range of new substances are being investigated in lung cancer. These include monoclonal antibodies, antisense oligonucleotides, tyrosine-kinase inhibitors, cyclo-oxygenase inhibitors, farnesyltransferase-inhibitors and proteasome inhibitors (Fietkau, 2004).

4.8.2.2 Stage III B

Only in T4N0 disease radical operation should be the goal (Battafarano et al., 2002). Otherwise, in stage IIIB disease, chemoradiotherapy is recommended. As in stage IIIA, cisplatin-based chemotherapy is the standard treatment. Patients with extensive local disease, metastatic pleuritis, metastasis in supraclavicular nodes, impaired pulmonary function or poor performance status are not eligible for chemoradiotherapy. Concurrent chemoradiotherapy seems to be associated with better survival compared with sequential chemotherapy and radiotherapy. Surgery may be an option in selected patients after induction chemotherapy (Jett et al., 2003).

4.8.3 Stage IV disease

Approximately one third of all lung cancer patients have stage IV disease at diagnosis (Tanoue, 2008). Here surgery has usually no role, and only palliative chemotherapy and radiotherapy can be applied (Fairchild et al., 2008). The exceptions for M1 patients eligible for surgery are those with an operable primary tumor and solitary brain metastasis which may be operated (Posther and Harpole, 2006). The aim of palliative treatment is to relieve symptoms, improve quality of life and possibly extend survival (Chao et al., 2002, p. 683–689).

4.9 Treatment of small cell lung cancer

SCLC is characterized by rapid dissemination and is considered a systemic disease (Ohe et al., 2004). Most SCLC patients are therefore unresectable and only very seldom may surgery be used in small limited tumors. SCLC is sensitive to chemotherapy, which is

thus the cornerstone of treatment in SCLC (Stupp et al., 2004). The combination of cyclophosphamide-adriamycin-vincristine (CAV) has been successfully used in the treatment of SCLC for more than 30 years (Schiller, 2001). Nowadays, the standard of care for good performance status patients is a combination of etoposide with cisplatin or carboplatin (Bayman et al., 2009).

4.9.1 Limited disease

One third of patients present with limited stage SCLC (Jassem, 2007). In limited disease, combining radiotherapy with chemotherapy significantly improves response rates (Sher et al., 2008); on the other hand, the impact on survival is still unclear (Jassem, 2007). Results of several trials suggest that early and concurrent radiotherapy with chemotherapy is optimal (Socinski and Bogart, 2007). The optimal dose of radiotherapy is not defined, but doses up to 70 Gy have been successfully employed without complications (Bayman et al., 2009). A 20% 5-year survival rate has been achieved with early radiotherapy (Bayman et al., 2009). As the brain represents a site of frequent recurrence in SCLC, prophylactic brain irradiation is recommended for all patients achieving complete response after chemotherapy and/or radiotherapy (Decker and Wilson, 2008). Despite the initial high response rate to chemotherapy, however, the duration of response is usually short. Refractory SCLC has a poor prognosis, but second line chemotherapy may be beneficial for some patients (Simon and Turrisi, 2007). The role of new targeted therapies in the treatment of SCLC still needs to be clarified (Bayman et al., 2009).

4.9.2 Extensive disease

For extensive stage small cell lung cancer, chemotherapy is the mainstay of treatment (Bayman et al., 2009). Cisplatin-based combination chemotherapy with either etoposide or irinotecan is currently the standard regimen in the USA as well as in Europe. The standard treatment comprises 4–6 cycles of chemotherapy given at 3-week intervals. 20–30% achieve complete response in extensive stage disease with a median overall survival of 10 months (Simon and Turrisi, 2007). The role of radiotherapy in extensive stage SCLC is mostly palliative; however, its role as consolidation treatment should still be explored (Bayman et al., 2009).

4.10 Radiotherapy

Radiotherapy is one of the main treatment modalities in cancer, in both the curative and palliative treatment setting (Jassem, 2007). This is in fact applied in one third of all lung cancer cases (Delaney et al., 2003). The delivering of fractionated radiotherapy was already introduced in the early 1900s. Fractionation refers to the dose per treatment, number of treatments in one day, as well as overall treatment time (Simon and Turrisi, 2007). The most frequently employed fractionation size is 1.8–2.5 Gy from Monday to Friday. This allows adequate tumor control without excessive risk of acute or late adverse events. Hyperfractionation means that more than one treatment per day is given, this allowing higher total doses with small dose fractions. Shortening the overall

treatment time by giving 2 Gy fractions more than 10 Gy/week is termed accelerated fractionation. This reduces the overall treatment time and increases the probability of tumor control for a given total dose; it is however, associated with increased acute toxicity (Chao et al., 2002, p. 17–19, Bayman et al., 2009).

In general, fractionated radiotherapy reduces the risk of permanent damage to normal tissue. There are differences in the tumor killing effects of treatment as well as acute and late adverse events with different fractionation schemes. Acute effects are more dependent on the total treatment time than late effects, whereas late effects are mostly affected by the total radiation dose and fractionation size (Chao et al., 2002, p. 23, Para et al., 2009). The base of the lungs seems to be more radiosensitive than the apex and the left more sensitive than the right (Para et al., 2009).

In lung cancer, the standard mode of radiotherapy delivery is external beam radiotherapy. Three-dimensional computerized tomography treatment planning devices are used to plan radical-aimed treatment. The clinical target volume (CTV) should include the lung cancer tumor itself and affected lymph node areas. Depending on the treatment fields, critical organs include medulla, healthy lung tissue, esophagus and heart. Three-year local tumor control rates have been 40–60% in patients treated with 60–70 Gy of radiotherapy (Decker and Wilson, 2008).

The aim of radiotherapy is to deliver a predefined radiation dose to the tumor and adjacent lymph nodes, sparing as much normal tissue as possible (Para et al., 2009). Multiple beams and oblique portals are required in order to achieve an adequate tumor dose. The most frequently employed dose is 60 Gy for the treatment of NSCLC (Jassem, 2007). In addition to destroying tumor cells, radiotherapy also damages normal healthy cells. The tolerance level of the normal tissues to irradiation is the main dose-limiting factor for radiotherapy in different tumor types. Although lung cancer irradiation is fairly well tolerated, treatment is often associated with short-term adverse events such as skin erythema and irritation. Acute reactions may also include esophagitis, cough, and fatigue. Chemoradiotherapy increases the risk of esophagitis. Late reactions may include pneumonitis, pulmonary fibrosis, esophageal stricture, cardiac sequelae, spinal cord myelopathy and brachial plexopathy (Chao et al., 2002, p. 23–25). The most prominent dose-limiting factor of lung cancer radiotherapy is acute radiation pneumonitis (Vujaskovic et al., 2002).

Remarkable progress has been made in the field of radiotherapy during last the two decades. New radiotherapy techniques include altered fractionation patterns, intensity-modulated radiation therapy (IMRT), tomotherapy, stereotactic radiotherapy and hadron therapy. Studies suggest that these new techniques may improve treatment results, as they allow delivery of a higher total radiotherapy dose (Schild and Bogart, 2006, Ball, 2008). Brachytherapy is a suitable alternative for the treatment of large, localized tumors which are unresectable. A further interesting research field for the future is the combination of targeted therapies and radiotherapy (Schild and Bogart, 2006).

Radiotherapy can be administered also as a palliation to treatment, e.g. to treat hemoptysis or pain or for symptom relief in brain and bone metastases (Jassem, 2007, Chao et al., 2002, p. 683–689).

4.11 Radiation pneumonitis

The lung is among the most radiosensitive visceral organs and radiation pneumonitis is a fairly common treatment-related adverse event and a distinct clinical entity. The conventional dosing causes symptomatic pneumonitis and lung fibrosis in 5–20% of patients, with higher values seen in patients treated with combined modality treatments (Vujaskovic et al., 2002). Acute radiation pneumonitis usually begins after 30 Gy of irradiation, and the risk seems to increase with increasing cumulative doses of radiation to normal lung tissue (Mehta, 2005, Park et al., 2007). Much research has been carried out in the radiotherapy field in order to determine suitable indicators or biomarkers which would discriminate among patients with either high or low risk of radiation-induced pulmonary complications. Up to now, no reliable markers have been defined (Novakova-Jiresova et al., 2004). Characteristic symptoms of radiation pneumonitis include dry cough, shortness of breath and occasional mild fever. The chest radiograph often reveals an infiltrate and the area affected might be larger than the original irradiated area (Kocak et al., 2005). The lung fibrosis phase starts 4–6 months after radiotherapy treatment. Clinical symptoms are rapidly alleviated by low-dose corticosteroid treatment (Chao et al., 2002, p. 317).

It has been suggested that in addition to dysregulation of various cytokines, e.g. IL-6 and TGF-β1, an oxidant/antioxidant imbalance locally in the lower respiratory tract may be responsible for radiation pneumonitis (Hill, 2005). Studies have shown that an increase in plasma IL-6 levels and also a decrease in IL-10 levels correlate with the occurrence of radiation pneumonitis (Chen et al., 2002, Arpin et al., 2005). However, in another study, no such association was detected (Barthelemy-Brichant et al., 2004).

4.12 Chemotherapy

Alkylating agents were discovered as far back as the 1940´s. The term adjuvant chemotherapy refers to systemic treatment administered after the primary treatment of a tumor, for example surgery or radiotherapy (DeVita et al., 2005, p. 297). Neoadjuvant chemotherapy denotes chemotherapy administered prior to the planned treatment, for example surgery or radiotherapy for patients with localized tumors. Primary chemotherapy refers to chemotherapy administered as the primary treatment. A common practice is combination chemotherapy, as the administration of several chemotherapeutic regimens provides many advantages over the single-agent approach. Firstly, combination chemotherapy provides maximal cell destruction with acceptable adverse events. It also provides broader coverage of possible resistant cell lines and prevents the formation of new such lines (DeVita et al., 2005, p. 297–298). The main challenges in the field of chemotherapy have been its toxicity for normal tissues as well as the development of resistance to it.

Many chemotherapeutic regimens exert their effects through the oxidative pathway. Such drugs are the anthracyclins (e.g. adriamycin, epiadriamycin and daunorubicin), procarbazine, bleomycin, vincristine, cyclophosphamide, etoposide and mitomycin (Faure et al., 1996). Most anticancer agents exert their effects through the initiation of apoptosis (Lopaczynski and Zeisel, 2001).

Although the current practice for NSCLC treatment includes ciplatin- or carboplatin-based combination therapy, none of the newer generation agents such as vinorelbine, gemcitabine, docetaxel or paclitaxel in addition of platinum has emerged as the golden standard (Fossella et al., 2003). Cisplatin acts by interfering with DNA replication and forms DNA adducts which activate signal transduction pathways (DeVita et al., 2005, p. 347–348, Halliwell, 2007). It evinces activity against various solid tumors, including lung cancer. Nephrotoxicity, neurotoxicity, ototoxicity, vomiting and nausea are the most common adverse events in cisplatin treatment (DeVita et al., 2005, p. 355). For SCLC, besides etoposide-platinum treatment, cyclophosphamide-adriamycin-vincristine (CAV) treatment is a commonly used modality (Schiller et al., 2001). As CAV combination chemotherapy was also the treatment for SCLC patients in study III, a detailed description is given of the chemotherapeutic agents involved.

4.12.1 Adriamycin

Anthracyclins have been in clinical use since the 1960s. Adriamycin (doxorubicin) belongs to the anthracycline antibiotics and is originally isolated from *Streptomyces peucetius* fungus. Adriamycin consists of an amine sugar attached to an anthraquinone ring (Faure et al., 1996). Its antitumor action is based on its inhibition of the topoisomerase II enzyme and subsequent binding to DNA. Adriamycin generates free radicals, as the reduction of one electron in its B ring leads to the formation of a semiquinone radical. It is readily oxidized under aerobic conditions due to donation of an unpaired electron, which results in a superoxide radical. The drug is highly lipophilic with a fairly long half-life in the body, and the metabolites are eliminated through the bile (Singal et al., 2000). Adriamycin is also known to cause lipid peroxidation and protein oxidation in cancer patients (Faber et al, 1995, DeAtley et al., 1998).

Adriamycin is one of the most effective and widely used chemotherapeutic regimens in both solid tumors and hematological malignancies (DeAtley et al., 1999, Singal et al., 2000). However, treatment with it may be limited by acute and chronic adverse events. The most common acute sequelae, myelosuppression, nausea and vomiting, are manageable and rarely dose-limiting. The most seriously constraining event in adriamycin treatment is the development of acute and chronic cardiac toxicity, including cardiomyopathy, the occurrence of which correlates with the total dose administered (Singal et al., 2000). Research has provided evidence that the adverse events associated with adriamycin are also driven in part by the production of free radicals, especially the hydroxyl radical (Faure et al., 1996). Apoptosis, lipid peroxidation, production of free radicals and inhibition of nucleic acid and protein synthesis have been suggested to explain the noxious cardiac effects of this drug (DeAtley et al., 1999, Singal et al., 2000). The total dose administered should therefore be limited to 500 mg/m^2.

4.12.2 Vincristine

Vincristine, vinblastine and vindesine belong to the group of vinca-alkaloids. These are naturally occurring or semisynthetic compounds found in the Catharanthus roseus plant. Vinca-alkaloids are dimeric molecules comprising two multiringed units, the catharanthine nucleus and the vindoline nucleus. The cytotoxic action of the vinca-alkaloids constitutes disruption of microtubules following subsequent dissolution of

microtubules and cell arrest in mitosis (Rivera-Fillat et al., 1988). The antitumor action of vincristine also involves peroxidative damage (Schlaifer et al., 1994).

The toxicity and clinical activity of the vinca-alkaloids varies widely. Vincristine has a large volume of distribution, high clearance rates and a long terminal half-life (approximately 23–85 hours). It binds extensively to plasma proteins and also to blood elements such as platelets. Vincristine is metabolized and excreted primarily by the hepatobiliary system (DeVita et al., 2005, p. 395).

Vincristine is widely used in combination with other chemotherapeutic agents in non-Hodgkin's and Hodgkin´s lymphoma, leukemias and lung cancer as well as in solid tumors. Peripheral neurotoxicity is the main adverse effect of this group of drugs. Other adverse events may include constipation, abdominal cramps, paralytic ileus, urinary retention and hypertension. Neutropenia is, however, the main dose-limiting adverse event (DeVita et al., 2005, p. 397).

4.12.3 Cyclophosphamide

Cyclophosphamide belongs to the alkylating agents and was originally developed from mustard gas. Its main effect is attributable to its active metabolite phosphoramide mustard. Phosphoramide mustard forms DNA crosslinks between and within DNA strands, and binds to DNA, thus modifying it chemically. It also interferes with DNA replication and transcription. Cyclophosphamide is converted by hepatic microsomal enzymes to 4-hydroxycyclophosphamide. Combination chemotherapy including cyclophosphamide has been shown to reduce the levels of various antioxidants and antioxidative enzyme (Subramaniam et al., 1994, DeVita et al., 2005, p. 333). Both in vitro and in vivo studies have shown that cyclophosphamide treatment induces lipid peroxidation and hyperlipidemia (Berrigan et al., 1987, Muralikrishnan et al., 2001).

Cyclophosphamide is the most widely used antitumor alkylating agent. It evinces broad activity in cancers, and is used in combination chemotherapy in a variety of leukemias and lymphomas as well as in multiple myeloma, sarcoma, and breast and lung cancer. It can be administered either orally or intravenously. Leukopenia is the main dose-limiting adverse event. Other adverse events comprise alopecia and hemorrhagic cystitis. With high doses, cardiotoxicity may occur (DeVita et al., 2005, p. 333, 340).

4.12.4 Biological treatment of lung cancer

The complexity of cancer development and proliferation makes it necessary to intervene at multiple stages and mechanisms to target cancer. Current treatment practice including surgery, chemotherapy and radiotherapy provides only limited improvement in the poor prognosis of lung cancer, and newer targeted therapies are needed to improve survival. The aim of targeted therapy is to yield better treatment results with less toxicity than with conventional chemotherapeutic agents (Cascone et al., 2007).

Several pathways are recognized to be involved in cancer cell proliferation and tumor invasion. The key signaling pathway is the epidermal growth factor receptor (EGFR) pathway (Gridelli et al., 2003). The EGFR family consists of four structurally similar receptor tyrosine kinase proteins: ErbB-1 (known as EGFR), ErbB-2 (HER-2), ErbB-3 (HER-3) and ErbB-4 (HER-4). All of these proteins include

extracellular, transmembrane and intracellular domains (Gridelli et al., 2003). The EGFR pathway contributes to various processes related to cancer development and progression, apoptosis, angiogenesis and metastasis (Ciardiello and Tortora, 2001). The EGFR tyrosine kinase inhibitors are reversible competitors with ATP for binding of the intracellular domain of tyrosine kinase (Gridelli et al., 2003). The first EGFR tyrosine kinase inhibitor (TKI) investigated was gefitinib; however, one randomized phase III trial showed no survival advantage between gefitinib and placebo in advanced NSCLC patients who had previously received one or two chemotherapy agents (Thatcher et al., 2005). Erlotinib is another EGFR-TKI which is an oral quinazoline derivative (Gridelli et al., 2003). Erlotinib has shown a survival advantage in the 2nd or 3rd line treatment of non-small cell lung cancer (Shepherd et al., 2005). Cetuximab is a human-mouse chimeric anti-EGFR monoclonal antibody which has shown survival benefit in a phase III trial in NSCLC (Pirker et al., 2008).

There are two kinds of approaches to target VEGF: monoclonal antibodies and vascular endothelial growth factor receptor tyrosine kinase inhibitors (VEGFR-TKIs). Monoclonal antibodies targeted against VEGF have also been examined in advanced lung cancer. The first agent discovered in this group was bevacizumab, an anti-VEGF recombinant humanized monoclonal antibody which blocks the binding of all VEGF isoforms (Gridelli et al., 2003). Bevacizumab has been shown to improve survival and progression-free survival significantly in one randomized phase III trial comparing paclitaxel-carboplatin alone or with bevacizumab in recurrent or advanced non-small cell lung cancer (Sandler et al., 2006). However, serious adverse events (such as bleeding) limit the usage of bevacizumab, and current ongoing trials will clarify its role in the treatment of lung cancer.

One further possibility is to target the various receptors in VEGF: VEGFR-1, VEGFR-2 and VEGFR-3. The first two mediate angiogenesis and VEGF-3 is involved in lymphogenesis. Agents in this group include sorafenib, sunitinib and vandetanib. Sorafenib is a novel oral kinase inhibitor of Raf-1 which also evinces activity against vascular endothelial growth factor receptors 2 and 3, as well as platelet-derived growth factor β and c-KIT. Sunitinib is an inhibitor for vascular endothelial growth factor receptors 1, 2 and 3, c-KIT and the platelet-derived growth factor receptor. It has both antitumor and antiangiogenic activities (Cascone et al., 2007).

Other new targeted therapies include matrix-metalloproteinases (MMPs) which are zinc-dependent endopeptidases responsible for the degradation of extracellular matrix, which promotes the angiogenesis and growth of tumors. MMPs have also been examined in lung cancer, without however, any substantial results (Gridelli et al., 2003). Another approach is via proteasome inhibition. Bortezomib is a small molecule which is a reversible proteasome inhibitor. A recent phase II trial has shown that adding bortezomib to gemcitabine-carboplatin treatment prolongs survival in advanced non-small cell lung cancer (Davies et al., 2009). The mammalian target of rapamycin (mTOR) is a serine threonine kinase which regulates cell growth and cell-cycle progression. Phase I/II trials are ongoing in NSCLC (Cascone et al., 2007). However, most of these new targeted therapies currently lack sufficient evidence to justify their routine use.

5. AIMS OF THE STUDY

This study was designed to explore the clinical significance of oxidative stress in lung cancer and the associations between antioxidants, cytokines, markers of oxidative and nitrosative stress and adverse events, response to treatment and overall survival in lung cancer patients. The specific aims were:

1. to assess markers of oxidative damage to DNA in lung cancer patients and evaluate the effects of radiotherapy and chemotherapy on them (study I)

2. to explore local and systemic oxidative stress and the oxidant effects of radiotherapy for lung cancer (study II)

3. to evaluate various antioxidants and parameters of oxidative and nitrosative stress caused by free radical-generating combination chemotherapy (study III)

4. to develop a non-invasive method to determine local lipid peroxidation caused by lung cancer radiotherapy (study IV)

5. to investigate the local and systemic effects of radiotherapy on different cytokines (study V)

6. The studies also set out to clarify whether the examined markers predict adverse events, response to treatment and overall survival (studies I–V)

6. MATERIAL AND METHODS

6.1 Lung cancer patients

These studies were conducted with newly diagnosed, untreated patients who had histologically or cytologically confirmed lung cancer. Altogether 74 lung cancer patients were included in the series. Of these, six were excluded from the final analysis for the following reasons: for three the analysis failed due to technical reasons, one had no cancer in the final pathology report, one patient had metastasis of renal cell carcinoma instead of primary lung tumor and one had non-Hodgkin lymphoma. Thus 68 patients were eligible for the study. As three of the patients served as pilot cases in the exhaled air collection study, 65 lung cancer patients were included in the study material. The control group consisted of 66 participants without cancer history. (Table V)

Patients were treated at Tampere University Hospital, except for two who entered the chemotherapy study (III) from Turku University Hospital. The patients and controls were included between June 1998 and July 2003. The intended follow-up time of this study was 72 months or to the death of the patient. Verbal and written information was given to all participants and they gave a written informed consent. The general inclusion criteria for lung cancer patients were: histologically or cytologically confirmed lung cancer and Karnofsky performance status of ≥70%. The general exclusion criteria were: previous cancer, except basalioma or carcinoma cervix in situ, serious cardiac, metabolic or liver disease, gout, regular high-dose vitamin supplementation during three months prior to the study or occasional vitamin supplementation during the week prior to inclusion in the study, and regular allopurinol or acetylcysteine treatment. In addition the following exclusion criteria were set: forced expiratory volume in one second (FEV_1) under 1.5 liters (in radiotherapy studies) and serious acute infection, inflammatory bowel disease or acute ischemia (V).

6.2 Control group

Altogether 66 non-cancer controls participated in the study. 36 of them were patients referred for bronchoscopy for reasons other than cancer (mainly prolonged cough), and who served as control patients in oxidative damage to DNA study (I) and the bronchoscopy studies (II+V). In the exhaled pentane study, 30 healthy hospital employees served as control subjects (IV).

A detailed description of the study groups is given in Table V.

Table V. *Characteristics of lung cancer patients and controls. Values are means (range) or numbers (percentages).*

	Lung cancer patients n=65		**Controls n=66**	
Age (years)	65.8	(48–83)	50.6	(18–75)
Males	52	(80.0)	35	(53.0)
BMI (kg/m^2)	24.4	(17.0–43.6)	25.6	(18.5–39.2)
FEV_1 (% of predicted)[1]	68.8	(21–102)	89.4	(65–108)
Smoking				
Non-smokers	3	(4.6)	39	(59.1)
Ex-smokers	21	(32.3)	13	(19.7)
Smokers	41	(63.1)	14	(21.2)
Pack-years of smoking[2]	37.2	(5.0–100)	28.5	(7.5–68)
Histological or cytological diagnosis				
Squamous cell carcinoma	38	(58.5)		
Adenocarcinoma	6	(9.2)		
Small cell carcinoma	20	(30.7)		
Not defined	1	(1.5)		
Stage of lung cancer				
I/IIA-B	3	(4.6)		
IIIA	12	(18.5)		
IIIB	19	(29.2)		
IV	30	(46.2)		
Not defined	1	(1.5)		
Karnofsky performance status				
70	6	(9.2)	0	(0)
80	16	(24.6)	0	(0)
90	31	(47.7)	3	(4.5)
100	12	(18.5)	63	(95.5)

[1] n=37 and n=20

[2] smokers and ex-smokers, n=57 and n=13

6.3 Pretreatment evaluation and follow-up of lung cancer patients

Pretreatment evaluation of the lung cancer patients included physical examination, bronchoscopy, chest radiography, chest and upper abdominal computerized tomography, urinanalysis, full blood count and serum chemistry. Electrocardiograms were obtained of all chemotherapy patients. Samples from bronchoalveolar washings were obtained from all bronchoscopy participants for tuberculosis cultivation. Abdominal ultrasound and skeletal scintigraphy were performed as clinically required. Diagnosis and TNM classification by UICC criteria (Sobin and Wittekind, 1997) of the cancer patients were based on findings from physical examination, bronchoscopy, chest radiography and chest and upper abdominal computerized tomography. No PET was available at the time when patients were included in the studies.

The full blood count included analysis of total red blood cells, hemoglobin, hematocrite, red blood cell indices, total white blood cells, white blood cell differential count and platelets. Serum chemistry analysis covered serum C-reactive protein, sodium, potassium, creatinine, albumin, alanine transferase, aspartate transferase and alkaline phosphatase. Urinanalysis was performed with a Urisys urine analyzer (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland) and comprised analysis of red and white blood cells and proteins (albumin). In addition, serum carcinoembryonic antigen (CEA) was obtained from all lung cancer patients and neuron-specific enolase (NSE) from all small cell lung cancer patients.

Karnofsky performance status, patient´s weight and smoking habits were registered at baseline and thereafter at each chemotherapy cycle in chemotherapy patients and in radiotherapy patients two weeks and three months after initiation of radiotherapy. Thereafter, the follow-up of patients was carried out according to current practice at Tampere University Hospital. The follow-up time varied from three weeks to 72 months.

6.3.1 Baseline questionnaire

Data on occupation, family history, symptoms, other diseases, medication and smoking were collected on a standardized questionnaire modified from the ATBC study (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study) (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994). (See appendix, page 143)

All participants in the exhaled pentane study (IV) also completed a detailed diet questionnaire over the three days prior to exhaled pentane collections.

6.3.2 Criteria for smoking

Smokers were defined either as current smokers or as smokers who had stopped smoking less than 6 months previously. Ex-smokers were subjects who had stopped smoking more than 6 months previously and non-smokers were subjects who had never smoked. Lifetime cigarette consumption was expressed as pack years. Pack years was defined as cigarette packs smoked per day multiplied by the number of years smoked (Erhola et al., 1998).

6.4 Treatments

Lung cancer patients were treated according to clinical guidelines (Sandler and Buzaid, 1992). 33 patients received radiotherapy, eight of whom received two or three cycles of cisplatin-based neoadjuvant chemotherapy; 16 small cell lung cancer patients received cyclophosphamide, adriamycin, vincristine (CAV)-chemotherapy. Two patients were operated after the diagnosis without further treatment; five received various other chemotherapeutic regimens and nine symptomatic treatment consisting of palliative radiotherapy and non-chemotherapy cancer treatment.

6.4.1 Radiotherapy

Twenty-seven patients underwent three-dimensional computer tomography (CT)-based treatment planning by Cad Plan (version 6.23, Varian, Varian Medical Systems Inc, Palo Alto, CA, USA). They were treated in the supine position with arms raised above their heads. Gross tumor volume (GTV) was delineated based on assessment of abnormalities in CT scan. The planning target volume (PTV) included the tumor and adjacent lymph nodes with adequate safety margins.

Six of the patients received palliative radiotherapy with two anterior-posterior fields. In five, the fractionation size was 3 Gy given five times in a week and in study IV one patient received radiotherapy 4 Gy/fraction 2 fractions/week to a total dose of 40 Gy.

Radiotherapy was delivered by a linear accelerator (Varian Clinac 2100 C/D, Varian Medical Systems Inc., Palo Alto, CA, USA) using 18 MV photons at the Radiotherapy Unit of Tampere University Hospital. Radiotherapy was usually given at two or three Gy fractions five times a week, from Monday to Friday. The mean radiation dose delivered was 46.6 Gy (range 30.0 Gy–60.0 Gy), corresponding to 4 ½ weeks´ duration of treatment.

6.4.2 Chemotherapy

In the chemotherapy study (III), SCLC patients received CAV chemotherapy comprising of adriamycin (doxorubicin, 50 mg/m^2), vincristine (1,5 mg/m^2, maximum dose 2 mg each time) and cyclophosphamide (750 mg/m^2). The treatment was administered intravenously; first adriamycin over 30 minutes, thereafter vincristine as a bolus and finally cyclophosphamide over 30 minutes. The planned number of cycles was six, with three-week intervals. Antiemetic medication, usually a 5-HT$_3$ antagonist, was given ½ hour before start of chemotherapy. Three liters of isotonic fluids was administered concomitantly with chemotherapeutic regimens during the first and second cycles. Ten patients (63%) also received 300 mg allopurinol and 6–12 mg sodium bicarbonate before each chemotherapy cycle to prevent the deleterious effects of possible tumorlysis. No colony-stimulating factors were used during the treatment.

6.5 Evaluation of performance status, adverse events and clinical response

Performance status was evaluated prior to, during and after chemotherapy and radiotherapy treatment according to the criteria of Karnofsky and Burchenal (1949). Adverse events were evaluated in the chemotherapy group at baseline and three weeks after each chemotherapy cycle according to World Health Organization (WHO) criteria (Miller et al., 1981). During and after radiotherapy treatment, all adverse events were assessed according to the Late Effects on Normal Tissue- Subjective, Objective, Management, Analytic (LENT-SOMA) scale and WHO criteria (Miller et al., 1981, LENT-SOMA tables, 1995). Radiation pneumonitis was scored according to the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC) acute radiation morbidity scoring criteria (Cox et al., 1995).

Response to treatment was assessed based on physical examination, laboratory tests and chest radiography after every two cycles of chemotherapy in the chemotherapy group (III). In the radiotherapy patients the response was based on physical examination and chest radiography and/or chest and upper abdominal computerized tomography. Evaluation of response was made three months after radiotherapy, and thereafter according to clinical practice. The responses to treatment were evaluated in all studies according to the criteria of WHO (WHO Handbook for Reporting Results of Cancer Treatment, 1979, Miller et al., 1981).

6.5.1 Definitions for response

The World Health Organization (WHO) definitions for responses were as follows: complete response (CR) was defined as the disappearance of all known disease determined by two observations not less than 4 weeks apart; partial response (PR) was defined as a 50% or more decrease in the total tumor load of those lesions that have been measured to evaluate the effect of treatment by two observations not less than 4 weeks apart. Thus, PR covered bidimensional lesions greater than or equal to 50% decrease in tumor area (multiplication of the longest diameter by the greatest perpendicular diameter) and in multiple lesions a 50% decrease in the sum of the products of the perpendicular diameters of the lesions. In addition no appearance of new lesions or progression of any lesions must occur. No change (NC) was defined as 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 (Miller et al., 1981). However, the RECIST criteria are nowadays commonly used to assess response to treatment (Eisenhauer et al., 2009).

Patients evincing complete or partial response were classified as responders and those showing no change or progressive disease were classified as non-responders. Progression-free survival was defined as time from inclusion in the study until progression of disease, and overall survival as time from inclusion in the study until death (DeVita et al., 2005, p. 485).

6.5.2 Ethical considerations

All studies were conducted according to the guidelines of the Declaration of Helsinki. The Ethics Committee of the Pirkanmaa Hospital District approved the studies. The chemotherapy study (III) was also approved by the Ethics Committee of the Hospital District of Southwest Finland. Participation in the studies was voluntary and refusal had no impact on patient's treatment. Verbal and written information was given to the participants and written informed consent was obtained from each participant. Patients or controls were not paid for participation in the study.

6.6 Schedule of study procedures

A summary of all studies with patient numbers, treatments and study procedures is presented in Table VI.

Table VI. *Summary of study settings*

Study number	Study groups	Treatment	Parameters measured	Collection time-points
I	65 lung cancer patients 36 non-cancer control patients	radiotherapy (n=33), CAV chemotherapy (n=16), other treatments (n=16)	*Urine*: 8-oxoGua and 8-oxodG *Whole blood*: nuclear 8-oxodG	*Urine (RT patients)*: baseline, at 8 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy, 3 months after RT *Blood (RT patients)*: baseline, at 20 Gy, 3 months after RT *Urine (CT patients)*: baseline and 19 hours after start of CT at 1st, 2nd and 6th CT cycle *Blood (CT patients)*: baseline, 3, 5 and 19 hours after the onset of CT during 1st, 2nd and 6th CT cycle *Urine and blood (control patients)*: baseline
II	36 lung cancer patients[1] 36 non-cancer control patients[2]	radiotherapy (n=20), other treatments (n=16)	*Serum/plasma and BAL fluid*: vitamin C, urate, thiols, nitrite, nitrite+nitrate *Serum/plasma*: vitamin E, GSH, TRAP, alpha- and gamma-tocopherol, proteins, oxidized proteins, TBARS, conjugated dienes	*Serum/plasma (patients)*: baseline, at 20 Gy, 3 months after treatment *BAL fluid (patients)*: baseline, at 20 Gy *Serum/plasma and BAL fluid (control patients)*: baseline
III	16 lung cancer patients[1]	CAV chemotherapy (n=16)	*Serum/plasma*: vitamin C, alpha- and gamma-tocopherol, urate, thiols, TRAP, proteins, oxidized proteins, TBARS, conjugated dienes, nitrite, nitrite+nitrate	*Serum/plasma (patients)*: baseline, 3, 5 and 19 hours after start of CT during 1st, 2nd and 6th CT cycle
IV	11 lung cancer patients[1] 30 healthy controls	radiotherapy (n=11)	*Exhaled air*: pentane *Serum*: TBARS, conjugated dienes	*Exhaled air (patients)*: before RT, 30 and 120 min after start of RT on days 1, 4 and 5, and at 30 and 40 Grays *Serum (patients)*: baseline, day 1, 4, 5, at 30 and 40 Gy *Exhaled air (controls)*: baseline
V	36 lung cancer patients[1] 36 non-cancer control patients[2]	radiotherapy (n=20), other treatments (n=16)	*Serum and BAL fluid*: VEGF, TNFα, IL-1β, IL-6, IL-8, IL-12, IL-18	*Serum (patients)*: baseline, at 20 Gy, 3 months after treatment *BAL fluid (patients)*: baseline, at 20 Gy *Serum and BAL fluid (control patients)*: baseline

[1] subgroup of patients from study I
[2] same control patient population as in study I
CAV = cyclophosphamide, adriamycin, vincristine
RT = radiotherapy
CT = chemotherapy
Gy = Gray

In these studies, various samples were collected for oxidative stress marker analysis: serum and plasma samples, bronchoalveolar lavage (BAL) samples, urine samples and exhaled air samples. The guidelines followed in all studies are set out in section 6.6.1 below.

6.6.1 Bronchoscopy and bronchoalveolar lavage (BAL)

Bronchoscopy was performed by an experienced bronchoscopist and the procedure followed the guidelines recommended by the American Thoracic Society and European Society of Pneumology (Sokolowsky et al, 1987, Klech and Pohl, 1989). During bronchoscopy the subjects were awake and breathed spontaneously. Premedication with intramuscular atropine sulphate (0.5–0.7 mg) and 5 mL topical lidocain (20 mg/mL) anesthesia was administered to the nasal airways and posterior pharyngeal wall. The patients were monitored with an Ohmeda 3800 oximeter (Louisville, CO, USA) during the procedure. A flexible bronchoscope was wedged into the segmental or subsegmental level of the left upper or right middle lobe.

The bronchial washings, consisting of five times 20 mL of 0.9% sterile saline (+37ºC) mixed with Addiphos buffer (20 mL buffer to 500 mL saline, Fresenius Kabi, Uppsala, Sweden), were instilled through the bronchoscope and aspirated via gentle suction. The first aspirated sample was used for the standard cytological examination, and the second and subsequent yields were used for antioxidant and cytokine assays. Each recovered lavage volume was recorded. The bronchial washings were collected in a centrifuge tube (10 mL), immediately cooled on ice and protected from light. The samples were transferred immediately to the laboratory and centrifuged at 500 rpm for 15 min at 4ºC and stored at –70ºC until analysis. The samples for ascorbic acid analysis were mixed (1:10) with 5% metaphoshoric acid and isoascorbate. Cells were stained with the May-Grűnwald Giemsa (MGG) and Papanicolau stains and fixed with 50% ethanol. Bronchoalveolar lavation was performed on only one side of the lungs to prevent complications from the procedure. Diagnosis of the patients was made by brush cytology or transbronchial lung biopsy during the first diagnostic bronchoscopy. A second bronchoscopy was performed on 15 patients receiving radiotherapy and if possible on the same segment in the irradiated lung as at diagnosis.

6.6.2 Collection of urine and blood samples

For analysis of markers of oxidative damage to DNA, six hours´ overnight urine was collected (collection time usually 12 p.m. – 6 a.m.). The total volume obtained was measured and two 20 mL plastic tubes were separated. The samples were stored at –20ºC until later analysis. For other urinanalysis, 20 mL of urine was collected into a vacuum tube.

The blood samples for oxidative stress markers were taken as follows: peripheral venous blood samples were collected using a Venoject blood collection system (Terumo, Leuven, Belgium). Two tubes (10 mL each) of blood were collected: one was used for serum and one for plasma analysis. The serum samples were collected in sterile tubes, the plasma samples in cooled, sterile tubes containing ethylene diaminetetraacetic acid (EDTA). In addition, whole blood samples were collected in EDTA tubes in study I. The plasma samples were protected from light and centrifuged at 2800g for 10 minutes,

after which the plasma specimen for ascorbic acid analysis was mixed (1:10) with 5% metaphosphoric acid and isoascorbate. Serum cytokine samples were centrifuged at 3000g for 10 min. The samples were immediately frozen and stored at –70ºC until analysis.

Blood samples for other laboratory measurements (full blood count, serum C-reactive protein, sodium, potassium, albumin, alanine transferase, aspartate transferase and alkaline phosphatase) were collected according to instructions from the Centre for Laboratory Medicine of the Pirkanmaa Hospital District.

6.6.3 Markers of oxidative damage to DNA (study I)

The whole lung cancer patient population (n=65) and 36 non-cancer control patients also included in the bronchoscopy study served as the study population. Altogether 33 of the patients received radiotherapy, 16 adriamycin-containing chemotherapy, and 16 received other treatments (operation, other chemotherapy or palliative).

Urinary 8-oxoguanine (8-oxoGua) and the corresponding nucleoside 8-oxo-2´-deoxyguanosine (8-oxodG) as well as whole blood 8-oxodG were determined in all participants at baseline. In addition, urine samples were collected from patients receiving radiotherapy at 8 Gy, 10 Gy, and 20 Gy. When possible, samples were also obtained at 30 Gy and 40 Gy, and three months after the start of radiotherapy. In addition to baseline, blood samples were collected twice: at 20 Gy during radiotherapy and three months after radiotherapy.

In the case of chemotherapy patients, urine and blood samples were obtained at baseline and urine samples also 19 hours after administration of the first chemotherapeutic drug. The samples were collected during the first, second and last (usually sixth) chemotherapy cycle. Blood samples were also collected from six patients during the actual chemotherapy treatment (at baseline 12 a.m., 3 p.m., 5 p.m. and 7 a.m. the following morning) at cycles 1, 2 and 6.

6.6.4 Antioxidant bronchoalveolar lavage (BAL) study (study II)

A total of 36 lung cancer patients and 36 non-cancer control patients were enrolled for the study. Bronchoscopy and bronchoalveolar lavage (BAL) were performed on both patients and controls as a diagnostic procedure. Twenty patients received radiotherapy, and fifteen of them underwent a second bronchoscopy two weeks after the start of radiotherapy, at a radiotherapy dose of 18–22 Gy.

Various antioxidants (ascorbic acid, urate, thiols), nitrite and nitrite+nitrate were investigated locally in BAL fluid and systemically in the blood. The total peroxyl radical trapping antioxidant potential (TRAP), vitamin E, alpha- and gamma-tocopherol, glutathione and several parameters of oxidative stress (proteins, oxidized proteins, TBARS, conjugated dienes) were analyzed from the blood. The samples were collected from patients receiving radiotherapy at baseline, and during and three months after radiotherapy. From the 36 non-cancer control patients and 16 patients receiving treatments other than radiotherapy, only baseline BAL fluid and blood samples were obtained.

6.6.5 Chemotherapy study (study III)

Sixteen patients with SCLC were enrolled for the study. A common combination chemotherapy at the time of the study accrual, CAV (cyclophosphamide, adriamycin, vincristine) treatment, was given to all patients (Clark and Ihde, 1998). The chemotherapy was administered according to standard protocol during one day with three weeks interval. Dose reductions were done according to guidelines.

Different antioxidants such as ascorbic acid, alpha- and gamma-tocopherol, urate, thiols, TRAP, proteins, oxidized proteins, TBARS, conjugated dienes, nitrite and nitrite+nitrate were evaluated in the study. Blood samples were collected at baseline, usually at 12 a.m. and thereafter 3, 5 and 19 hours after administration of the first chemotherapeutic drug. The samples were obtained during the first, second and last (usually sixth) chemotherapy treatment.

6.6.6 Exhaled pentane study (study IV)

Fourteen untreated lung cancer patients were enrolled in this study. Three of them served as pilot patients for the study to determine appropriate sampling time-points, and 11 patients were included in the actual study population; 30 healthy individuals from hospital employees served as the control group.

Before exhaled pentane collections between 7.30 a.m. and 12 noon, all individuals had been fasting for 12 hours and resting for half an hour. Each participant was seated and used nose clips. The subjects breathed through a non-rebreathing Ruben valve to prevent inhaling ambient air. Initially, the participants were required to breathe hydrocarbon-free air for 4 minutes to wash contaminating hydrocarbons from their lungs. Hydrocarbon-free air collected in an 25-L impermeable gas bag was prepared with an AS80 air purifier (Signal Instruments, Camberley, UK). During wash-out period the exhaled air was discarded. After washout, the participants were instructed to inspire to total lung capacity and then to exhale slowly to the residual volume. The exhaled gas was collected through a sterile gauze into an impermeable 750 mL Quintron gas collection bag (Model QT00841-P, Quintron, Milwaukee, WI, USA) connected to a disposable 400 mL gasbag (Model QT000843-P, Quintron, Milwaukee, WI, USA), used to discard dead space air. The Quintron gas collection bags were used only once.

Exhaled pentane samples were collected from lung cancer patients before radiotherapy, and 30 and 120 min after the start of radiotherapy on days 1, 4 and 5, and at 30 and 40 Grays, if possible. Exhaled pentane samples were collected once from controls. Patients fasted for two hours between exhaled air collections on each collection day. During each exhaled pentane collection day two samples of purified hydrocarbon-free background air were collected into identical Quintron gas collection bags. Samples for serum TBARS and conjugated dienes were obtained once from patients on each exhaled air collection day.

6.6.7 Cytokine study (study V)

Consecutive patients scheduled for bronchoscopy at the Department of Respiratory Medicine of Tampere University Hospital and fulfilling the inclusion criteria were

enrolled in the study. The same lung cancer patient population as well as 36 non-cancer control patients were also examined in the antioxidant BAL study (II).

The levels of several cytokines such as vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, IL-8, IL-12 and IL-18 in the BAL fluid and serum of lung cancer patients and non-cancer control patients were examined. The first bronchoscopy was performed on both patients and controls as a diagnostic procedure before any treatment was given. In the case of lung cancer patients, the samples were taken at baseline. In addition, from patients receiving radiotherapy, serum and BAL fluid samples were obtained during radiotherapy and serum samples three months after radiotherapy. Control patients provided one set of samples.

6.7 Measurement methods

6.7.1 BAL and plasma ascorbic acid

BAL and plasma ascorbic acid concentrations were determined in metaphosphoric acid-stabilized samples by ion-paired reversed-phase HPLC coupled with an electrochemical (EC) detector. The chromatography system comprised a Hewlett-Packard 1090 HPLC (Hewlett-Packard Co., 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 LC-18-DB HPLC column (250 mm×4.6 mm, 5 μm particle) and a Supelguard LC-18-DB guard column (20 mm×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 (Salminen and Alfthan, 2008). The coefficient of variation between the series was 5.5%. Results are expressed as μmol/L. Analyses were made at Biomarker Laboratory and Laboratory of Analytical Biochemistry, National Public Health Institute, Helsinki, Finland.

6.7.2 BAL and serum urate

BAL and serum urate were determined by an enzymatic method (Konelab, Thermo Fisher Scientific, 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). Results are expressed as μmol/L. All samples were analyzed at Biomarker Laboratory and Laboratory of Analytical Biochemistry, National Public Health Institute, Helsinki, Finland.

6.7.3 BAL and plasma protein thiols

Total thiols were determined from 400 μL of BAL fluid or 100 μL of plasma using a spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (Ellmann's reagent) according to the method described by Hu (1994). The coefficient of variation between the series was 6.6%. Results are expressed as μmol/L. Analyses were performed at Biomarker Laboratory and Laboratory of Analytical Biochemistry, National Public Health Institute, Helsinki, Finland.

6.7.4 BAL and plasma NOx and nitrite

To measure nitrite+nitrate (NOx) concentrations, vanadium (III) chloride (VCl_3) in hydrochloric acid was used to convert nitrite and nitrate to nitric oxide (NO), which was then quantified by the ozone-chemiluminescence method (Braman and Hendrix, 1989, Kotikoski et al., 2002). The samples were first treated with two volumes of ethanol at –20ºC for 2 h to precipitate proteins. A sample of 20 µL was injected into a cylinder containing a saturated solution of VCl_3 in 1 M HCl at 95ºC, 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 limits were 1.5 µmol/L for NOx and 0.2 µmol/L for nitrite. Plasma and BAL NOx and nitrite were analyzed at the Medical School, University of Tampere, Tampere, Finland.

6.7.5 BAL and serum cytokines

Tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6 and IL-8 levels in the serum and BAL fluid were measured using a PeliKine compact TM ELISA kit (Central Laboratories of the Netherlands Red Cross, Amsterdam, the Netherlands) which are a sandwich-type of enzyme immunoassays. The sensitivity of the IL-1β assay is 0.8–1.5 pg/mL, 0.2–0.4 pg/mL for IL-6 assay and 1–3 pg/mL for TNFα and IL-8 assays. The expected concentrations of TNFα, IL-1β, IL-6 and IL-8 in fresh serum and plasma from healthy individuals are <10 pg/mL, <5 pg/mL, <20 pg/mL and <10 pg/mL, respectively.

Serum and BAL fluid vascular endothelial growth factor (VEGF) and interleukin-12 levels were determined by a quantitative sandwich-type enzyme immunoassay (R&D Systems Inc., Minneapolis, MN, USA). The minimum detectable dose of both VEGF and IL-12 is <5 pg/mL.

Serum and BAL fluid interleukin-18 levels were measured with a Human IL-18 ELISA Kit (Medical and Biological Laboratories Co. Ltd, Nagoya, Japan), which measures human IL-18 by sandwich ELISA. The assay uses two monoclonal antibodies against different epitopes of human IL-18. The sensitivity of the assay is 12.5 pg/mL.

All serum and BAL fluid cytokine samples were assayed in duplicate. Results were expressed as picograms of cytokine per milliliter. Analyses were made at the Centre for Laboratory Medicine, Pirkanmaa Hospital District, Tampere, Finland.

6.7.6 BAL fluid cell counts

Total and differential cell counts were determined by microscopy in a Bürker´s chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) on fixed BAL fluid samples. The determination of albumin in BAL fluid was performed immunonephelometrically using a Dade Behring BN II Nephelometer and reagents from Dade Behring GmbH (Marburg, Germany). BAL proteins were measured colorimetrically (Cobas Integra, F. Hoffmann-La Roche Ltd, Basel, Switzerland) by the Biuret method (Doumas et al., 1981a+b, Tietz 1995, p. 518–519). Analyses were made at the Centre for Laboratory Medicine, Pirkanmaa Hospital District, Tampere, Finland.

6.7.7 Plasma glutathione

Total glutathione in plasma (GSSG+GSH) was determined by an enzymatic recycling reaction (Baker et al., 1990). Plasma was used as sample instead of acidified whole blood. Thawed plasma samples were kept in an ice bath and diluted 5-fold with cold distilled water, whereafter the diluted plasma was added without delay to a reaction mixture. Production of 5-thio-2-nitrobenzoic acid was measured photometrically at 414 nm in an iEMS microtiter plate reader (Thermo Electron Corp., Helsinki, Finland). The analyses were performed at Oy Jurilab Ltd, Kuopio, Finland.

6.7.8 Plasma alpha-tocopherol and gamma-tocopherol

Here 0.4 mL of a 50% ethanolic solution containing ascorbic acid and butylated hydroxytoluene (BHT) and 50 µL of the internal standard tocol was added to 50 µL of plasma. 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. Alpha- and gamma-tocopherol were separated with an Inertsil ODS-3 column (2.1×100 mm, 3 µm, GL Sciences Inc., Tokyo, Japan). The mobile phase was methanol, 0.3 mL/min; 5 µL was injected into the column and the tocopherols detected by their fluorescence at 292/324 nm. Peak height/internal standard ratios were compared to the ratios of reference plasma with values traceable to NIST-certified serum standards, 968b (National Institute of Standardization and Technology, Gaithersburg, MD, USA) (Catignani and Bieri 1983, Anttolainen et al., 1996).

The coefficient of variation between the series was 5.2%. Results are expressed as mg/L. The samples were analyzed at Biomarker Laboratory and Laboratory of Analytical Biochemistry, National Public Health Institute, Helsinki, Finland.

6.7.9 Serum vitamin E

Serum vitamin E was measured by chromatographic methods as described elsewhere (Porkkala-Sarataho et al., 1996). In brief, vitamin E was extracted from 200 µL of serum with 5 mL of hexane and 1 mL of ethane. After centrifugation (5 min at 3000 rpm), the top layer was separated and evaporated to dryness under nitrogen and the residue dissolved in 200 µL of mobile phase (acetonitrile-ethanolchloroform, 47 + 47 + 6 by vol). Samples were injected into a C18-column and detected at 294 nm. The analyses were performed at Oy Jurilab Ltd, Kuopio, Finland.

6.7.10 Serum TRAP

The antioxidant potential of serum samples (total peroxyl radical trapping antioxidant potential, TRAP) was estimated by their potency in resisting 2,2'-azobis (2-amidinopropane hydrochloride) (ABAP; Polysciences Inc., Warrington, PA, USA) -induced peroxidation (Alanko et al., 1993). 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 (5-amino-2,3-dihydro-1,4-phtalazinedione, 0.5 mg/mL; Bio-Orbit Ltd., Turku, Finland) and 20 µL of serum sample were mixed in the cuvette and the assay initiated with 0.05 mL of ABAP (83 mg/mL). Chemiluminescence in duplicate

cuvettes at 37ºC was measured until a peak value for each sample was detected. Peroxyl radical trapping capacity was defined by the half-peak time-point. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Aldrich Chem. Co., Milwaukee, WI, USA) served as standard radical scavenger (Ahotupa et al., 1997). Results are expressed as µmol/L. The analyses were made at MCA Research Laboratory Ltd, Turku, Finland.

6.7.11 Serum proteins

Serum proteins were analyzed colorimetrically (Cobas Integra, F. Hoffmann-La Roche Ltd, Basel, Switzerland) by the Biuret method, in which divalent copper reacts with the peptide bonds of proteins under alkaline conditions to form the characteristic pink to purple biuret complex (Doumas et al., 1981a+b, Tietz 1995, p. 518–519). Sodium potassium tartrate prevents copper hydroxide precipitation and potassium iodide prevents the autoreduction of copper. The color intensity is proportional to the protein concentration. It is assessed by measuring the increase in absorbance at 552 nm. The intra-assay coefficient of variation was 0.88% and the interassay 1.80%. Results are expressed as g/L. Analyses were made at the Centre for Laboratory Medicine, Pirkanmaa Hospital District, Tampere, Finland.

6.7.12 Serum protein oxidation

Protein carbonyl determinations were carried out as described by Reznick and Packer (1994). The sample was treated with 2,4-dinitrophenylhydrazine and trichloroacetic acid (TCA). The protein was washed in ethyl acetate–ethanol (1:1 vol/vol) and dissolved in guanidine hydrochloride. The 2,4-dinitrophenylhydrazine and ethyl acetate were obtained from Merck KGaA (Darmstadt, Germany), guanidine hydrochloride from Fluka (Buchs, Switzerland) and TCA from Riedel-de Haën (Seelze, Germany). Protein carbonyl content was quantitated 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). Results are expressed as µmol/L. The analyses were made at MCA Research Laboratory Ltd, Turku, Finland.

6.7.13 Serum TBARS

For the analyses of thiobarbituric acid-reactive substances (TBARS) 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 (Bird and Draper, 1984). 1,1,3,3-Tetraethoxypropane purchased from Sigma Chemical Co. (St. Louis, MO, USA) was used as standard. The coefficient of variation for within-assay was 8.2%, and for between-assay 8.8%. Results are expressed as µmol/L. The analyses were made at MCA Research Laboratory Ltd, Turku, Finland.

6.7.14 Serum conjugated dienes

For the measurement of diene conjugation, lipids extracted from serum samples (100 µL) by chloroform-methanol (2:1 vol/vol), dried under a nitrogen atmosphere and

then redissolved in cyclohexane, were analyzed spectrophotometrically (at 234 nm) as previously described (Corongiu et al, 1983). The coefficient of variation for within-assay was 4.4%, and for between-assay 4.5%. Results are expressed as μmol/L. The analyses were made at MCA Research Laboratory Ltd, Turku, Finland.

6.7.15 Urinary 8-oxoGua and 8-oxodG

A total of 0.5 nmol of [$^{15}N_3$, ^{13}C] 8-oxoguanine (8-oxoGua), 0.05 nmol of [$^{15}N_5$] 8-oxo-2′-deoxyguanosine (8-oxodG) and 10 μL of acetic acid (HPLC grade, Sigma, St. Louis, MO, USA) were added to 2 mL of human urine. The isotopic purity of the applied standards was 97.65% and 95%, respectively. The isotopically labeled standard of 8-oxoGua was the kind gift of Dr M. Dizdaroglu from the National Institute of Standard and Technology, Gaithersburg, USA. The standard of 8-oxodG was prepared from [$^{15}N_5$] dGTP according to the procedure described by Bialkowski and Kasprzak (1998) with addition of an alkaline phosphatase digestion step. After centrifugation (2000g, 10 min), supernatant was filtered through a Millipore GV13 0.22 μm syringe filter and 500 μL of this solution was injected onto an HPLC system. In the pilot study isotopically labeled internal standards of unmodified compounds (1 nmol of [$^{13}C_3$] Gua and 1 nmol of [$^{15}N_5$] dG) were added to the urine samples to monitor fractions containing both of these compounds and to avoid overlapping of the peaks containing the modified and unmodified base/nucleoside. The isotopic purity of the applied standards was 96.4% and 98.0% respectively.

Urine high-performance liquid chromatography (HPLC; Supelcosil LC18 column, 250 mm×10 mm, 5 μm equipped with a Supelguard LC18 guard column, 20 mm× 4.6 mm; Supelco, Sigma Aldrich, Poznan, Poland) purification of 8-oxoGua and 8-oxodG was performed according to the method described by Gackowski and associates (2001, 2003). Chromatography/mass spectrometry (CG/MS) analysis was as described by M. Dizdaroglu (1994), adapted for additional [$^{15}N_5$] 8-oxoGua analyses (m/z 445 and 460 ions were monitored; these ions represent the masses of characteristic ions of the base shifted in the mass spectra according to the extent of labeling). The urinary biomarkers were normalized to the concentration of creatinine. The concentration of creatinine in urine was measured using a creatinine kit Sigma Diagnostics (Sigma Aldrich, Poznan, Poland), where measurement is based on Jaffe reaction with picric acid. Results are expressed as nmol/mmol creatinine. All analyses were made at the Department of Clinical Biochemistry at Nicolaus Copernicus University, Bydgoszcz, Poland.

6.7.16 Nuclear 8-oxodG

DNA from whole blood was isolated by a non-enzymatic method (Lahiri and Nurnberger, 1991). Samples were stored at –70ºC before hydrolysis and HPLC analysis, which took place no later than 1 week from DNA purification.

The amount of 8-oxo-2'-deoxyguanosine was determined using HPLC equipped with an electrochemical detector, and deoxyguanosine was determined with a UV detector. The nucleosides were separated by C18 reverse-phase column (Phenomenex Luna C18(2), 4.6 mm×150 mm, 3 μm, Phenomenex Inc., Torrance, CA, USA). The elution solution was 50 mM citric acid-sodium citrate buffer, pH 5.2, with 10% methanol (HPLC grade) and 2 mM NaCl, the flow rate was 0.8 mL/min and the column

was maintained at 30ºC in a column oven. The cell potential of the electrochemical detector was 680 mV and the range-value was 0,1 nA/V. The absorbance of the UV-detector was 290 nm, which increased the amount of deoxyguanosine which could be quantitated. The retention time for 8-oxodG was 9,3 min and for dG 7,0 min. After 12 minutes of elution at a flow rate of 0.8 mL/min the column was washed at a higher flow rate of 1.7 mL/min for 40 min. After washing, the system was allowed to re-equilibrate for 8 min at a running flow rate. 8-oxodG and dG standards were injected before and after the samples. The 8-oxodG concentration was expressed as the ratio of 8-oxodG per 10^5 dG. The analyses were made at the MCA Research Laboratory Ltd, Turku, Finland.

6.7.17 Exhaled pentane

The exhaled air samples and the samples taken from purified hydrocarbon-free background air were transferred into adsorbent tubes containing graphitized carbon (Carbopack B, N930-7002/ Perkin Elmer; Perkin Elmer Corp., Norwalk, CT, USA) before analysis. An air volume of 0.8 L was pumped at 150 mL/min from each Quintron gas collection bag into the sampling tubes. The tubes were analyzed with a Perkin Elmer ATD 400 thermal desorber and a gas chromatograph (HP 5890, Hewlett-Packard, Palo Alto, CA, USA) equipped with a HP 5970A quadruple mass-selective detector. The following conditions were used for thermal desorption: the temperature for desorption was 300ºC for 10 min with a desorb flow of 25 mL/min and an inlet split flow of 12 mL/min. The cold trap (Tenax TA; 60/80 mesh) was kept at –30ºC during the first desorption and at 300ºC for 5 min during the final desorption. The outlet split flow was adjusted to 10 mL/min. The valve and line temperatures were 200ºC.

The sample was transferred from the cold trap directly to the analytical column PLOT Al_2O_3/KCl (30 m×0.32 mm, 5 µm; Chrompack, Middelburg, the Netherlands) with a carrier gas (helium) at a column pressure of 3.7 psi. The gas chromatograph (GC) oven temperature was programmed as follows: 50ºC for 1 min, increased by 5ºC/min to 130ºC, held for 1 min, increased by 12ºC/min to 180ºC, then held for 18 min. The temperature of the transfer line between the GC and the mass-selective detector was 225ºC. The retention time of pentane was 8.2 min. Mass-selective detection was based on the electron impact ionization mode and the ions (m/z) 43, 57 and 72 were monitored. The area of the base peak (m/z 43) was used for quantification.

Calibration standards were made by injecting 1 µL of calibration solution (n-pentane in methanol) into the sampling tubes and sucking air through the tubes for 2 min. The blank samples were prepared correspondingly by injecting 1 µL of methanol into the sampling tubes. The calibration curve was obtained after subtracting the peak area of the blanks from the peak areas of the calibration standards. The concentration of pentane (ng/L) was then calculated for all samples. The concentration of pentane in the expired air of the study subjects was obtained by subtracting its concentration in purified hydrocarbon-free background air samples from the corresponding concentrations in exhaled air samples. The pentane concentration was expressed as ng/liter. All exhaled air samples were analyzed at the Finnish Institute of Occupational Health, Helsinki, Finland.

6.8 Statistics

6.8.1 Primary and secondary variables

The primary variables were urinary 8-oxoGua, urinary 8-oxodG, and nuclear 8-oxodG (study I), oxidative stress markers in blood and in bronchoalveolar lavage fluid (study II), oxidative stress markers in blood (studies II, III), exhaled pentane concentration (IV) and cytokines in serum and in bronchoalveolar lavage fluid (study V). The variables were measured at baseline for cancer patients and controls, and during radiotherapy or chemotherapy treatment.

The results are given as means or geometric means with 95% confidence intervals, or as medians with interquartile ranges (III). If the distribution was skewed to the right, the values were logarithmically transformed before analysis and the results given as geometric means.

Secondary variables were serum TBARS and conjugated dienes (IV). In addition, the occurrence of adverse events, response to treatment and survival time were estimated in all studies.

6.8.2 Group comparisons

Independent samples t-test was used as an unadjusted analysis when the lung cancer patients were compared to controls (I, II, IV, V) at baseline before any treatment. The results for group comparisons are given as mean difference with 95% confidence interval. For logarithmically transformed variables the group comparisons are given as ratios of patients/controls with 95% confidence intervals. The distributions of age, gender and smoking were different in patients and controls and they were also associated with most of the primary variables. Age (as continuous variable), gender and smoking (smoker vs. non-smoker) were therefore considered possible confounding variables and were included, when appropriate, as covariates or random factors in multivariable linear models (I, II, IV, V). Hypertension (V), FEV_1 (II), body mass index (V), and histological diagnosis and stage of cancer (IV) were also included as factors and covariates, when appropriate. The interaction terms between the explaining factors were not included in the multivariable models.

6.8.3 Within-patient changes during treatment

The within-patient changes in the primary variables from baseline to certain measuring points during the treatment period were analyzed using t-test for paired samples (II, V) or non-parametric Wilcoxon's signed ranks test (IV), when appropriate. ANOVA for repeated measures (I, II, IV) or Friedman's ANOVA (I), when appropriate, was used to test the time effect in the primary variables during the treatment period. In study III, due to the high number of repeated measurements, the analyses were performed using the following procedure: first, the repeated baseline (at 0 h, before chemotherapy) measurements during the 1^{st}, 2^{nd} and 6^{th} cycles were analyzed by Friedman's non-parametric ANOVA. Then, in the case of a significant time effect, the Wilcoxon signed ranks test was used for paired comparisons (1^{st} vs. 2^{nd}, 1^{st} vs. 6^{th} and 2^{nd} vs. 6^{th}). The same method was applied to the within-cycle results at 0, 3, 5 and 19 hours. First,

Friedman's ANOVA was used in 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 time effect results were significant.

6.8.4 The effect of demographic characteristics

Associations between subject characteristics and the primary variables were studied in order to assess the need for an adjusted group comparison (I, II, IV, V). Pearson´s correlation and non-parametric Spearman's rank correlation were used to study the associations between continuous demographic characteristics (age, FEV_1, BMI and pack years of smoking) and the primary variables. Spearman's rank correlation was also used for associations between primary variables and other laboratory measurements at baseline. In study I, both the primary variables and the other laboratory measurements were repeated simultaneously during the treatment period. The method given by Bland and Altman (1995) was used to calculate the correlation coefficient for repeated measurements. Independent samples t-test, ANOVA, non-parametric Mann-Whitney U test and Chi-squared test were used for associations between dichotomous or dichotomized demographic characteristics (gender, other diseases, histology, stage of disease and smoking) and the primary variables.

6.8.5 Baseline levels of primary and secondary variables vs. adverse events, response to treatment and survival

The distributions of the primary variables at baseline were divided into two groups (median as cut-off point) or three groups (tertiles T1, T2 and T3, study V) to describe the baseline level. The occurrence of adverse events, response to treatment and survival time were compared between these groups (I–V). The log-rank test was used to compare the survival curves and Kaplan-Meier's method was used to estimate the median survival times in these groups and to plot the survival curves. Fisher's exact test was used to study the association between the baseline level of the primary variables, adverse events and response to treatment. In study V, Cox's regression analysis was performed to compare the tertiles T2 and T3 to the lowest tertile T1. The results are given as odds ratios (OR) with 95% confidence intervals.

P-values less than 0.05 were considered statistically significant. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, release 15.0) software (SPSS Inc., Chicago, IL, USA).

7. RESULTS

7.1 Study subjects demographics

Of the 65 lung cancer patients, 52 (80%) were male and 13 (20%) female. The control group consisted of 35 (53%) males and 31 (47%) females. Most (n=38) of the patients had squamous cell carcinoma (58%), 20 (31%) had small cell lung cancer, six (9%) had adenocarcinoma and in one (2%) case the histology remained unclassified.

In addition to cancer, twenty-three (35%) of the patients had hypertension, fourteen (22%) had cardiac disease, eight (12%) had chronic arrhythmia, five (8%) had asthma, four (6%) had mild or moderate chronic obstructive pulmonary disease (COPD), two (3%) had diabetes and two (3%) had rheumatic disease. None of the patients had chronic bronchitis, tuberculosis or asbestosis. None of the control patients were diagnosed with cancer; however, eleven had hypertension, four had asthma, one had chronic bronchitis, one mild COPD, one cardiac disease, one diabetes and one had rheumatic disease. The definition for COPD followed the guidelines from the European Lung White Book (Loddenkemper, 2003), according to which mild COPD is defined as $FEV_1/FVC<70\%$ and $FEV_1 \geq 80\%$ of predicted. Moderate COPD is defined as $FEV_1/FVC<70\%$ and $50\% \leq FEV_1 \geq 80\%$ of predicted.

In the bronchoscopy group, 80% of the non-cancer control patients were diagnosed as having idiopathic prolonged cough and received no further treatment. Of 22 (61%) of these, a histological diagnosis was obtained according to which 10 had normal histology, 10 had inflammation, one had squamous metaplasia and one was operated on for a benign papilloma. According to cytological analysis four of the control patients had normal cytology, four had squamous metaplasia and two had inflammation.

In the bronchoscopy group, the mean instilled volume at the 1st bronchoscopy was 111 mL (SD 26.7) in patients and 101 mL (SD 8.2, p=0.033) in non-cancer control patients and the recovery of saline 55 mL (SD 21.0) in patients and 67 mL (SD 11.9, p=0.005) in non-cancer control patients. There were no statistically significant differences between the groups in the total cell counts of BAL fluid samples, but lung cancer patients had a higher neutrophil count than non-cancer control patients (3% vs. 1%, p=0.002). There were no significant complications during or after the bronchoscopies.

7.2 Occurrence of adverse events

Of 33 patients receiving radiotherapy, twenty-two (67%) experienced grade I/II adverse events during treatment: esophagitis (n=12), dysphagia (n=6), cough (n=4), fatigue (n=5) and fever (n=4). No serious adverse events were seen during or after the treatment. Ten patients (30%) developed symptomatic radiation pneumonitis according to

RTOG/EORTC criteria. Eight (24%) patients showed mild/moderate (grade I/II) lung toxicity, whereas two (6%) suffered from grade III radiation pneumonitis.

Of 16 patients receiving CAV chemotherapy, 13 (81%) experienced adverse events during treatment. In three of the patients (19%), transient grade III hematological toxicity occurred, and grade I/II hematological toxicity was noted in nine (56%). Other adverse events reported were total alopecia (n=7), nausea (n=4), fatigue (n=4) and dyspnea (n=4) (Table VII).

Table VII. *Adverse events during treatments, response to treatments and overall survival of all lung cancer (LC) patients. RT=radiotherapy, CT=chemotherapy*

	All LC patients (n=65)		**RT-treated patients (n=33)**		**CT-treated patients (n=16)**	
	N	**(%)**	**N**	**(%)**	**N**	**(%)**
Adverse events during RT/CT (n=49)	35	(71 %)	22	(67 %)	13	(81 %)
Response to RT/CT treatment (n=49)						
Complete or partial response	37	(76 %)	24	(73 %)	12	(75 %)
No change or progressive disease	12	(24 %)	9	(27 %)	4	(25 %)
Survival[1]						
1 year	24	(37 %)	11	(33 %)	10	(63 %)
3 years	4	(15 %)	4	(12 %)	4	(25 %)
5 years	4	(6 %)	0	(0 %)	2	(13 %)
Median survival (months)	15.8		9.2		13.0	
range	0.4–72.0		0.4–58.8		2.5–70.1	
95% CI	11.4 to 20.2		6.0 to 12.4		9.6 to 16.4	

[1] Kaplan-Meier estimates

7.3 Response to treatment

A total of 33 patients received radiotherapy in these studies. After a mean radiation dose of 46.6 Gy (range 30.0–60.0 Gy), 24 patients achieved complete or partial response (73%) and 9 had stable or progressive disease (27%).

Altogether 75% of the patients completed all six CAV chemotherapy cycles. No significant treatment delays occurred. The mean total doses delivered during each cycle were as follows: adriamycin 91 mg (range 80–110 mg), cyclophosphamide 1360 mg (range 1050–1650 mg) and vincristine 2 mg. The mean total doses delivered overall were 540 mg of adriamycin, 8160 mg of cyclophosphamide and 12 mg of vincristine.

After six cycles of treatment, one patient (6.3%) achieved complete response, 11 (68.8%) a partial response and two (12.5%) had stable disease. In two of the patients (12.5%), the disease progressed during the treatment, for which reason the CAV chemotherapy was discontinued after the first cycle (one patient) or after two cycles of chemotherapy (one patient). The overall response rate was 75%.

7.4 Survival

The planned follow-up time for the patient group was 72 months. None of the patients was lost to follow-up. At the end of the study, one patient (1.5%) was alive, and 64 (98.5%) had died. The median overall survival was 15.8 months (95 % CI 11.4–20.2 months, range 0.4–72.0 months). The one-year survival rate was 37%, the three-year survival rate 15% and the five-year survival rate 6%.

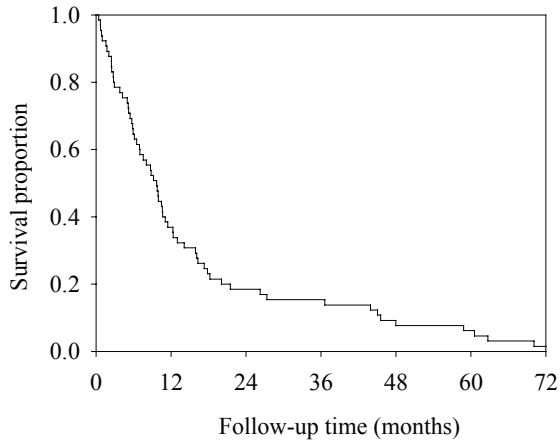

Figure 4. *Kaplan-Meier survival curve for lung cancer patients (n=65)*

7.5 Markers of oxidative damage to DNA (study I)

7.5.1 Baseline situation

Lung cancer patients and non-cancer control patients showed no significant differences in the excretion of urinary 8-oxoguanine (8-oxoGua, geometric mean 9.79 vs. 11.21 nmol/mmol creatinine, p=0.264) or of 8-oxo-2'-deoxyguanosine (8-oxodG, geometric mean 2.17 vs. 2.11 nmol/mmol creatinine, p=0.816) at baseline. The difference remained non-significant even after adjusting for age, gender and smoking (p=0.148 and p=0.422, for 8-oxoGua and 8-oxodG, respectively). A significant positive correlation between urinary 8-oxoGua and 8-oxodG excretion (R=0.463, p<0.001) was observed at baseline.

Lung cancer patients had significantly lower nuclear 8-oxodG levels than the non-cancer control patients at baseline (geometric mean 1.27 vs. 2.54 8-oxodG/10^5dG, p=0.003), which, however, was lost after adjusting for age, gender and smoking (p=0.145) (Original publication number I, page 1085, Table 2).

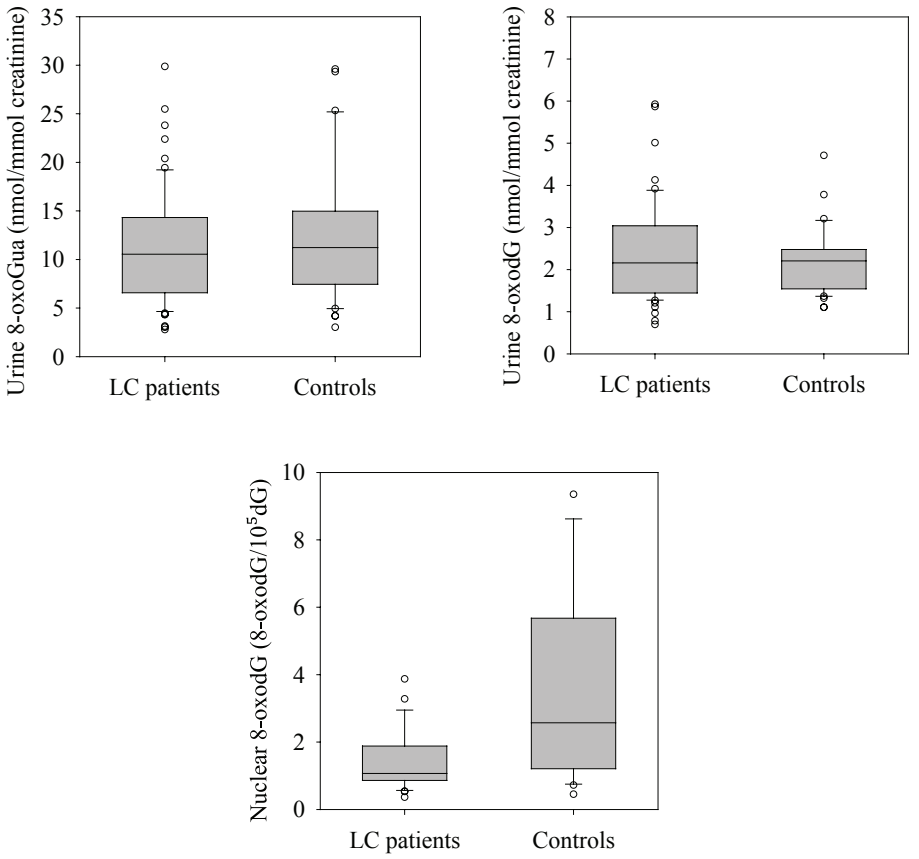

Figure 5. *Box-plot figure for urinary 8-oxoGua (8-oxoguanine, nmol/mmol creatinine), urinary 8-oxodG (8-oxo-2'-deoxyguanosine, nmol/mmol creatinine) and nuclear 8-oxodG (8-oxo-2'-deoxyguanosine, 8-oxodG/10^5dG) at baseline in lung cancer (LC) patients (n=63) and non-cancer control patients (n=36). The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90^{th} and 10^{th} percentiles. Outliers are given as circles. Outside the figure are the following outliers: urinary 8-oxoGua 80.6 nmol/mmol creatinine in the control group, urinary 8-oxodG 28.1 nmol/mmol creatinine in the patient group and 9.48 nmol/mmol creatinine in the control group, nuclear 8-oxodG 12.8 8-oxodG/10^5dG in the patient group and 14.4 8-oxodG/10^5dG in the control group.*

However, differences emerged for urinary 8-oxoGua when the patient group was divided into small cell (SCLC) and non-small cell (NSCLC) cancer patients, albeit still without significance (p=0.127). Comparing SCLC patients to non-cancer control patients, a statistically significant difference was found in 8-oxoGua (8.17 vs. 11.21 nmol/mmol creatinine, p=0.05). SCLC patients tended also to have lower urinary 8-oxoGua values compared to NSCLC patients (8.17 vs. 10.65 nmol/mmol creatinine, p=0.09). The differences in urinary 8-oxodG were non-significant between the tumor types (p=0.606). After adjustment for age, gender and smoking, the group differences for urinary 8-oxoGua expanded, and the difference between groups was almost significant (p=0.073). The adjusted geometric mean for urinary 8-oxoGua was 7.35 nmol/mmol creatinine in SCLC patients, 9.60 nmol/mmol creatinine in NSCLC patients and

16.97 nmol/mmol creatinine in non-cancer control patients. The significantly lower nuclear 8-oxodG levels in patients compared to non-cancer control patients at baseline were attributable to NSCLC patients, who tended to have lower values than SCLC patients (1.10 vs. 1.70 8-oxodG/10^5dG, p=0.136) and had significantly lower nuclear 8-oxodG values compared to non-cancer control patients (1.10 vs. 2.54 8-oxodG/10^5dG, p=0.001) (Unpublished data).

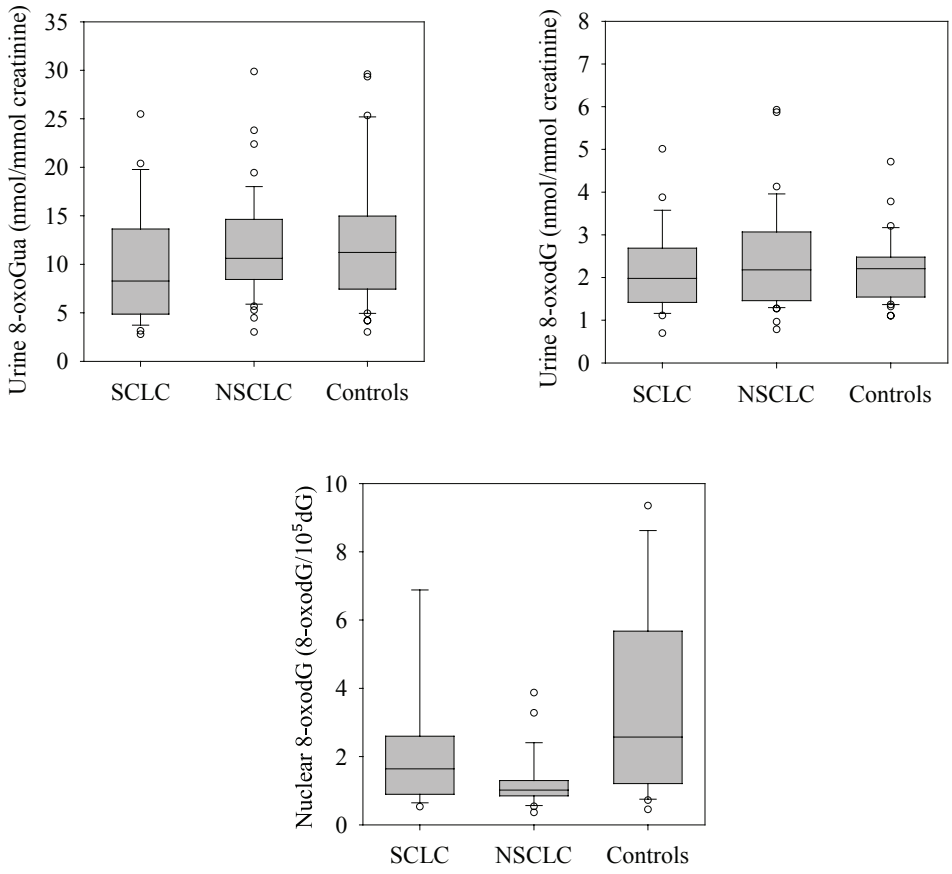

Figure 6. *Box-plot figures for urinary 8-oxoGua (8-oxoguanine, nmol/mmol creatinine), urinary 8-oxodG (8-oxo-2'-deoxyguanosine, nmol/mmol creatinine) and nuclear 8-oxodG (8-oxo-2'-deoxyguanosine, 8-oxodG/10^5dG) at baseline in SCLC (n=20), NSCLC patients (n=43) and non-cancer control patients (n=36). The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are given as circles. Outside the figure are the following outliers: urinary 8-oxoGua 80.6 nmol/mmol creatinine in the control group, urinary 8-oxodG 28.1 nmol/mmol creatinine in the NSCLC group and 9.48 nmol/mmol creatinine in the non-cancer control group, nuclear 8-oxodG 12.8 8-oxodG/10^5dG in the SCLC group and 14.4 8-oxodG/10^5dG in the non-cancer control group.*

7.5.2 Radiotherapy treatment

The levels of urinary 8-oxodG increased significantly during the first week of radiotherapy (1.74 at baseline, 2.62 at day 4 and 2.87 nmol/mmol creatinine at day 5, $p<0.001$). The geometric mean of 8-oxoGua was 9.84 at baseline, 11.38 on day 4 and 9.01 nmol/mmol creatinine on day 5 ($p=0.366$).

There were no significant changes at 20 Gy, 30 Gy or 40 Gy when compared to baseline. However, a tendency to decrease was recorded in the levels of urinary 8-oxoGua and 8-oxodG three months after radiotherapy (11.11 at baseline vs. 11.20 at two weeks vs. 8.72 at 3 months, $p=0.479$ for 8-oxoGua and 2.40 vs. 2.35 vs. 1.79 nmol/mmol creatinine, $p=0.389$, for 8-oxodG, respectively). There was also a significant positive correlation between urinary 8-oxoGua and 8-oxodG levels during radiotherapy, when all repeated measurements were included ($R=0.532$, $p<0.001$). The levels of nuclear 8-oxodG increased two weeks (1.39 at baseline vs. 1.57 8-oxodG/10^5dG) and three months after start of radiotherapy (1.39 vs. 1.57 vs. 3.36 8-oxodG/10^5dG). Due to the small number of samples (n=4), testing was not carried out for statistical significance.

7.5.3 Chemotherapy treatment

The baseline values of all three markers of oxidative damage to DNA tended to increase during chemotherapy; however, without statistical significance (7.66 vs. 8.68 vs. 11.12 nmol/mmol creatinine, $p=0.226$ for urinary 8-oxoGua; 1.61 vs. 1.69 vs. 2.28 nmol/mmol creatinine, $p=0.150$ for urinary 8-oxodG; 1.98 vs. 3.74 vs. 4.32 8-oxodG/10^5dG, $p=0.311$ for nuclear 8-oxodG at cycles 1, 2 and 6, respectively). A significant increase was recorded in nuclear 8-oxodG levels between the first and second chemotherapy cycle (2.06 vs. 3.99 8-oxodG/10^5dG, $p=0.043$), and urinary 8-oxodG levels during the sixth chemotherapy cycle (2.28 at baseline and 2.91 nmol/mmol creatinine 19 hours later, $p=0.009$).

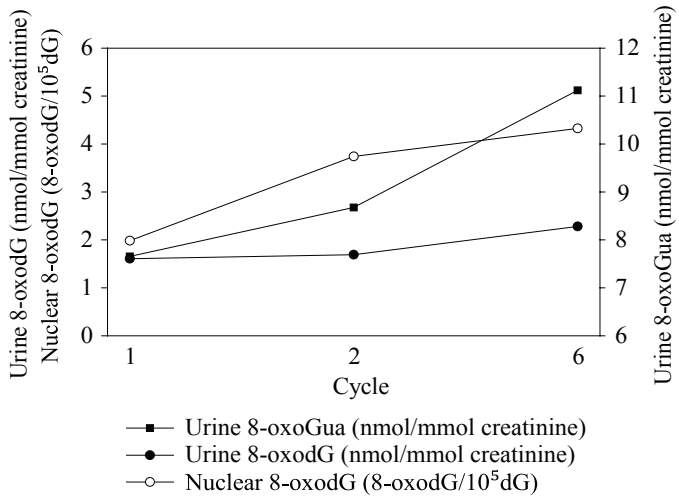

Figure 7. *Geometric means for urinary 8-oxoGua (8-oxoguanine, nmol/mmol creatinine, n=10), urinary 8-oxodG (8-oxo-2'-deoxyguanosine, nmol/mmol creatinine, n=10) and nuclear 8-oxodG (8-oxo-2'-deoxyguanosine, 8-oxodG/10^5dG, n=6) at baseline at chemotherapy cycles 1, 2 and 6.*

7.6 Antioxidant BAL study (study II)

Lung cancer patients had significantly higher levels of serum urate (241, 95% CI 210–272 vs. 116, 95% CI 91–141 μmol/L, p<0.001) and plasma nitrite (0.591, 95% CI 0.548–0.634 vs. 0.181, 95% CI 0.136–0.225 μmol/L, p<0.001) and lower levels of plasma thiols (303, 95% CI 283–322 vs. 364, 95% CI 338–390 μmol/L, p<0.001) and serum oxidized proteins (2.85, 95% CI 2.46–3.23 vs. 4.30, 95% CI 4.06–4.55 μmol/L, p<0.001) compared to non-cancer control patients at baseline. After adjustment for age, FEV_1, gender and smoking, significant differences persisted for urate, nitrite and oxidized proteins (p=0.016, p<0.001 and p=0.027, respectively); however, no differences were noted in plasma thiol levels (p=0.651).

At baseline, higher levels of BAL fluid urate (11.18, 95% CI 7.76–16.11 vs. 6.45, 95% CI 4.77–8.72 μmol/L, p=0.019) and thiols (4.40, 95% CI 2.69–7.21 vs. 1.48, 95% CI 1.12–1.95 μmol/L, p<0.001) and lower levels of NOx (0.87, 95% CI 0.77–0.97 vs. 1.34, 95% CI 1.12–1.60 μmol/L, p<0.001) were observed in patients compared to non-cancer control patients, being however, non-significant after appropriate adjustments (p=0.069, p=0.378 and p=0.979 for BAL urate, thiols and NOx).

No significant differences were detected in plasma ascorbic acid (p=0.529), plasma glutathione (p=0.636), serum vitamin E (p=0.264), serum conjugated dienes (p=0.420), serum TRAP (p=0.751), serum TBARS (p=0.855), plasma NOx (p=0.482), serum proteins (p=0.054), BAL ascorbic acid (p=0.293) or BAL nitrite (p=0.176) between patients and non-cancer control patients at baseline. The concentrations of TRAP in BAL fluid were undetectable at baseline (Original article II, page 651, Table II).

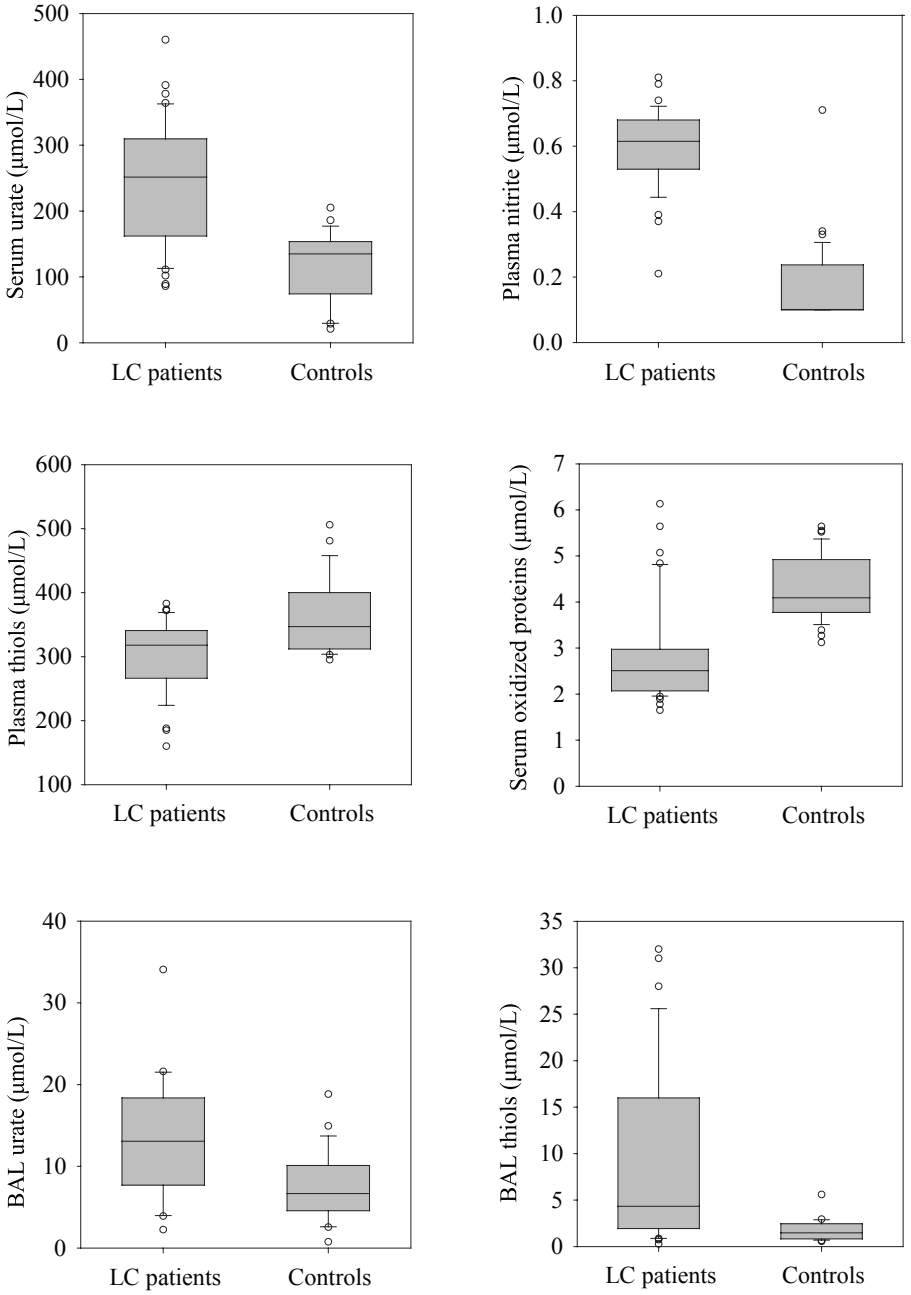

Figure 8. *Box-plot figures for systemic and local oxidative stress markers (µmol/L) at baseline in lung cancer (LC) patients (n=17–36) and non-cancer control patients (n=21–33). The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are given as circles.*

During radiotherapy, a trend towards an increase was shown in plasma alpha-tocopherol (6.43 vs. 8.86 mg/L, p=0.056), serum urate (220 vs. 255 µmol/L, p=0.064) and serum oxidized proteins (2.64 vs. 3.29 µmol/L, p=0.088) after two weeks from the start of treatment. The levels of serum urate (214 vs. 280 µmol/L, p=0.044), serum conjugated dienes (53.4 vs. 64.6 µmol/L, p=0.034) and serum TBARS (4.58 vs. 5.64 µmol/L, p=0.004) had risen significantly and those of plasma alpha-tocopherol nearly significantly (6.53 vs. 9.70 mg/L, p=0.055) three months after radiotherapy. For other plasma/serum antioxidants and markers of oxidative stress, changes during or after radiotherapy were non-significant.

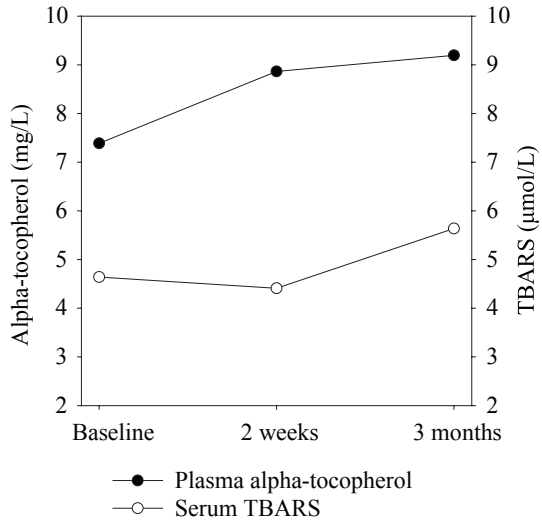

Figure 9. *Mean levels of plasma alpha-tocopherol (mg/L) and serum TBARS (µmol/L) at baseline, after two weeks of radiotherapy and three months after radiotherapy. The numbers of patients are 17, 8 and 7 for plasma alpha-tocopherol and 36, 16 and 18 for serum TBARS at baseline, 2 weeks and 3 months, respectively.*

Thiols, ascorbic acid, urate, nitrite and NOx were the markers analyzed in BAL fluid both at baseline and during radiotherapy. After two weeks of radiotherapy, nearly significant increases occurred in BAL fluid urate (geometric mean 7.30 µmol/L vs. 13.91 µmol/L, p=0.083) and thiols (geometric mean 3.34 µmol/L vs. 4.85 µmol/L, p=0.069). However, no differences emerged in other markers measured in BAL fluid during radiotherapy.

7.7 Chemotherapy study (study III)

The baseline levels (measured before the start of chemotherapy) of alpha- and gamma-tocopherol increased significantly between the first and sixth chemotherapy cycles (7.86 mg/L, IQR 7.03–8.88 at 1st cycle, 8.07 mg/L, IQR 7.18–9.02 at 2nd cycle and 10.10 mg/L, IQR 8.16–11.07 at 6th cycle, p=0.045 for alpha-tocopherol and 0.77 mg/L, IQR 0.51–1.22 at 1st cycle, 0.76 mg/L, IQR 0.58–1.36 at 2nd cycle and 0.97 mg/L, IQR 0.57–1.82 at 6th cycle, p=0.041 for gamma-tocopherol, respectively). The baseline levels of plasma NOx decreased from the 1st to the 6th cycle (35.3 µmol/L, IQR 28.5–43.0 at 1st cycle, 28.9 µmol/L, IQR 24.4–43.1 at 2nd cycle and 28.4 µmol/L, IQR 23.6–44.1 at 6th cycle, p=0.020). Changes in other baseline-values antioxidants or markers of oxidative or nitrosative stress were not significant during the follow-up at the 1st, 2nd and the 6th chemotherapy cycle.

Decreases in various antioxidants and markers of oxidative and nitrosative stress appeared throughout the whole study period. However, the most notable changes in various oxidative stress markers occurred during the first chemotherapy cycle, when significant decreases were seen in the levels of urate (318 vs. 297 vs. 290 vs. 307 µmol/L, p<0.001), ascorbic acid (57.7 vs. 45.0 vs. 48.8 vs. 48.8 µmol/L, p=0.021), proteins (69.5 vs. 67.5 vs. 69.0 vs. 64.0 g/L, p=0.004), TBARS (5.65 vs. 4.62 vs. 5.06 vs. 5.51 µmol/L, p=0.012) and TRAP (925 vs. 876 vs. 828 vs. 925 µmol/L, p=0.001 at baseline, 3, 5 and 19 hours after the start of chemotherapy, respectively).

A significant decrease occurred during all cycles in serum urate (p<0.001 at 1st cycle, p=0.002 at 2nd cycle and p=0.005 at 6th cycle) and proteins (p=0.004 at 1st cycle, p=0.014 at 2nd cycle and p=0.004 at 6th cycle) measured from baseline at 12 noon to 7 a.m. the following day. In addition, a significant decrease was seen in serum TRAP (p=0.038) during the second cycle. An increase occurred in serum oxidized proteins 19 hours after initiation of chemotherapy during the 2nd cycle (p=0.009). During the last (6th) cycle, the levels of serum conjugated dienes (p=0.022) and plasma alpha-tocopherol (p=0.008) decreased (Original article III, page 1240, Table 2 and page 1241, Figure 2).

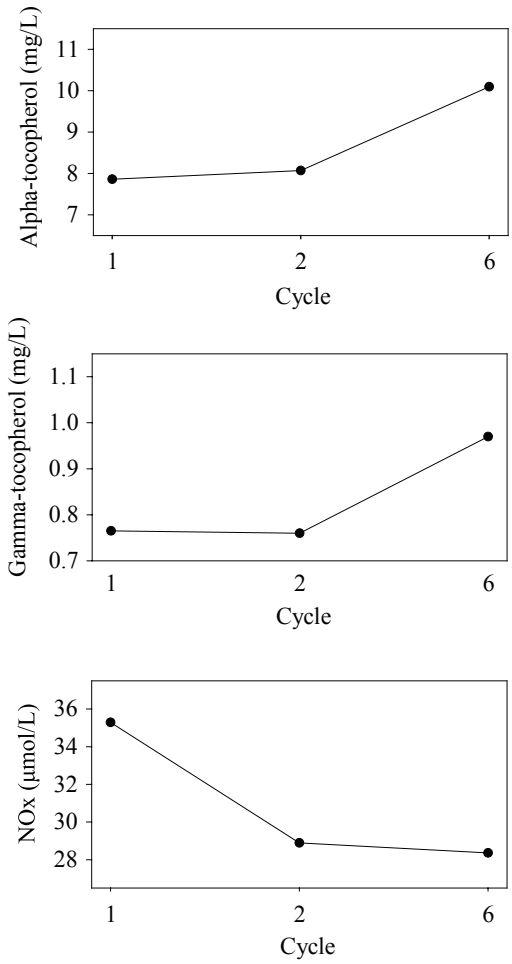

Figure 10. *Median levels of plasma alpha-tocopherol (mg/L), gamma-tocopherol (mg/L) and NOx (µmol/L) at baseline during 1st, 2nd and 6th chemotherapy cycle. The numbers of patients are 15, 13 and 10 for plasma alpha-tocopherol, 14, 13 and 10 for gamma-tocopherol and 16, 13 and 10 for NOx at 1st, 2nd and 6th cycles, respectively.*

7.8 Exhaled pentane study (study IV)

The geometric mean for exhaled pentane in the patients was 1.69 ng/L (95% CI 1.14–2.50 ng/L) and in the controls 0.96 ng/L (95% CI 0.66–1.39 ng/L, p=0.082) at baseline. As gender had an influence on exhaled pentane levels in the control group (1.43 ng/L in men vs. 0.48 ng/L in women, p=0.002), the results were adjusted accordingly (adjusted geometric mean 1.73 ng/L, 95% CI 1.05–2.86 ng/L and 0.83 ng/L, 95% CI 0.61–1.13 ng/L for patients and controls, respectively). The patients/controls ratio was 2.08 ng/L, 95% CI 1.15–3.76 ng/L, p=0.017.

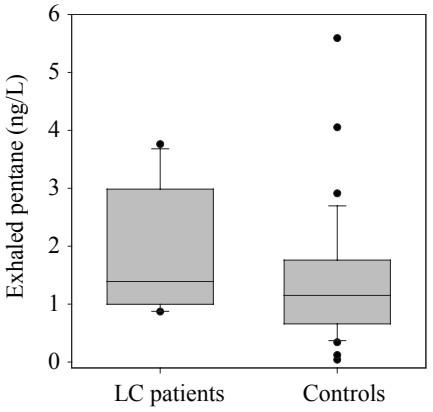

Figure 11. *Box-plot figures for exhaled pentane (ng/L) in lung cancer (LC) patients (n=11) and controls (n=30) at baseline. The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are given as circles.*

The mean concentration of thiobarbituric acid-reactive substances (TBARS) was 3.10 µmol/L (SD 2.10, range 1.16–6.62 µmol/L) and of conjugated dienes 46.8 µmol/L (SD 14.4, range 32.0–66.4 µmol/L) in patients at baseline.

There was a tendency for exhaled pentane levels to decrease during the first day of radiotherapy; the geometric mean concentrations were 1.69 ng/L before radiotherapy, 1.50 ng/L at 30 min, and 1.24 ng/L at 120 min after the start of radiotherapy. However, these changes were not statistically significant (the median change between baseline and 120 minutes was –0.40 ng/L, p=0.075). No significant changes occurred in the levels of exhaled pentane later during the first week of radiotherapy. At 30 Gy of radiotherapy, the mean levels of exhaled pentane were not significantly changed from baseline to 30 min and to 120 min. Due to the small number of samples, no testing for statistical significance was appropriate at 30 Gy or 40 Gy.

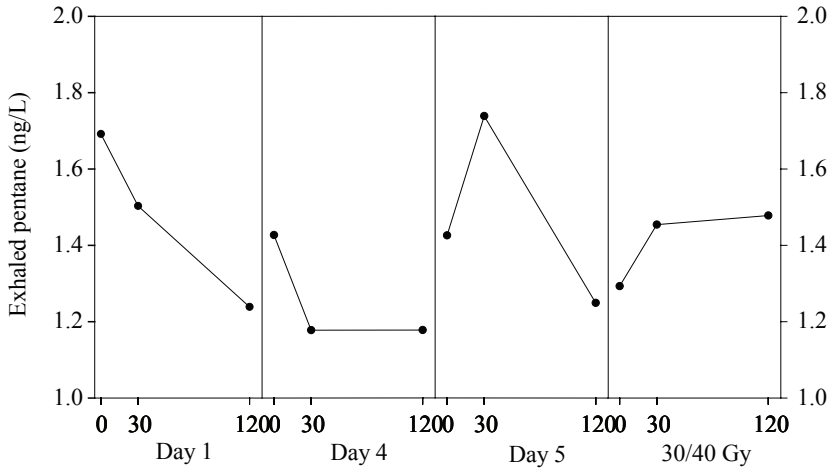

Figure 12. *Geometric means for exhaled pentane (ng/L) during 120 min after start of radiotherapy on days 1 (n=11), 4 (n=10), 5 (n=8) and 30/40 Gy (n=6).*

The levels of serum conjugated dienes decreased significantly during the first week of radiotherapy (48.2 µmol/L at day 1, 43.9 µmol/L at day 4 and 42.7 µmol/L at day 5, p=0.014). However, no significant differences were observed in the levels of serum TBARS during the first week of radiotherapy (3.03 µmol/L at day 1, 2.88 µmol/L at day 4 and 2.88 µmol/L at day 5, p=0.946). Testing for conventional statistical significance was not performed at 30 Gy or 40 Gy due to the small sample size.

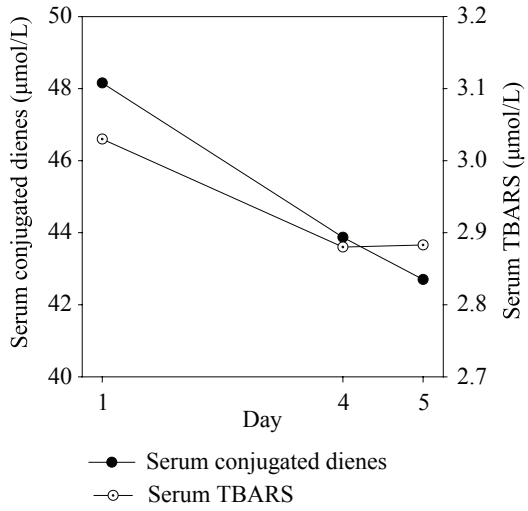

Figure 13. *Means for serum conjugated dienes (µmol/L) and serum TBARS (µmol/L) at day 1 (baseline), day 4 and day 5 (n=7) during radiotherapy.*

7.9 Cytokine study (study V)

The patients had significantly higher concentrations of serum IL-6 (10.60, 95% CI 1.27–162.00 vs. 2.55, 95% CI 0.90–96.10 pg/mL, p<0.001), IL-8 (15.76, 95% CI 3.11–170.00 vs. 8.63, 95% CI 2.12–29.40 pg/mL, p=0.001) and IL-18 (254.1, 95% CI 116.0–1509.0 vs. 197.0, 95% CI 118.0–360.0 pg/mL, p=0.015) than the non-cancer control patients at baseline. The patients also tended to have higher levels of serum VEGF compared to non-cancer control patients (216.1, 95% CI 18.2–1262.0 vs. 149.6, 95% CI 20.5–845.0 pg/mL, p=0.098). After adjustment for smoking, gender, hypertension, age and BMI, the differences in serum IL-6 and IL-8 between patients and non-cancer control patients persisted (p<0.001 and p=0.030, respectively). Measurable levels of TNFα were detected in 33.3% of the patients and in 13.9% of the non-cancer control patients, and of IL-12 in 16.7% of the patients and in 5.6% of the non-cancer control patients. The corresponding odds ratios were 0.32 (95% CI 0.10–1.04, p=0.058) and 0.29 (95% CI 0.06–1.57, p=0.152) for TNFα and IL-12.

The levels of IL-6 (2.97, 95% CI 0.25–175.00 vs. 1.55, 95% CI 0.50–14.20 pg/mL, p=0.021), IL-8 (31.79, 95% CI 2.66–735.00 vs. 16.48, 95% CI 1.33–216.00 pg/mL, p=0.018) and IL-18 (15.70, 95% CI 6.49–47.20 vs. 7.80, 95% CI 0.24–37.70 pg/mL, p=0.002) in the BAL fluid were significantly higher, and the levels of IL-1β significantly lower (0.10, 95% CI 0.01–2.80 vs. 0.41, 95% CI 0.01–13.10 pg/mL, p=0.006) among the lung cancer patients compared to non-cancer control patients at baseline. However, after appropriate adjustments, only BAL fluid IL-6 remained significantly higher among the patients compared to non-cancer control patients (p=0.039). No significant differences were observed in BAL fluid VEGF levels (32.42 vs. 44.42 pg/mL, p=0.344) between patients and non-cancer control patients. Measurable levels of TNFα were detected in 25.0% of the patients and 57.1% of the non-cancer control patients, of IL-1β in 27.8% of the patients and in 91.4% of the non-cancer control patients and of IL-12 in 50.0% of the patients and in 65.7% of the non-cancer control patients. The corresponding odds ratios were 0.25 (95% CI 0.09–0.69, p=0.006), 0.04 (95% CI 0.01–0.14, p<0.001) and 0.52 (95% CI 0.20–1.36, p=0.180) for TNFα, IL-1β and IL-12, respectively. (Original article V, page 33, Table 2+3)

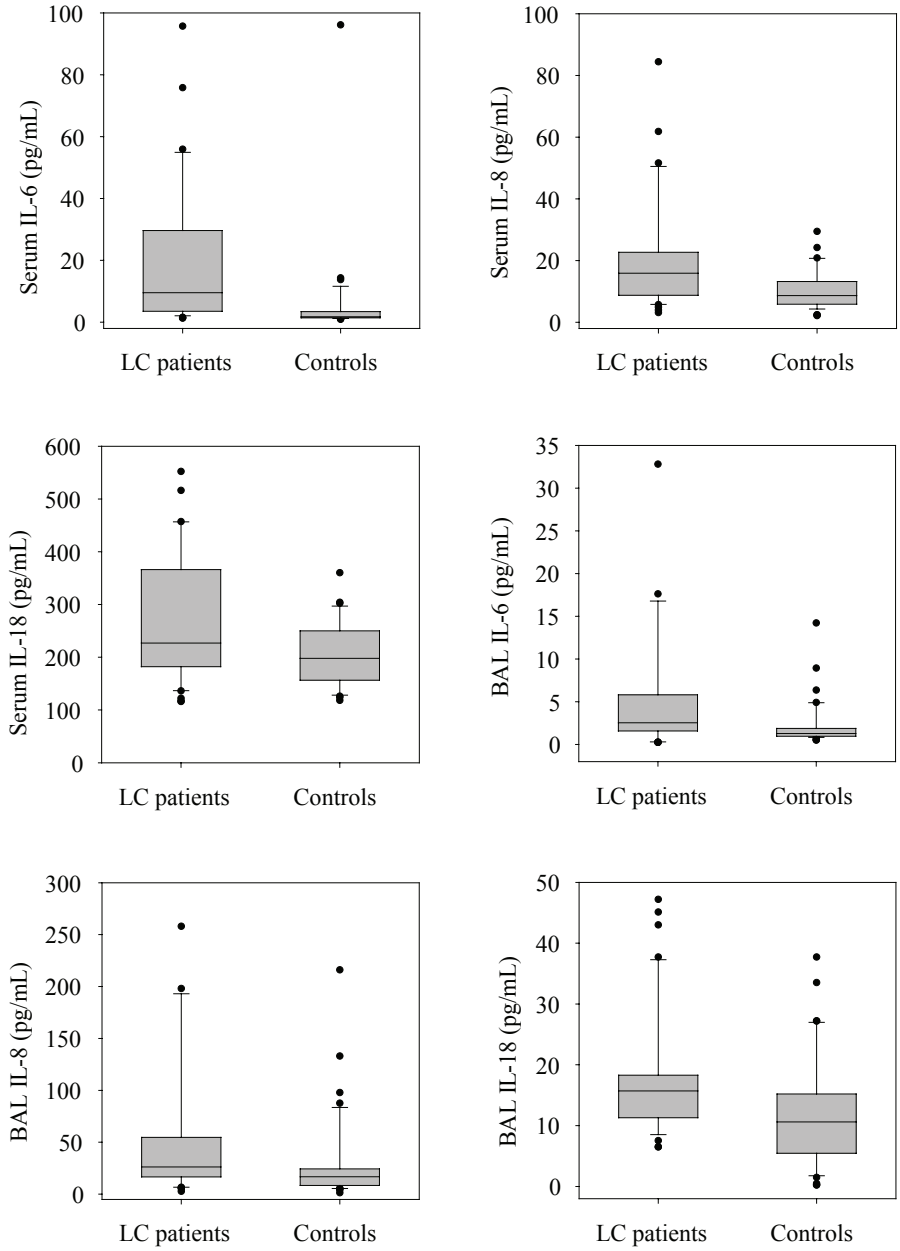

Figure 14. *Box-plot figures for IL-6, IL-8 and IL-18 in serum and in BAL fluid at time of diagnosis in lung cancer (LC) patients (n=36) and non-cancer control patients (n=36). The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are given as circles. In patients seven outliers are outside the figure: serum IL-6 162, serum IL-8 170, serum IL-18 1509, BAL fluid IL-6 175 and 94.3, and BAL fluid IL-8 734 and 620 pg/mL.*

The changes in serum VEGF, IL-6, IL-8 and IL-18 concentrations from baseline to the end of radiotherapy and to three months from the start of radiotherapy were non-significant. The respective geometric mean levels were 184.5 vs. 150.3 vs. 207.4 pg/mL (p=0.124 after radiotherapy and p=0.948 after three months) for VEGF, 7.95 vs. 8.27 vs. 10.50 pg/mL (p=0.756 and p=0.890) for IL-6, 13.37 vs. 10.08 vs. 18.61 pg/mL (p=0.407 and p=0.348) for IL-8 and 241.9 vs. 241.8 vs. 313.8 pg/mL (p=0.254 and p=0.077) for IL-18.

The geometric mean level of IL-6 in the BAL fluid increased after two weeks of radiotherapy (2.72, range 0.25–32.80 pg/mL vs. 6.38, range 1.04–42.40 pg/mL, p=0.037). The levels of BAL fluid VEGF, IL-8 and IL-18 also increased during radiotherapy, although not statistically significantly (23.86, range 1.10–728.0 vs. 30.63, range 1.10–799.0 pg/mL, p=0.621 for VEGF, 26.99, range 5.66–620.0 vs. 46.08, range 3.49–807.0 pg/mL, p=0.260 for IL-8 and 15.21, range 6.49–47.20 vs. 19.68, range 4.46–157.0 pg/mL, p=0.382 for IL-18, respectively). No significant changes occurred in BAL fluid TNFα, IL-1β or IL-12 during radiotherapy.

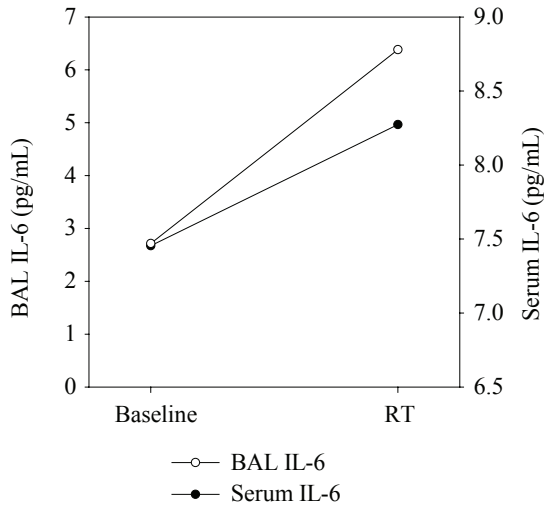

Figure 15. *Geometric means for IL-6 in serum (n=15) and in the BAL fluid (n=14) for lung cancer patients at baseline and during radiotherapy (RT)*

7.10 Associations with subjects´ demographics

There was no evidence of associations between antioxidants, cytokines, oxidative and nitrosative stress marker levels and other diseases (cardiac disease, hypertension, chronic arrhythmia, diabetes, asthma, COPD, chronic bronchitis or rheumatic disease) in studies I–IV. In study V, hypertension was a possible confounding factor and was taken into account in the statistical analysis. Neither had any of the other diseases an effect on the antioxidants, oxidative or nitrosative stress parameters during chemotherapy or

radiotherapy (II–IV). Nor did neoadjuvant chemotherapy affect markers of oxidative damage to DNA (I).

There was a significant positive correlation between age and baseline levels of urinary 8-oxoGua and 8-oxodG in lung cancer patients (R=0.37, p=0.003 and R=0.28, p=0.03, respectively) (I). Over 65-year-old patients had higher levels of urinary 8-oxoGua (11.33 vs. 7.95 nmol/mmol creatinine, p=0.008) and 8-oxodG (2.42 vs. 1.85 nmol/mmol creatinine, p=0.062) compared to those under 65. No significant correlations in respect of age were noted among non-cancer control patients (I).

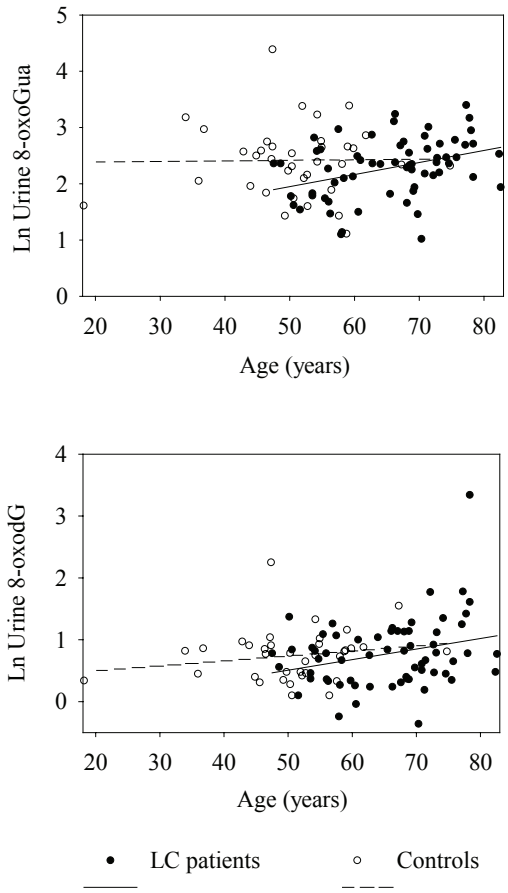

Figure 16. *Relation between age and logarithmically transformed urinary 8-oxoGua and urinary 8-oxodG (nmol/mmol creatinine) with regression lines in lung cancer (LC) patients (n=63) and non-cancer control patients (n=36).*

Women tended to have higher urinary and nuclear DNA damage marker levels compared to men among non-cancer control patients in study I. However, the only statistically significant difference was seen in urinary 8-oxoGua at baseline (13.97 vs. 8.51 nmol/mmol creatinine for women and men, respectively, p=0.02). Gender also affected the results in the exhaled air collection study; in the healthy control group the

exhaled pentane values were 1.43 ng/L in men vs. 0.48 ng/L in women, p=0.002. In both of these studies, gender was taken into account in the statistical analyses.

Smoking had no effect on the urinary marker levels of oxidative damage to DNA in study I when comparing smokers and non-smokers (p=0.123 for 8-oxoGua and p=0.227 for 8-oxodG, respectively). However, there was a significant difference in baseline nuclear 8-oxodG values between smokers (1.65 8-oxodG/10^5dG) and non-smokers (4.31 8-oxodG/10^5dG) in non-cancer control patients (p=0.018). In lung cancer patients, the corresponding values were 1.26 vs. 1.29 8-oxodG/10^5dG (p=0.926).

In the antioxidant BAL study (II) there were no significant differences in antioxidants or oxidative stress marker levels between smokers and non-smokers among the patients. However, smokers had significantly higher levels of BAL fluid thiols (2.73 vs. 1.47 µmol/L, p=0.022), serum urate (177 vs. 106 µmol/L, p=0.035), serum TBARS (5.06 vs. 4.36 µmol/L, p=0.042) and serum nitrite (0.25 vs. 0.14 µmol/L, p=0.017) and lower levels of plasma ascorbic acid (59 vs. 88 µmol/L, p=0.011) in the non-cancer control patients. Also among the non-cancer control patients, the number of pack years smoked correlated negatively with plasma ascorbic acid (R=–0.452, p=0.014).

Smokers had lower levels of serum IL-6, IL-8 and IL-18 compared to non-smokers and ex-smokers (p=0.041, p=0.003 and p=0.038, respectively) in study V. BAL fluid VEGF levels were likewise lower among smokers compared to non- and ex-smokers (p<0.001**)**. In contrast, higher BAL fluid IL-18 levels were detected in smokers compared to ex- and non-smokers (p=0.026). (V)

Smoking had no significant effect on the results in studies III–IV.

7.11 Associations with stage of disease

Stage IV lung cancer was associated with higher urinary DNA damage marker levels than stage I–III cancer (11.50 vs. 8.78 nmol/mmol creatinine, p=0.044 and 2.58 vs. 1.91 nmol/mmol creatinine, p=0.034 for 8-oxoGua and 8-oxodG, respectively) (I). The interaction between the tumor type (SCLC vs. NSCLC) and the stage of cancer was non-significant for all DNA damage markers (p=0.801, p=0.460 and p=0.706 for urinary 8-oxoGua, urinary 8-oxodG and nuclear 8-oxodG).

Stage I–III SCLC patients tended to have lower levels of urinary 8-oxoGua and of urinary 8-oxodG (geometric mean 6.60 vs. 9.76 and 1.79 vs. 2.16 nmol/mmol creatinine, respectively) compared to stage IV SCLC patients at baseline. In addition, stage I–III NSCLC patients tended to have lower levels of urinary 8-oxoGua and of urinary 8-oxodG (geometric mean 9.46 vs. 13.01 and 1.94 vs. 2.94 nmol/mmol creatinine, respectively) compared to stage IV NSCLC patients at baseline. (Unpublished data)

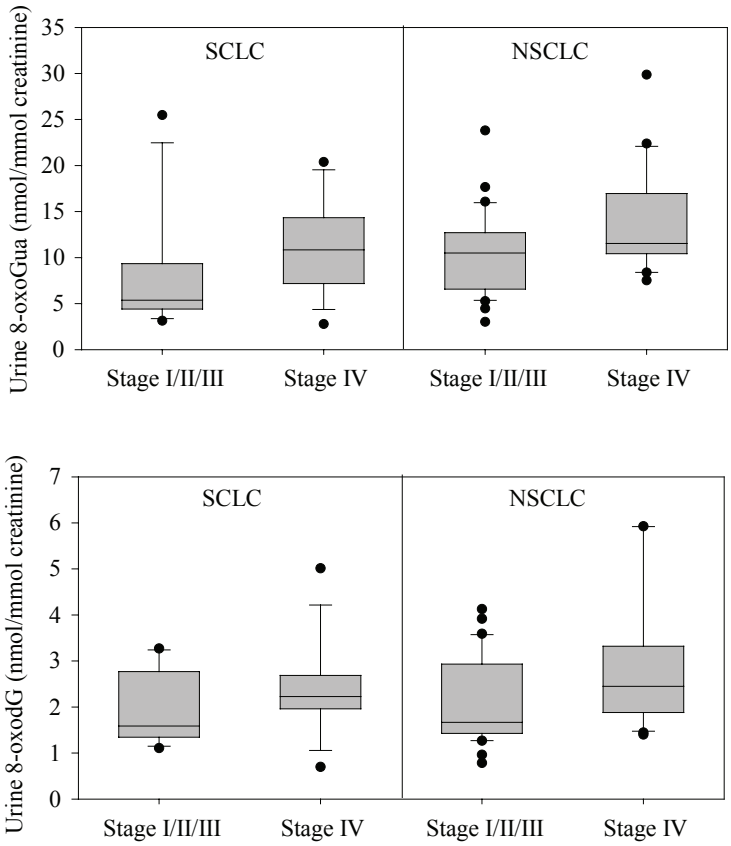

Figure 17. *Box-plot figure for urinary 8-oxoGua (8-oxoguanine, nmol/mmol creatinine) and urinary 8-oxodG (nmol/mmol creatinine) in small cell lung cancer (SCLC) patients with stage I/II/III (n=7) and stage IV (n=12) and in non-small cell lung cancer (NSCLC) patients with stage I/II/III (n=27) and stage IV (n=16). The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Outliers are given as circles. One outlier in urinary 8-oxodG lies outside the figure (value 28.11 nmol/mmol creatinine in NCSLC; stage IV).*

Likewise higher BAL thiol (6.29 vs. 2.54 µmol/L, p=0.063), BAL nitrite (0.21 vs. 0.15 µmol/L, p=0.021) and lower serum oxidized proteins (2.36 vs. 3.39 µmol/L, p=0.005) and TBARS (4.37 vs. 4.94 µmol/L, p=0.067) levels were recorded in stages I, II or III compared to those with stage IV disease (II).

7.12 Associations with other laboratory measurements

There were significant positive correlations between urinary 8-oxodG levels and serum neutrophils count in the lung cancer patients and the whole study group (Spearman's rank correlation R=0.40, p=0.001 and R=0.24, p=0.021, respectively), and also urinary 8-oxodG and serum C-reactive protein levels (R=0.31, p=0.016 and R=0.19, p=0.060, in lung cancer patients and whole study group, respectively). (I)

Plasma thiols correlated positively with blood lymphocytes (R=0.69, p=0.009) and blood hemoglobin (R=0.57, p=0.026), and negatively with blood leukocytes and neutrophils (R=−0.51, p=0.054 and R=−0.63, p=0.020, respectively). There were significant positive correlations between BAL ascorbic acid and plasma ascorbic acid (R=0.34, p=0.005), BAL thiols and both serum urate and serum alpha-tocopherol (R=0.48, p=0.001 and R=0.71, p=0.031, respectively), and a negative correlation between plasma ascorbic acid and plasma (R=−0.45, p=0.008) and BAL thiols (R=−0.38, p=0.052) at baseline. (II)

There were positive correlations between serum TRAP and serum urate (R=0.80, p<0.001), plasma nitrite and serum conjugated dienes and serum TBARS (R=0.51, p=0.043 and R=0.62, p=0.010, respectively), and plasma NOx and plasma alpha-tocopherol (R=0.53, p=0.043). Negative correlations were noted between plasma ascorbic acid and serum oxidized proteins (R=−0.52, p=0.039), serum TBARS and plasma thiols (R=−0.68, p=0.004), and plasma nitrite and plasma gamma-tocopherol (R=−0.59, p=0.026) (III).

Positive correlations were also found between serum and BAL fluid IL-6 and IL-8 levels (R=0.34, p=0.040 for serum and R=0.47, p=0.004, for BAL fluid). Serum IL-6 also correlated positively with serum IL-18 and VEGF (R=0.30, p=0.078 and R=0.48, p=0.003, respectively). The number of neutrophils in BAL fluid correlated positively with BAL fluid IL-6 and IL-8 levels (R=0.35, p=0.035 and R=0.37, p=0.025, respectively) (V).

7.13 Associations with adverse events

In the study of oxidative damage to DNA adverse events were not significantly associated with the baseline levels of the markers studied (I). Nor were there significant associations between adverse events and baseline levels of antioxidants or markers of oxidative and nitrosative stress in the antioxidant BAL study or chemotherapy study (II–III).

In the exhaled pentane study, the occurrence of radiation pneumonitis was associated with baseline exhaled pentane levels. If the pretreatment exhaled pentane levels were below the median (<1.39 ng/L), one out of five patients developed radiation pneumonitis, but if the pretreatment levels were above the median, four out of six patients developed radiation pneumonitis. Due to the small sample size, testing for statistical significance was not appropriate (IV).

In the cytokine study, moreover, adverse events tended to be more frequent when baseline BAL fluid lL-8 levels were below median (<26.2 pg/mL, p=0.077), this finding however, lacking statistical significance (study V).

7.14 Associations with response to treatment

The response to treatment was not significantly associated with changes in urinary 8-oxoGua, urinary 8-oxodG or nuclear 8-oxodG from baseline to the last measurement in study I. Likewise in the antioxidant BAL study no significant associations were seen between response to treatment and baseline antioxidant or oxidative stress marker levels

(II). Exhaled pentane or serum lipid peroxidation markers had no significant associations with response to treatment (IV).

In the chemotherapy study, both baseline plasma ascorbic acid and serum oxidized proteins showed an almost significant association with response to treatment. If baseline plasma levels of ascorbic acid were above the median (>57.7 μmol/L), patients evinced a better response to treatment than if the levels were below the median (p=0.077). Patients with baseline levels of serum oxidized proteins above the median (>3.32 μmol/L) had poorer response to treatment than those with baseline levels below (p=0.077). (III) Also a trend towards better response was evidenced in the cytokine study in patients with higher than median (>9.52 pg/mL) baseline IL-6 levels (p=0.077). (V)

7.15 Associations with overall survival

The overall survival time tended to be longer when the baseline level of nuclear 8-oxodG was highest (>1.34 8-oxodG/10^5dG), when nuclear 8-oxodG values were divided into tertiles, this albeit without statistical significance (p=0.182). The mean survival time was 8.4 months in the lowest tertile, 14.6 months in the middle tertile and 19.5 months in the highest tertile (I).

Baseline BAL and plasma thiols seemed to be associated with overall survival in the antioxidant BAL (II) and chemotherapy study (III). When baseline BAL thiol levels were above the median concentration of 4.34 μmol/L, the survival time tended to be longer (9.9 months vs. 6.1 months, p=0.051) (II). If baseline plasma thiol levels were lower than median (<0.306 μmol/L), median survival was 5.5 months, whereas if baseline thiol levels were above the median, median survival was 10.0 months (p=0.008). There was also a tendency towards better overall survival (8.8 months vs. 5.7 months, p=0.068) in patients with baseline levels of plasma NOx above the median (>35.3 μmol/L). (III)

Also in the exhaled pentane collection study, a significant association between pretreatment exhaled pentane levels and overall survival was verified (p=0.003). If pretreatment pentane levels were below the median (<1.39 ng/L), the median survival of the patients was 5.2 months (95% CI 0–10.5 months), but if the levels were above the median, the median survival was 16.1 months (95% CI 10.1–22.1 months). An almost significant association was noted between the serum lipid peroxidation marker TBARS and overall survival. Lower levels of TBARS predicted better survival (17.3 months vs. 2.8 months, p=0.051). (IV)

Overall survival was significantly inversely associated with baseline serum and BAL fluid IL-8 (p=0.035 and p=0.027, respectively) and serum VEGF (p=0.012) levels: higher baseline serum and BAL fluid IL-8 and serum VEGF levels were associated with poorer survival (V).

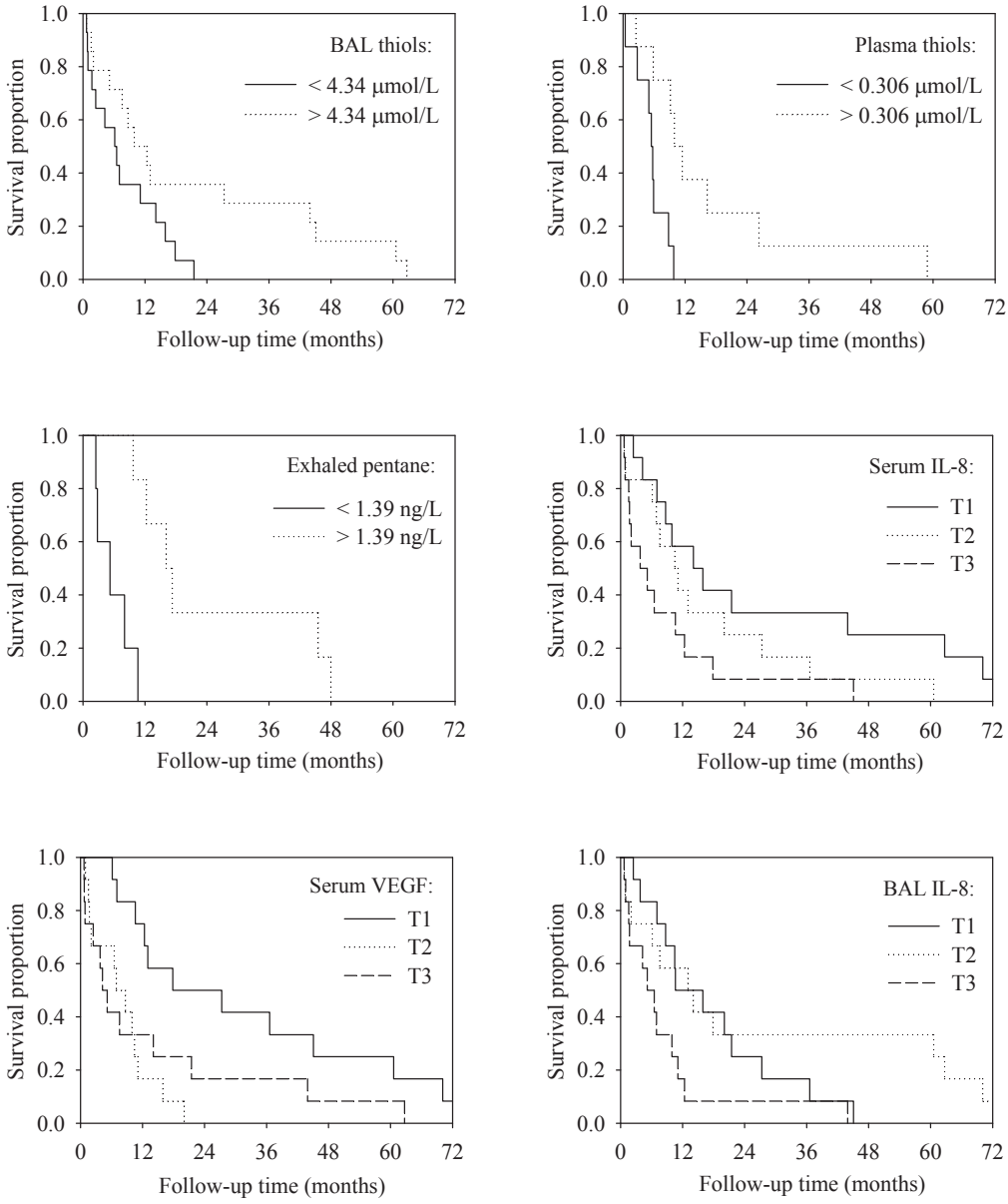

Figure 18. *Kaplan-Meier curves for patients with different baseline levels of BAL thiols (log-rank p=0.051, study II), plasma thiols (p=0.008, study III), exhaled pentane (p=0.003, study IV), serum IL-8, serum VEGF and BAL fluid IL-8 (p=0.035, p=0.012 and p=0.027, respectively, study V). The baseline values are divided into three groups (tertiles T1, T2 and T3) or into two groups (<median and >median).*

8. DISCUSSION

To the author´s knowledge this is the largest study to have evaluated local oxidant/antioxidant status in lung cancer by measuring different antioxidants, cytokines and markers of oxidative and nitrosative stress among lung cancer patients and non-cancer controls. The systemic levels of these markers were also monitored as well as changes in these marker levels among lung cancer patients during chemotherapy and radiotherapy. The study also for the first time evaluated the associations between various antioxidants, cytokines and oxidative and nitrosative stress markers and adverse events, response to treatment and survival with a planned follow-up time of six years.

8.1 Markers of oxidative damage to DNA (study I)

No significant differences were detected here in the levels of urinary 8-oxoGua and urinary and nuclear 8-oxodG between cancer patients and non-cancer control patients at baseline, which accords with some previous studies (Erhola et al., 1997b, Honda et al., 2000). However, one study has shown that the amount of modified base, but not the nucleoside, excreted into the urine was higher in cancer patients compared to healthy controls (Rozalski et al., 2002). There are several reports suggesting that inflammation leads to the production of ROS, which subsequently can damage DNA (Cooke et al., 2006, Siomek et al., 2006). The control patients in this study were patients referred for bronchoscopy for prolonged cough and were not completely healthy controls. A later analysis revealed that none of them had cancer but many had mild acute or chronic pulmonary inflammation. The significant positive correlation found between urinary 8-oxodG levels and serum neutrophils count ($R=0.24$, $p=0.021$), and the tendency towards a correlation between urinary 8-oxodG and serum C-reactive protein levels ($R=0.19$, $p=0.060$) would indicate that inflammation may partly explain the lack of difference in markers of oxidative damage to DNA between the two groups. In fact, some findings suggest that inflammation is indeed associated with elevated levels of oxidative DNA damage markers (Cooke et al., 2003). However, in the present study SCLC patients tended to yield lower urinary 8-oxoGua values compared to NSCLC patients and non-cancer control patients ($p=0.09$ and $p=0.05$, respectively). Also NSCLC patients had significantly lower nuclear 8-oxodG values compared to non-cancer control patients ($p=0.001$). These data are in conflict with those of our previous study; however, the small number of patients in both studies prevents any reliable subgroup analysis (Erhola et al., 1997b).

One earlier study detected an association between tumor mass and urinary 8-OHdG levels (Honda et al., 2000), which is supported by our finding that lung cancer patients with stage IV disease have higher urinary oxidative damage marker levels than patients with stage I–III disease ($p=0.044$ and $p=0.034$ for urinary 8-oxoGua and 8-oxodG,

respectively). In this study over 65-year-old patients had higher levels of urinary 8-oxoGua ($p=0.008$) and 8-oxodG ($p=0.062$) compared to younger patients, which is again in accord with some previous findings (Loft et al., 2006). As the amount of muscle mass is also known to influence the urinary 8-oxodG level, it may explain our finding of elevated urinary 8-oxodG levels among women compared to men at baseline (Loft et al., 2006).

In the present series, a significant increase was observed in urinary 8-oxodG levels during the first week of radiotherapy ($p<0.001$), this possibly due to a radiotherapy-induced increase in oxidative stress. Several previous studies have reported similar observations; namely an increase in urinary 8-OHdG levels seen during and after total body irradiation (Tagesson et al., 1995, Bergman et al., 2004). However, another study has shown that urinary excretion of 8-oxodG might also be attributed to increased cell death (Gedik et al, 2002). It has been speculated that disrupted cells undergo liquid peroxidation faster than healthy cells, and thus would also sustain oxidative DNA damage faster (Mei et al., 2005).

One of the first studies to explore oxidative damage to DNA during radiotherapy reported an increase in lymphocyte 8-oxodG after treatment (Bialkowski et al., 1996). Those findings support our results here as we found a clinically significant increase in nuclear 8-oxodG levels at 20 Gy of radiotherapy and three months after radiotherapy. However, the limitations of the study by Bialkowksi and co-workers are that only four patients were involved and the measurements were made at baseline and after a total radiotherapy dose of 30 Gy, no measurements being made during radiotherapy. Another study examined 23 prostate, breast and head and neck lung cancer patients at baseline and one week after routinely fractionated radiotherapy. In line with our observations, there was a two-fold increase in lymphocyte 8-oxodG/dG levels after radiotherapy, which, however, was not statistically significant due to wide inter-individual variations (Zwingmann et al., 1999).

No significant changes were seen in urinary 8-oxoGua levels during radiotherapy. Oxidative DNA base lesions are mostly removed by two repair pathways: base excision repair (BER) and nucleotide excision repair (NER) (Cooke et al., 2003). It has been suggested that the urinary excretion of 8-oxoGua and 8-oxodG may reflect different DNA damage repair mechanisms, namely, BER and NER. The difference in repair pathways may explain why no changes were seen in the modified base excretion during radiotherapy in our study (Gackowski et al., 2001, Rozalski et al., 2002). It is also known that different oxidative stress markers may behave quite differently in respect of their appearance in the urine after whole-body oxidative stress (Bergman et al., 2004). Individuals may also vary in their radiation sensitivity and capacity to repair DNA damage, which might influence the extent of modification in lymphocyte DNA or urinary DNA damage markers (Setlow et al., 1983). However, the actual repair of DNA damage was not assessed in this present study.

In our study, the levels of urinary markers of oxidative damage to DNA, especially urinary 8-oxodG, tended to decrease three months after start of radiotherapy ($p=0.479$ and $p=0.389$ for urinary 8-oxoGua and 8-oxodG, respectively). This might be due to a positive response to treatment, which leads to a diminution in tumor mass, as a previous study has suggested that lower urinary 8-OHdG levels reflect a decrease in tumor mass (Honda et al., 2000). Nonetheless, a limitation of this present study was that the actual tumor volume was not evaluated at baseline or later in the course of radiotherapy.

Only a few studies have explored markers of oxidative damage to DNA during chemotherapy. According to our findings, adriamycin-based chemotherapy induces oxidative damage to DNA, as significant increases were noted in nuclear 8-oxodG after the first chemotherapy cycle ($p=0.043$) and urinary 8-oxodG during the last ($p=0.009$). It is also possible that increased cell death and DNA turnover might contribute to the increase seen in oxidative DNA damage parameters (Mei et al., 2005). One study has reported elevated urinary 8-oxoGua and 8-oxodG levels after cisplatin-containing chemotherapy (Siomek et al., 2006). In contrast, several studies have reported that urinary 8-oxodGuo levels did not increase after adriamycin treatment (Erhola et al., 1997b, Faure et al., 1998), while urine 5-(hydroxymethyl) uracil levels increased significantly 24 hours after administration of adriamycin (Faure et al., 1998). It has been suggested that repair of 8-oxodG in DNA and its elimination in urine are relatively slow processes, and therefore the increase seen in nuclear 8-oxodG levels during radiotherapy might not have reflected urinary biomarkers (Mousseau et al., 2005). In general, it has been evidenced that the excretion of DNA damage repair products in the urine represents the average rate of DNA damage in the whole body, whereas measuring lymphocyte DNA damage expresses the damage to the cells at a certain moment (Wu et al., 2004). Measurement of several different markers of oxidative damage to DNA is thus to be recommended.

8.2 Antioxidant BAL study (study II)

According to the present findings lung cancer is associated with higher baseline levels of serum urate and plasma nitrite and lower levels of serum oxidized proteins compared to non-cancer control patients ($p=0.016$, $p<0.001$ and $p=0.027$, respectively). Urate is one of the most important antioxidants in human plasma. It has an essential role in protecting cells against damage caused by ROS. Thus, elevated levels of urate among cancer patients may be explained as a compensatory mechanism against oxidative stress (Ames et al., 1981). In fact, previous studies have found elevated levels of plasma urate and serum nitrite among cancer patients compared to controls (Burgaz et al., 1996, Gönenç et al., 2001). Also increased production of nitric oxide is held to protect cells against oxidants (Gönenç et al., 2006). Nitric oxide is oxidized to nitrite in plasma and therefore measurement of plasma nitrite levels reflects well the overall production of nitric oxide (Bryan and Grisham, 2007). Urate is also a major contributor to plasma total peroxyl radical trapping antioxidant capacity (TRAP), and a significant correlation between these two values ($R=0.47$, $p=0.004$) was observed at baseline in the present study, a finding also previously reported (Dürken et al., 2000). Our previous study noted lower TRAP levels in lung cancer patients compared to controls (Erhola et al., 1998). In the present study likewise, the levels of TRAP tended to be lower in lung cancer patients ($p=0.751$, $p=0.388$ after adjustments), although significance was altained only for protein thiols ($p<0.001$, $p=0.651$ after adjustments) and urate ($p<0.001$, $p=0.016$ after adjustments), components of plasma TRAP.

The present non-cancer control patients had been referred for bronchoscopy due to chronic cough and may thus have had augmented RTLF antioxidant status due to increased glandular secretion, plasma leakages and cellular adaptation as a response to the chronic inflammation and oxidative stress caused by their chronic cough (Cross

et al., 2002). This may partly explain why no differences between the groups were evidenced in other antioxidants and markers of oxidative stress. Interestingly, lower baseline serum TBARS levels were associated with limited disease (stages I–III) in contrast to extensive disease (p=0.067), although this finding lacked statistical significance. However, it may be explained in the light of a previous study showing that the plasma malondialdehyde concentration decreases with decreasing tumor size (Gedik et al., 2002). Even though smoking had no impact on the results in the patient population, smokers had significantly higher levels of BAL thiol (p=0.022), serum urate (p=0.035), serum TBARS (p=0.042) and serum nitrite (p=0.017) and lower levels of plasma ascorbic acid (p=0.011) compared to non-smokers in the non-cancer control patients. Also among the non-cancer control patients, the number of pack years smoked correlated negatively with plasma ascorbic acid (R= –0.452, p=0.014). It is known that both acute and chronic cigarette smoking induce enhanced production of neutrophils and promote lipid peroxidation, which has been evidenced both locally in the lungs and systemically in the blood (Morrison et al., 1999). We cannot thus exclude the possibility that smoking may have had an impact on the results.

The tendency of BAL urate (p=0.083) levels to rise during radiotherapy may be attributed to the movement of urate onto the lung surface to protect alveolar cells against the oxidative stress caused by radiotherapy (Behndig et al., 2006). It is also possible that the tumor cell destruction due to radiotherapy increases the levels of urate, as various antioxidants may be released from internal cell pools after appropriate stimulus such as irradiation (Gordon et al., 1995). There was also a tendency for BAL thiols (p=0.069) to rise during treatment, which may be understood in the light of a report showing that radiotherapy causes oxidation of membrane protein SH groups (Edwards et al., 1984). A study by DeVries and co-workers (2001) evidenced that irradiation of granulocytes with a single dose of 2 Gy showed no change in the production of reactive oxygen species, whereas single doses of 4 Gy and 8 Gy resulted in a reduction of 15%, and 32 Gy in a 25% reduction in the production of ROS. This suggests that local defence mechanisms are fairly sufficient to counter the deleterious actions of irradiation despite rather high doses.

There was a tendency for the levels of plasma alpha-tocopherol (p=0.056), serum urate (p=0.064) and serum oxidized proteins (p=0.088) to increase two weeks after the start of radiotherapy. Alpha-tocopherol is the main lipid-soluble free radical scavenger in membrane lipids. One previous study investigating rat liver microsomes detected an increase in alpha-tocopherol levels and glutathione peroxidase activity during radiotherapy, suggesting that low-dose radiation induces inhibitory actions against radiation-induced lipid peroxidation (Yukawa et al., 2005). The increase in serum oxidized proteins during radiotherapy might be attributed to oxidative endothelial damage following irradiation (Lenz et al., 1999). The increase in urate, again, may be attributed to increased cell death as it is known that urate is released from dying cells, a factor which also helps to stimulate immune response (Halliwell and Gutteridge, 2007, p. 155). In general, enhanced antioxidative defense mechanisms reflect the ability of tumor cells to counter the oxidative stress caused by radiotherapy. Interestingly, serum and BAL compartment oxidative stress markers seem to behave quite independently in relation to an external stimulus such as radiotherapy.

In the case of the significant rise in the levels of serum conjugated dienes and TBARS (p=0.034 and p=0.004) three months after radiotherapy it is possible

that radiotherapy induces lipid peroxidation with some degree of tissue damage (Konukoğlu et al., 1998). Previous studies have likewise reported elevated levels of lipid peroxidation markers during radiotherapy (Konukoğlu et al., 1998, Sabitha and Shyamaladevi, 1999). The rise in lipid peroxidation markers may indicate to a defect in the antioxidative defense mechanisms, especially in antioxidant enzymes as suggested elsewhere (Yukawa et al., 2005). It may also imply that the main antioxidant scavenger systems are consumed during radiotherapy, this leading to enhanced lipid peroxidation.

8.3 Chemotherapy study (study III)

The antitumor actions of many chemotherapeutic regimens are exerted through the oxidative pathway. For example, the formation of free radicals is one of the main mechanisms by which adriamycin attacks DNA (Faure et al., 1996). The cardiotoxic effects of adriamycin are also believed to involve free radical formation (Singal et al., 2000). Adriamycin has likewise been shown to induce protein oxidation (DeAtley et al., 1998). In this study, in addition to adriamycin two other chemotherapeutic agents were administered: cyclophosphamide and vincristine, both known to involve oxidative metabolism (Patel, 1990, Schlaifer et al., 1994). These free radical-generating agents may also lower antioxidant enzyme concentrations in plasma during chemotherapy (Subramaniam et al., 1994), which, however, was not the subject of the present study.

Significant decreases were demonstrated in all major antioxidants and markers of oxidative stress throughout the whole treatment period. The most marked decreases were seen in serum urate, serum proteins and serum TRAP. Urate is the end-product of purine metabolism and an important extracellular water-soluble antioxidant. It is known to inhibit lipid peroxidation and may act to preserve ascorbic acid (Davies et al., 1986, Abiaka et al., 2001). Thus the decreases seen in urate may be attributed to enhanced oxidative stress resulting from chemotherapy. As all the patients received 3 liters of isotonic fluids during each chemotherapy cycle, the decrease in urate might also be due to increased renal excretion following the high fluid load. Other factors possibly contributing to the lowering of urate levels are cytolysis, the influence of different pharmacological agents, and biliary excretion (Dűrken et al., 2000). In fact, administration of allopurinol in this study may partly account of the diminution in urate, as allopurinol is an inhibitor of xanthine-oxidase (Hayden and Tyagi, 2004).

Our previous study evidenced a reduction in TRAP, reflecting a quantitative chain-breaking antioxidant capacity, eight hours after the beginning of the first adriamycin infusion. This reduction was mainly due to the reduction in plasma urate and ascorbic acid (Erhola et al., 1996). Our current study corroborates this, as significant reductions were seen in TRAP and urate at all measured time-points (at baseline 12 noon., 3 p.m., 5 p.m. and 7 a.m. the following day, $p=0.001$ and $p<0.001$, respectively) during the first chemotherapy cycle. Also during the first chemotherapy cycle, plasma ascorbic acid decreased 3 hours after initiation of treatment ($p=0.041$). Previous studies have shown that ascorbic acid and urate have a critical protective role against oxidative stress, and lipid peroxidation occurs only after ascorbic acid is totally depleted (Cross et al., 1992, Mudway et al., 1999). Thus, the reductions in the levels of protective antioxidants imply an increase in oxidative burden or a failure in antioxidative defense mechanisms.

Significant reductions in many antioxidants and markers of oxidative stress also took place during the second and sixth chemotherapy cycles. The most notable changes occurred in urate and TRAP. In accord with our results, a previous study has indicated a decline in serum urate levels during high-dose chemotherapy (Dűrken et al., 2000).

Our previous study suggests that alpha-tocopherol is the final main antioxidant to be consumed after oxidative damage (Erhola et al., 1996). In our present series, the levels of alpha-tocopherol decreased significantly during the last chemotherapy cycle, a finding which may also result from its enhanced breakdown (Clemens et al., 1990). However, this study indicated, for the first time, that the baseline levels of alpha-tocopherol and gamma-tocopherol increase from first to last chemotherapy cycle ($p=0.045$ and $p=0.041$, respectively), while those of conjugated dienes have a tendency to rise ($p=0.092$). There was also a significant decrease in baseline levels of plasma NOx from the 1st to the 6th chemotherapy cycle ($p=0.020$). No significant changes in the baseline levels of TBARS were found between the first and last cycles, which is understandable considering that TBARS is a somewhat unspecific marker of lipid peroxidation (Vasankari et al., 1995, Halliwell, 2007). The tendency towards an increase in conjugated dienes, an early marker of lipid peroxidation, at the end of chemotherapy might be attributed to the cumulative dose of cyclophosphamide and vincristine, as both these agents are known to cause peroxidative damage (Patel, 1990, Schlaifer et al., 1994). It is equally possible that chain-breaking antioxidants protected against lipid peroxidation at the beginning of chemotherapy and were consumed at the end of treatment.

After six cycles of treatment, ten (91%) out of eleven patients evinced complete or partial response to chemotherapy. An interesting hypothesis is that both the increase in plasma tocopherols as well as the decrease in serum NOx during chemotherapy may be associated with a positive response to treatment, as derivatives of alpha-tocopherol have been shown to increase apoptosis and reduce cell proliferation (Jia et al., 2008). The decrease of NOx may also be related to this, as nitric oxide is known to have a role in the initiation of apoptosis in vitro (Ellis et al., 1998). Also enhanced lipid peroxidation, which we noted at the end of the chemotherapy period, has been shown to induce apoptosis (Hockenbery et al., 1993). However, larger studies are needed to confirm this hypothesis.

8.4 Exhaled pentane study (study IV)

There has been increasing interest in non-invasive monitoring of respiratory tract inflammation and oxidative stress. Exhaled air analysis has many advantages: measurements are non-invasive, simple and repeatable. Analysis may also be performed on patients with severe airflow obstruction or other lung diseases where more invasive techniques are not possible (Kharitonov and Barnes, 2002). Exhaled pentane represents an indirect indicator of lipid peroxidation, and measurement of it is considered highly sensitive (Urso and Clarkson, 2003). Exhaled ethane is produced during peroxidation of omega-3 fatty acids and pentane is derived from omega-6 fatty acids (i.e. linoleic and arachidonic acid) (Loiseaux-Meunier et al., 2001). We chose measurement of exhaled pentane instead of ethane, as measurement of the latter is associated with increased variability (Aghdassi et al., 2003). Nevertheless, measuring exhaled hydrocarbons involves a number of pitfalls, e.g., ambient air contamination during sample collection,

storage and concentration, and technical difficulties in analysis (Knutson et al., 1999, Loiseaux-Meunier et al., 2001). These are discussed in detail in the original manuscript (IV, p. 971–972). However, in this study possible confounding factors were excluded by reliable and well-controlled exhaled air collection and analysis. Special care was taken in the handling and storage of the samples.

This study showed that lung cancer is associated with elevated levels of exhaled pentane ($p=0.017$), which may be attributed to an excess of oxidative stress and the lipid peroxidation burden caused by cancer (Taysi et al., 2003). In fact, other studies have shown that cancer itself is associated with increased lipid peroxidation (Konukoğlu et al., 1998, Sabitha and Shyamaladevi, 1999).

The levels of exhaled pentane tended to decrease during the first radiotherapy day (1.69 ng/L at baseline and 1.24 ng/L 120 min later, $n=11$, $p=0.075$) and the first week of radiotherapy (1.73 ng/L at baseline on day 1 and 1.43 ng/L at baseline on day 5, $n=8$, $p=0.361$). Several explanations for this are conceivable: it is possible that exhaled pentane levels may decrease as a consequence of the destruction of tumor cells following radiotherapy, as it is known that tumor cells may initially be the source of lipid peroxidation (Zieba et al., 2000). On the other hand, one previous study has suggested that the onset of lipid peroxidation might be delayed after irradiation stimulus and the elevation in lipid peroxidation markers might thus have occurred at a later stage during or after radiotherapy (Umegaki et al., 2001). However, no significant changes were observed in the levels of exhaled pentane later during radiotherapy treatment. The levels of conjugated dienes decreased significantly during the first week of radiotherapy ($p=0.014$). The decrease in serum lipid peroxidation markers may be attributed to the induction of intracellular antioxidants as a response to irradiation (Yukawa et al., 2005). Irradiation may also reduce tumor cell metabolism or intracellular activity.

It is also possible that the fractionation scheme we used induces oxidative stress only mildly and patients´ antioxidative defense mechanisms were able to counter the lipid peroxidation burden induced by radiotherapy. The suggestion that fractionated irradiation reduces lipid peroxidation is supported by an in vitro study by Haidenberger and colleagues (2003) showing that small fractionation schemes produce less lipid peroxidation than single higher radiotherapy fractions. Another in vitro work has suggested that fractionated radiation induces apoptosis through caspase-3 and reactive oxygen species-mediated apoptosis (Bucci et al., 2006). It is known that the biological damage (i.e. anti-tumoral effect) produced by irradiation is mainly mediated directly based on interactions between the ionizing particles and DNA molecules, whereas the amount of lipid peroxidation induced by radiotherapy may primarily result from indirect effects of irradiation (Beir, 1990, p. 20). Thus, the effect of radiotherapy might not be compromised even though the antioxidative defense mechanisms act against radiation-induced lipid peroxidation (Haidenberger et al., 2003). Obviously larger studies are needed to explore this matter.

It was also observed here that when pretreatment exhaled pentane levels were below the median (<1.39 ng/L), one out of five patients developed radiation pneumonitis, while at pretreatment levels above the median, four out of six developed radiation pneumonitis. Due to the small number of patients, testing for conventional statistical significance was not appropriate. Nevertheless, we consider this finding clinically significant and more extensive studies should be undertaken to clarify this matter.

8.5 Cytokine study (study V)

Research has confirmed the importance of cytokines in cancer biology. The serum levels of various cytokines are elevated in lung cancer patients as well as in other cancer types (Yanagawa et al., 1995, Martín et al., 1999, Chen et al., 1999, Vuoristo et al., 2000, Kallio et al., 2001, Benoy et al., 2002). A less widely investigated topic is the local production of cytokines in lung cancer. However, a few studies report higher levels of BAL fluid VEGF, IL-1β, IL-6 and TNFα in lung cancer patients compared to patients with benign lung disease (Arias-Díaz et al., 1994, Beinert et al., 1999 & 2000, Matanić et al., 2003). Our findings are consistent with these data. We recorded elevated levels of serum and BAL fluid IL-6 and serum IL-8 in lung cancer patients ($p<0.001$, $p=0.039$ and $p=0.030$, respectively) at the time of diagnosis compared to non-cancer control patients. One explanation for this may be that tumor cells produce immunosuppressive molecules which influence the formation of cytokines (Staal-van den Brekel et al., 1998). The positive correlations between BAL fluid IL-6 and IL-8 levels and the number of neutrophils in BAL fluid ($R=0.35$, $p=0.035$ and $R=0.37$, $p=0.025$, respectively) suggest that inflammation may partly account for the elevation of cytokines levels. It is in fact known that IL-8 may be derived from inflammatory cells which infiltrate and surround the tumor, or from the tumor itself. Conversely, the tumor cells may attract neutrophils by releasing chemokines, such as IL-8 (Luppi et al., 2007). The serum and BAL fluid levels of VEGF did not differ between patients and non-cancer control patients ($p=0.808$ and $p=0.103$). In addition, we monitored serum and BAL fluid levels of other cytokines (TNFα, IL-1β and IL-12). Most values were below detection limits implying that these cytokines are not valuable tools for assessing the biopathological profile of lung cancer patients. It is previously been shown that patients with SCLC have significantly lower levels of cytokine production compared to NSCLC patients (Matanić et al., 2003). The small size of our patient population precluded any subgroup analysis performed according to the histological type of the cancer.

It was observed that smokers had significantly lower VEGF levels in BAL fluid ($p<0.001$) compared to non-smokers and ex-smokers. A previous study has correspondingly demonstrated that expression of VEGF in alveolar macrophages as well as in BAL fluid is significantly lower in current smokers compared to older smokers. This may imply that the biological availability of VEGF may be impaired in smokers (Nagai et al., 2005).

There was a significant increase in the levels of BAL fluid IL-6 during radiotherapy ($p=0.037$). On the other hand the tendency of IL-8 and IL-18 in BAL fluid to rise during radiotherapy failed to reach statistical significance. It is known from in vitro models that there is considerable individual variability in the secretion of interleukins, which may explain why only IL-6 but no other cytokine was elevated during radiotherapy (Barthelemy-Brichant et al., 2004). IL-6 belongs to the inflammatory cytokines and the rise in its levels may be associated with the inflammatory reaction caused by radiotherapy. A few studies have shown that the number of macrophages increases shortly after irradiation, and these produce various cytokines (Vignaud et al., 1994, Rabbani et al., 2005). It is equally possible that the increase in BAL fluid IL-6 during radiotherapy may be associated with damage to the alveolar compartment due to radiotherapy, thus enabling a leakage of inflammatory mediators (Staal-van den Brekel

et al., 1998). Many inflammatory markers may have a stronger signal in the BAL fluid that in the blood, which would corroborate the present results as no elevation of the serum levels of cytokines was seen during radiotherapy.

Cytokines may also contribute to both acute and late stage radiation damage (Rabbani et al., 2005). Although no notable associations were seen between baseline levels of various cytokines and adverse events during the treatment, the occurrence of adverse events tended to be more frequent as baseline serum IL-8 were below the median (p=0.077).

8.6 Associations with overall survival

There was no mortality among the patients during the time-period when the samples were collected for the study. However, due to the nature of lung cancer, survival was quite short; the 5-year survival rate was only 6% in this study. The median overall survival of the patients was 15.8 months (95% CI 11.4–20.2 months, range 0.4–72.0 months). It emerged that higher baseline plasma and BAL thiols were associated with better overall survival in the chemotherapy (III) and the antioxidant BAL (II) study (p=0.008 and p=0.051, respectively). One earlier study has also explored this matter in head and neck cancer patients receiving radiotherapy. After 36 months of follow-up, patients with elevated post-radiotherapy total plasma glutathione values survived longer than those whose values were below the median (Bøhn et al., 2006). According to an in vitro study, different human lung cancer cell lines have different redox properties and, furthermore, cells with higher expressions of thioredoxin were more susceptible to the anticancer actions of chemotherapeutic drugs (Ceccarelli et al., 2008). Interestingly, it is known that protein p53 is involved in the induction of apoptosis and may be regulated by changes in protein thiols (Dean et al., 1997). A valuable addition here would have been to examine changes in the GSH/GSSG ratio, an approach commonly used as an indicator of the redox status of the cell (Halliwell and Gutteridge, 2007, p. 121).

A marker rarely investigated in association with the overall survival of cancer patients is NOx. In the chemotherapy study there was a tendency towards better overall survival (8.8 months vs. 5.7 months, p=0.068) when baseline plasma NOx levels were above the median (>35.3 μmol/L). One explanation for this may be induction of apoptosis by actions of nitric oxide, as one in vitro work has suggested that nitric oxide induces apoptosis (Rishi et al., 2007).

In the exhaled air collection study, a significant association between pretreatment exhaled pentane levels and overall survival was observed (p=0.003). An interesting situation arises here, as cancer itself is associated with increased lipid peroxidation (Seven et al., 1999, Bakan et al., 2003); yet, enhanced lipid peroxidation seemed to serve as a prognostic marker for survival in our study. One recent study has suggested that the end-products of lipid peroxidation inhibit tumor growth in vivo (Nowak and Janczak, 2006). Another study showed that factors which increase lipid peroxidation can increase apoptosis of pre-cancerous and cancerous cells, an observation also supported by an in vitro work (Das, 2002, Gago-Dominguez and Castelao, 2006). Thus, even though the amount of lipid peroxidation might be elevated among lung cancer patients, at the same time, through other still partly unknown mechanisms, this might serve as a prognostic

factor. However, due to the small number of patients (n=11), the predictive power of exhaled pentane should be confirmed in larger studies.

A finding not previously reported is the association between BAL fluid IL-8 and overall survival (p=0.027) seen in the cytokine study (V). Also higher baseline serum IL-8 levels were associated with shorter survival (p=0.035). This finding is supported by one previous report evidencing poorer survival in chronic lymphocytic leukemia patients who had high pretreatment plasma IL-8 levels (Wierda et al., 2003). IL-8 is known to act by multiple mechanisms on tumor cell proliferation, angiogenesis and migration, which is in accord with our finding (Xie, 2001).

A number of earlier studies have reported serum VEGF to predict survival in lung cancer patients (Salven et al., 1998, Chen et al., 2003, Hasegawa et al., 2005). This was confirmed here in that higher baseline serum VEGF levels were associated with poorer survival (p=0.012). One recent meta-analysis has also suggested that overexpression of VEGF is associated with poor prognosis in lung cancer patients, although data have been contradictory, possibly partly due to diversity in analysis techniques (Zhan et al., 2009). There has also been a lack of prospective studies, which is one advantage of the study in question. It has been speculated that elevated VEGF levels cause disruption in the vasculature within the tumor, followed by an increase in interstitial pressure which may thereby dampen the effects of chemotherapy, leading to poorer response (Jain, 2001).

8.7 Comments on study subjects

The study has several limitations which are discussed in connection with the subjects and the methods and their possible influence on results and conclusions.

The patient and control groups in these studies differed by various factors. Firstly, the mean age for the patients was 65.8 years and for the controls 50.6 years. In our material, the healthy controls in the exhaled pentane study were recruited from among hospital employees at Tampere University Hospital; however, the controls in the bronchoscopy studies had been referred for bronchoscopy for reasons other than cancer, mainly prolonged cough. It is thus natural that the median age of the control group is lower compared to the patient group, who represent rather well the overall lung cancer patient population (Devesa et al., 2005). None of the controls had been diagnosed as having cancer at the time of bronchoscopy or at the control visit six months later. Neither had they serious lung diseases, four of them, however, had asthma, one had chronic bronchitis and one mild COPD. For ethical reasons it was not possible for us to enrol healthy volunteers for the bronchoscopy study, which is an invasive procedure. Also, it would have been particularly difficult and time-consuming to recruit healthy controls who would match by sex, age and smoking habits to the lung cancer patients. In the lung cancer group 80% were male and 63% were smokers. These figures accord fairly well with the demographics of lung cancer patients in general. Also the distribution of lung cancer stages was in line with the common pattern among lung cancer patients (75% of the patients had stage III–IV disease) (Devesa et al., 2005).

Thirty-eight of the patients had squamous cell carcinoma, six had adenocarcinoma and twenty had SCLC. Although adenocarcinoma has become a more common histological tumor type, it is usually more peripherally located and is seldom visualized in bronchoscopy, which may partly explain the low number of adenocarcinomas in our

patient population (Devesa et al., 2005, Pirozynski, 2006). However, we did not have the statistical power in this small number of patients to draw correlations between histological type of cancer and oxidative stress marker levels.

The data on smoking were collected with a standardized questionnaire filled in by all patients and controls. Thereafter, all participants were interviewed. We may therefore consider the information obtained to be as reliable as possible. In this study, there were more smokers in the lung cancer patient group than in the control group. Nevertheless, smoking had no significant impact on most of the study results, and we may therefore consider that smoking is not a major confounding factor for the results. However, smoking was taken into account in statistical analyses, when appropriate. Moreover, patients with severe COPD and those more prone to the deleterious effects of smoking were rejected in these studies by excluding patients with FEV_1 less than 1.5 liters. However, the nature of the study precludes any solid conclusions as to the effect of smoking on the results.

In order to exclude other confounding factors, patients with gout, serious cardiac, metabolic or hepatic disease were excluded, as well as those receiving regular allopurinol or acetylcysteine treatment. None of the patients or controls had taken vitamin or herbal supplementation three months prior to entry into the study. Nevertheless, significant differences persisted in age, sex, BMI, FEV_1 and smoking between the groups. On the other hand, BMI or patients other diseases had no effect on oxidative stress marker levels in these studies. Also possible confounding variables were taken into account in statistical analysis by adjusting results for such factors.

One major limitation in this study was the small number of patients. For example, only 11 patients participated in the exhaled pentane study. However, with these 11 patients, 30 healthy controls and purified background air samples, a total of 233 samples were collected and analyzed. Adding more patients to the cohort would have required a much longer recruitment time and extra resources. All in all, however, the major strength in the series was that all adverse events and responses to treatment were carefully recorded and the patients were followed up for at least 6 years. Neither was any patient lost to follow-up. Thus, despite the small number of patients in the substudies, we consider the clinical relevance of these results high.

8.8 Comments on methodology

To assess oxidative stress comprehensively, we measured several aspects of oxidative damage in this study: markers of oxidative damage to DNA, various vitamins such as ascorbic acid, alpha- and gamma-tocopherol, urate, thiols, the total radical trapping antioxidant capacity (TRAP) and markers of oxidative stress (oxidized proteins, proteins, TBARS, conjugated dienes) and nitrosative stress (nitrite, nitrite+nitrate). In the case of bronchoscopy patients we also measured a wide array of cytokines in both BAL fluid and serum. We also developed a totally new analytical method to explore exhaled pentane as a marker of lipid peroxidation. These markers of oxidative stress are among those most commonly employed to investigate free radical-mediated physiological and pathological conditions (Morabito et al., 2004). However, there is currently no consensus as to which methods are most useful and accurate while also being sufficiently specific. Only a few studies have made a direct comparison of the existing methods. For this reason, most of

the current methods cannot provide absolute reference values (Pryor and Stone, 1993). It is known that cellular antioxidants are under homeostatic control and thus a decrease in one particular antioxidant can be compensated by an increase in another (Kasapoglu and Özben, 2001). Thus, comprehensive evaluation of the antioxidant system is essential.

In this study, no tissue samples were analyzed for oxidative stress marker levels. However, monitoring accessible surrogate elements such as blood cells or bronchoalveolar lavage (BAL) fluid also provides a means of investigating the oxidative status related to lung cancer and its treatments. In fact, only a few clinical studies related to cancer have employed bronchoscopy and BAL due to the rather laborious technique and possible risks involved. For ethical reasons, it was not possible to perform an invasive procedure like bronchoscopy on healthy controls and therefore the controls in studies II and V were patients scheduled for bronchoscopy for benign reasons. Additional tissue sampling in these cases would have added to the potential risks of the procedure and was therefore not undertaken.

There are a number of typical methodological issues unique to studies examining markers of oxidative stress. Firstly, the possibility of artefactual oxidation of the samples exists, which can occur during normal sample handling, processing and analysis (Mayne, 2003). We therefore ensured careful handling of the samples, and special attention was paid to the handling of BAL fluid samples, which were immediately placed in +4ºC and protected from light. Also the collection of ascorbic acid and vitamin E samples were meticulously dealt with; the samples were protected from light and metaphosphoric acid was added to those intended for ascorbic acid analysis. As it is also known that cytokines may vary in their stability and may be influenced by storage temperature or blood collection tube, all samples were collected according to the manufacturer´s instructions and handled with special care.

There are nonetheless limitations in some of the analysis methods. Analyzing TRAP is a rather time-consuming method and also considered not sufficiently specific. TRAP measures practically all chain-breaking antioxidants, e.g. antioxidative enzymes and transition metal binding proteins. It gives quantitative results only as it measures the amount of peroxyls eliminated and does not provide information on the reactivity of antioxidants (Aejmelaeus et al., 1997). Wayner and associates also noted a confounding factor when analyzing plasma TRAP: the greater the plasma dilution was, the higher were also TRAP values (Wayner et al., 1985). However, this has been taken into account when using the chemiluminescence method (Uotila et al., 1994, Ahotupa et al., 1997).

The TBARS test has been widely used in clinical practice to explore free radical-mediated lipid peroxidation and oxidative stress in various pathological conditions. The TBARS test is highly sensitive; however, it has often been criticized for its lack of specificity, which is most conspicuous when using human plasma (De Zwart et al., 1999). Due to its unspecificity, some investigators suggest that the TBARS test should not be employed in clinical studies (Halliwell, 2007). Some thiobarbituric acid-reactive constituents of plasma, e.g. bilirubin and sialic acid, are also reactive towards TBA. Moreover, eicosanoid presynthesis is known to produce endoperoxide precursors of malondialdehyde (MDA). Drugs which inhibit eicosanoid biosynthesis, for example corticosteroids and especially anti-inflammatory drugs, can interfere with the plasma TBARS test. In addition, the usage of EDTA tubes may underestimate the levels of TBARS, and samples should therefore be collected in sterile tubes (Lapenna et al., 2001).

Another confounding factor for the TBARS test is elevated levels of room temperature, as high temperatures may artificially increase concentrations of MDA (De Zwart et al., 1999). Nonetheless, the TBARS test is sensitive for detecting lipoperoxidation aldehydes such as alkenals and alkadienals (Lapenna et al., 2001). In general, measuring for example conjugated dienes as a marker of lipid peroxidation instead of measuring TBARS has been extensively studied (Ahotupa and Vasankari, 1999). Conjugated dienes are also less sensitive to compensatory antioxidant mechanisms than other lipid peroxidation markers (Vasankari et al., 1995)

The most frequently investigated marker of oxidative damage to DNA is 8-oxoguanine, 8-oxoGua. There are several means of measuring oxidative damage to DNA: 1) chromatographic methods (including GC-MS/MS, HPLC-ECD, and LC-MS/MS), 2) the comet assay, 3) slot-blot assays and 4) immunohistochemistry. However, ESCODD (European Standards Committee on Oxidative DNA Damage) has reported discrepancies ranging over three orders of magnitude in oxidative DNA damage marker levels using these techniques. The highest levels have been seen using the GC-MS methodology, this being explained as due to oxidation during the analysis. For this reason, GC-MS is recommended only for studies analyzing DNA adducts. HPLC-ECD is considered to be a highly sensitive method. LC-MS/MS offers some advantages over HPLC-ECD, for example information on the structure and possibility for an internal standard (Cooke et al., 2002, Powell et al., 2005).

Since urinary expression of oxidative lesions depends on sufficient renal excretion, renal impairment may influence the levels of these markers. In order to overcome this problem, the urinary measurements of 8-oxoGua and 8-oxodG were here corrected for creatinine (Cooke et al., 2002, Rozalski et al., 2004). It has also been argued that urinary levels of 8-oxodG may represent cell death and not actual repair. However, in vitro and clinical studies have concluded that urinary 8-oxodG levels are independent of cell death (Cooke et al., 2002). Some findings have also suggested that diet might be a confounding factor for oxidative DNA damage analysis (Halliwell, 2002). Nevertheless, human studies involving HPLC prepurification followed by gas chromatography with isotope dilution/mass spectrometry detection, which we also employed in this study, have shown that urinary excretion of 8-oxoGua and 8-oxodG are not dependent on diet (Gackowski et al., 2001, Rozalski et al., 2004). In general, an assay which can determine both 8-oxoGua and 8-oxodG may represent the amount of cellular oxidative DNA damage better than single oxidative DNA marker analysis (Gackowski et al., 2001).

It is of note, that all the samples in these studies were collected according to the instructions from the originator of the measurement methods. Also the different laboratories which analyzed the samples here are well-established and certified establishments with many years of experience in analyzing antioxidants and markers of oxidative and nitrosative stress. In general, we did our best to minimize any possible confounding effects involved in sample collection and handling as well as in laboratory analysis.

8.9 Comments on the results of the study

The findings in these series are in agreement with those in a number of earlier studies, showing that lung cancer is associated with enhanced oxidative stress and upregulation

of certain cytokines (Yanagawa et al., 1995, Erhola et al., 1997a, Martín et al., 1999, Taysi et al., 2003, Rozalski et al., 2002, Bakan et al., 2003). It seems that cells react to the increased oxidative stress by enhancing the local and systemic production of protective antioxidants. We also noted a disturbance in the pro-oxidative/antioxidative balance in patients undergoing chemotherapy or radiotherapy. The cellular damage caused by lung cancer treatments may lead to release of intracellular antioxidative substances or enhancement of antioxidative defenses.

The findings here underline the importance of assessing appropriate markers when analyzing the local and systemic oxidative status of lung cancer. These results also bring out the importance of local markers, and demonstrate that BAL fluid may be a useful medium for analyzing local cytokine and antioxidant production in lung cancer patients and non-cancer control patients. Our studies also stress the utility of serial markers in elucidating the oxidative status of lung cancer patients during cancer treatments. The relationships with adverse events, response to treatment and survival in lung cancer patients may be examined more thoroughly by analyzing a number of antioxidants, cytokines and markers of oxidative and nitrosative stress.

This study utilized various approaches to examine oxidative stress:

1) direct evaluation of the levels of certain antioxidants and cytokines
2) measurement of end-products of oxidative damage, e.g. TBARS, conjugated dienes and oxidized proteins
3) evaluation of non-invasive markers of oxidation products such as exhaled pentane
4) investigation of the oxidized, repair end products excreted in the urine, e.g. markers of oxidative damage to DNA
5) measurement of the total peroxyl radical trapping antioxidant potential of the body as well as analysis of various markers of oxidative and nitrosative stress.

In this study we assessed the value of exhaled pentane in predicting the occurrence of symptomatic radiation pneumonitis in the lung cancer patient group receiving conventional thoracic radiotherapy. Although exhaled pentane has aroused widespread interest as a non-invasive index of lipid peroxidation, there are significant limitations to the methods used to analyze pentane in the past (Mendis et al., 1995). Thus, the results must be interpreted with caution and additional indicators of oxidative stress should be measured simultaneously.

To our knowledge, this is the largest study hitherto to evaluate the associations between adverse events and responses to treatment with numerous antioxidants and markers of oxidative stress, and the first to report a six-year follow-up of patients. The results imply that plasma and BAL thiols, exhaled pentane, serum and BAL fluid IL-8 and serum VEGF levels may be associated with overall survival in lung cancer patients. The role of NOx and TBARS in relation to survival needs to be clarified in larger studies. Even though overall survival can never be predicted based on any laboratory tests, it would be beneficial to gain information on the possible prognosis of the disease. However, additional studies should also be undertaken to explore the possible relationship between oxidative stress markers and adverse events and response to treatment.

9. CONCLUSIONS

These studies contribute to existing knowledge of the association between ROS and lung cancer, and possibly offer a better clarification of the oxidant/antioxidant status of lung cancer patients. In vivo evidence was provided that oxidative stress is present during and after lung cancer treatments as demonstrated in the reduced levels of certain antioxidants and elevated levels of markers of oxidation. However, judging from our findings it seems that the oxidative stress caused by radiotherapy and chemotherapy is quite adequately counterbalanced by antioxidant defense mechanisms, even though significant lipid peroxidation does occur at the end of treatments. Our study also showed that changes in the levels of cytokines and oxidative stress markers in the BAL fluid during radiotherapy are independent of systemic changes in these markers.

The main findings in this series were:

1) Advanced lung cancer stage is associated with higher marker levels of urinary damage to DNA. Both radiotherapy and chemotherapy seem to enhance oxidative damage to DNA. (I)
2) Lung cancer patients have higher baseline levels of serum urate, plasma nitrite and lower levels of serum oxidized proteins than non-cancer control patients; however, no significant differences were shown in oxidative stress markers in bronchoalveolar lavage fluid before lung cancer therapy as compared to non-cancer control patients. Radiotherapy induces lipid peroxidation observed after the treatment, which may imply that the main antioxidant scavenger systems are consumed during radiotherapy. (II)
3) Adriamycin-containing combination chemotherapy causes significant oxidative stress, as evidenced by reduced levels of protective antioxidants. Chemotherapy treatment seems to enhance lipid peroxidation at the end of treatment. (III)
4) Lung cancer would appear to be related to enhanced lipid peroxidation. Exhaled pentane levels tended to decrease during the first radiotherapy day, and levels of conjugated dienes decreased significantly during the first week of radiotherapy, which may be attributed to enhanced antioxidant defense mechanisms countering the deleterious effects of radiotherapy. (IV)
5) Lung cancer patients had significantly higher levels of serum and BAL fluid IL-6 and serum IL-8 compared to non-cancer control patients. Radiotherapy caused a significant increase in BAL fluid IL-6 levels which might be attributed to enhanced radiotherapy-related inflammation and damage to cells or increased cell death. (V)
6) Higher baseline exhaled pentane levels tended to be associated with an increased occurrence of radiation pneumonitis. These higher levels were also significantly associated with better overall survival (IV). Plasma and BAL fluid thiols and serum and BAL fluid IL-8, in addition to serum VEGF, seem to be prognostic factors for survival. (II, III and V)

The challenge for future research remains to establish which antioxidants are relevant markers for cancer-associated oxidative stress, and which of the wide range of current oxidative stress markers should be employed. There is an intensive ongoing search for markers which would identify the patients most likely to encounter adverse events from cancer treatments as well as those who would benefit most from cancer treatments. Our findings imply that some of the cytokines and oxidative stress markers may predict the occurrence of adverse events as well as predict the time of relapse. As more information accrues, results may have some implications for the treatment of lung cancer patients and evaluation of prognosis based on markers. However, such tests need to be standardized and validated in larger prospective clinical trials.

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APPENDIX

KYSELYLOMAKE POTILAILLE JA KONTROLLEILLE

Potilaan koodi:

Lomakkeen täyttäjä:

1. Mikä on siviilisäätynne?

   1. Naimisissa tai avoliitossa
   2. Naimaton
   3. Asumuserossa tai eronnut
   4. Leski

2. Mikä on peruskoulutuksenne?

   1. Vähemmän kuin kansakoulu
   2. Kansa- tai kansalaiskoulu
   3. Oppikoulua alle keskikoulun
   4. Keskikoulu
   5. Osa lukiota
   6. Ylioppilas

3. Kuinka pitkä on ammattikoulutuksenne (edellisessä kysymyksessä peruskoulutuksen jälkeen)?

   _____

4. Missä ammatissa olette toiminut pisimmän ajan viimeisen 20 vuoden aikana (mahdollisimman tarkasti, esim. automyyjä, metallityön opettaja; tarvittaessa entinen ammatti, esim. entinen maalari)? Kuvatkaa myös mitä teette tai teitte työssänne.

   ammatti:

   työn kuvaus:

LÄÄKÄRINTUTKIMUKSET JA SAIRAUDET

5. Milloin olette viimeksi käynyt oireiden tai sairauden vuoksi lääkärissä?

   1. Viimeksi kuluneen kuukauden aikana
   2. Kuukausi – puoli vuotta sitten
   3. Puoli vuotta – vuosi sitten
   4. 1–5 vuotta sitten
   5. yli 5 vuotta sitten
   6. Ei koskaan

6. Onko teillä todettu syöpää?

   1. Ei
   2. Kyllä; missä elimessä? _____

7. Onko suvussanne (vanhemmat, sisarukset, isovanhemmat, tädit, enot, sedät) esiintynyt syöpäsairauksia?

   1. Ei
   2. Kyllä; kenellä, missä elimessä? _____

8. Onko LÄÄKÄRI TODENNUT Teillä seuraavia sairauksia?

	Ei	Kyllä
sydänveritulppa eli sydäninfarkti	___	___
sepelvaltimotauti eli angina pectoris	___	___
sydämen vajaatoiminta	___	___
sydämen rytmihäiriö	___	___
laajentunut sydän	___	___
sydämen läppävika	___	___
verenpainetauti, korkea verenpaine	___	___
alaraajan valtioahtauma, katkokävely	___	___
aivohalvaus, -verenvuoto, -veritulppa	___	___
alaraajan laskimotukos	___	___
pinnallinen laskimotukos/-tulehdus	___	___
keuhkoveritulppa eli keuhkoembolia	___	___
keuhkoastma	___	___
keuhkolaajentuma eli keuhkoemfyseema	___	___
krooninen keuhkoputken tulehdus, bronkiitti	___	___
homepölykeuhko	___	___
nivelreuma	___	___
muu reumatismi	___	___
jänne- tai jännetupentulehdus	___	___
nivelkuluma, nivelrikko	___	___
maha- tai pohjukaissuolihaava	___	___
sappikivet, sappikivitauti	___	___
haimatulehdus	___	___
maksakirroosi eli maksan kovettumistauti	___	___
krooninen munuaisten toiminnanvajaus	___	___
eturauhasen eli prostatan liikakasvu	___	___
krooninen/toistuva eturauhasen tulehdus	___	___
psoriasis eli hilsetystauti	___	___
valoihottuma	___	___
yliherkkyys- eli allerginen ihottuma	___	___
muu pitkäaikainen ihottuma, mikä? _____		
harmaakaihi	___	___
viherkaihi	___	___
sokeritauti eli diabetes	___	___
muu pitkäaikainen sairaus, vika tai vamma, mikä? _____		

9. Onko teillä VIIMEKSI KULUNEEN NELJÄN KUUKAUDEN aikana ollut seuraavia sairauksia, oireita tai vaivoja?

	Ei	Kyllä
voimakasta väsymystä rasituksessa	___	___
ruokahaluttomuutta	___	___
unettomuutta	___	___
pahoinvointia	___	___
muistin heikentymistä	___	___
keskittymisvaikeuksia	___	___
päänsärkyä	___	___
huimausta	___	___
näön huononemista	___	___
jännittyneisyyttä, hermostuneisuutta	___	___
masentuneisuutta, alakuloisuutta	___	___
vatsakipuja	___	___
närästystä	___	___
ilmavaivoja	___	___
ummetusta	___	___
ripulia	___	___
sukupuolista kyvyttömyyttä	___	___
ihon hilseilyä	___	___
ihon kutinaa	___	___
ihon keltaisuutta	___	___
hiusten lähtöä	___	___
turvotusta jaloissa	___	___
suonenvetoa	___	___
öisin nk. levottomat jalat	___	___
nivelsärkyä	___	___
lihassärkyä	___	___
lonkkakipua kävellessä	___	___
polvikipua kävellessä	___	___
nenäverenvuotoa	___	___
mustelmia	___	___

10. Yleiskunto
Millainen on yleiskuntonne kehitys ollut viimeisen vuoden aikana

1. Parantunut ___
2. Pysynyt samana ___
3. Laskenut vähän ___
4. Laskenut paljon ___

11. Paino
Onko painonne laskenut pysyvästi viimeisen vuoden aikana?

1. Ei ___
2. Alle 2kg ___
3. 3–5kg ___
4. Yli 5kg ___

12. Hengenahdistuksen esiintyminen

12.1. Esiintyykö Teillä hengenahdistusta kiiruhtaessanne tasaisella maalla tai kävellessänne portaita tai loivaa ylämäkeä?

Ei ___
Kyllä ___

12.2. Onko Teidän pysähdyttävä hengenahdistuksen takia kävellessänne omaa vauhtia tasamaalla?

Ei ___
Kyllä ___

13. Yskösten esiintyminen
Esiintyykö Teillä tavallisesti ysköksiä (lima) heti aamulla herättyänne tai ylösnoustuanne? (Ota huomioon ensimmäisen savukkeen tai ulosmenon aiheuttama yskös. Ota huomioon myös nielty yskös. Nenälimaa ei oteta huomioon)

Ei __
Kyllä __

14. Veriyskä?
Onko Teillä ollut kolmen viimeisen vuoden aikana yskässä verta tai veriyskää?

Ei ___
Kerran ___
Useammin ___

15. Tupakointi

15.1. Montako vuotta olette säännöllisesti tupakoineet?

____ vuotta

15.2. Koska olette tupakoineet viimeksi?

1. Eilen tai tänään
2. Kaksi päivää – puoli vuotta sitten
3. Yli puoli vuotta sitten

15.3. Kuinka paljon poltatte nykyisin keskimäärin päivässä?

1. Valmissavukkeita ___ kpl/pv
2. Itse käärittyjä ___ kpl/pv
3. Piipullista ___ kertaa/pv
4. Sikareja ___ kpl/pv

15.4. Kuinka monta savuketta olette polttaneet keskimäärin päivässä niinä vuosina, joina poltitte säännöllisesti?

Savukkeita/sikareita/piipullista päivässä _____

15.5. Vedättekö yleensä tupakansavua henkeen?

1. En lainkaan
2. Vähän
3. Kohtalaisesti
4. Syvälle

15.6. Missä iässä aloititte säännöllisen tupakoinnin (ensimmäisen kerran tupakoitte joka päivä tai lähes joka päivä vähintään vuoden ajan)

_____ vuoden iässä

15.7. Mikäli poltatte valmissavukkeita, merkitkää allaolevasta listasta se savukemerkki, jota pääasiassa poltatte.

01 Armiro, regular	17 Form, Menthol	33 Klubi 22
02 Armiro, mild	18 Form, light	34 L & M
03 Barclay	19 Form, light Menthol	35 Marlboro
04 Belmont 2002	20 Form, Special	36 Marlboro, Menthol
05 Belmont, filter	21 Gauloises Plain	37 Marlboro, Lights
06 Belmont, Mild	22 Gauloises Filtro	38 Multifilter, Long size
07 Belmont, Menthol	23 Gold Dollar	39 Newport
08 Boston, filter	24 Hudson	40 North State, plain
09 Camel	25 Kent, Box	41 North State, filter
10 Colt, long size	26 Kent, Deluxe	42 Punaraita (North State)
11 Colt, lights	27 Kevytsavuke 5	43 Siniraita (North State)
12 Colt Internat.	28 Kevytmenthol 5	44 Partner
13 Dunhill	29 Kevytsavuke 3	45 Salem, Lights
14 Dunhill, Menthol	30 Ultra Kevyt 1	46 Smart
15 Form, Extra Light	31 Kevytsavuke S	47 Työmies
16 Form, Medium Tar	32 Klubi 77	48 Winston

49 Muu merkki, mikä? _____

Kuinka monta vuotta olette polttaneet tätä savukemerkkiä? _____ vuotta

16. Lääkitys
Onko Teillä käytössänne säännöllisesti lääkärin määräämiä lääkkeitä? Lyhyitä (alle 2 viikon) lääkekuureja ei huomioida.

Merkitkää myös kuinka paljon nykyisin otatte lääkkeitä päivässä sekä kuinka monta vuotta olette sitä käyttänyt.

1. Ei
2. Kyllä, lääkkeen nimi: nykyinen päiväannos käytön pituus (vuosia)

_____ _____ _____

_____ _____ _____

_____ _____ _____

_____ _____ _____

_____ _____ _____

17. Vitamiini- ja hivenainevalmisteet

Oletteko viimeksi kuluneen 3 kuukauden aikana käyttänyt mitään vitamiini- tai hivenainevalmisteita?

1. En
2. Kyllä, valmisteen nimi päiväannos

_____ _____

_____ _____

18. Kiitos vastaamisesta. Mikäli Teillä on ongelmia jonkin kohdan täyttämisessä, jättäkää se tyhjäksi. Voimme palata kysymykseen myöhemmin.

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Impact of radiotherapy and chemotherapy on biomarkers of oxidative DNA damage in lung cancer patients

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Abstract

Objectives: To assess oxidative damage to DNA during lung cancer (LC) treatments.
Design and methods: Urinary levels of 8-oxoguanine (8-oxoGua) and levels of 8-oxo-2′-deoxyguanosine (8-oxodG) from urine and whole blood were determined in 36 non-cancer controls and 65 LC patients before any treatments. Samples were also obtained of LC patients during and after radiotherapy (RT, $n=33$) and chemotherapy (CT, $n=16$).
Results: Stage IV LC patients had higher urinary 8-oxoGua and 8-oxodG levels than patients with stage I–III disease ($p=0.044$ and $p=0.034$, respectively). Urinary 8-oxodG levels increased during the first week of RT ($p<0.001$). Nuclear 8-oxodG increased during RT and 3 months after start of RT. Nuclear 8-oxodG levels also rose between the first two CT cycles ($p=0.043$), and urinary 8-oxodG levels during the sixth CT cycle ($p=0.009$).
Conclusions: Urinary DNA damage biomarker levels may be associated with LC stage. Both RT and CT increase the parameters of DNA oxidation.

Keywords: 8-oxoGua; 8-oxodG; Lung cancer; Radiotherapy; Chemotherapy; Oxidative DNA damage

Introduction

Ionizing radiation generates several free radicals, most important of which is the hydroxyl radical. The hydroxyl radical is very reactive and it can attack DNA bases and damage proteins and lipids [1]. Damage to DNA produces numerous pyrimidine- and purine-derived lesions e.g. 8-oxoguanine (8-oxoGua). 8-oxoGua and the corresponding nucleoside 8-oxo-2′-deoxyguanosine (8-oxodG, also 8-OHdG) are the most common oxidative DNA lesions examined [2]. 8-oxoGua is excreted into the urine without further metabolism, and constitutes a suitable biomarker of generalized cellular oxidative stress [3,4].

Anthracyclines, including adriamycin, exert their cytotoxic effects by inhibition of topoisomerase II, which generates DNA strand breaks leading to apoptosis [5,6]. Anthracyclines also produce free radicals, especially hydroxyl radicals, which may explain partly the adverse effects of the anthracyclines [6].

Abbreviations: 8-oxoGua, 8-oxoguanine; 8-oxodG, 8-oxo-2′-deoxyguanosine; LC, lung cancer; CT, chemotherapy; RT, radiotherapy; HPLC, high-performance liquid chromatography; CG/MS, chromatography/mass spectrometry.

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Elevated levels of DNA damage biomarkers in the urine occur in lung, colorectal, gynecological, bladder, prostate, and breast cancer and in lymphoma patients [4,7–12]. In a previous study, we reported increased urinary 8-OHdG levels after radiotherapy (RT) and chemotherapy (CT) in lung cancer (LC) patients [7]. In a few other papers oxidative DNA damage during cancer treatment has also been studied, but the study populations have been small [12–14].

Some studies suggest that urinary 8-OHdG excretion might be a useful tool to predict responses to treatment or patient survival, but no actual long-term follow-up of patients has been carried out to test this hypothesis [7,14,15].

Based on our earlier finding of elevated urinary 8-oxodG levels during LC treatments [7] we decided to evaluate a broader spectrum of DNA damage biomarkers, urinary 8-oxoGua, 8-oxodG and nuclear 8-oxodG, in a larger study with LC patients and non-cancer controls. We have investigated oxidative DNA damage markers during RT and CT, and how these marker levels are associated with adverse events, response to treatment and overall LC patient survival.

Materials and methods

Study group and procedures

Sixty-five LC patients and 36 non-cancer control patients were enrolled. The patients were invited consecutively to enter the study; they were referred to the Department of Respiratory Medicine or Department of Oncology at the Tampere University Hospital. The inclusion criteria for the patients were: histologically or cytologically confirmed LC and Karnofsky performance status >70%. The inclusion criteria for the controls were: patients referred for bronchoscopy due to prolonged cough. The exclusion criteria for all were: serious acute infection, serious cardiac, metabolic or hepatic disease, unstable angina pectoris, forced expiratory volume in one second (FEV_1) <1.5 L and gout. Pretreatment evaluation of the LC patients consisted of physical examination, bronchoscopy, chest radiography, chest and upper abdominal computerized tomography, urinalysis, full blood count and serum chemistry. Samples from bronchoalveolar suction were obtained of most participants for tuberculosis cultivation. Skeletal scintigraphy was performed as clinically needed. The diagnosis and TNM classification by UICC criteria [16] of the cancer patients was based on findings from physical examination, bronchoscopy, chest radiography and chest and upper abdominal computed tomography. Most (n=38) of the patients had squamous cell carcinoma (59%), 20 (31%) had small cell lung cancer, six (9%) had adenocarcinoma and for one (2%) patient the histology remained unclassified.

LC patients were treated according to clinical guidelines [17]. Blood and urine samples were collected once of non-cancer control patients and of LC patients at baseline before any treatments. In addition, urine samples were collected of patients receiving RT at 8 Gray (Gy), 10 Gy, 20 Gy and at 30 Gy and 40 Gy as well as 3 months after start of RT, if possible. Blood samples were collected at 20 Gy and 3 months after RT. The mean number of urine samples collected from RT patients (n=33) was three (range 1–5) and of blood samples 2 samples (range 1–3). Of the small cell lung cancer (SCLC) patients (n=16) receiving CT, blood and urine samples were collected before start of CT and urine samples also 19 h after the administration of first chemotherapeutic drug. The samples were collected during the first, second and last (usually sixth) CT cycle. To assess short-term oxidative stress on DNA, blood samples were collected of six patients during the treatment (at baseline 12 AM, 3 PM, 5 PM and 7 AM the following morning) during the first, second and sixth CT cycle. The mean number of urine samples collected from CT patients was 5 (range 1–7) and of blood samples 7 (range 1–14).

None of the patients or controls had taken vitamins or herbal supplementation during 3 months prior to the study. Data on smoking, other diseases, medication and symptoms were collected on a standardized questionnaire modified from the ATBC (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study) [18]. The characteristics of the two groups are shown in Table 1.

Ethics

The Ethics Committee of the Tampere University Hospital approved the study and written informed consent was obtained of each participant.

Radiotherapy

Twenty-seven patients underwent three-dimensional computer tomography-based treatment planning by CadPlan

Table 1
Characteristics of lung cancer patients and controls. Values are means (range) or numbers (percentages).

	Lung cancer patients n=65 (%)	Controls n=36 (%)
Age in years (range)	65.8 (48–83)	50.8 (18–75)
Males/females	52/13	16/20
Body mass index, kg/m^2, (range)	24.4 (17.0–43.6)	26.3 (18.5–39.2)
FEV_1, % of predicted [a], (range)	68.8 (21–102)	89.4 (65–108)
Smoking		
Non-smokers	3 (4.6)	18 (50.0)
Ex-smokers	21 (32.3)	6 (16.7)
Smokers	41 (63.1)	12 (33.3)
Pack-years of smokers or ex-smokers	37.2 (5.0–100)	28.5 (7.5–68)
Histological diagnosis squamous cell ca	38 (58.5)	
Adenoca	6 (9.2)	
Small cell ca	20 (30.7)	
Unclassified	1 (1.5)	
Stage of lung cancer		
I/IIA–B	3 (4.6)	
IIIA	11 (16.9)	
IIIB	21 (32.3)	
IV	30 (46.1)	
Karnofsky performance status		
70	6 (9.2)	0 (0)
80	16 (24.6)	0 (0)
90	31 (47.7)	3 (8.3)
100	12 (18.5)	33 (91.7)

[a] n=37 and n=20.

(Varian, Varian Medical Systems Inc., Palo Alto, CA, USA), six patients received palliative radiotherapy with two anterior–posterior fields. The planning target volume (PTV) included the tumor and adjacent lymph nodes with adequate safety margins.

Radiotherapy with 18 MV photons was applied by a linear accelerator (Varian, Varian Clinac 2100 C/D, Varian Medical Systems Inc., Palo Alto, CA, USA) at the Radiotherapy Unit of Tampere University Hospital. Fractions of 2 or 3 Grays (Gy) were given five times a week and one patient received RT 4 Gy/fraction, 2 fr/week. The mean radiation dose delivered was 46.6 Gy (range 30.0 Gy–60.0 Gy) corresponding to 4 1/2 weeks duration of treatment.

Chemotherapy

Sixteen SCLC patients received CAV chemotherapy; adriamycin (doxorubicin, 50 mg/m^2), vincristine (1.5 mg/m^2, maximum dose 2 mg each time) and cyclophosphamide (750 mg/m^2) intravenously. Adriamycin was always infused first over 30 min, after that vincristine as a bolus and finally cyclophosphamide over 30 min. The chemotherapy treatment was administered during the first day of the cycle; consecutive cycles were repeated every 3 weeks (second CT cycle starting on day 22). The planned number of chemotherapy cycles was six corresponding to 4 1/2 months duration of treatment.

Urine and blood collection

Six hours overnight urine was collected (collection time usually 12 PM–6 AM) and the total volume was measured. Two 20 mL plastic tubes were separated and stored at −20 °C until analysis.

Peripheral venous blood samples were collected into ethylene diamine tetra-acetic acid (EDTA) tubes after the patient had fasted overnight. A Venoject blood collection system (Terumo, Leuven, Belgium) was used. Whole blood samples were stored at −70 °C until analysis.

Urinalysis

Urine sample preparation was done as previously described [8]. Urine high-performance liquid chromatography (HPLC; Supelcosil LC18 column, 250 mm × 10 mm, 5 μm equipped with Supelguard LC18 guard column, 20 mm × 4.6 mm; Supelco, Sigma Aldrich, Poznan, Poland) purification of 8-oxoGua and 8-oxodG was performed according to the method described by Gackowski et al. [19,20]. Chromatography/mass spectrometry (CG/MS) analysis was performed according to the method described by M. Dizdaroglu [21], adapted for additional [$^{15}N_5$] 8-oxoGua analyses (m/z 445 and 460 ions were monitored). The urinary biomarkers were normalized to the concentration of creatinine. The concentration of creatinine in urine was measured using a creatinine kit by Sigma Diagnostics (Sigma Aldrich, Poznan, Poland), where creatinine measurement is based on Jaffe's reaction with picric acid. All analysis were done at the Department of Clinical Biochemistry at Nicolaus Copernicus University, Bydgoszcz, Poland.

Nuclear 8-oxodG analysis

DNA from whole blood was isolated by a non-enzymatic method [22]. The amount of 8-oxo-7,8-dihydro-2′-deoxyguanosine was determined using HPLC equipped with an electrochemical detector and deoxyguanosine was determined with a UV-detector [23,24]. The nucleosides were separated by a C18 reverse-phase column (Phenomenex Luna C18, 3 mm, 4.6 × 150 mm; Phenomenex, Torrance, CA, USA). The 8-oxodG concentration was expressed as the ratio of 8-oxodG per 10^5 dG. All analysis were done at the MCA Research Laboratory, Turku, Finland.

Evaluation of adverse events and response to treatment

During and after RT, all adverse events were evaluated according to the criteria of the World Health Organization (WHO) and Lent Soma Table [25,26]. The adverse events during CT and the responses to treatments were evaluated according to WHO criteria [25,27]. Complete response (CR) was defined as the disappearance of all known disease determined by two observations not less than 4 weeks apart; partial response (PR) was defined as a 50% or more decrease in the total tumor mass of those lesions measured during two observations not less than 4 weeks apart; no change (NC) was defined 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 25% or greater increase in the size of one or more measurable lesions or appearance of new lesions. [25,27].

All cancer patients were followed at the Tampere University Hospital during and after their treatments.

Statistics

Urine 8-oxoGua (nmol/mmol creatinine), urine 8-oxodG (nmol/mmol creatinine) and nuclear 8-oxodG (8-oxodG/10^5dG) were the primary variables. The distributions of these biomarkers were skewed to the right and were thus logarithmically (ln) transformed before analysis. The results are given as geometric means with 95% confidence interval. Independent samples *t*-test was used when the LC patients were compared to controls. ANOVA and Chi-squared test was used to study the associations between a subject's characteristics and the primary variables. As age, gender and smoking seemed to be associated with the primary variables, they were included as covariates or factors in general linear models (adjusted group comparisons). ANOVA for repeated measures or Friedman's test, when appropriate, was used to test the change in the primary variables during the treatment (time-effect). Sperman's rho was used for associations between primary variables and laboratory measurements and Pearson's correlation coefficient was used for associations between primary variables and other continuous variables at the baseline. All repeated measurements during the

treatment period were included to study the associations between urine and nuclear biomarkers during the study period [28]. The distributions of the primary variables at baseline were divided into two categories (median as a cut off point) or three categories (tertiles T1, T2 and T3) and were compared with the incidence of adverse events, response to the treatment and survival. The Log-rank test was used to compare survival curves. Data were analysed with the SPSS statistical software (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

Sixty-five LC patients and 36 non-cancer control patients were entered into the study. 52 of the patients were male, 13 female whereas control patients included 16 males and 20 females. None of them were diagnosed with tuberculosis. Thirty-three of the patients received RT; of these patients eight received two or three cycles of cisplatin-based chemotherapy before RT. 16 SCLC patients received CAV chemotherapy as treatment. Of these SCLC patients, twelve (75%) received the planned six chemotherapy cycles and in two of the patients (13%) the treatment was discontinued due to progressive disease.

Two patients were operated on after the diagnosis without further treatments, five patients received various CT regimens and 9 received symptomatic treatment; of these patients, only baseline samples were obtained.

None of the control patients were diagnosed with cancer, three (8%) had asthma, two (6%) had both chronic bronchitis and mild chronic obstructive pulmonary disease (COPD), one (3%) had COPD and one (3%) control patient was operated on for a benign papilloma. All other control patients (80%) were diagnosed as having prolonged cough and required no further treatment.

The patient and control groups differed with respect to some main characteristics (Table 1), and thus age, gender and smoking were taken into account and the DNA damage marker analyses were adjusted accordingly.

The adverse events during and after radiotherapy or chemotherapy, response to treatments and overall survival of lung cancer patients are in Table 3.

DNA damage biomarkers at baseline

Urine samples

There were no significant differences in the excretion of 8-oxoGua to the urine between LC patients and non-cancer control patients (geometric mean 9.79, 95% CI 8.56–11.20 vs. 11.21, 95% CI 9.00–13.95 nmol/mmol creatinine, $p=0.264$) or of 8-oxodG (geometric mean 2.17, 95% CI 1.88–2.50 vs. 2.11, 95% CI 1.84–2.43 nmol/mmol creatinine, $p=0.816$). Adjustment for age, gender and smoking did not change this ($p=0.148$ and $p=0.422$, for 8-oxoGua and 8-oxodG, respectively) (Table 2). There was a significant positive correlation between urinary 8-oxoGua and 8-oxodG excretion ($R=0.463$, $p<0.001$) at baseline.

Blood samples

LC patients had significantly lower nuclear 8-oxodG levels than the non-cancer control patients at baseline (geometric mean 1.27, 95% CI 1.00–1.61 vs. 2.54, 95% CI 1.64–3.95 8-oxodG/10^5 dG, $p=0.003$), but after adjustment for age, gender and smoking, the difference between the groups disappeared ($p=0.145$).

DNA damage biomarkers during and after radiotherapy

Urine samples

There was a statistically significant increase in urinary 8-oxodG levels during the first week of RT (1.74; 95% CI 1.36–2.24 at baseline, 2.62; 95% CI 2.15–3.19 at day 4 and 2.87; 95% CI 1.98–4.17 nmol/mmol creatinine at day 5, $p<0.001$). The geometric mean of 8-oxoGua was 9.84 at baseline, 11.38 on day 4 and 9.01 nmol/mmol creatinine on day 5 ($p=0.366$) (Fig. 1).

There were no significant changes after 2 weeks of RT or at 30 or 40 Gy, but a slight, albeit non-significant, decrease in both

Table 2
Urine and nuclear levels of oxidative DNA damage biomarkers in lung cancer patients and controls at baseline.

	Patients		Controls		Patients/controls		*p*
	($n=63$)		($n=36$)				
	G mean	(95% CI)	G mean	(95% CI)	Ratio	(95% CI)	
Urine 8-oxoGua							
Unadjusted [a]	9.79	(8.56–11.20)	11.21	(9.00–13.95)	0.87	(0.69 to 1.11)	0.264
Adjusted [b]	10.13	(8.58–11.96)	12.70	(10.18–15.84)	0.80	(0.59 to 1.09)	0.148
Urine 8-oxodG							
Unadjusted	2.17	(1.88–2.50)	2.11	(1.84–2.43)	1.03	(0.83 to 1.27)	0.816
Adjusted	2.10	(1.80–2.45)	2.35	(1.92–2.89)	0.89	(0.67 to 1.18)	0.422
Nuclear 8-oxodG [c]							
Unadjusted	1.27	(1.00–1.61)	2.54	(1.64–3.95)	0.50	(0.32 to 0.78)	0.003
Adjusted	1.47	(1.06–2.05)	2.30	(1.50–3.53)	0.64	(0.35 to 1.17)	0.145

Results are given as unadjusted and as adjusted geometric means (95% CI), and the group comparison is given as ratio (95% CI) of patients/controls.
[a] ANOVA, using logarithmically transformed values.
[b] ANCOVA, adjusted for current smoking (no/yes), gender and age.
[c] $n=34$ in patients and $n=20$ in controls.

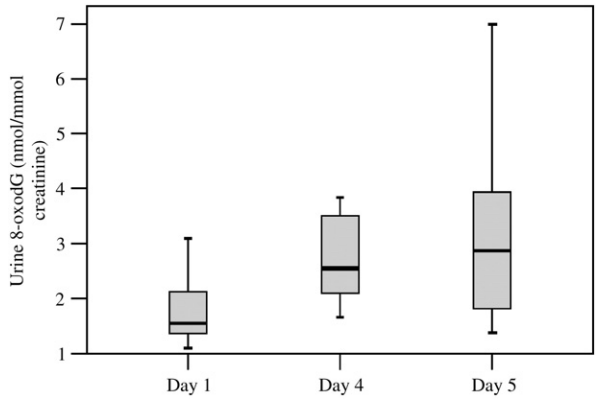

Fig. 1. Box-plot figure for urine 8-oxodG (8-oxo-2′-deoxyguanosine, nmol/mmol creatinine) during the first week of radiotherapy ($n=10$). The boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the maximum and the minimum.

8-oxoGua and 8-oxodG (11.11 vs. 8.72, $p=0.479$ and 2.40 vs. 1.79 nmol/mmol creatinine, $p=0.389$, respectively) levels did occur 3 months after RT (Fig. 2). There was a significant positive correlation between urinary 8-oxoGua and 8-oxodG levels also during RT, when all repeated measurements are included ($R=0.532$, $p<0.001$).

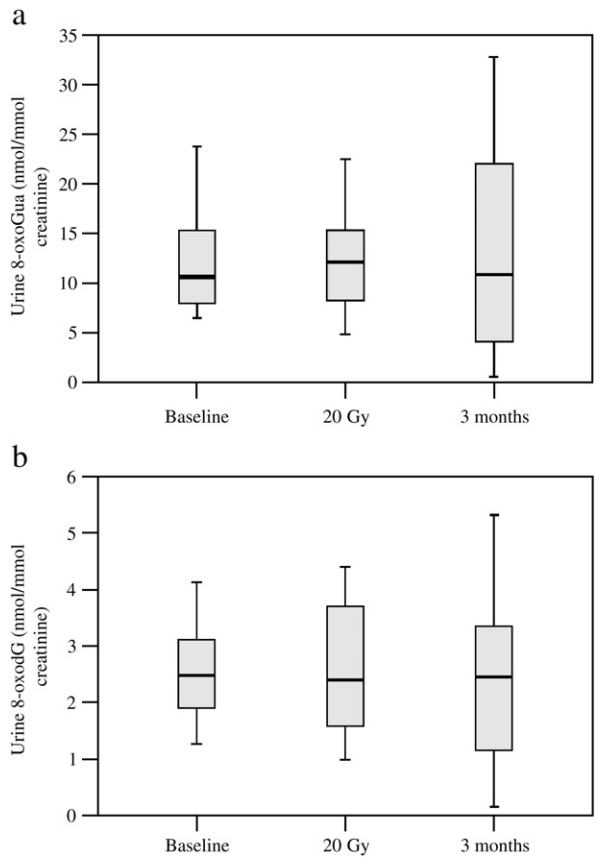

Fig. 2. (a) Box-plot figure for urine 8-oxoGua (8-oxoguanine, nmol/mmol creatinine) at baseline, during radiotherapy at 20 Gy and after 3 months ($n=12$). (b) Box-plot figure for urine 8-oxodG (8-oxo-2′-deoxyguanosine, nmol/mmol creatinine) at baseline, during radiotherapy at 20 Gy and after 3 months ($n=12$).

Blood samples

The levels of nuclear 8-oxodG increased 2 weeks after radiotherapy (1.39 at baseline vs. 1.57 8-oxodG/10^5 dG at 20 Gy) as also at 3 months after RT compared to baseline (1.39 vs. 3.36 8-oxodG/10^5 dG). Due to the small amount of samples, testing for statistical significance was not appropriate.

DNA damage biomarkers during chemotherapy

Urine samples

The baseline values of urinary 8-oxoGua and 8-oxodG increased during CT treatment, from the first to the last (sixth) CT cycle; however, without statistical significance. The geometric means before administration of CT at 1st, 2nd and 6th cycle were 7.66, 8.68 and 11.12 nmol/mmol creatinine for 8-oxoGua ($p=0.226$) and 1.61, 1.69 and 2.28 nmol/mmol creatinine for 8-oxodG ($p=0.150$, Fig. 3). Urinary biomarkers increased also during each CT cycle, from start of CT at 12.00 AM to 7 AM on the following morning. However, the only significant change was recorded for 8-oxodG during the

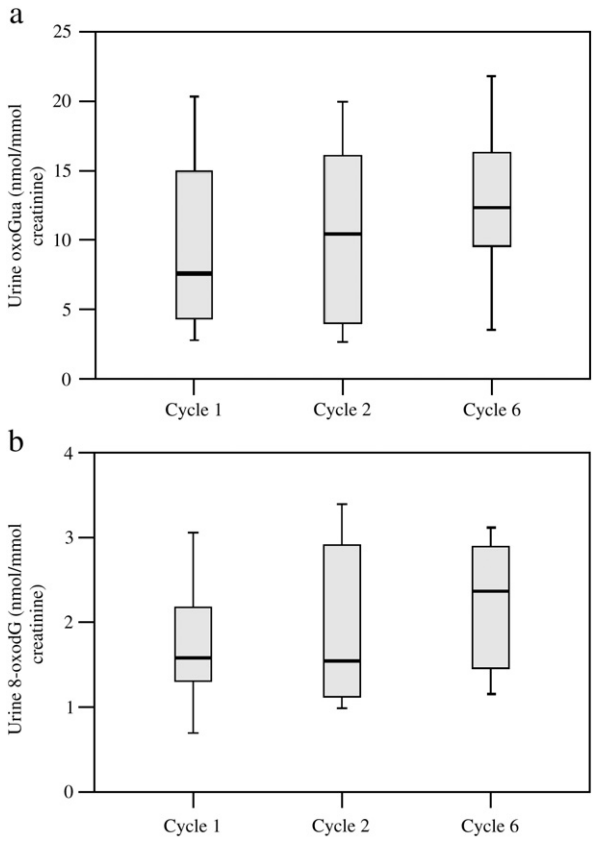

Fig. 3. (a) Box-plot figure for urinary 8-oxoGua (8-oxoguanine, nmol/mmol creatinine) during chemotherapy cycles ($n=10$). (b) Box-plot figure for baseline levels of urinary 8-oxodG (8-oxo-2′-deoxyguanosine, nmol/mmol creatinine) during chemotherapy cycles ($n=10$). The chemotherapy treatment was administered during the first day of the cycle; consecutive cycles were repeated every 3 weeks up to a maximum of six cycles. Measurements were performed during the first, second and sixth chemotherapy cycle. Outside the figure one outlier: 5.75 nmol/mmol creatinine.

sixth CT cycle (2.28 at baseline and 2.91 nmol/mmol creatinine 19 h from start of CT, $p=0.009$).

Blood samples

The concentration of nuclear 8-oxodG/10^5dG increased during CT treatment, although not statistically significantly. The geometric means at the 1st, 2nd and 6th cycle were 1.98, 3.74 and 4.32 for nuclear 8-oxodG/10^5 dG ($p=0.311$). The baseline nuclear 8-oxodG concentration increased significantly between the first and second CT cycle (2.06 vs. 3.99 8-oxodG/10^5 dG, $p=0.043$). During the first cycle, in three of the patients 8-oxodG/10^5 dG levels increased and in three on the patients the levels decreased during the treatment when samples were collected at 12 AM, 3 PM, 5 PM and 7 AM the following morning. During the second ($n=5$) and sixth ($n=4$) CT cycle, the levels decreased in four of the patients during the CT treatment. In one patient, the 8-oxodG/10^5 dG levels increased during second CT treatment. Due to the small amount of samples, testing for statistical significance was not appropriate.

Association between DNA damage biomarkers and other demographics

LC patients with stage IV disease had significantly higher urinary DNA damage biomarker levels than patients with stage I, II or III disease; 11.50 vs. 8.78 nmol/mmol creatinine ($p=0.044$) and 2.58 vs. 1.91 nmol/mmol creatinine ($p=0.034$), for 8-oxoGua and 8-oxodG, respectively.

There were no statistically significant associations between DNA damage biomarker levels and other diseases (cardiac disease, hypertension, chronic arrhythmia, diabetes, asthma, COPD, chronic bronchitis or rheumatic disease). Nor did neoadjuvant chemotherapy affect biomarker levels.

There was a significant positive correlation between age and baseline urinary 8-oxoGua and 8-oxodG in patients ($R=0.365$, $p=0.003$ and $R=0.28$, $p=0.03$, respectively). Over 65 years old patients had higher urinary 8-oxoGua (11.33 vs. 7.95 nmol/mmol creatinine, $p=0.008$) and 8-oxodG (2.42 vs 1.85 nmol/mmol creatinine, $p=0.062$) levels compared to patients under 65 years of age. No significant correlations in relation to age were observed among control patients.

Women tended to have higher urinary and nuclear DNA damage biomarker levels compared to men among non-cancer controls. However, statistically significant difference was detected only in urinary 8-oxoGua at baseline (13.97 for women and 8.51 nmol/mmol creatinine for men, $p=0.02$).

There were no differences in the urinary DNA damage biomarker levels between smokers and non-smokers ($p=0.123$ and $p=0.227$ for 8-oxoGua and 8-oxodG, respectively). However, smokers had lower nuclear 8-oxodG levels ($p=0.005$) compared to non-smokers.

There were significant positive correlations between urinary 8-oxodG levels and serum neutrophils count in the LC patients and in whole study group ($R=0.403$, $p=0.001$ and $R=0.236$, $p=0.021$, respectively), and urinary 8-oxodG and serum C-reactive protein levels ($R=0.306$, $p=0.016$ and $R=0.191$, $p=0.060$, respectively).

Table 3
Adverse events during radiotherapy or chemotherapy, response to treatments and overall survival of lung cancer patients.

	Patients ($n=65$)	
Adverse events during RT/CT ($n=49$)	35	(71%)
Response to RT/CT treatment ($n=49$)		
Complete or partial response	37	(76%)
No change or progressive disease	12	(24%)
Survival		
1 year	24	(37%)
3 years	10	(15%)
5 years	3	(6%)
Median (range) of survival (months)	15.7	(0.4–70.1)
95% CI	11.3 to 20.0	

Association between DNA damage biomarkers and adverse events, response to treatment and overall survival

The adverse events were not significantly associated with the baseline levels of DNA damage biomarkers.

The response to treatment was not associated with changes in urinary 8-oxoGua, urinary 8-oxodG or nuclear 8-oxodG from baseline to the last measurement.

The planned follow-up time of the patient group was 72 months. None of the patients was lost to follow-up. At the end of the study, one patient (1.5%) was alive, and 64 (98%) had died.

The median overall survival of the patients was 15.7 months (95% CI 11.3–20.0 months, range 0.4–70.1 months). The one-year survival rate was 37%, the three-year survival rate 15% and the five-year survival rate 6%. (Table 3) Although not significantly, the overall survival time tended to be longer when the baseline level of nuclear 8-oxodG was highest (>1.34 8-oxodG/10^5 dG), when nuclear 8-oxodG values were divided into tertiles. The mean survival time was 8.4 months in the lowest tertile, 14.6 months in the middle tertile and 19.5 months in the highest tertile ($p=0.182$).

Discussion

The major findings in this study are:

a) LC patients with stage IV disease have higher urinary DNA damage biomarker levels than patients with stage I–III disease ($p=0.044$ and $p=0.034$ for urinary 8-oxoGua and 8-oxodG, respectively).
b) There was a significant increase in the levels of urinary nucleoside form, 8-oxodG, during the first week of RT ($p<0.001$). The nuclear 8-oxodG levels had a tendency to increase at 20 Gy during RT and 3 months after RT.
c) The levels of DNA damage biomarkers had a tendency to increase during CT treatment, and a significant increase was seen in nuclear 8-oxodG between first and second CT cycles ($p=0.043$) and in urinary 8-oxodG during the last CT cycle ($p=0.009$).
d) There were no statistically significant associations between DNA damage biomarkers and adverse events during the treatments, responses to treatment or overall survival.

We observed that LC patients with stage IV disease have higher urinary DNA damage biomarker levels at baseline than patients with stage I–III disease ($p=0.044$ and $p=0.034$ for 8-oxoGua and 8-oxodG respectively), which is supported by a previous study suggesting that urinary 8-OHdG levels are associated with tumor mass of patients with hematological malignancies [14]. The present study focused on a solid tumor (LC) and there were no significant baseline differences in the levels of urinary 8-oxoGua and urinary and nuclear 8-oxodG between the cancer patients and non-cancer control patients — a finding which is in accordance with previous studies [7,14]. The control patients in this study had prolonged cough and, in addition to cancer, several pathological conditions, including acute and chronic inflammation, are associated with elevated levels of oxidative DNA damage markers [29,30]. The positive correlations between urinary 8-oxodG levels and serum neutrophils count ($R=0.236$, $p=0.021$) and serum C-reactive protein levels ($R=0.191$, $p=0.060$) in the whole study group support the hypothesis that inflammation might partly explain why no significant differences were noted between the lung cancer and control groups at baseline.

There was a significant positive correlation between age and baseline urinary 8-oxoGua and 8-oxodG levels in LC patients ($R=0.365$, $p=0.003$ and $R=0.28$, $p=0.03$, respectively). Patients over 65 years of age had higher urinary 8-oxoGua ($p=0.008$) and 8-oxodG ($p=0.062$) levels than younger patients. Also women had higher urinary 8-oxoGua levels at baseline ($p=0.02$) than men among the non-cancer control patients. It is known that urinary 8-oxodG levels are also affected by muscle mass. A study based on urine collection by spot samples reported higher urinary 8-oxodG values in women than men [31]. This might also partly explain the higher levels among patients above the age of 65 years [31]. Strenuous exercise is known to affect urinary 8-oxodG levels [32], however, all LC cancer patients receiving CT were hospitalized during the collection of urine. The first urine sample was collected on the day after bronchoscopy both of LC patients receiving RT and of the non-cancer control patients. Therefore it is unlikely that exercise could have influenced the biomarker levels.

Contradictory data has been published on the effect of smoking on DNA damage biomarkers. Many studies have reported that smokers have higher oxidative DNA damage levels compared to non-smokers [33–37]; however there are several studies reporting that smoking has no effect on the urinary DNA damage biomarkers [38–42]. In this study, smoking had an effect on nuclear 8-oxodG levels in the non-cancer control patients but no differences were noted in the patient group between smokers and non-smokers.

Since age, gender and smoking influenced urinary DNA damage biomarker levels and smoking had influence on nuclear 8-oxodG levels, the DNA damage marker analyses were adjusted for statistical analysis accordingly. It is unlikely that the results are influenced by the diet as human studies using the same methodology as in this study have shown that urinary excretion of 8-oxoGua and 8-oxodG do not dependent on diet [19,43]. We also excluded subjects with serious acute infection, serious cardiac, metabolic or hepatic disease, forced expiratory volume in 1 s (FEV_1) ≤ 1.5 L and gout which are conditions known to affect the amount of oxidative stress and thus oxidative DNA damage. The known other diseases the study subjects had (cardiac disease, hypertension, chronic arrhythmia, diabetes, asthma, COPD, chronic bronchitis or rheumatic disease) or neoadjuvant chemotherapy before RT had no effect on any of the biomarker levels.

A significant increase in urinary 8-oxodG levels during the first week of RT ($p<0.001$) was recorded. There was also a tendency for urinary 8-oxoGua levels to increase during RT. The increase in urinary DNA damage biomarker levels may be attributed to enhanced oxidative stress caused by RT. Urinary excretion of 8-oxodG may also originate from the DNA of dead cells [44]. This finding is in agreement with the report of Bergman et al. [45], who observed a prompt increase in urinary 8-OHdG levels during and immediately after total body irradiation of eleven patients with hematological malignancies. Also Tagesson et al. [12] reported that urinary excretion of 8-OHdG increases after whole body irradiation. Interestingly, the only significant increase during RT did occur in urinary 8-oxodG, and not in 8-oxoGua, in the present study.

There was a clinically significant increase in nuclear 8-oxodG levels during and 3 months after RT. This is consistent with earlier observations of higher levels of lymphocyte 8-oxodG after RT [46]. A two-fold, albeit non-significant, increase in lymphocyte 8-oxodG/dG ratio has been reported after RT [47].

In this study, all DNA damage biomarkers tended to increase during adriamycin-containing CT; however, a statistically significant increase took place in nuclear 8-oxodG levels after the first CT cycle ($p=0.043$). It has been shown that epirubicin containing CT damages cellular DNA and produces the mutagenic modified base 8-oxodG [48]. Mei et al. [10] reported an increase in urinary 8-oxodG levels immediately after CT. The increase in urinary 8-oxodG levels after CT may be due to increased production of reactive oxygen species (ROS) caused by CT, but it could also be attributed to increased cell death and, hence, DNA turnover [10].

A previous study of ours and some other studies have suggested that there may be an association between urinary DNA damage biomarkers and response to treatment or overall survival, although these studies have been of too short duration to prove the point [7,14,15]. To our knowledge, the present study is the first one to report a six-year follow-up of the patients and the largest one to evaluate the association between adverse events and responses to treatment with three different DNA damage biomarkers. In contrast to previous studies, we found no significant associations between DNA damage biomarkers and adverse events during the treatments, responses to treatment or overall survival.

Conclusions

This study appears to be the largest one so far to investigate three different DNA oxidation biomarkers during RT and CT for

LC. The results suggest that oxidative DNA damage biomarkers may be associated with LC stage. This study confirms that both RT and CT increase the oxidative burden, and it demonstrates the importance of frequent, repetitive measurements when assessing DNA oxidation during cancer therapy. Larger studies are warranted to explore possible associations between oxidative DNA damage biomarkers and response to treatment and overall survival.

Acknowledgments

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Local and systemic oxidant/antioxidant status before and during lung cancer radiotherapy

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Abstract

To examine local and systemic oxidative status of lung cancer (LC) and oxidant effects of radiotherapy (RT), this study evaluated antioxidants and markers of oxidative and nitrosative stress in bronchoalveolar lavage (BAL) fluid and in the blood of 36 LC patients and 36 non-cancer controls at baseline and during and after RT for LC. LC patients had higher baseline serum urate, plasma nitrite and lower serum oxidized proteins than controls ($p = 0.016$, $p < 0.001$ and $p = 0.027$, respectively), but BAL fluid oxidative stress markers were similar. RT tended to raise some antioxidants, however, significant increases were seen in serum urate, conjugated dienes and TBARS ($p = 0.044$, $p = 0.034$ and $p = 0.004$, respectively) 3 months after RT. High urate at baseline may compensate against the oxidative stress caused by LC. RT shifts the oxidant/antioxidant balance towards lipid peroxidation, although the antioxidant defense mechanisms of the body appear to counteract the increased oxidative stress rather effectively.

Keywords: *Oxidative stress, BAL fluid, lung cancer, radiotherapy, antioxidants, nitrosative stress*

Abbreviations: *BAL, bronchoalveolar lavage; LC, lung cancer; RT, radiotherapy*

Introduction

Lung cancer (LC) is the leading cause of cancer-related deaths in the Western world [1]. LC is divided into two main groups: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Treatment is based on the staging of the cancer and on the performance status of the patient. Surgery is the main treatment for limited disease. Advanced NSCLC is mainly treated with radiotherapy (RT) and chemoradiotherapy [2–4].

Radiation generates primary radicals by transferring energy to certain cellular components, e.g. water [5]. Reactive oxygen species (ROS) are formed in this process and they mediate the anti-tumour effects of RT [6]. A delicate situation occurs at the tissue level when oxidative stress is a desired effect against malignant cells; yet the amount of oxidative stress should be kept in balance to prevent permanent damage to normal cells.

An increasing number of studies have been published where bronchoalveolar lavage (BAL) has been used as a window to assess the oxidative status of the lungs, e.g. in asthma, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and after

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exposure to ozone and diesel [7–12]. However, there are only a limited number of clinical studies involving BAL in LC patients. Melloni et al. [13] reported increased glutathione and reduced superoxide dismutase levels in BAL fluid of LC patients compared to non-cancer controls. The levels of vascular endothelial growth factor in BAL fluid are elevated in LC patients before and during radio-chemotherapy [14,15]. RT raises the concentration of interleukin-6 and TGF-β_1 in the BAL fluid of LC patients [16].

The respiratory tract lining fluid (RTLF) contains a variety of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase), small non-enzymatic antioxidants (vitamin C, E, A and glutathione) and other compounds like albumin, ceruloplasmin and transferrin. RTLF plays an important role in protecting the cells from external oxidants, e.g. tobacco smoke and air pollutants. The fluid obtained in connection with therapeutic or diagnostic BAL provides a sample of the cellular and non-cellular components from the site of the respiratory epithelium itself [7,11,17].

To explore the RTLF status and the systemic oxidative stress, we determined the levels of a number of antioxidants (ascorbic acid, vitamin E, alpha- and gamma-tocopherol, urate, thiols, glutathione), the total antioxidant capacity (TRAP) and several parameters of oxidative and nitrosative stress (proteins, oxidized proteins, TBARS, conjugated dienes, nitrite, nitrite + nitrate) in BAL fluid and blood of lung cancer patients and non-cancer controls. These markers were evaluated at baseline and during and after RT of lung cancer. We also examined whether these markers predict adverse events, response to treatment and overall survival of LC patients.

Material and methods

Study groups and procedures

Thirty-six histologically or cytologically confirmed LC patients and 36 non-cancer controls were enrolled at the Department of Respiratory Medicine. The inclusion criteria for both groups were: Karnofsky performance status of $\geq 70\%$ and no serious acute infection. The exclusion criteria were: serious cardiac, metabolic or hepatic disease, forced expiratory volume in 1 s (FEV_1) ≤ 1.5 l, regular allopurinol or acetylcystein medication or gout. The controls were recruited among the patients referred for bronchoscopy due to prolonged cough. The characteristics of the two groups are shown in Table I.

Neither the patients nor the controls had taken vitamin or herbal supplementation within 3 months prior to the study. Data on smoking, other diseases, medication and symptoms were collected on a standardized questionnaire modified from the ATBC (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study) study [18]. Pre-treatment evaluation of the LC patients consisted of a physical examination, chest radiography, bronchoscopy, chest and upper abdominal computerized tomography, urinanalysis, full blood count and serum chemistry. Abdominal sonography and bone scintigraphy were performed as needed. Smokers were defined either as current smokers or as smokers who had stopped smoking less than 6 months previously, ex-smokers as subjects who had stopped smoking more than 6 months ago and non-smokers had never smoked. The lifetime cigarette consumption was expressed as pack years (cigarette packs smoked/day × years smoked).

Table I. Characteristics of lung cancer patients and controls. Values are means (SD; range) or numbers (percentages).

	Lung cancer patients ($n=36$)	Controls ($n=36$)
Age (years)	66.9 (9.1; 47–82)	50.8 (9.9; 18–75)
Males	29 (80.6)	16 (44.4)
BMI (kg/m^2)	23.7 (3.2; 17.0–31.8)	26.3 (5.2; 18.5–39.2)
FEV1 (% of predicted)[a]	74.1 (11.8; 58.0–94.0)	89.4 (11.5; 65–108)
Smoking		
Non-smokers	2 (5.6)	18 (50.0)
Ex-smokers	10 (27.8)	6 (16.7)
Smokers	24 (66.7)	12 (33.3)
Pack-years of smokers or ex-smokers	35.3 (23.0; 5.0–96)	28.5 (17.7; 7.5–68)
Histology		
Squamous cell cancer	33 (91.7)	
Adenocarcinoma	1 (2.8)	
Small cell cancer	2 (5.6)	
Stage of lung cancer		
IB	1 (2.8)	
IIIA	6 (16.7)	
IIIB	12 (33.3)	
IV	17 (47.2)	
Karnofsky performance status		
70	4 (11.1)	0 (0)
80	8 (22.2)	0 (0)
90	15 (41.7)	3 (8.3)
100	9 (25.0)	33 (91.7)

[a] $n=13$ and $n=20$.

This study was conducted according to the guidelines of the Declaration of Helsinki. Written informed consent was obtained from each participant to a study protocol approved by the ethics committee of the Tampere University Hospital.

Bronchoscopy and BAL samples

All patients and controls underwent bronchoscopy and BAL as a diagnostic procedure. Patients receiving RT underwent a second bronchoscopy and BAL 2 weeks after start of RT (at 18–22 Gray). During RT BAL was performed on the irradiated lung and on the same segment as at diagnosis, if possible. All bronchoscopies were carried out by the same experienced bronchoscopist (SS) according to

standardized methods [19,20]. During bronchoscopy the subjects were awake and breathed spontaneously. All subjects received pre-medication with intramuscular atropine sulphate (0.7 mg) and topical lidocain anaesthesia to the nasal airways and posterior pharyngeal wall. A flexible bronchoscope was wedged into the segmental or subsegmental level of the left upper lobe or right middle lobe. Five-times 20 ml of sterile saline (37°C) mixed with Addiphos buffer (4% v/v, Fresenius Kabi, Uppsala, Sweden) was instilled through the bronchoscope. The fluid was immediately recovered by gentle suction after each instillation [19,20]. Due to ethical reasons, BAL was performed only on one side of the lungs at each bronchoscopy.

The BAL fluid samples were immediately protected from light and put on ice. The samples were centrifuged at 500 rpm for 15 min at 4°C and stored at −70°C until analysis. The samples for ascorbic acid analysis were mixed (1:10) with 5% metaphoshoric acid and isoascorbate. Cells were stained with the May-Grűnwald Giemsa (MGG) and Papanicolau stains and fixed with 50% ethanol.

Blood samples

All patients underwent laboratory testing at baseline. In addition to oxidative stress markers the tests included: full blood count, alkaline phosphatase, alanine transferase, aspartate transferase, creatinine, serum C- reactive protein, sodium and potassium.

The blood samples for oxidative stress markers were taken as follows: Peripheral venous blood samples were collected using a Venoject blood collection system (Terumo, Leuven, Belgium). Two tubes (10 ml each) of blood were obtained, one for serum preparation and one for plasma analysis. The serum samples were collected into sterile tubes and the samples intended for plasma analysis were collected to cooled, sterile tubes containing ethylenediaminetetraacetic acid (EDTA). The samples were protected from light and centrifuged at 2800 g for 10 min after which the plasma specimen for ascorbic acid analysis was mixed (1:10) with 5% metaphoshoric acid and isoascorbate. The samples were frozen immediately and stored at −70°C until analysis.

Blood samples were collected of all patients at baseline. The second set of blood samples coincided with second bronchoscopy during RT at 18–22 Gray and the third 3 months after start of RT.

Radiotherapy

Twenty out of 36 patients received RT as a treatment. Sixteen patients received RT based on three-dimensional CT-based treatment planning by Cad Plan (version 6.23, Varian Medical Finland, Varian Medical Systems Inc). Fractions (fr), usually of 2 Gray (Gy), were given five times a week. Four patients received palliative RT with two anterior-posterior fields 3 Gy/fr five times a week. The planning target volume (PTV) contained the primary tumour and adjacent lymph nodes with adequate margins. RT with 18 MV photons was applied by a linear accelerator (Varian Clinac 2100 C/D, Varian Medical Systems Inc., Palo Alto, CA) at the Radiotherapy Unit of the Tampere University Hospital. The mean radiation dose delivered was 46.9 Gray (range 30.0–60.0 Gy).

Analyses

BAL fluid and plasma thiols. Thiols in 400 μl of BAL fluid or 100 μl of plasma were determined with Ellman's reagent as described [21]. The coefficient of variation between the series was 6.6%.

BAL fluid and plasma ascorbic acid. BAL and plasma ascorbic acid were determined in metaphosphoric acid (5%) stabilized samples by HPLC with electrochemical detection [22]. The coefficient of variation between the series was 5.5%.

BAL fluid and serum urate. BAL and serum urate were analysed by an enzymatic method (Thermo Fisher Scientific Oy, Vantaa, Finland) using uricase, peroxidase and ascorbate oxidase. The coefficient of variation between the series varied from 1.4–2.3% and the accuracy (bias) was +1.4%, as assessed by an external quality programme (Labquality Ltd, Helsinki, Finland).

BAL fluid and plasma nitrite and Nox. Concentrations of nitrite and Nox (nitrite + nitrate) were measured by the ozone-chemiluminescence method [23,24]. The detection limit for nitrite was 0.2 μmol/L and 1.5 μmol/L for Nox.

Plasma tocopherols. Alpha- and gamma-tocopherols were analysed as described [25,26]. The coefficient of variation between the series was 5.2%. The results are expressed as mg/L.

Serum vitamin E. Serum vitamin E was measured by chromatographic methods as described [27].

Plasma glutathione. Total glutathione in the plasma (GSSG + GSH) was determined by an enzymatic recycling reaction [28].

Serum protein oxidation, diene conjugation, TBARS, TRAP. Protein carbonyl determinations were carried out as described [29]. Two different methods (diene conjugation, thiobarbituric acid reactive substances, TBARS) were used to estimate serum levels of lipid peroxides. Conjugated dienes were analysed spectrophotometrically at 234 nm [30]. For the analyses of TBA-reactive substances the absorbances were measured at 535 nm [31]. The antioxidant potential

of the samples (total peroxyl radical trapping antioxidant potential, TRAP) was estimated by their potency to resist 2,2′-azobis(2-amidinopropane) (ABAP) induced peroxidation [32].

The concentrations of BAL and plasma thiols, BAL and plasma ascorbic acid, BAL and serum urate, BAL and plasma nitrite and Nox, serum vitamin E, plasma glutathione, serum protein oxidation, serum diene conjugation, serum TBARS and serum TRAP are expressed as μmol/L.

Serum proteins. Serum proteins were analysed by a colorimetric end-point measurement following the Biuret method [33,34]. The intra-assay coefficient of variation was 0.88% and the inter-assay 1.80%. The results are expressed as g/L.

BAL fluid cell counts. The total cell and differential cell counts were determined by microscopy of fixed BAL fluid samples. BAL fluid albumin was measured by nephelometry and proteins by colorimetry.

Evaluation of adverse events and response to treatment

During and after the RT, all adverse events were evaluated according to the criteria of the World Health Organization (WHO) and Lent Soma Table [35,36]. The responses to treatment were evaluated according to the criteria of WHO [35].

Statistics

The systemic and local oxidative stress markers were the primary variables of the study. The results are given as means or geometric means with 95% confidence intervals. The distributions of some oxidative stress markers were skewed to the right and were thus logarithmically (ln) transformed before analysis. The *t*-test for independent samples was used to compare patients and controls. Since there were differences in demographic variables, the adjusted comparison (ANCOVA) includes age and FEV_1 (%) as continuous covariates and gender and smoking (smoker vs non-smoker) as random factors. The interaction terms between the explaining factors are not included in the multivariable models. The group comparisons are presented as mean difference or as a ratio (patients/controls) with 95% confidence intervals. The within-patient changes in oxidative stress markers were analysed with the *t*-test for paired samples and ANOVA for repeated measures. Spearman's and Pearson's correlations were calculated to express the associations between oxidative stress markers, age and pack-years of smoking. The *t*-test for independent samples was used to compare different demographic groups (grouping by smoking, gender, other diseases, stage of disease). The oxidative stress markers at baseline were divided into two groups ($<$ median and $>$ median) and the Kaplan-Meier method was applied to plot the survival curves. The log-rank test was used to compare the survival distributions. The survival times are given as medians with 95% confidence interval. The Mann-Whitney's U-test was used for variables with a non-normal distribution and the χ^2-test was used for categorical variables. *p*-values of less than 0.05 were considered statistically significant. The statistical analyses were performed with the SPSS (release 15.0) software (SPSS inc. Chicago, IL).

Results

Patients and controls

Thirty-six LC patients entered to the study and 36 non-cancer patients served as controls (Table I). The diagnosis of LC was confirmed histologically in 27 of the patients and for the remaining nine patients the diagnosis was based on class V BAL cytology and imaging results. Twenty of the LC patients received radiotherapy, 15 of whom underwent a second bronchoscopy during RT. Two patients were treated only by surgery after the diagnosis, six received various chemotherapy regimens and nine received symptomatic treatment. Of these 16 patients only baseline samples were obtained.

The final diagnosis for the controls showed that none of them had cancer, three (8%) had asthma, two (6%) chronic bronchitis and mild chronic obstructive pulmonary disease (COPD), one (3%) COPD and one (3%) control patient was operated on for a benign papilloma. All other control patients (80%) were diagnosed as having idiopathic prolonged cough and had no further treatment.

The patient and control groups differed with respect to some major main characteristics (Table I) and thus the results were adjusted for age, FEV_1, gender and smoking. There were no significant associations between any of the other diseases (cardiac disease, diabetes, chronic arrhythmia or rheumatic disease) with regard to oxidative stress marker levels.

The mean instilled BAL fluid volume was 111 ml for LC patients and 101 ml for controls ($p = 0.033$); the recovery volumes were 55.3 ml and 67.3 ml ($p = 0.005$), respectively, at first bronchoscopy. There were no statistically significant differences in the total cell counts of BAL fluid samples between the groups, but LC patients had a higher neutrophil count than controls (3% vs 1%; $p = 0.002$). There were no significant complications during or after the bronchoscopies.

Systemic oxidative stress markers at baseline

The LC patients had significantly higher levels of urate (241 μmol/L, 95% CI 210–272 μmol/L vs 116 μmol/L, 95% CI 91–141 μmol/L, $p < 0.001$) and nitrite (0.591 μmol/L, 95% CI 0.548–0.634 μmol/L vs

0.181 μmol/L, 95% CI 0.136–0.225 μmol/L, $p < 0.001$) than the controls at baseline. The LC patients had significantly lower levels of thiols (303 μmol/L, 95% CI 283–322 μmol/L vs 364 μmol/L, 95% CI 338–390 μmol/L, $p < 0.001$) and oxidized proteins (2.85 μmol/L, 95% CI 2.46–3.23 μmol/L vs 4.30 μmol/L, 95% CI 4.06–4.55 μmol/L, $p < 0.001$) than the controls at baseline.

After adjustment for age, FEV_1, gender and smoking, significant differences persisted for urate, nitrite and oxidized proteins ($p = 0.016$, $p < 0.001$ and $p = 0.027$, respectively), whereas significance was lost for thiol levels ($p = 0.651$) (Figure 1).

There were no significant differences in ascorbate (73.3 vs 78.3 μmol/L, $p = 0.529$), glutathione (geometric mean 0.79 vs 0.89 μmol/L, $p = 0.636$), vitamin E (26.7 vs 29.8 μmol/L, $p = 0.264$), conjugated dienes (51.8 vs 54.8 μmol/L, $p = 0.420$), TRAP (947 vs 963 μmol/L, $p = 0.751$), TBARS (4.64 vs 4.60 μmol/L, $p = 0.855$), Nox (28.6 vs 26.5 μmol/L, $p = 0.482$) or proteins (69.0 vs 72.0 g/L, $p = 0.054$) between the two groups at baseline (Table II).

Local oxidative stress markers in BAL fluid at baseline

Cancer patients had significantly higher levels of urate (geometric mean 11.18 μmol/L, 95% CI 7.76–16.11 μmol/L vs 6.45 μmol/L, 95% CI 4.77–8.72 μmol/L, $p = 0.019$) and thiols (geometric mean 4.40 μmol/L, 95% CI 2.69–7.21 μmol/L vs 1.48, 95% CI 1.12–1.95 μmol/L, $p < 0.001$) and lower levels of Nox (geometric mean 0.87 μmol/L, 95% CI 0.77–0.97 μmol/L vs 1.34 μmol/L, 95% CI 1.12–1.60 μmol/L, $p < 0.001$) than controls at baseline. However, after adjustment for age, FEV_1, gender and smoking, significance was lost for the difference of

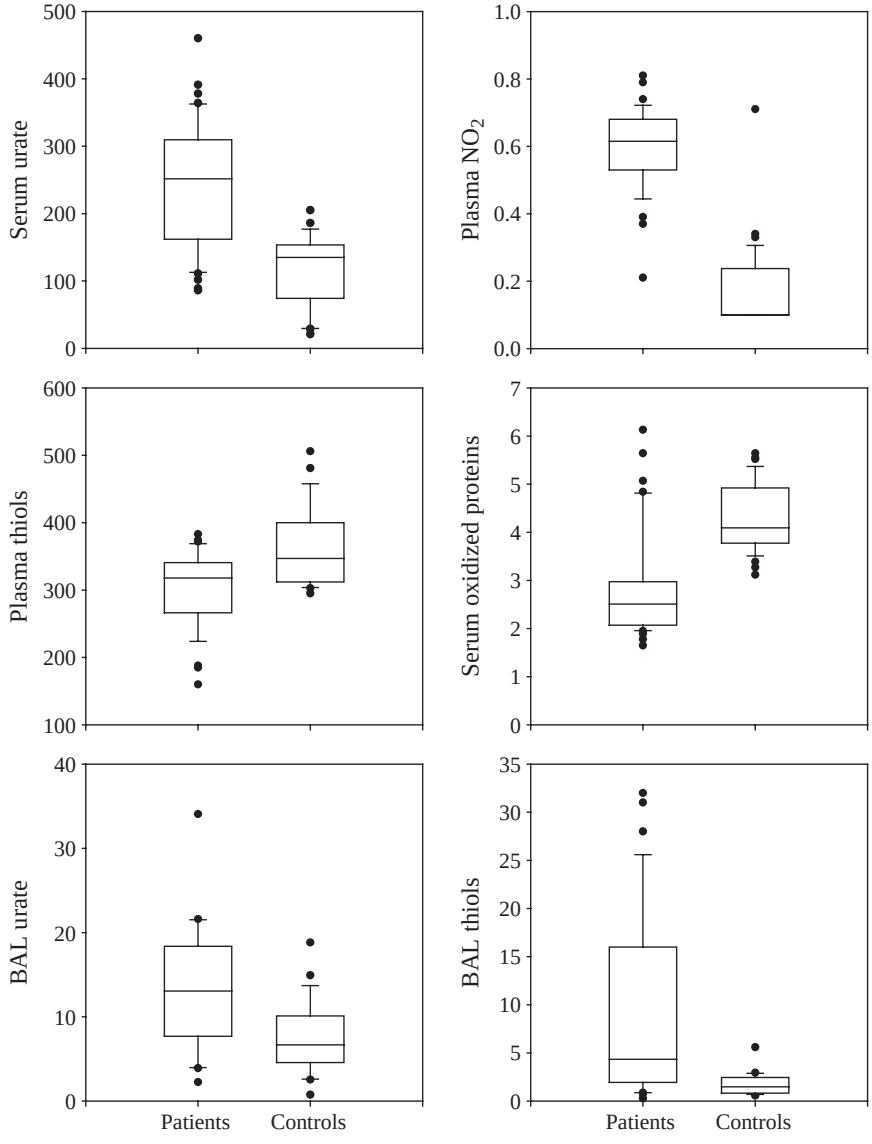

Figure 1. Box-plot figures of systemic and local oxidative stress markers (μmol/L) at baseline in patients and controls. The boxes indicate the lower and upper quartiles and the central line us the median. Whiskers above and below the box indicate the 90th and 10th percentile. Outliers are given as filled circles (●).

Table II. Antioxidant variables at baseline as mean (95% CI) or geometric mean (95% CI) for patients and controls. The crude and adjusted group comparison is given as mean difference (95% CI) or as ratio patients/controls (95% CI). Measuring units: mg/L for plasma tocopherols, g/L for serum protein, μmol/L for other variables.

			Patients vs Controls			
			Crude[a]		Adjusted[b]	
	Patients	Controls	M (95% CI)	*p*	M (95% CI)	*p*
Plasma						
Alphatocopherol	7.39 (6.01–8.76)	NA				
Gammatocopherol	0.80 (0.55–1.04)	NA				
Ascorbate	73.3 (61.9–84.8)	78.3 (67.0–89.7)	−5.0 (−20.8 to 10.8)	0.529	6.8 (−37.1 to 50.7)	0.753
Thiols	303 (283–322)	364 (338–390)	−61 (−92 to −31)	<0.001	20.6 (−73.3 to 114.6)	0.651
Nitrite	0.591 (0.548–0.634)	0.181 (0.136–0.225)	0.41 (0.35 to 0.47)	<0.001	0.32 (0.18 to 0.46)	<0.001
Nox	28.6 (23.8–33.4)	26.5 (22.8–30.2)	2.1 (−3.9 to 8.1)	0.482	7.0 (−10.2 to 24.2)	0.410
Serum						
Vitamin E	26.7 (22.1–31.3)	29.8 (26.3–33.2)	−3.0 (−8.5 to 2.4)	0.264	−14.4 (−33.1 to 4.3)	0.121
Urate	241 (210–272)	116 (91–141)	125 (86 to 164)	<0.001	173 (37 to 310)	0.016
Proteins	69.0 (66.1–71.9)	72.0 (70.3–73.7)	−3.00 (−6.05 to 0.05)	0.054	−2.25 (−7.48 to 2.98)	0.377
Oxid. proteins	2.85 (2.46–3.23)	4.30 (4.06–4.55)	−1.46 (−1.91 to −1.01)	<0.001	−1.12 (−2.10 to −0.14)	0.027
Conjugated dienes	51.8 (46.7–57.0)	54.8 (49.5–60.1)	−2.9 (−10.2 to 4.3)	0.420	−6.2 (−24.4 to 11.9)	0.486
TRAP	947 (872–1022)	963 (895–1030)	−16 (−115 to 83)	0.751	−89 (−299 to 120)	0.388
TBARS	4.64 (4.33–4.95)	4.60 (4.26–4.93)	0.04 (−0.41 to 0.49)	0.855	−0.78 (−1.95 to 0.38)	0.180
BAL nitrite	0.182 (0.156–0.209)	0.206 (0.183–0.229)	−0.024 (−0.06 to 0.01)	0.176	−0.013 (−0.07 to 0.05)	0.659
			Ratio (95% CI)	*p*	Ratio (95% CI)	*p*
Plasma glutathione[c]	0.79 (0.51–1.22)	0.89 (0.65–1.23)	0.89 (0.53 to 1.48)	0.636	0.72 (0.15 to 3.54)	0.667
BAL thiols[c]	4.40 (2.69–7.21)	1.48 (1.12–1.95)	2.98 (1.71 to 5.19)	<0.001	2.00 (0.39 to 10.17)	0.378
BAL ascorbate[c]	0.53 (0.29–0.94)	0.79 (0.47–1.34)	0.67 (0.31 to 1.43)	0.293	1.37 (0.16 to 11.98)	0.769
BAL urate[c]	11.18 (7.76–16.11)	6.45 (4.77–8.72)	1.73 (1.10 to 2.73)	0.019	2.43 (0.92 to 6.43)	0.069
BAL Nox[c]	0.87 (0.77–0.97)	1.34 (1.12–1.60)	0.65 (0.53 to 0.79)	<0.001	1.01 (0.62 to 1.63)	0.979

[a]Independent samples *t*-test, [b]The adjusted comparison (ANCOVA) includes age and FEV_1 (%) as continuous covariates and gender and smoking (smoker vs non-smoker) as random factors, [c]Geometric mean (95% CI); the values were logarithmically (ln) transformed before analysis.

these markers between the patients and controls ($p = 0.069$, $p = 0.378$ and $p = 0.979$ for BAL urate, thiols or Nox). Nor were there significant differences in the levels of BAL ascorbate or nitrite between LC patients and controls (0.53 μmol/L vs 0.79 μmol/L, $p = 0.293$ and 0.18 μmol/L vs 0.21 μmol/L, $p = 0.176$, respectively). The concentrations of TRAP in BAL fluid samples were undetectable (Table II, Figure 1).

Systemic oxidative stress markers during and after radiotherapy

Although not significant, there was a trend towards an increase in alpha-tocopherol (6.43 vs 8.86 mg/L, $p = 0.056$), urate (220 vs 255 μmol/L, $p = 0.064$) and oxidized proteins (2.64 vs 3.29 μmol/L, $p = 0.088$) after 2 weeks of RT. There was also a slight, but non-significant, reduction in glutathione, thiols, TRAP, TBARS, proteins, Nox and vitamin E. Non-significant increases were noted in ascorbate, gamma-tocopherol and conjugated dienes during RT. The levels of nitrite remained unchanged during RT (Figure 2).

Three months after RT, the levels of urate (214 vs 280 μmol/L, $p = 0.044$), conjugated dienes (53.4 vs 64.6 μmol/L, $p = 0.034$) and TBARS (4.58 vs 5.64 μmol/L, $p = 0.004$) had risen significantly. A nearly significant increase was noted in alpha-tocopherol ($p = 0.055$). The changes in the levels of other antioxidants and markers of oxidative and nitrosative stress 3 months after RT were non-significant.

Local oxidative stress markers in BAL fluid during radiotherapy

There was an almost significant increase in urate (geometric mean 7.30 μmol/L vs 13.91 μmol/L, $p = 0.083$) and thiols (geometric mean 3.34 μmol/L vs 4.85 μmol/L, $p = 0.069$) after 20 Gy of RT compared to baseline. No notable changes took place for BAL ascorbate, nitrite or Nox during RT.

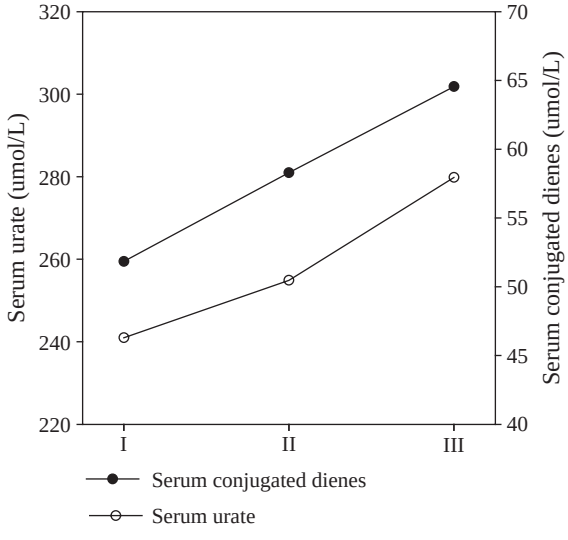

Figure 2. The mean levels of serum conjugated dienes and serum urate (μmol/L) at baseline (I), after 2 weeks of radiotherapy (II) and 3 months after radiotherapy (III).

Oxidative stress markers and lung cancer stage

Higher BAL thiol (6.29 μmol/L vs 2.54 μmol/L, $p = 0.063$) and BAL nitrite (0.21 μmol/L vs 0.15 μmol/L, $p = 0.021$) levels and lower serum oxidized proteins (2.36 μmol/L vs 3.39 μmol/L, $p = 0.005$) and serum TBARS (4.37 μmol/L vs 4.94 μmol/L, $p = 0.067$) levels were recorded in stages I–III compared to stage IV disease.

Association between oxidative stress markers, adverse events and response to RT

Overall toxicity during RT was mild and none of the patients experienced any serious adverse events. Thirteen (65%) patients experienced gr I/II adverse events during RT: esophagitis ($n = 9$), cough ($n = 4$), fatigue ($n = 4$) and fever ($n = 4$). Five patients (25%) developed symptomatic radiation pneumonitis. The occurrence of adverse events during RT was not significantly associated with any baseline oxidative stress marker levels.

Twenty-five per cent of the LC patients achieved complete and 56.3% partial response to RT, whereas 6.3% had no change and 12.5% had progressive disease. The response to treatment was not significantly associated with any baseline oxidative stress marker levels.

Association between oxidative stress markers and overall survival

The planned follow-up time of the patient group was 72 months. None of the patients was lost to follow-up. At the end of the study, one patient (3%) was alive and 35 (97%) had died. The median survival time of the patients was 9.9 months (95% CI 5.4–14.4 months). The survival time tended to be longer when the patient's baseline BAL thiol levels were above the median concentration of 4.34 μmol/L (9.9 months vs 6.1. months, $p = 0.051$) (Figure 3).

Associations between oxidative stress markers and other demographics

There were no significant differences in oxidative stress markers between smokers and non-smokers among the patients. However, among the controls, smokers had significantly higher levels of BAL thiol (2.73 vs 1.47 μmol/L, $p = 0.022$), serum urate (177 vs 106 μmol/L, $p = 0.035$), serum TBARS (5.06 vs 4.36 μmol/L, $p = 0.042$) and serum nitrite (0.25 vs 0.14 μmol/L, $p = 0.017$) and lower levels of plasma ascorbate (59 vs 88 μmol/L, $p = 0.011$). Among the controls, the number of pack years smoked correlated

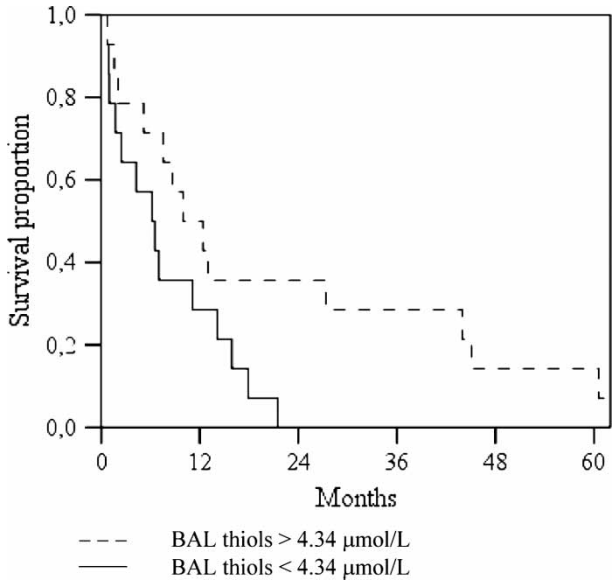

Figure 3. Kaplan-Meier survival curves for patients with baseline BAL thiols >4.34 μmol/L ($n=14$) vs patients with BAL thiols <4.34 μmol/L ($n=14$). Log-rank test $p=0.051$.

negatively with plasma ascorbic acid ($R=-0.452$, $p=0.014$).

There was a trend favouring an association between weight loss prior to diagnosis and overall survival, but this finding lacked statistical significance ($p=0.769$). If patients had lost weight less than 2 kg prior to lung cancer diagnosis, the median overall survival was 12.4 months (95% CI 9.2–15.6 months); if weight loss was 3–5 kg median survival was 8.7 months (95% CI 4.0–13.4 months) and if weight loss was more than 5 kg, median survival was 3.8 months (95% CI 0–11.5 months).

There were significant positive correlations between BAL ascorbic acid and plasma ascorbic acid ($R=0.342$, $p=0.005$), BAL thiols and both serum urate ($R=0.481$, $p=0.001$) and serum alpha-tocopherol ($R=0.714$, $p=0.031$) and a negative correlation between plasma ascorbic acid and both plasma and BAL thiols at baseline ($R=-0.446$, $p=0.008$ and $R=-0.377$, $p=0.052$, respectively) (Figure 4).

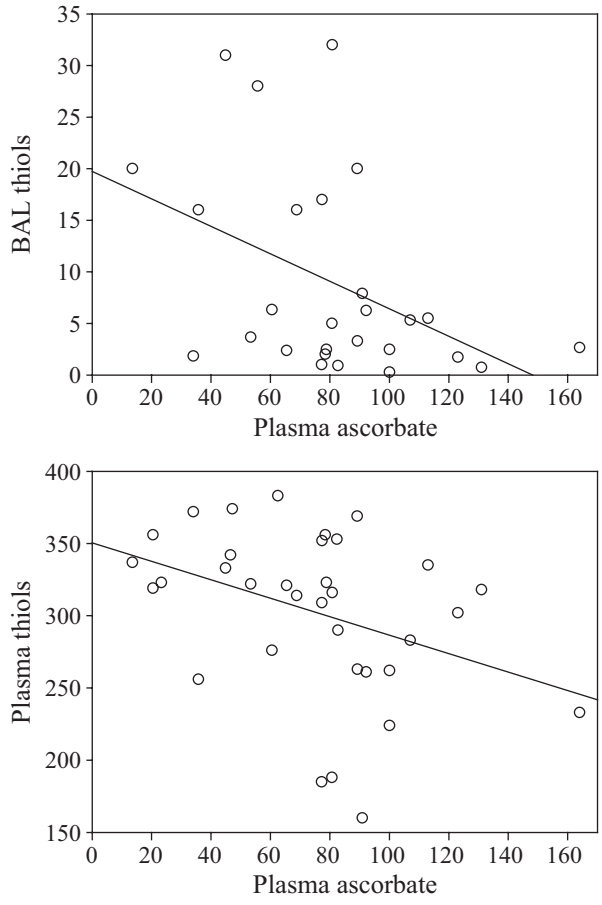

Figure 4. Scatterplots and regression lines for associations between patients plasma ascorbate (μmol/L) vs BAL thiols (μmol/L), Spearman $R=-0.377$, $p=0.052$ and plasma thiols (μmol/L), $R=-0.446$, $p=0.008$. One outlier with plasma ascorbate 60.5 vs BAL thiols 11.9 is outside the figure.

Discussion

This study shows that lung cancer is associated with enhanced circulating concentrations of urate and nitrite ($p<0.001$ for both), which is in accordance with previous findings [37,38]. Urate, which is the end product of purine metabolism, is one of the major antioxidants in human plasma and it may play an essential role in protecting cells against free-radical induced damage. Thus, elevated levels of urate may signify a compensatory mechanism to oxidative stress [39]. It is also shown that both cancer and RT are associated with increased oxidative damage to DNA [40,41] and thus hyperuricemia might be also partly due to increased purine metabolism through the effects of xanthine oxidase as a consequence of RNA-DNA breakdown [42].

Nitric oxide (·NO) is involved in many physiological processes and has an extremely short half-life [43]. However, the blood ·NO level does not necessarily reflect the ·NO status of the tissues. In plasma and other physiological fluids ·NO is oxidized to nitrite, whereas in the whole blood ·NO and nitrite are oxidized to nitrate [44]. Increased production of nitric oxide may protect the cells from oxidative stress and this might explain the elevated levels of nitrite among LC patients compared to controls ($p<0.001$, Table II) [45]. On the other hand, production of a potent oxidant and cytotoxic molecule, peroxynitrite in the reaction of ·NO with the superoxide anion may lead to increased biochemical reactivity and a wide range of damaging effects.

Increased free radical production, decreased activity of the antioxidant defense mechanisms or enhanced consumption of antioxidants lead to oxidative stress [46]. The total antioxidant capacity has been used to measure oxidative stress in whole body

[45,47]. In a previous study we reported significantly lower total peroxyl radical trapping antioxidant potential (TRAP) levels in LC patients compared to healthy controls [48]. Although also the present study noted decreased levels of TRAP in LC patients compared to controls (Table II), the difference between the two groups was not significant. This is in agreement with a previous study [49]. Yet, the known components of TRAP, besides urate, tended to be lower in the LC group than controls: protein SH-groups (thiols) ($p < 0.001$, $p = 0.651$ after adjustment for age, FEV_1, gender and smoking), ascorbic acid ($p = 0.53$, $p = 0.753$ after adjustment) and vitamin E ($p = 0.26$, $p = 0.121$ after adjustment). We also recorded a significant positive correlation between plasma TRAP and serum urate, which corroborates a previous similar observation [50].

The antioxidant levels in BAL fluid of lung cancer patients have not been the subject of very much research. After adjustment for age, FEV_1, gender and smoking, no significant differences were noted between LC patients and non-cancer controls in BAL fluid oxidative stress markers, although LC patients tended to have higher levels of BAL urate ($p = 0.069$). As bronchoscopy was performed on the control patients because of prolonged cough, it is possible that they had altered mucus secretion due to hypertrophy in mucus secreting glandula caused by chronic, hyperplastic bronchitis appearing as prolonged cough. This may partly explain why no differences were seen in BAL oxidative stress markers between LC patients and non-cancer controls. The levels of BAL ascorbate seen in this study are in conformity with previous studies [7,12,51]. The levels of BAL urate are higher than reported in some previous studies [7,12,51]; however, in alignment with the levels obtained of lung transplant recipients [52]. The method we used to analyse BAL and plasma thiols measures all small molecular weight as well as protein thiols, which explains the higher levels compared to studies reporting only glutathione/reduced glutathione levels [12,13].

We observed a tendency of BAL urate ($p = 0.083$) and thiols ($p = 0.069$) to rise during RT, a finding not reported previously. Although not significant, the levels of plasma alpha-tocopherol ($p = 0.056$), serum urate ($p = 0.064$) and serum oxidized proteins ($p = 0.088$) tended to increase after 2 weeks of RT. The elevated levels of urate both locally and systemically during RT might be attributed to enhanced cell necrosis following RT; it is known that urate is released from dying cells [46]. This increase in urate during RT is in accordance with previous findings [48]. It has been shown that RT also produces thiol radicals, which can react with ascorbic acid or ·NO [46]. The elevated levels of BAL urate might also be attributed to the movement of urate onto the lung surface to protect against oxidative stress caused by RT [12]. The elevation of oxidized proteins levels during RT might be a causal factor in an early event of oxidative endothelial cell damage and also a marker of enhanced RT-related oxidative stress [53]. Aside from urate, the changes in BAL fluid antioxidant levels during RT seem to be independent of systemic changes in these markers.

Lipid peroxidation might be one of the main causes of damage during RT and previous studies have reported increased lipid peroxidation marker levels during RT [54,55]. RT is also known to cause oxidation of membrane protein SH- groups, which may explain the present finding of elevated levels of serum oxidized proteins and BAL thiols during RT [56].

Thiobarbituric-acid-reactive substances (TBARS) are considered to be markers of lipid peroxidation in tissues and the plasma, although rather unspecific [46,57–59]. During the early phase of lipid peroxidation the double bonds of polyunsaturated fatty acids (PUFAs) are rearranged and conjugated dienes are formed. Conjugated dienes have been widely studied as an index of lipid peroxidation and are less sensitive to the compensatory antioxidant mechanisms than the other lipid peroxidation markers [59,60]. The significantly elevated levels of serum conjugated dienes ($p = 0.034$) and TBARS ($p = 0.004$) 3 months after RT imply that the main antioxidants scavenger systems are consumed during RT, leading to enhanced lipid peroxidation after RT [45]. Previous studies have shown that the onset of lipid peroxidation caused by RT may be delayed [61]. In the present study lower baseline serum TBARS levels were measured in limited disease (stages I/II/III) compared to extensive disease (stage IV, $p = 0.067$), which is supported by a previous study reporting reduced plasma malondialdehyde concentration with decreasing tumour size [62].

To our knowledge, this is the largest study so far to evaluate different antioxidants and parameters of oxidative and nitrosative stress in lung cancer patients and during RT for lung cancer. No previous clinical follow-up studies have been performed that have recorded oxidative stress markers in relation to response to RT and overall survival of LC patients. We found no associations between any of the oxidative stress marker baseline levels and adverse events during or after RT or response to treatment. However, a borderline significant association was recorded between higher BAL thiol levels and longer overall survival. A recent study observed that head and neck carcinoma patients who have a higher than median concentration of glutathione in plasma survive longer [63]. Measuring also the ratio of oxidized glutathione to reduced glutathione in this study would have added beneficial information to the hypothesis that increased glutathione is associated with overall survival; this is one limitation of our finding. We also noted

that the LC stage was associated with baseline BAL thiol levels: higher local thiol levels were seen in stage I–III LC compared to stage IV LC ($p = 0.063$). Although these associations are of borderline statistical significance, it is also possible that the longer survival of some of the patients in this study is related to limited stage of the disease. Obviously, larger studies are needed to discriminate cause and effect in this respect.

Conclusions

This study supports strongly the hypothesis that LC is associated with increased oxidative stress. The findings suggest that antioxidant responses may serve as a protective mechanism against production of ROS during RT. The cellular damage caused by RT may also result in release of intracellular antioxidative substances. This study indicates that oxidative stress caused by RT may be counterbalanced by local lung antioxidant systems; however, notable lipid peroxidation does occur after RT. None of the examined oxidative stress markers prognosticated adverse events during RT or the response to treatment. However, the results imply that BAL thiols may be associated with overall survival of LC patients.

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Preliminary results of this study have been presented as an oral presentation at the 10th Biennial meeting of the SFRRI in Kyoto, Japan (2000) and as a poster presentation at the Annual Meeting of the SFRR- Europe in Rome, Italy (2001).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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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.

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, nitrite+nitrate; 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 gammatocopherol, 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, gout, 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.

Table 1
Patient characteristics.

		Lung cancer patients	
		$n=16$	
Age (years)		65.3	(8.7; 50–78)
Males		14	(87.5)
BMI (kg/m^2)		25.8	(5.7; 19.8–43.6)
FEV1 (% of predicted)		65.8	(14.7; 41–85)
Smoking	Ex-smokers	6	(37.5)
	Current smokers	10	(62.5)
	Pack-years	44.1	(24.2; 12.5–100)
Stage of lung cancer	III	6	(37.5)
	IV	10	(62.5)
Karnofsky performance status	70	2	(12.5)
	80	3	(18.8)
	90	10	(62.5)
	100	1	(6.3)

Values are means (SD; range) or absolute values (percentages).

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 isoascorbate. 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

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/m^2, vincristine 1.5 mg/m^2 (maximum dose 2 mg each time) and cyclophosphamide 750 mg/m^2. 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-HT$_3$ 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 of 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 CT cycle starting on day 22). The planned number of chemotherapy cycles was six corresponding to 4 1/2 months duration of treatment.

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 total tumor mass of 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 (VCl_3) 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 VCl_3 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,

Germany), guanidine hydrochloride from Fluka (Buchs, Switzerland) and TCA from Riedel-de Haen (Seelze, Germany). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was purchased from Bio-Orbit Ltd. (Turku, Finland) and 2,2′-azobis(2-amidinopropane)HCl (ABAP) from Polysciences Inc. (Warrington, PA, 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 (2:1 vol/vol), 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 divalent copper reacts 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 autoreduction 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 ranks 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

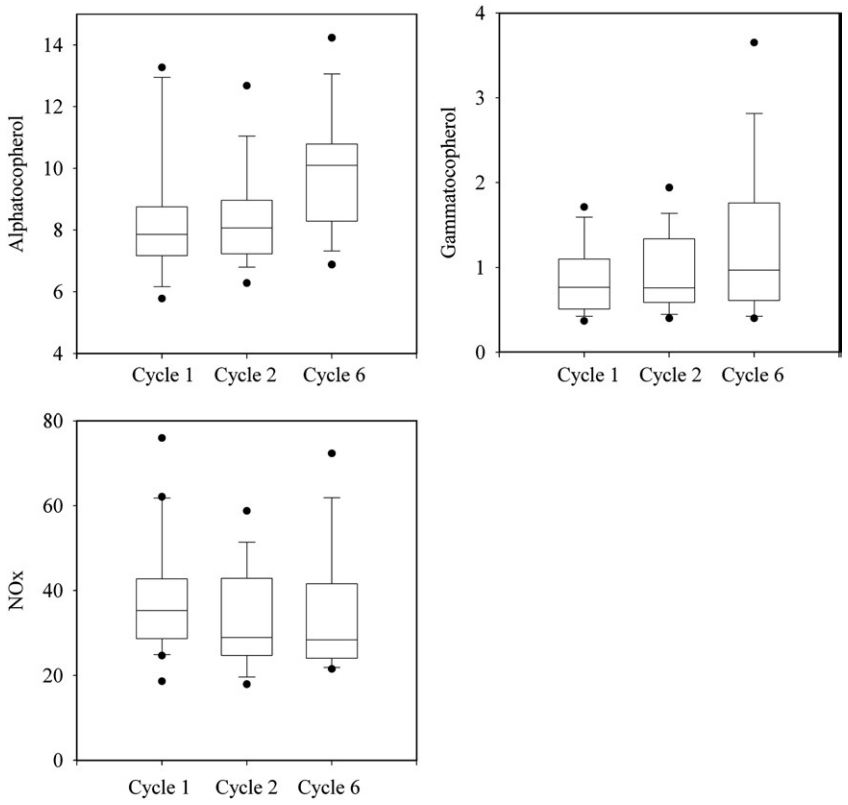

Fig. 1. Box-plot figures for alphatocopherol (mg/L, $p = 0.045$), gammatocopherol (mg/L, $p = 0.041$) and NOx (μmol/L, $p = 0.020$) at baseline during cycles 1 (before CT infusion), 2 and 6. The boxes indicate the lower and upper quartiles and the central line is the median. The "whiskers" above and below the box indicate the 90th and 10th percentiles. Outliers are given as filled circles (●).

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.

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

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.
[a] 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 ($p=0.045$ and $p=0.041$, 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 and −65 μmol/L for urate, −5.7, −2.3 and −4.3 μmol/L for ascorbic acid, −3.0, −2.0 and −5.0 g/L for proteins, −0.53, −0.42 and −0.14 μmol/L for TBARS and −49, −97 and −49 μmol/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 ($p=0.002$, $p=0.038$, 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 and −29 μmol/L for urate, −49, −49 and −49 μmol/L for TRAP and −5.0, −1.0 and −4.5 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 and −8.5 μmol/L for urate, −0.6, −0.2 and −0.8 mg/L for alphatocopherol, −4.0, −3.0 and −5.0 g/L for proteins and −0.8, −5.6 and −6.4 μmol/L for conjugated dienes (Fig. 2).

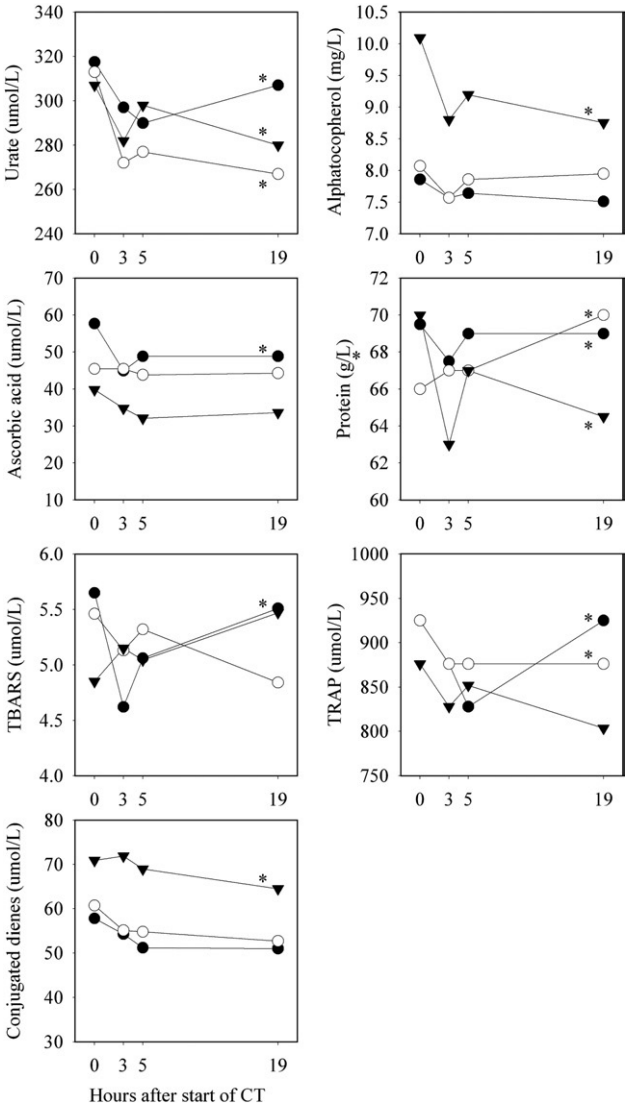

Fig. 2. Median values for urate (μmol/L), alphatocopherol (mg/L), ascorbic acid (μmol/L), proteins (g/L), TBARS (μmol/L), TRAP (μmol/L) and conjugated dienes (μmol/L) at baseline (0 h), 3, 5 and 19 h after the start of CT during the 1st (●), 2nd (○) and 6th (▼) cycles. The chemotherapy treatment was administered during the first day of the cycle; consecutive cycles were repeated every 3 weeks up to a maximum of six cycles. Measurements were performed during the first, second and sixth chemotherapy cycles. * Significant change during the cycle.

Association between oxidative stress markers and other demographics

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

thiols ($R=-0.683$, $p=0.004$), and plasma nitrite and plasma gammatocopherol ($R=-0.592$, $p=0.026$).

Plasma thiols correlated positively with blood lymphocytes ($R=0.693$, $p=0.009$) and blood hemoglobin ($R=0.571$, $p=0.026$), and negatively with blood leukocytes and neutrophils ($R=-0.506$, $p=0.054$ and $R=-0.634$, $p=0.020$, respectively).

Smoking or concurrent illnesses (cardiac disease, chronic arrhythmia, hypertension or chronic obstructive pulmonary disease) had no significant effect on the antioxidants or oxidative and nitrosative stress parameters during any of the CT cycles.

Association between oxidative stress markers and adverse events, response to treatment and overall survival

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).

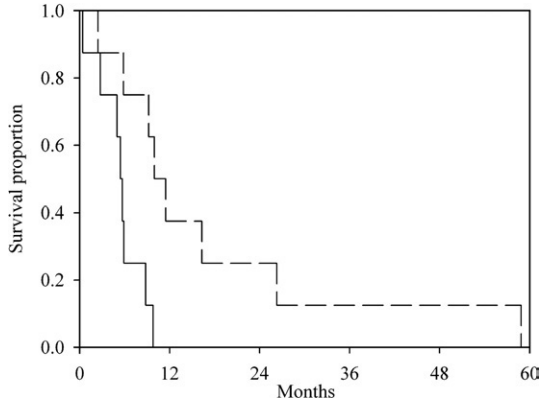

Fig. 3. Kaplan–Meier survival curves for patients with baseline thiols during the first chemotherapy cycle >0.306 μmol/L ($n=8$) vs. patients with baseline thiols <0.306 μmol/L ($n=8$). Log rank test $p=0.008$.

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 (.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 NO [54]. Elevated levels of 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.

Acknowledgments

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Exhaled pentane as a possible marker for survival and lipid peroxidation during radiotherapy for lung cancer—a pilot study

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Abstract

To examine lipid peroxidation during radiotherapy (RT), exhaled pentane samples were collected from 11 lung cancer patients before RT and 30 and 120 min after the start of RT on days 1, 4 and 5 and at 30 and 40 Grays, if possible. Exhaled pentane samples were collected once from 30 healthy controls. Serum thiobarbituric-acid-reactive substances (TBARS) and conjugated dienes (CD) were obtained from patients on each exhaled air collection day. Lung cancer patients had higher exhaled pentane levels than controls (1.73 ng/L vs 0.83 ng/L, $p = 0.017$). Exhaled pentane levels tended to decrease during the first RT day ($p = 0.075$) and levels of CD decreased during the first week of RT ($p = 0.014$). Higher pre-treatment pentane levels predicted better survival ($p = 0.003$). Elevated exhaled pentane levels before RT may be due to the lipid peroxidation burden associated with cancer. The decrease of lipid peroxidation markers during RT may be attributable to enhanced antioxidant defense mechanisms.

Keywords: *Exhaled pentane, lipid peroxidation, lung cancer, radiotherapy, TBARS, conjugated dienes*

Abbreviations: *RT, radiotherapy; TBARS, thiobarbituric-acid-reactive substances; CD, conjugated dienes*

Introduction

Lung cancer is the leading cause of cancer mortality worldwide [1]. In early stage non-small cell lung cancer (NSCLC), surgery is currently the most effective treatment modality. However, ~75% of patients are not eligible for curative resection and are usually referred to radiotherapy (RT) [2].

Oxidative mechanisms may have a role in the initiation, promotion and progression of carcinogenesis and many cancers are associated with increased production of reactive oxygen species (ROS) [3]. The tumour cell killing mechanism of RT is mainly based on the formation of ROS [4]. Ionizing radiation interacts with water and this generates highly reactive free radicals which can react with lipids, proteins, carbohydrates and DNA causing damage [5]. Polyunsaturated fatty acids (PUFAs), consisting mainly of n-3 and n-6 PUFAs, are located in the cell membranes and are particularly sensitive to free radical damage [6]. Pentane is formed during peroxidation of

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n-6 PUFAs such as 9,12,15-linoleic acid and arachidonic acid. Ethane is formed during n-3 fatty acid peroxidation. Ethane and pentane are volatile hydrocarbons readily excreted in the breath [6,7].

Previous studies have evaluated exhaled ethane or pentane as a marker of *in vivo* lipid peroxidation in different disease conditions, e.g. asthma, obstructive sleep apnea, chronic obstructive pulmonary disease (COPD), cystic fibrosis, interstitial lung disease, Crohn's disease, ischemic heart disease and in critically ill patients and pre-term infants [8–17].

Thiobarbituric acid (TBA) reacts with aldehydes such as malondialdehyde (MDA) and thus thiobarbituric-acid-reactive substances (TBARS) are considered as markers of lipid peroxidation in tissues and plasma [18]. During the early phase of lipid peroxidation the double bonds in PUFAs are rearranged, with conjugated dienes being formed. Conjugated dienes are also widely studied as an index of lipid peroxidation and are less susceptible to compensatory antioxidant mechanisms than other lipid peroxidation markers [19].

Only a few studies have explored exhaled hydrocarbons in cancer. According to Hietanen et al. [20], breast cancer patients have increased exhaled pentane production compared to healthy controls. The effects of RT on exhaled hydrocarbons have previously been examined in only one patient in a unique RT treatment situation, namely during total body irradiation. In this case report exhaled ethane levels increased on day 2 of treatment and this was associated with clinical toxicity. However, the pre-treatment exhaled ethane levels on day 3 and 4 decreased below pre-treatment exhaled ethane levels on day 1, suggesting upregulation of endogenous or exogenous antioxidant defences due to RT [21]. As a previous study showed exhaled ethane to be unsuitable for the detection of lung cancer we decided to measure exhaled pentane in this study [22].

The aim of this pilot study was to develop a non-invasive exhaled air collection method to explore lipid peroxidation in lung cancer patients, healthy controls and during RT of patients with lung cancer. We also set out to evaluate the associations between exhaled pentane levels and serum lipid peroxidation markers, adverse events and overall survival of lung cancer patients.

Materials and methods

Study groups

Eleven newly diagnosed lung cancer patients and 30 healthy hospital employees at Tampere University Hospital participated in the study. The inclusion criteria for lung cancer patients were: histologically or cytologically confirmed lung cancer, Karnofsky performance status $\geq 70\%$ and planned RT treatment. The exclusion criteria for all participants were: acute respiratory infection, unstable angina pectoris, asthma, serious hepatic, pulmonary or cardiac disease, gout, inflammatory bowel disease, previous cancer (except basalioma or carcinoma cervix *in situ*), marked impairment in pulmonary function (forced expiratory volume in 1 s below 1.5 L), previous RT and regular allopurinol or acetylcysteine medication. Pre-treatment evaluation of cancer patients consisted of a physical examination, bronchoscopy, chest radiography, chest and upper abdominal computerized tomography, full blood count and serum chemistry. Bone scintigraphy and abdominal ultrasound were performed when clinically indicated. None of the patients or controls had taken any vitamins or herbal supplementation during the preceding 3 months. Data on smoking, other diseases, medication and symptoms were collected in a standardized questionnaire [23]. All participants also completed a detailed diet questionnaire over the 3 days prior to the exhaled pentane collections. Smokers were defined either as current smokers or as smokers who had quit less than 6 months previously, ex-smokers as subjects who had quit more than 6 months ago and non-smokers as never smokers. Lifetime cigarette consumption was expressed as pack years (cigarette packs smoked/day $\times$ years smoked).

The characteristics of the two groups are shown in Table I.

Ethics

This study was conducted according to the guidelines of the Declaration of Helsinki. The local Ethics Committee approved the study and written informed consent was obtained from each participant.

Radiotherapy

Ten patients underwent three-dimensional computer tomography-based treatment planning by CadPlan (version 6.23, Varian, Varian Medical Systems Inc, Palo Alto, CA); one patient received palliative RT with two anterior posterior fields. The planning target volume (PTV) included the tumour and adjacent lymph nodes, with adequate safety margins.

Radiotherapy with 18 MV photons was applied by linear accelerator (Varian). Fractions of usually 2 Gy were given five times a week; two patients received RT 3 Gy/fraction five times weekly up to a total dose of 30 Gy and one patient received RT 4 Gy/fraction 2 days/week up to a total dose of 40 Gy. The mean radiotherapy dose delivered was 46.72 Gy (range 30.0–60.0 Gy).

Collection of exhaled pentane

Before collection of exhaled pentane all individuals had been fasting for 12 h and resting for 30 min.

Table I. Characteristics of lung cancer patients and controls. Values are means (range) or numbers (percentages).

	Patients ($n=11$)		Controls ($n=30$)	
Males	7	(64%)	19	(63%)
Mean (range) age, years	63.8	(50–82)	50.5	(34–62)
Mean (range) BMI, kg/m^2	24.6	(17–33)	24.8	(20–29)
Current smokers	7	(64%)	2	(7%)
Ex-smokers	3	(27%)	7	(23%)
Non-smokers	1	(9%)	21	(70%)
Karnofsky performance status				
80	4	(36%)	0	(0%)
90	6	(55%)	0	(0%)
100	1	(9%)	30	(100%)
Mean (range) FEV_1, % of predicted	72.6	(61–81)		
Histological classification				
Squamous cell ca	4	(36%)		
Adenocarcinoma	4	(36%)		
Small cell lung ca	2	(18%)		
Unclassified	1	(9%)		
Stage				
IA	1	(9%)		
IIB	1	(9%)		
IIIA	5	(45%)		
IIIB	2	(18%)		
IV	2	(18%)		

The exhaled air collections were performed between 7.30 am and 12 am. Each individual was seated, wore nose clips and breathed through a non-rebreathing Ruben valve to prevent inhaling ambient air. The system presented no resistance to inspiration or expiration. The individuals were required to breathe hydrocarbon-free air for 4 min to wash contaminating hydrocarbons out of their lungs. Hydrocarbon-free air was prepared using an AS80 air purifier (Signal Instruments, Camberley, UK) and collected in a 25-L impermeable gas bag. After the washout period, each individual was instructed to rapidly inspire to total lung capacity and then to slowly exhale to residual volume. The exhaled gas was collected through a sterile gauze into an impermeable 750 mL Quintron gas collection bag (Model QT00841-P, Quintron, Milwaukee, WI) connected to a disposable 400 mL gasbag (Model QT000843-P, Quintron), which was used to discard dead space air. Gas collection bags were not reused.

The gas collection procedure was repeated three times during each measurement day. The pre-treatment exhaled pentane sample was taken before RT was initiated; the subsequent samples were taken 30 min and 120 min after start of RT on day 1. Patients fasted between these exhaled air collections. This procedure was repeated on RT Day 4 (or after 6 Gy of RT), Day 5 (or after 8 Gy of RT) and on RT days of 30 Gy and 40 Gy, if possible. During each exhaled pentane collection day two samples of purified hydrocarbon-free background air were also collected in identical Quintron gas collection bags. Exhaled air samples were collected once from the controls.

The gas collection bags were stored in a refrigerator ($+4^\circ$C) for a maximum of 20 h before analysis. Altogether 233 gas samples (including samples from patients and controls and background air) were collected and analysed in this study.

Collection of blood samples

All lung cancer patients underwent laboratory testing at baseline. In addition to lipid peroxidation markers the tests included full blood count, alkaline phosphatase, alanine transferase, aspartate transferase, creatinine, serum C-reactive protein, sodium and potassium.

Peripheral venous blood samples were collected after overnight fasting. A Venoject blood collection system (Terumo, Leuven, Belgium) was used. The blood was collected into sterile tubes and centrifuged at 3000 g for 10 min, after which serum samples were obtained and stored at -70°C until analysis. Blood samples were collected from lung cancer patients before treatment on RT day 1, 4, 5 and at 30 Gy and 40 Gy, if possible. Serum thiobarbituric-acid-reactive substances (TBARS) and conjugated dienes were analysed.

Analysis of exhaled pentane

The exhaled air samples and the samples taken from purified hydrocarbon-free background air were transferred into adsorbent tubes containing graphitized carbon (Carbopack B, N930-7002/Perkin Elmer; Perkin Elmer Corp., Norwalk, CT) before analysis. An air volume of 0.8 L was pumped at 150 mL/min from each Quintron gas collection bag into the

sampling tubes. The sample tubes were analysed with a Perkin Elmer ATD 400 thermal desorber and a gas chromatograph (HP 5890, Hewlett-Packard, Palo Alto, CA) equipped with a HP 5970A quadruple mass-selective detector. The following conditions were used for thermal desorption: the temperature for desorption was 300°C for 10 min with a desorb flow of 25 mL/min and an inlet split flow of 12 mL/min. The cold trap (Tenax TA; Perkin Elmer 60/80 mesh) was kept at −30°C during the first desorption and at 300°C for 5 min during the final desorption. The outlet split flow was adjusted to 10 mL/min. The valve and line temperatures were 200°C.

The sample was transferred from the cold trap directly to the analytical column PLOT Al_2O_3/KCl (30 m × 0.32 mm × 5 μm; Chrompack, Middelburg, Netherlands) with a carrier gas (helium) at a column pressure of 3.7 psi. The gas chromatograph (GC) oven temperature was programmed as follows: 50°C for 1 min, increase by 5°C/min to 130°C, hold for 1 min, increase by 12°C/min to 180°C, then hold for 18 min. The temperature of the transfer line between the GC and mass-selective detector was 225°C. The retention time of pentane was 8.2 min. The mass-selective detection was based on the electron impact ionization mode and the ions (m/z) 43, 57 and 72 were monitored. The area of the base peak (m/z 43) was used for quantification.

Calibration standards were made by injecting 1 μL of calibration solution (n-pentane in methanol) into the sampling tubes and by sucking air through the tubes for 2 min. The blank samples were prepared correspondingly by injecting 1 μL of methanol into the sampling tubes. The calibration curve was obtained after subtracting the peak area of the blanks from the peak areas of the calibration standards. The concentration of pentane (ng/L) was then calculated for all samples. The concentration of pentane in the expired air of the study subjects was obtained by subtracting the concentration of pentane in purified hydrocarbon-free background air samples from the corresponding concentrations in exhaled air samples. The concentration of pentane was expressed as ng/L. All exhaled air samples were analysed at the Finnish Institute of Occupational Health, Helsinki, Finland.

Measurement of serum TBARS and conjugated dienes

For analyses of thiobarbituric-acid-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. 1,1,3,3-Tetraethoxypropane purchased from Sigma Chemical Co. (St.Louis, MO) was used as a standard [24].

For the measurement of diene conjugation, lipids extracted from serum samples (100 μL) by chloroform-methanol (2:1 vol/vol), dried under nitrogen atmosphere and then redissolved in cyclohexane, were analysed spectrophotometrically (at 234 nm) as described [25]. All analyses were done at the MCA Research Laboratory, Turku, Finland.

Evaluation of adverse events and response to treatment

During and after the RT, adverse events were evaluated according to the criteria of the World Health Organization (WHO) and Lent Soma Table [26,27]. Radiation pneumonitis was scored according to the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC) acute radiation morbidity scoring criteria [28]. Treatment responses were evaluated according to the WHO criteria [26].

Statistics

The exhaled pentane concentration was the primary outcome measure. TBARS and conjugated dienes were the secondary variables. The distribution of exhaled pentane was skewed to the right and was logarithmically ($\log_e$) transformed before analysis; the concentrations are given as geometric means. The patients were compared to controls using the *t*-test for independent samples and, due to logarithmic transformation, the results are given as ratios (patients/controls) with 95% confidence intervals. Analysis of covariance (ANCOVA), including gender as a categorical covariate, was also conducted, as gender proved to be a significant factor explaining the level of pentane in healthy controls. The within-patient changes in pentane concentration were calculated from 0 min (pre-treatment) to 120 min after onset of radiotherapy on all RT days and the changes from 0 min to 120 min were analysed using Wilcoxon's signed ranks test. The time-effect in TBARS and conjugated dienes during the first week of RT treatment was analysed using the ANOVA for repeated measures. The effects of age, gender, smoking, histological diagnosis and stage of cancer on the dependent variables were analysed using Spearman's rank correlation and analysis of variance, when appropriate. Pre-treatment exhaled pentane, TBARS and conjugated dienes were dichotomised (< median or > median) and the Kaplan-Meier method was used to estimate survival time in groups with lower vs higher exhaled pentane, TBARS and conjugated dienes. The log rank test was used to compare the groups. *P*-values below 0.05 were considered statistically significant. Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences for Windows), version 15.0 (SPSS Inc, Chicago, IL).

Results

Study groups

Eleven lung cancer patients and 30 healthy controls entered the study. None of the patients or controls had asthma, chronic bronchitis, tuberculosis or asbestosis. Two (18%) of the patients had mild chronic obstructive pulmonary disease. Four of the patients (36%) had squamous cell carcinoma, four (36%) had adenocarcinoma, two (18%) had small cell lung cancer and one patient (9%) had histologically unclassified cancer. The majority ($n = 9$, 82%) had stage III–IV disease. There were seven (64%) current smokers in the patient population, whereas two (7%) of the controls were smokers. All the smokers refrained from smoking for at least 14 h prior to the exhaled pentane collections. Four patients (36%) received two or three cycles of cisplatin- based chemotherapy before RT treatment. The detailed diet questionnaire revealed no significant differences in dietary intakes between the lung cancer patients and healthy controls. Smoking, histological diagnosis, stage of cancer, diet or neoadjuvant chemotherapy had no effect on lipid peroxidation marker levels. However, gender did affect exhaled pentane levels in the control group and thus the effect of gender was taken into account by adjusting the results accordingly.

Lipid peroxidation markers before radiotherapy

The exhaled pentane distribution was skewed to the right. The geometric mean for the patients was 1.69 ng/L (95% CI 1.14–2.50 ng/L) and for the controls 0.96 ng/L (95% CI 0.66–1.39 ng/L) at baseline ($p = 0.082$). As gender affected exhaled pentane levels in the control group (1.43 ng/L in men vs 0.48 ng/L in women, $p = 0.002$), the effect of gender was taken into account. The gender-adjusted geometric mean for the patients at baseline was 1.73 ng/L (95% CI 1.05–2.86 ng/L) and for the controls 0.83 ng/L (95% CI 0.61–1.13 ng/L). The patients/controls ratio for exhaled pentane was 2.08 (95% CI 1.15–3.76 ng/L), $p = 0.017$ (Figure 1).

The mean concentration of thiobarbituric-acid-reactive substances (TBARS) was 3.10 μmol/L (SD = 2.10, range = 1.16–6.62 μmol/L) and of conjugated dienes 46.8 μmol/L (SD = 14.4, range = 32.0–66.4 μmol/L) in patients before RT. There were no significant correlations between the baseline values for serum TBARS, conjugated dienes and exhaled pentane.

There was a significant negative correlation between pre-treatment serum TBARS levels and blood haemoglobin count ($R = -0.743$, $p = 0.035$) in lung cancer patients. No statistically significant associations were noted between lipid peroxidation markers and other laboratory measurements in lung cancer patients.

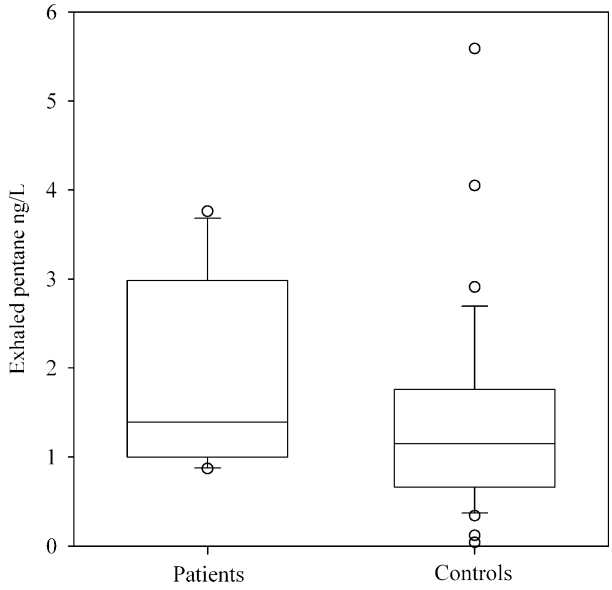

Figure 1. The boxplot figure for the distribution of pentane (ng/L) in patients ($n = 11$) and controls ($n = 30$). Patients vs controls: non-adjusted $p = 0.082$, gender-adjusted $p = 0.017$. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. Values outside the 90th and 10th percentiles are labelled as outliers (o).

Lipid peroxidation markers during radiotherapy

There was a tendency for exhaled pentane levels to decrease during the first week of RT. The greatest change in exhaled pentane levels occurred during the first RT day, when the geometric mean concentrations were 1.69 ng/L before RT, 1.50 ng/L at 30 min and 1.24 ng/L at 120 min after the start of RT. However, these changes were not statistically significant (median change between baseline and 120 min was −0.40 ng/L, $p = 0.075$). (Figure 2, Table II) At the day of 30 Gy of RT (corresponding to 3 weeks of RT treatment with the conventional 2 Gy/day fractionation scheme), the exhaled pentane levels were unchanged. Due to the small number of samples, no testing for statistical significance was appropriate at 30 Gy or 40 Gy.

The mean levels of conjugated dienes decreased significantly during the first week of RT ($p = 0.014$). The mean concentration (μmol/L) of conjugated dienes was 48.2 on day 1, 43.9 on day 4 and 42.7 on day 5. The concentration of TBARS (μmol/L) remained unchanged during the first week of RT (3.03 on day 1, 2.88 on day 4 and 2.88 on day 5, $p = 0.946$). Again, the small number of samples obtained at 30 Gy and 40 Gy precluded any testing for statistical significance.

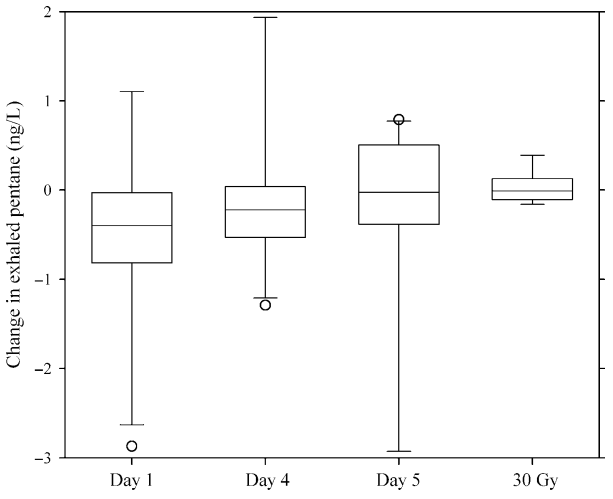

Figure 2. Box-plot figure for changes in exhaled pentane (ng/L) from 0 min to 120 min on RT day 1 ($n=11$), day 4 ($n=10$) and day 5 ($n=8$) and at 30 Gy ($n=5$). Six outliers are outside the figure.

Lipid peroxidation markers and adverse events

Eight (73%) of the patients experienced adverse events during RT. The most common adverse events were dysphagia ($n=6$), esophagitis ($n=3$), infection ($n=2$) and fatigue ($n=1$). No serious adverse events were noted during or after the treatment. Five patients (45%) developed symptomatic radiation pneumonitis according to RTOG/EORTC criteria. Three (27%) patients showed mild/moderate lung toxicity (grade I pneumonitis in two patients and grade II in one patient). Two patients (18%) suffered from grade III radiation pneumonitis. If the pre-treatment exhaled pentane levels were below the median (1.39 ng/L), one out of five patients developed radiation pneumonitis, but if the pre-treatment exhaled pentane levels were above the median, four out of five patients developed radiation pneumonitis. There were no other associations between pre-treatment exhaled pentane levels or levels of serum TBARS and conjugated dienes in relation to any other adverse events (Table III).

Table II. Exhaled pentane (ng/L) in patients before radiotherapy and after 30 min and 120 min from the onset of RT on Days 1, 4 and 5 and at 30 and 40 Gy.

Day	Min	Geometric mean (range)
1 ($n=11$)	0	1.69 (0.87–3.76)
	30	1.50 (0.38–8.95)
	120	1.24 (0.52–3.42)
4 ($n=10$)	0	1.43 (0.68–2.25)
	30	1.18 (0.65–2.26)
	120	1.18 (0.41–5.53)
5 ($n=8$)	0	1.43 (0.47–4.98)
	30	1.74 (0.81–6.80)
	120	1.25 (0.77–2.85)
30 Gy ($n=5$)	0	1.20 (0.63–2.08)
	30	1.20 (0.53–2.93)
	120	1.20 (0.62–2.31)
40 Gy ($n=2$)	0	1.32 (0.92–1.90)
	30	2.33 (1.40–3.87)
	120	2.12 (1.08–4.15)

Lipid peroxidation markers and overall survival

The median overall survival of the patients was 10.7 months (range 2.5–48.0 months). At the end of the planned follow-up period of 60 months all patients had died. There was a statistically significant association between pre-treatment exhaled pentane levels and overall survival ($p=0.003$). If pre-treatment pentane levels were below the median (1.39 ng/L), the median survival of the patients was 5.2 months (95% CI 0–10.5), but if pentane levels were above the median, the median survival was 16.1 months (95% CI 10.1–22.1) (Figure 3). There was an almost significant association between lower baseline serum TBARS levels and better overall survival (17.3 months, 95% CI 0–54.1 vs 2.8 months, 95% CI 0–10.8, $p=0.051$).

Discussion

There has been increasing interest in non-invasive monitoring of respiratory tract inflammation and oxidative stress. Several non-cancer studies have explored either exhaled ethane or pentane as markers of *in vivo* lipid peroxidation [6,7]. Previous studies have suggested that exhaled pentane is a simple and objective non-invasive marker of inflammation [9]. To our knowledge, this is the first study evaluating exhaled pentane before and during radiotherapy for lung cancer. According to this study lung cancer patients have significantly higher exhaled pentane levels than healthy controls. This is probably due to the increased oxidative stress and especially lipid peroxidation burden associated with cancer [3]. Phillips et al. [29] have created a predictive model to diagnose lung cancer using exhaled breath analysis and they identified nine volatile organic compounds, one of which was pentane, as markers of lung cancer.

This study shows that levels of exhaled pentane tended to decrease during the first day of radiotherapy ($p=0.075$). This might be due to tumour cell destruction following RT. Irradiation might also reduce the

Table III. Adverse events during radiotherapy, response to treatments and overall survival of lung cancer patients.

	Patients ($n=11$)
Adverse events during RT	8 (73%)
Response to RT treatment	
Complete or partial response	8 (73%)
No change or progressive disease	3 (27%)
Median (range) of survival (months)	10.7 (2.5–48.0)

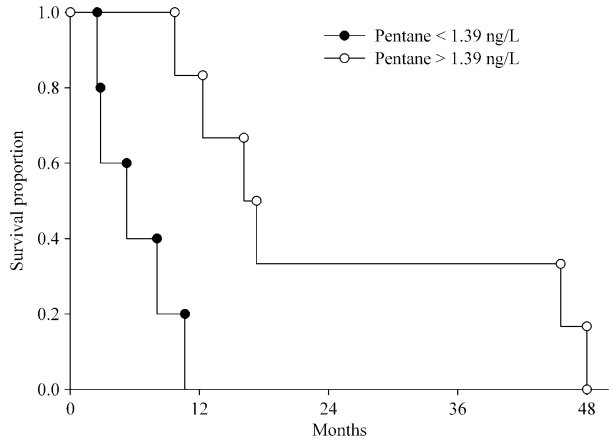

Figure 3. Kaplan-Meier survival plots for patients with baseline pentane <1.39 ng/L ($n=5$) and >1.39 ng/L ($n=6$). Log-rank test $p=0.003$.

tumour cell metabolism or intracellular activity, thus decreasing the amount of lipid peroxidation. However, no significant changes were noted later during the course of RT. The slight decrease, albeit non-significant, in exhaled pentane levels during the first week of radiotherapy might be attributed to a decrease in tumour mass, as a previous study has suggested that tumour tissue itself might be a source of ROS and lipid peroxidation [30]. The decrease of exhaled pentane is supported by a decrease of serum conjugated dienes ($p=0.014$) during the first week of RT. However, the levels of TBARS remained unchanged ($p=0.946$). Previous studies have also found a relationship between serum lipid peroxidation markers and exhaled pentane [31]. The decrease of lipid peroxidation markers during RT might be associated with induction of intracellular antioxidants, which has been reported as an adaptive mechanism towards low-dose radiation [32].

In vitro it has been shown that reactive oxygen species release is highest after a single dose of 12 Gy of irradiation, whereas the usual fractionation 9×2 Gy up to a total dose of 18 Gy produces the lowest absolute release of ROS [33]. Thus the production of ROS caused by RT might be diminished by fractionated irradiation, which is the current practice in treating cancer patients with radiotherapy. It is known that aerobic cells and tissues have sophisticated antioxidant defence systems, including both enzymatic and non-enzymatic components, which counter the actions of ROS [5]. Therefore, it is possible that the radiation fractionation scheme we used only mildly induced the formation of ROS and the patients' own antioxidative defence mechanisms were able to counter this. As an *in-vitro* study evidenced that the onset of lipid peroxidation might be delayed [34], it is also possible that a noteable increase in lipid peroxidation markers occurred at a later stage during the course of radiotherapy. However, the small number of patient samples at 30 Gy and 40 Gy in this study precludes any reliable analysis of lipid peroxidation markers at the end of radiotherapy.

As there are no published studies on exhaled pentane measurement during RT for lung cancer, selection of the exhaled air collection timepoints in this study was based on a three-patient pilot study we performed previously (data not shown). In an *in-vitro* study by Benderitter et al. [35], human erythrocyte membranes were exposed to 0, 2, 4 and 8 Gy of irradiation. The investigators noted that the malondialdehyde (MDA) concentration in the erythrocyte membrane had increased significantly 3 h after irradiation ($p<0.001$). The same study showed that the n-6 phosphatidylethanolamine (PE) fatty acids series, especially PE arachidonic acid, fell drastically after radiation exposure. Another study found that accumulation of neutrophils was first seen 6 h after exposure to irradiation and the number of neutrophils increased up to 24 h post-4 Gy of gamma irradiation [36]. On the other hand, the formation of conjugated dienes increased only 4 days after irradiation in the lipoproteins of rats exposed to neutrons/gamma irradiation [37]. This data reflects the difficulty of accurate timing and therefore it is also possible that the time points we used (before treatment, 30 min and 120 min after RT on days 1, 4, 5 and at 30 Gy and 40 Gy) are not optimal. Exhaled pentane measurements also after RT treatment would have added beneficial information, but were not possible to perform in this pilot study.

Measuring exhaled pentane has often been criticized because of the numerous technical difficulties involved [7,38]. The most common problems are the influence of ambient air hydrocarbons during collection, storage and analysis of the samples and inadequate sensitivity of chromatography, as there are no generally accepted standards for sampling, preconcentrating or analysing exhaled pentane [39,40]. In this study we excluded possible confounding factors by a well-designed and controlled expired air collection method and analysis. It is known that effective removal of ambient air hydrocarbons before collection is essential [16]. The 4-min wash-out period used in our study is known to adequately remove residual ambient hydrocarbons from the lungs [39,40]. Breathing hydrocarbon-free air for a longer time has not been shown to provide any additional benefit [41]. Because it is also important to avoid contamination of the breath sample, only suitable materials should be used for sampling and storage [7]. Previous studies show that ethane and pentane levels are stable for up to 6 days of storage in sample tubes; however, the data suggests that storage in gas collection bags should be limited to 48 h [7,42]. Accordingly, the samples in this study were stored in the bags for a maximum of 20 h. Although Quintron gas collection bags are reusable and specially designed for alveolar air collection, to avoid any contamination the bags were not reused. As diet is

also known to affect exhaled pentane levels, all the measurements in this study were performed after overnight fasting [38]. In light of previous study findings suggesting that smoking causes an immediate increase in exhaled pentane levels, the subjects in this study refrained from smoking for at least 14 h before the exhaled air collections [43]. However, it is known that both acute and chronic cigarette smoking induce enhanced production of neutrophils and promote lipid peroxidation, which has been evidenced both locally in the lungs and systemically in blood [44]. Thus, we cannot exclude that smoking may have an impact on the results.

In this study, five out of 11 patients developed radiation pneumonitis, which was more likely if pre-treatment exhaled pentane levels were higher than the median. However, due to the small number of patients, testing for conventional statistical significance was not appropriate. Nevertheless, the investigators consider this finding clinically significant and larger studies should be performed to obtain its statistical significance. On the contrary, no significant associations were noted between other adverse events and the levels of exhaled pentane or serum lipid peroxidation markers.

To our knowledge, this is the first time an association has been demonstrated between exhaled pentane levels and overall survival of lung cancer patients. Those with pre-treatment exhaled pentane levels above the median survived longer than patients with levels below the median ($p = 0.003$). This observation is in agreement with an *in vivo* study showing that the end products of lipid peroxidation might inhibit tumour growth [45]. A recent study reported that head and neck carcinoma patients with higher than the median value of post-radiotherapy glutathione, as a marker of generalized oxidative stress, survive longer [46]. Although this effect might be mediated through redox-sensitive thiol-containing proteins, it possesses an interesting hypothesis [47]. It has also 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 [48]. Polyunsaturated fatty acids have also been shown to be anti-angiogenic and are thus able to suppress tumours [48]. Interestingly, a borderline significant association was recorded between lower TBARS levels and longer overall survival. If serum TBARS levels were below the median, the patients survived longer ($p = 0.051$) than if serum TBARS levels were higher than the median.

Conclusions

This study showed that lung cancer is associated with elevated levels of exhaled pentane, which may be attributed to an excess lipid peroxidation burden caused by cancer. No significant changes were noted in the levels of exhaled pentane during radiotherapy treatment; however, the levels of conjugated dienes decreased significantly during the first week of radiotherapy. Despite the small patient population, this study suggests that higher exhaled pentane levels are associated with the occurrence of radiation pneumonitis. This is also the first study to report an association between pre-treatment exhaled pentane levels and overall survival, although the small sample size means that larger studies should be performed to confirm this finding.

Although analysis of exhaled pentane levels might prove an interesting tool, the difficulties and possible confounding factors associated with this method limit its use in larger settings. Finally, we suggest that measuring other oxidative and nitrosative stress markers as well as markers of oxidative DNA damage simultaneously with exhaled pentane could add beneficial information to the oxidant effects of radiotherapy.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Cytokines in bronchoalveolar lavage fluid and serum of lung cancer patients during radiotherapy — Association of interleukin-8 and VEGF with survival ☆

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ABSTRACT

Radiotherapy (RT) produces oxidative stress and local inflammation. This study aimed at clarifying the role of different cytokines (VEGF, TNFα, IL-1β, IL-6, IL-8, IL-12 and IL-18) in bronchoalveolar lavage (BAL) fluid and in the serum of lung cancer patients at baseline and during RT. Bronchoscopy and BAL were performed on 36 lung cancer patients and 36 controls for diagnosis; patients receiving RT had a second bronchoscopy during RT. Serum samples were obtained during RT and three months after RT. In this study lung cancer patients had higher levels of serum and BAL fluid IL-6 and serum IL-8 compared to controls ($p < 0.001$, $p = 0.039$ and $p = 0.030$, respectively). RT caused a significant increase of BAL fluid IL-6 ($p = 0.037$). There were no significant associations between baseline cytokine levels and adverse events or response to treatment. Higher baseline serum and BAL fluid IL-8 and serum VEGF levels ($p = 0.036$, $p = 0.027$ and $p = 0.014$, respectively) were associated with shorter survival. This study shows that lung cancer is associated with upregulation of IL-6 and IL-8. The increase of BAL fluid IL-6 during RT might be attributed to enhanced RT-related oxidative stress or increased cell death. Serum and BAL fluid IL-8 and serum VEGF might have a prognostic role in survival of lung cancer.

1. Introduction

Cytokines are important for several biological processes of malignant tumors. Cytokines are produced by both normal and tumor cells and act as paracrine and autocrine tumor cell growth factors [1]. Of the cytokines related to tumor biology, most interest has focused on vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, IL-8, IL-12 and IL-18.

VEGF is one of the most potent and specific angiogenic molecules. It is involved in endothelial cell proliferation and migration as well as increased vascular permeability [2,3]. Interleukin-6, a multifunctional cytokine secreted by lymphoid and nonlymphoid cells, is involved in the regulation of cellular functions including proliferation, apoptosis, angiogenesis and differentiation [4,5]. Interleukin-8 plays an important role as a neutrophil activator and chemoattractant [6]. It is produced by non-small cell lung cancer cells in vitro and in vivo and has been shown to contribute to tumor-induced angiogenesis in vivo [7]. IL-8 may play a role in enhancing tumor cell proliferation, tumor cell motility and adhesion [8]. Interleukin-18 induces synthesis of interferon-γ (IFN-γ) in T cells and natural killer (NK) cells. It is essential for host defences against infections, but may, at the same time, induce inflammatory disorders [9,10].

While it is known that lung cancer is associated with elevated systemic levels of certain cytokines [11–14], there is less data on the occurrence of cytokines locally, i.e., in the bronchoalveolar lavage (BAL) fluid of lung cancer patients. BAL provides a sample of the cellular and non-cellular components of the fluid lining the respiratory epithelium [15]. Studies so far have shown that TNFα, IL-6 and IL-1β occur at higher levels in the BAL fluid of lung cancer patients than of patients with benign lung disease [16,17].

☆ Preliminary results of this study have been presented as a poster presentation at International Association for the Study of Lung Cancer (IASCL) 10th World conference on Lung Cancer in Vancouver, Canada, 2003.

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Radiotherapy (RT) induces local inflammation and oxidative stress [18]. It also inhibits angiogenesis by damaging the endothelium [19]. Since changes in inflammatory markers after RT may emerge more clearly at the local level (in BAL fluid) than systemically (in serum) [18,20], we evaluated the occurrence of several cytokines in the BAL fluid and serum of lung cancer patients at baseline and during RT and compared the results with non-cancer controls. We examined their association with various clinicopathological variables and with the emergence of adverse events, with the response to treatment and with overall patient survival.

2. Materials and methods

2.1. Study subjects

Consecutive patients at the Department of Respiratory Medicine of the Tampere University Hospital were invited to participate in the study. The inclusion criteria for the patients were: histologically or cytologically confirmed lung cancer and Karnofsky performance status $\geqslant$70%. The inclusion criteria for the controls were: patients scheduled for bronchoscopy for diagnostic purposes because of prolonged cough (not cancer). Exclusion criteria of all participants were: serious cardiac, hepatic or metabolic disease, regular allopurinol or acetylcysteine medication, forced expiratory volume in one second (FEV_1) $\leqslant$1.5 liters or other contraindications for bronchoscopy. All consenting patients and controls were interviewed and examined by a pulmonologist (SS) and oncologist (MC). Altogether 36 lung carcinoma patients and 36 non-cancer controls were enrolled. The characteristics of the two groups are shown in Table 1.

Data on smoking, concomitant diseases, symptoms and medication were collected on a standardized questionnaire [21]. Diagnostic lung, mediastinal and upper abdominal computerized tomography was performed on all cancer patients for staging of the disease. Before treatment a physical examination was made and samples for urinanalysis, full blood count and serum chemistry were taken. Abdominal sonography and bone scintigraphy were performed as needed clinically. All patients and controls underwent bronchoscopy and BAL for diagnostic purposes before treatment. Patients receiving RT underwent a second bronchoscopy and BAL of the irradiated lung two weeks after start of RT (18–22 Gray). All bronchoscopies were performed in the same manner and in the same unit.

Table 1
Lung cancer patients and controls. Values are means (SD; range) or numbers (percentages).

	Lung cancer patients (n = 36)		Controls (n = 36)	
Age (years)	66.9	(9.1; 47–82)	50.8	(9.9; 18–75)
Males	29	(80.6)	16	(44.4)
BMI (kg/m^2)	23.7	(3.2; 17.0–31.8)	26.3	(5.2; 18.5–39.2)
FEV1 (% of predicted)[a]	74.1	(11.8; 58.0–94.0)	89.4	(11.5; 65–108)
Smoking				
Non-smokers	2	(5.6)	18	(50.0)
Ex-smokers	10	(27.8)	6	(16.7)
Smokers	24	(66.7)	12	(33.3)
Pack-years of smokers and ex-smokers	35.3	(23.0; 5.0–96)	28.5	(17.7; 7.5–68)
Histology				
Squamous cell ca	33	(91.7)		
Adenoca	1	(2.8)		
Small cell ca	2	(5.6)		
Stage of lung cancer				
IB	1	(2.8)		
IIIA	6	(16.7)		
IIIB	12	(33.3)		
IV	17	(47.2)		
Karnofsky performance status				
70	4	(11.1)	0	(0)
80	8	(22.2)	0	(0)
90	15	(41.7)	3	(8.3)
100	9	(25.0)	33	(91.7)

[a] n = 13 and n = 20.

2.2. Radiotherapy

Twenty out of 36 patients received RT as a treatment. Sixteen patients received RT based on three-dimensional CT-based treatment planning by Cad Plan (version 6.23, Varian Medical Finland, Varian Medical Systems Inc, Palo Alto, CA, USA), whereas four patients received palliative RT with two anterior–posterior fields. The planning target volume (PTV) contained the primary tumor and adjacent lymph nodes with adequate margins. RT with 18 MV photons was applied by a linear accelerator (Varian Clinac 2100 C/D, Varian Medical Systems Inc., Palo Alto, CA, USA) at the Radiotherapy Unit of the Tampere University Hospital. Fractions (fr) usually of 2 Gray (Gy) were given five times a week, four patients received RT 3 Gy/fr five times a week. The mean radiation dose delivered was 46.9 Gray (range 30.0–60.0 Gy).

2.3. Bronchoscopy and BAL

All bronchoscopies were carried out by the same experienced bronchoscopist (SS) according to a standardized method [22,23]. During bronchoscopy the patients were awake and breathed spontaneously. Topical lidocaine anesthesia and premedication with intramuscular atropine sulfate (0.7 mg) were used. The patients were monitored with an Ohmeda 3800 oximeter (Louisville, CO, USA) during the bronchoscopy. The bronchoscope was wedged into the segmental or subsegmental level of the left upper lobe or right middle lobe. 20 mL of sterile saline (37 °C) mixed with Addiphos buffer (20 mL of buffer to 500 mL sterile saline, Fresenius Kabi, Uppsala, Sweden), were instilled through the bronchoscope. The fluid was immediately recovered by gentle suction after each instillation [22,23]. This procedure was repeated five times and each recovered lavage volume was recorded. The collected BAL fluid samples were immediately protected from light and put on ice, centrifuged at 500 rpm for 15 min at 4 °C and stored at –70 °C until analysis. The cells were stained with the May-Grünwald Giemsa (MGG) and Papanicolau stains and fixed with 50% ethanol.

2.4. Blood samples

Peripheral venous blood samples were collected using a Venoject blood collection system (Terumo, Leuven, Belgium). The blood was collected in sterile tubes, centrifuged at 3000g for 10 min and stored at –70 °C until analysis. The first venous blood sample was drawn at the time of the first bronchoscopy. Subsequent samples were obtained of patients receiving RT at the time of the second bronchoscopy and three months after start of RT.

2.5. Measurements

Total cell and differential cell counts were determined by microscopy of fixed BAL fluid samples. The albumin in the BAL fluid was measured nephelometrically and proteins colorimetrically.

Tumor necrosis factor alpha (TNFα), interleukin-1β, IL-6 and IL-8 levels in the serum and BAL fluid were measured using a PeliKine compact™ ELISA kits (Central Laboratories of the Netherlands Red Cross, Amsterdam, The Netherlands) which are a sandwich-type of enzyme immunoassays. The sensitivity of the IL-1β assay is 0.8–1.5 pg/mL, 0.2–0.4 pg/mL for IL-6 assay and 1–3 pg/mL for

TNFα and IL-8 assays. The expected concentrations of TNFα, IL-1β, IL-6 and IL-8 in fresh serum and plasma of healthy individuals are <10 pg/mL, <5 pg/mL, <20 pg/mL and <10 pg/mL, respectively.

Serum and BAL fluid vascular endothelial growth factor (VEGF) and interleukin-12 levels were determined by a quantitative sandwich-type enzyme immunoassay (R&D Systems, Minneapolis, MN, USA). The minimum detectable dose of both VEGF and IL-12 is <5 pg/mL.

Serum and BAL fluid interleukin-18 levels were measured with a Human IL-18 ELISA Kit (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) which measures human IL-18 by sandwich ELISA. The sensitivity of the assay is 12.5 pg/mL.

All values were expressed as picograms of cytokine per milliliter. A total of 1442 samples were analyzed, all in duplicate.

2.6. Evaluation of adverse events and response to treatment

During and after the RT, adverse events were evaluated according to the criteria of World Health Organization (WHO) and Lent Soma Table [24,25]. Radiation pneumonitis was scored according to the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC) acute radiation morbidity scoring criteria [26]. The response to treatment was evaluated according to the criteria of WHO [24].

2.7. Ethics

This study was conducted according to the guidelines of the Declaration of Helsinki. The Ethics Committee of the Pirkanmaa Hospital District approved the study protocol and written informed consent was obtained of each participant.

2.8. Statistical analysis

The cytokine values are given as geometric means and ranges. Values below the detection limits were estimated using the value of (detection limit)/2. The distributions of the cytokine concentrations were skewed to the right and were logarithmically (ln) transformed before analysis. The *t*-test for independent samples was used to compare patients and controls. Group comparisons are presented as ratios (patients/controls) with the respective 95% confidence intervals. The Chi-squared test was used to compare the percentages of measurable levels of cytokines in patients and controls and the corresponding results are given as odds ratios with 95% confidence intervals. The Mann-Whitney *U*-test was used to compare the cell counts between the groups. Analysis of covariance (ANCOVA) was used to control for differences in the demographic characteristics. The possible confounding baseline factors were: current smoking (no/yes), gender, concomitant diseases (hypertension, no/yes), age and BMI. Current smoking, gender and hypertension were included as categorical factors, age and BMI were included as continuous covariates. The results were given as baseline-adjusted patients/controls ratios.

The within-patient changes in the cytokine concentrations during RT were analyzed using the *t*-test for paired samples. The results are given as ratios (e.g., after radiotherapy/baseline ratio). The Mann–Whitney *U*-test, the Kruskal Wallis test and the Spearman rank correlation were used to study the associations between patient characteristics and cytokine levels at baseline or the changes during RT. The associations between the cytokine concentrations at the time of diagnosis and survival were analyzed by Kaplan–Meier's survival analysis. The serum and BAL fluid levels of cytokines at the time of diagnosis were divided into tertiles (T1–T3, T1 representing the lowest cytokine level and T3 the highest), and the Kaplan–Meier method was applied to plot the survival curves by tertiles (T1, T2 and T3). The log-rank test was used to compare the survival distributions. The survival times are given as median with 95% confidence interval. In addition, Cox's regression analysis was performed to compare tertiles T2 and T3 to the lowest tertile T1. The results are given as odds ratios (OR) with 95% confidence intervals. *p*-Values of less than 0.05 were considered statistically significant. Statistical analyses were performed with the SPSS (release 15.0) software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Characteristics of patients and controls

Thirty-six lung carcinoma patients entered to the study and 36 non-cancer patients served as controls. The diagnosis of lung cancer was confirmed histologically in 27 and radiologically and cytologically in 9 of the patients. Twenty of the patients received radiotherapy; 15 underwent a second bronchoscopy during RT. Two patients were operated on after the diagnosis without further treatments, 5 patients received various chemotherapy regimens and 9 received symptomatic treatment. Of these 16 patients only baseline samples were obtained.

None of the controls were diagnosed with cancer; three (8%) had asthma, two (6%) chronic bronchitis and mild chronic obstructive pulmonary disease (COPD), one (3%) COPD and one (3%) control patient was operated on for a benign papilloma. All other control patients (80%) were diagnosed with idiopathic cough and had no further treatment.

The patient and control groups differed with respect to some main characteristics (Table 1), and thus smoking, gender, concomitant diseases (hypertension), age and BMI were taken into account and the cytokine analyses were adjusted for them. There were also more smokers in the patient group than among the controls (67% vs. 33%); yet smoking had no significant impact on cytokine levels. There were no significant associations between any of the concomitant diseases (cardiac disease, diabetes, chronic arrhythmia or rheumatic disease) and the various cytokine levels.

The mean instilled BAL fluid volume was 111 mL in the group of patients and 101 mL in the group of controls ($p = 0.033$), the recovery volumes were 55.3 and 67.3 mL ($p = 0.005$), respectively, at first bronchoscopy. There were no statistically significant differences in the total cell counts in the BAL fluid samples between the groups, although lung cancer patients had more neutrophils than controls (3% vs. 1%, $p = 0.002$). No serious complications occurred during or after any of the bronchoscopies.

3.2. Cytokine serum levels at baseline

The patients had significantly higher concentrations of serum IL-6 ($p < 0.001$), IL-8 ($p = 0.001$) and IL-18 ($p = 0.015$) than the controls at baseline (Fig. 1 and Table 2). The patients also tended to have higher levels of VEGF than controls ($p = 0.098$). After adjustment for smoking, gender, hypertension, age and BMI, the differences in IL-6 and IL-8 between patients and controls remained significant ($p < 0.001$ and $p = 0.030$, respectively). Measurable levels of TNFα were detected in 25.0% of the patients and in 57.1% of the controls, of IL-1β in 27.8% of the patients and in 91.4% of the controls, and of IL-12 in 50.0% of the patients and in 65.7% of the controls. The corresponding odds ratios were 0.25 (95% CI 0.09-0.69, $p = 0.006$), 0.04 (95% CI 0.01-0.14, $p < 0.001$) and 0.52 (95% CI 0.20-1.36, $p = 0.180$)for TNFα, IL-1β and IL-12, respectively.

3.3. Cytokine BAL fluid levels at baseline

The levels of IL-6 ($p = 0.021$), IL-8 ($p = 0.018$) and IL-18 ($p = 0.002$) in the BAL fluid were significantly higher among the

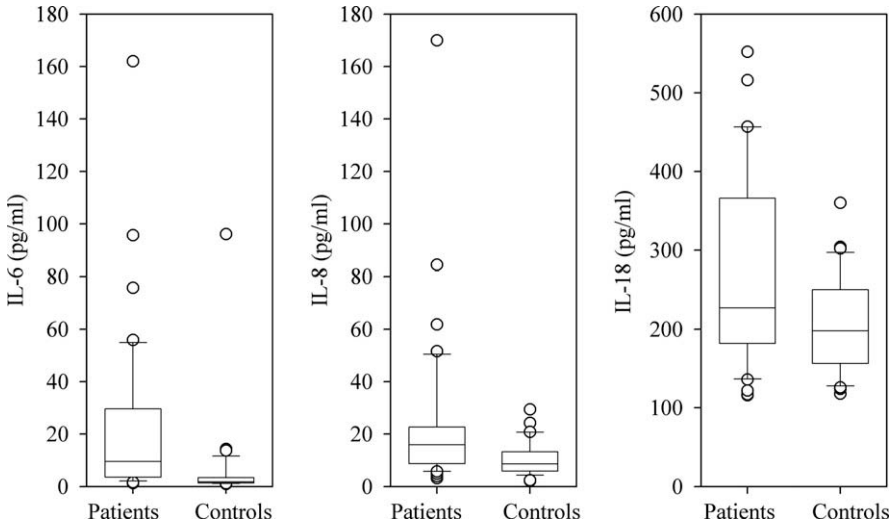

Fig. 1. IL-6, IL-8 and IL-18 in serum at time of diagnosis in lung cancer patients ($n = 36$) and controls ($n = 36$). One IL-18 outlier, 1509 pg/mL, in the patient group is outside the figure. Boxes indicate the lower and upper quartiles and the central line is the median. Whiskers above and below the box indicate the 90th and 10th percentiles. The outliers are plotted with circles.

Table 2
Serum levels of cytokines at baseline in patients and controls.

Cytokines (pg/mL)	Patients ($n = 36$)		Controls ($n = 36$)		Unadjusted patients/controls ratio			Adjusted[a] patients/controls ratio		
	G mean	Range	*G* mean	Range	Mean	95% CI	*p*	Mean	95% CI	*p*
IL-6	10.60	1.27–162.00	2.55	0.90–96.10	4.16	2.42–7.14	<0.001	6.10	2.69–13.83	<0.001
IL-8	15.76	3.11–170.00	8.63	2.12–29.40	1.83	1.27–2.62	0.001	1.72	1.06–2.80	0.030
IL-18	254.1	116.0–1509.0	197.0	118.0–360.0	1.29	1.05–1.58	0.015	1.23	0.93–1.62	0.144
VEGF	216.1	18.2–1262.0	149.6	20.5–845.0	1.44	0.93–2.24	0.098	1.09	0.56–2.12	0.808

[a] ANCOVA, adjusted for current smoking (no/yes), gender, hypertension, age and body mass index.

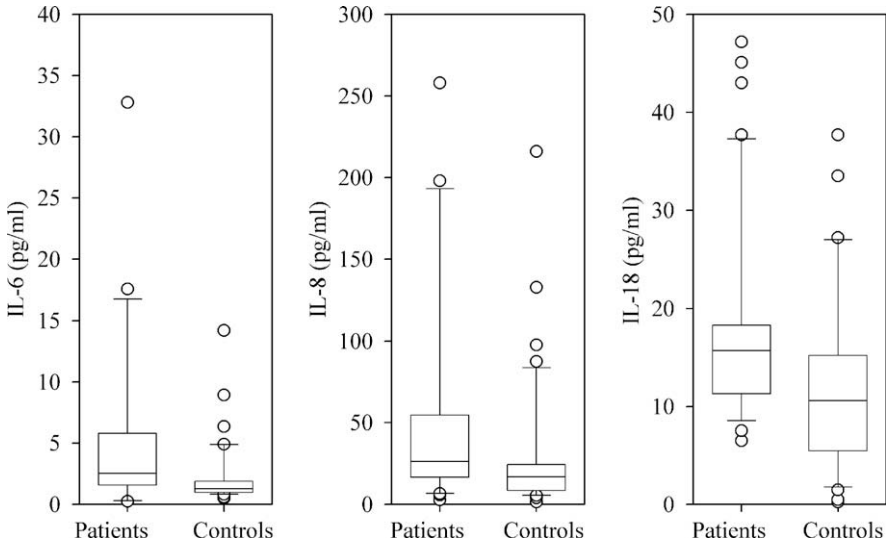

Fig. 2. IL-6, IL-8 and IL-18 in BAL fluid at time of diagnosis in lung cancer patients ($n = 36$) and controls ($n = 36$). The following outliers are outside the figure: IL-6 values 175 pg/mL and 94 pg/mL, and IL-8 values 735 pg/mL and 620 pg/mL.

Table 3
BAL fluid levels of cytokines at baseline in patients and controls.

Cytokines (pg/mL)	Patients ($n = 36$)		Controls ($n = 36$)		Unadjusted patients/controls ratio			Adjusted[a] patients/controls ratio		
	G mean	Range	*G* mean	Range	Mean	95% CI	*p*	Mean	95% CI	*p*
IL-6	2.97	0.25–175.00	1.55	0.50–14.20	1.92	1.11–3.33	0.021	2.43	1.05–5.64	0.039
IL-8	31.79	2.66–735.00	16.48	1.33–216.00	1.93	1.12–3.32	0.018	2.16	0.95–4.93	0.066
IL-18	15.70	6.49–47.20	7.80	0.24–37.70	2.01	1.30–3.12	0.002	1.73	0.92–3.25	0.085
IL-1β	0.10	0.01–2.80	0.41	0.01–13.10	0.24	0.09–0.65	0.006	0.27	0.06–1.21	0.086
VEGF	32.42	1.10–959.00	44.42	2.10–290.00	0.73	0.38–1.41	0.344	1.84	0.88–3.84	0.103

[a] ANCOVA, adjusted for current smoking (no/yes), gender, hypertension, age and body mass index.

lung cancer patients than controls at the time of diagnosis (Fig. 2 and Table 3). The levels of IL-1β were significantly lower among the patients than the controls ($p = 0.006$). The concentration of VEGF was not significantly different ($p = 0.344$). After adjusting for smoking, gender, hypertension, age and BMI, only IL-6 remained significantly higher among the patients ($p = 0.039$), while the differences regarding the concentrations of IL-8, IL-18, IL-1β and VEGF were nearly significant ($p = 0.066$, $p = 0.085$, $p = 0.086$ and $p = 0.103$). Measurable levels of TNFα were detected in 33.3% of the patients and 13.9% of the controls, and of IL-12 in 16.7% of the patients and in 5.6% of the controls. The corresponding odds ratios were 0.32 (95% CI 0.10–1.04, $p = 0.058$) and 0.29 (95% CI 0.06 – 1.57, $p = 0.152$) for TNFα and IL-12.

3.4. Cytokine serum levels during and after RT

The changes in serum VEGF, IL-6, IL-8 and IL-18 concentrations from baseline to two weeks and three months after start of RT were not significant (Table 4).

3.5. Cytokine BAL fluid levels during RT

The geometric mean level of IL-6 in the BAL fluid increased from 2.72 pg/mL (range 0.25–32.8 pg/mL) to 6.38 pg/mL (range 1.04–42.4 pg/mL) after two weeks of RT ($p = 0.037$) (Fig. 3). The levels of VEGF, IL-8 and IL-18 also increased during RT, although not statistically significantly ($p = 0.621$, $p = 0.260$ and $p = 0.382$, respectively). No notable changes took place for TNFα, IL-1β or IL-12 during RT (Table 5).

3.6. Cytokines and clinicopathological variables

There were no significant associations between the levels of the various cytokines and the stage of the disease, patient age or Karnofsky performance status. There were positive correlations between serum and BAL fluid IL-6 and IL-8 levels ($R = 0.344$, $p = 0.040$ and $R = 0.471$, $p = 0.004$, for serum and BAL fluid values respectively). Serum IL-6 also correlated positively with serum IL-18 and VEGF ($R = 0.298$, $p = 0.078$ and $R = 0.476$, $p = 0.003$, respectively). The number of neutrophils in BAL fluid correlated positively with BAL fluid IL-6 and IL-8 levels ($R = 0.352$, $p = 0.035$ and $R = 0.374$, $p = 0.025$, respectively).

3.7. Cytokines, adverse events and response to treatment

Overall toxicity during RT was mild and none of the patients experienced any serious adverse events. Thirteen (65%) patients experienced gr I/II adverse events during RT: esophagitis ($n = 9$), cough ($n = 4$), fatigue ($n = 4$) and fever ($n = 4$). Five patients (25%) developed symptomatic radiation pneumonitis according to the RTOG/EORTC scale. The occurrence of adverse events during RT was not significantly associated with any of the baseline cytokine levels, although adverse events tended to be more frequent when BAL fluid IL-8 was lower. Incidence of adverse events was 100% when IL-8 was < 26.2 pg/mL vs. 57% when IL-8 was > 26.2 pg/mL ($p = 0.077$).

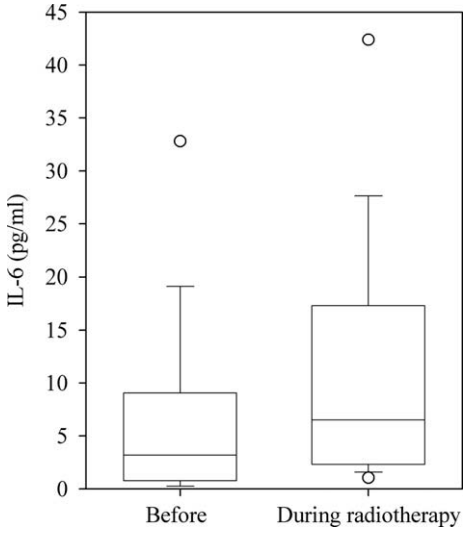

Fig. 3. IL-6 in the BAL fluid before and during radiotherapy in lung cancer patients ($n = 14$).

Twenty-five percent of the patients achieved complete and 56.3% partial treatment response, whereas 6.3% had no change and 12.5% had progressive disease. The response to treatment was not significantly associated with any baseline cytokine levels, although there was a trend to better response to treatment when base-line serum IL-6 levels was higher. Achievement of complete/partial response was 100% when IL-6 was > 9.52 pg/mL vs. 57% when IL-6 was < 9.52 pg/mL.

3.8. Cytokines and overall survival

The planned duration of follow-up of the patient group was 72 months. None of the patients was lost to follow-up. At the end of the study, one patient (3%) was alive, and 35 (97%) had died. The median overall survival time of the patients was 9.9 months (95% CI 5.4–14.4 months). The one-year survival rate was 41.2%, the three-year survival rate 19.4% and the five-year survival rate 7.4%. Overall survival was significantly inversely associated with baseline serum and BAL fluid IL-8 ($p = 0.036$ and $p = 0.027$, respectively) and serum VEGF ($p = 0.014$): higher baseline serum and BAL fluid IL-8 and serum VEGF levels were associated with poorer survival. When the T2 of serum IL-8 was compared to T1, the OR estimate of the risk of dying was 1.80 (95% CI 0.75–4.29) and when T3 was compared to T1 the value was 3.07 (95% CI 1.27–7.45). The corresponding estimates for BAL fluid IL-8 were 0.62 (95% CI 0.25–1.54) and 2.06 (95% CI 0.91–4.70) and for serum VEGF 3.55 (95% CI 1.42–8.84) and 2.37 (95% CI 1.01–5.52) (Fig. 4).

Table 4
Changes in serum levels of cytokines in patients during radiotherapy. The within-group changes from baseline to 'after radiotherapy' and from baseline to 'after 3 months' are given as ratios.

Cytokines (pg/mL)	Baseline ($n = 19$[a])		After radiotherapy ($n = 15$)		After 3 months ($n = 16$)		Ratio after radiotherapy/baseline			Ratio after 3 months/baseline		
	G mean	Range	*G* mean	Range	*G* mean	Range	Mean	95% CI	*p*	Mean	95% CI	*p*
IL-6	7.95	1.27–95.70	8.27	1.82–18.80	10.50	2.16–668.0	1.11	0.55–2.24	0.756	1.08	0.31–3.75	0.890
IL-8	13.37	3.11–170.0	10.08	3.98–36.70	18.61	5.79–362.0	0.84	0.55–1.29	0.407	1.39	0.67–2.86	0.348
IL-18	241.9	117.0–516.0	241.8	124.0–356.0	313.8	172.0–942.0	0.95	0.86–1.04	0.254	1.32	0.97–1.80	0.077
VEGF	184.5	28.4–1262.0	150.3	28.4–623.0	207.4	53.9–898.5	0.87	0.72–1.05	0.124	1.01	0.65–1.59	0.948

[a] Only patients with at least one follow-up measurement are included.

Table 5
Changes in BALF levels of cytokines in patients during radiotherapy. The within-group changes from baseline to 'after radiotherapy' are given as ratios.

Cytokines (pg/mL)	Baseline (n = 14)		After radiotherapy (n = 14)		Ratio after radiotherapy/baseline		
	G mean	Range	G mean	Range	Mean	95% CI	p
IL-6	2.72	0.25–32.80	6.38	1.04–42.40	2.35	1.06–5.20	0.037
IL-8	26.99	5.66–620.0	46.08	3.49–807.0	1.71	0.64–4.55	0.260
IL-18	15.21	6.49–47.20	19.68	4.46–157.0	1.29	0.70–2.39	0.382
VEGF	23.86	1.10–728.0	30.63	1.10–799.0	1.28	0.44–3.72	0.621

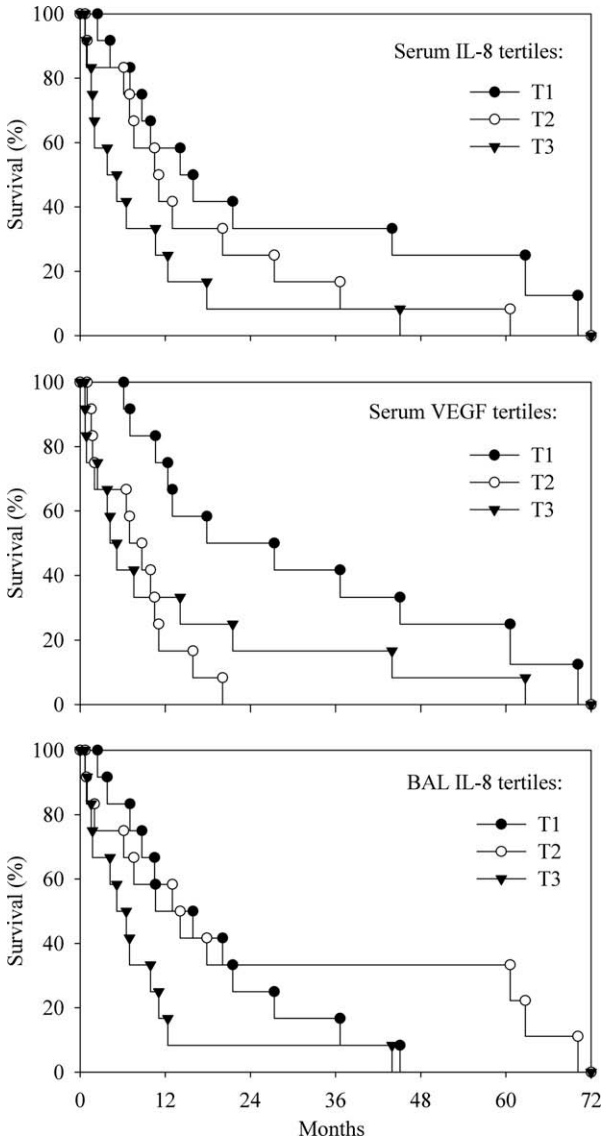

Fig. 4. Survival by serum IL-8, serum VEGF and BAL fluid IL-8 tertiles T1, T2 and T3 (log-rank test p = 0.036, p = 0.014 and p = 0.027, respectively). N = 12 in each group.

4. Discussion

This is apparently the largest study so far evaluating the local and systemic concentrations of an array of different cytokines among lung cancer patients at baseline and during RT. The results show that serum and BAL fluid IL-6 and serum IL-8 are higher in lung cancer patients than non-cancer controls ($p < 0.001$, $p = 0.039$ and $p = 0.030$, respectively) and implies that lung cancer itself upregulates the production of IL-6 and IL-8, and that BAL fluid IL-6 is further increased by the action of RT.

The cytokine levels locally in BAL fluid have not been the subject of very much research. Previous studies have reported higher levels of BAL fluid VEGF, IL-1β, IL-6 and TNFα in lung cancer patients compared to patients with benign lung disease [27–29,16,17]. This study supports these findings, as we recorded higher levels of BAL fluid IL-6 in patients compared to controls ($p = 0.039$) (Table 5).

We also found that BAL fluid IL-6 is further elevated during radiotherapy ($p = 0.037$), which is in conformity with a preliminary observation according to which BAL fluid TGF-β_1 and IL-6 levels rise during RT for lung cancer [30]. IL-6, together with some other cytokines, is a primary mediator of the inflammatory response [11,31], and thus the enhanced level of IL-6 during RT could be due to a local inflammatory reaction. It is equally possible, however, that IL-6 is produced by the immune system in response to RT or that the destruction of tumor cells releases IL-6. In vitro research has shown that IL-6 is produced locally by alveolar macrophages in response to an appropriate stimulus [32]. There is experimental evidence from animal studies that lung IL-6 immunoreactivity increases significantly after pulmonary radiation [33].

We also observed a tendency of VEGF, IL-8 and IL-18 in BAL fluid to rise during RT, a finding not reported previously (Table 5). In vitro studies have shown that there is considerable individual variation in the secretion of interleukins [31], which may explain why the only significant interleukin increase during RT was noted for IL-6. As the data increases, significance emerges, but this would require very large and tedious clinical studies of lung cancer patients.

A previous study has shown that the levels of VEGF in the serum remain unchanged in patients with pharyngeal and laryngeal cancer after 40 Gy of RT [34]. There were no marked changes in the levels of any of the cytokines in the serum of patients during or after RT in this study. Interestingly, but maybe not very surprisingly, the IL-8 concentrations of lung cancer patients were higher in BAL fluid than in the serum at baseline (31.8 pg/mL vs. 15.8 pg/mL, $p = 0.001$) and during RT (46.1 pg/mL vs. 10.2 pg/mL, $p = 0.003$). This implies that the expression of cytokines may be identified and followed with higher precision in the local tumor environment than systemically (Tables 2–5).

The baseline levels of certain cytokines have been associated with overall survival in some tumor types. High plasma and serum IL-8 and IL-6 have been associated with shorter survival of patients with chronic lymphocytic leukemia and squamous cell head and neck carcinoma [35–37]. Also, higher baseline serum VEGF levels have been associated with reduced survival of patients with small cell lung cancer [38–40]. According to other studies, high tumor levels of IL-8 mRNA, VEGF mRNA and protein as well as positive staining of tumor-cell VEGF-C and VEGFR-3 were associated with significantly shorter survival of lung cancer patients [41–43]. Our findings are consistent with these, as we noticed that higher baseline serum and BAL fluid IL-8 and serum VEGF levels were associated with reduced survival of lung cancer patients who primarily have squamous cell carcinoma. Of note, this study is the first one to establish that also IL-8 in the BAL fluid might be a prognostic factor for overall survival.

5. Conclusions

This study shows the importance of assessing the appropriate tumor markers when analyzing the local and systemic cytokine status of lung cancer. Based on the results, it seems that IL-6, IL-8, IL-18 and VEGF play an important role in the complex network of cytokines in lung cancer biology, whereas TNFα, IL-1β and IL-12 seem to be less important.

These results also stress the importance of local markers, and demonstrate that BAL fluid may be a useful medium for analyzing local cytokine production in lung cancer patients. Lung cancer is associated with upregulation of serum and BAL fluid IL-6 and serum IL-8. The changes in the different cytokines during RT are best evidenced at the level of the local tumor environment. IL-6 in the BAL fluid showed a secretion pattern compatible with an acute phase response to RT. None of the examined cytokines prognosticated adverse events during RT or the response to treatment. These data imply that serum and BAL fluid IL-8 and serum VEGF might have a prognostic role in survival of patients with lung cancer.

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