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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.

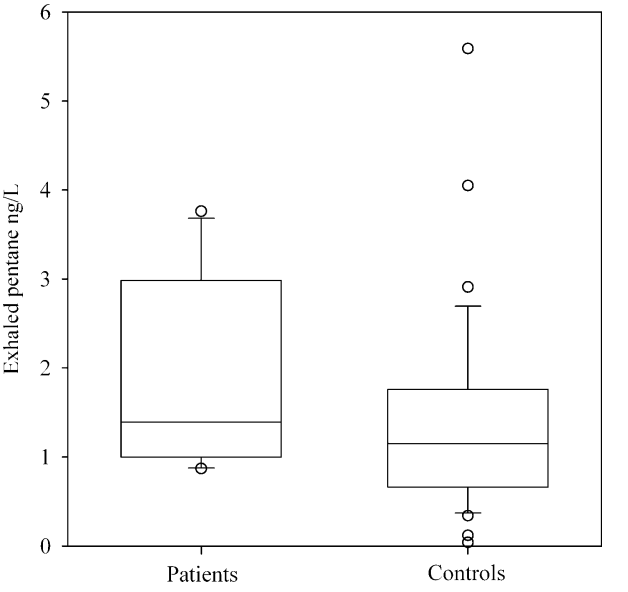

Figure 1. The boxplot figure for the distribution of pentane (ng/L) in patients ($n = 11$) and controls ($n = 30$). Patients vs controls: non-adjusted $p = 0.082$, gender-adjusted $p = 0.017$. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median 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.

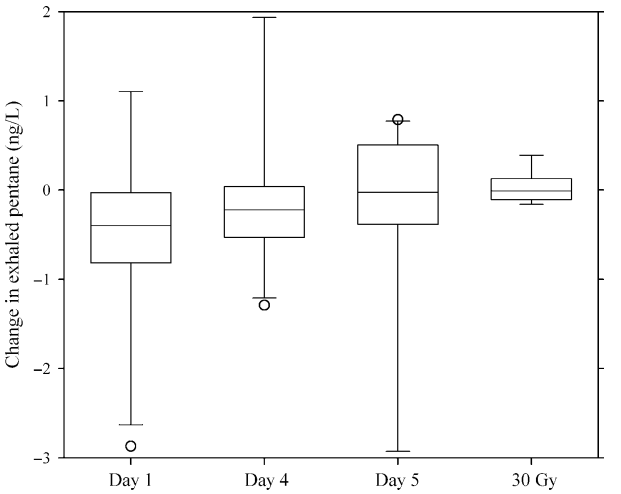

Figure 2. Box-plot figure for changes in exhaled pentane (ng/L) from 0 min to 120 min on RT day 1 ($n=11$), day 4 ($n=10$) and day 5 ($n=8$) and at 30 Gy ($n=5$). Six outliers are outside the figure.

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)

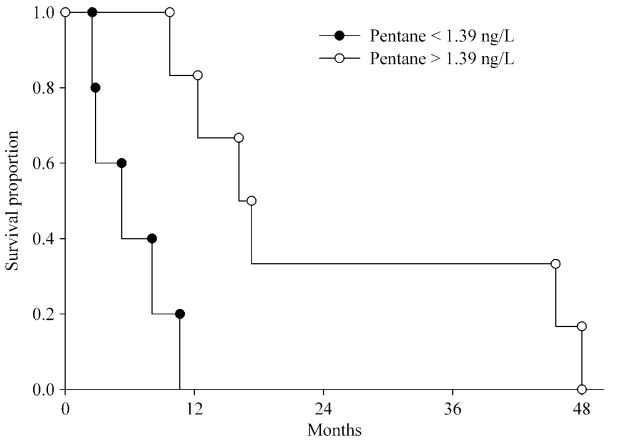

Figure 3. Kaplan-Meier survival plots for patients with baseline pentane <1.39 ng/L ($n=5$) and >1.39 ng/L ($n=6$). Log-rank test $p=0.003$.

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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