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Does radiotherapy increase oxidative stress? A study with nasopharyngeal cancer patients revealing anomalies in isoprostanes measurements

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Abstract
This study aimed to examine if exposure to ionizing radiation during clinical radiotherapy (RT) causes increased oxidative damage. Seven patients with nasopharyngeal cancer (NPC) who underwent RT took part in this controlled-trial study. Blood and urine samples were obtained for F_2-isoprostanes (F_2-IsoPs) measurement. Urinary F_2-IsoPs levels were elevated pre-treatment and remained high (but did not increase) during treatment, but decreased to the normal range after treatment. Plasma F_2-IsoPs decreased significantly after the start of treatment before rising midway through treatment. Levels decreased significantly to below baseline following treatment. However, the patients were observed to have substantially lower levels of plasma esterified arachidonic acid (AA) residues than controls. The data shows that NPC is associated with elevated F_2-isoprostanes in urine and in plasma after correction for decreased AA levels. RT did not increase these levels and, indeed, was associated with falls in F_2-IsoPs. The validity and usefulness of correction of plasma F_2-IsoPs for lowered AA levels is discussed.

Keywords: *Isoprostanes, free radicals, radiotherapy, nasopharyngeal cancer*

Introduction

Ionizing radiation causes damage to biomolecules, in part by causing the generation of highly reactive radicals such as hydroxyl radical, $OH^{\bullet}$ [1,2]. However, few studies have examined the degree of oxidative stress produced in humans by exposure to radiation, particularly during radiotherapy [2–4]. Radiotherapy (RT) involves the use of high dose ionizing radiation to deliver a cytotoxic level to the tumour whilst minimizing exposure to normal tissue. RT is used with or without chemotherapy as the primary curative treatment in certain cancers, e.g. nasopharyngeal carcinoma (NPC). However a substantial heterogeneity in patient response to radiation exists. This may be due to intrinsic differences in cellular radiosensitivity of individuals [3].

Measurement of prostaglandin (PG)-like substances that are produced by oxidation of arachidonic acid, such as F_2-isoprostanes (F_2-IsoPs), has been established as a reliable method to assess oxidative lipid damage *in vivo* [5]. This approach allows for quantification of oxidative lipid damage in a wide variety of human illnesses, including cardiovascular, pulmonary, neurological, renal and liver disease [5–7], and has led to the preliminary development of disease predictive tools based on combining parameters of oxidative damage with those of redox state, DNA-repair activity, antioxidant defences and body mass index [8,9].

To date, however, there have been few studies which have established the effect of radiotherapy on F_2-IsoPs. One previous study analysing the effect of

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radiotherapy for prostate cancer on urinary levels of F_2-IsoPs failed to show any effect of radiotherapy on urinary levels. However the authors acknowledged problems in their study which may have accounted for the negative result [10]. Another study demonstrated a correlation of change in levels of antioxidant enzymes with susceptibility of lung cancer patients to radiation pneumonitis [11]. The question is important as there are widespread speculations as to whether administration of antioxidants could be beneficial or deleterious in radiotherapy [12,13] and knowledge about oxidative damage levels may help inform the debate.

The aim of this study was to determine if radiotherapy given with curative intent will alter the levels of F_2-IsoPs in plasma and urine of NPC patients. A second aim was to examine if variation in F_2-IsoPs levels correspond to increasing incidence of acute toxicity and to rates of local control following treatment.

Methods and materials

Patient accrual

Patients with American Joint Committee on Cancer (AJCC) stage IIb to III, World Health Organization (WHO) type 3 nasopharyngeal cancer (NPC) were recruited. Under the consent of the Institutional Review board (National Healthcare Group, Singapore), blood samples from patients undergoing chemoradiotherapy (CRT) were used for this study. All patients had a staging magnetic resonance imaging (MRI) scan of the head and neck, computed tomography (CT) thorax and liver scan, bone scan and liver and renal function tests. Patients were excluded if they had received induction chemotherapy or prior radiotherapy, had an unfavourable Eastern Cooperative Oncology Group (ECOG) performance status (2 or greater) or had a past medical history of renal impairment, cardiac disease or liver failure.

Treatment, sample collection and clinical assessment

All patients were treated with intensity modulated radiotherapy (IMRT) delivered with multi-leaf collimation on a Siemens linear accelerator (Siemens Healthcare, Erlangen, Germany). An extended field IMRT technique was used to treat the primary tumour along with all the regional lymph nodes, including the supraclavicular nodes. This was achieved with a beam arrangement consisting of seven equispaced coplanar beams.

The prescribed dose was 69.96 Gray to the gross tumour volume (GTV) and positive neck nodes, 59.4 Gray to the clinical target volume (CTV 59.4), which included the GTV and high risk adjacent areas such as the entire nasopharynx, anterior half of the cilvus, skull base and level II, III and V cervical nodes and 54 Gray to bilateral uninvolved lower neck regions (CTV54). A margin of 10 mm was given to all volumes to account for potential microscopic disease and daily set-up variations. Dose to critical structures in the head and neck region such as the brainstem, optic nerves and optic chiasm was limited to 50–54 Gray in order to minimize the risk of long-term side-effects due to radiation, e.g. brainstem necrosis.

Radiotherapy was delivered in 33 fractions, once daily for 5 days a week (total 6.5 weeks) at 2.12 Gray/fraction/day to the GTV, 1.8 Gray/fraction/day to the CTV 59.4 and 1.64 Gray/fraction/day to the CTV 54 dose to surrounding critical structures.

All patients also received weekly low dose chemotherapy (IV cisplatin 40 mg/m^2) concurrent with RT. They were reviewed for toxicity once a week during treatment. Incidence and severity of mucositis, xerostomia and skin dermatitis were scored in accordance with the Common Toxicity Criteria for Adverse Events, version 3.

Follow-up reviews were conducted 1 week after completion of treatment, 6 weeks after treatment and then at 3 monthly intervals. Repeat imaging by either MRI or CT was assessed at 3 months post-RT for radiologic response to therapy.

Plasma and urine collection and analysis

Blood and urine samples were collected from patients once weekly within 30 min after treatment. Timing of sample collection and assessment was taken from the time the first sample was obtained during the radiotherapy planning CT scan (week 0). Radiotherapy was started 3 weeks later and the first sample collected during the first week of radiation (week 3). Radiotherapy was completed 6 weeks later and the last sample collected during the final week of radiation (week 8). Final sample collections were done at 6 weeks post-treatment (week 14) and at 3 months (12 weeks) post-treatment (week 20). A total of nine time points of blood and urine was collected from every patient.

Blood samples were obtained by venipuncture into EDTA blood tubes. They were then centrifuged at 3000 rpm for 10 min at 4°C and the plasma portion was aspirated and placed in microtubes pre-loaded with indomethacin and BHT to prevent artefactual oxidation of lipids [14]. The samples were then analysed for F_2-isoprostanes (F_2-IsoPs). A total of 2 ml of midstream urine was also collected for F_2-IsoPs analysis.

For extraction of total (free+esterified) form of F_2-IsoPs and total arachidonate, deuterated standard was added to 1 ml plasma which was then hydrolysed at 37°C for 30 min with 1 ml of 1 M potassium hydroxide prepared in methanol, to release the esterified

lipids [15,16]. Afterwards, 0.5 ml of methanol, 0.2 ml of 5 M HCl and 2.5 ml of 40 mM formic acid (pH 4.6) were further added and mixed. For measurement of free forms in plasma and urine for F_2-IsoPs deuterated standard and 1 ml of formic acid (40 mM, pH 4.5) was added to 1 ml of sample, mixed and then immediately processed by anionic solid phase extraction (SPE) [15,16]. For standardizing the dilution of urine, creatinine levels were measured using the Sigma (St Louis, Missouri, USA) diagnostic kit. The extracted samples were derivatized [15,16] for gas chromatography-mass spectrometry/negative chemical ionization (GC-MS/NCI) analysis. Quantitation was achieved by relating the peak area of the total and free forms of F_2-IsoPs and total arachidonate with their respective peaks of deuterated internal standard [15,16].

Statistical analysis

The data are expressed as the median ± SD. Changes in oxidative stress markers from baseline were compared using the Generalized Estimating Equation (GEE) models as described in Zeger and Liang [17]. These models help to correctly account for any inherent within-patient correlation in the parameters. For the GEE models, we specified the exchangeable correlation structure in all analyses. The naive statistical methods would include the *t*-tests and analysis of variance (ANOVA). However, the main assumptions for these tests include observations being independent, which is not true in this study, as the observations are repeated for the same patients. To study the impact of changes in covariates such as mucositis and xerostomia with each outcome, we specified an interaction term consisting of each covariate against week. Data analysis was performed using Stata V10.2 (Stata Corp, College Station, TX), with level of significance set at 5%.

Results

Seven male patients were enrolled in the trial and their characteristics are summarized in Table I. All patients completed the prescribed course of radiotherapy with chemotherapy and had samples taken at all time points. A group of age and sex matched controls ($n=7$) was also included in this study to serve as healthy reference controls and samples were taken at similar time points.

Urinary F_2-isoprostanes levels

As shown in Figure 1, baseline median urinary F_2-IsoPs levels standardized to creatinine, 1.04 ng/mg Cr (0.42–1.73), were significantly higher ($p=0.04$) than control levels, 0.61 ng/mg Cr (0.15–0.84) at all time points, before and during RT. There was no significant change in the levels during treatment. Upon completion of treatment, urinary F_2-IsoPs levels had become significantly lower than baseline levels, 0.58 ng/mg Cr (0.16–1.0) at week 14 ($p=0.04$) and 0.31 ng/mg Cr (0.18–0.84) at week 20 ($p=0.007$) and had moved into the range of the control subjects.

Table I. Patient characteristics.

	Patients	Control
Number	7	7
Age		
Mean	53	55
Range	42–68	50–63
ECOG performance status		
0	2	–
1	5	–
AJCC clinical stage		
IIb	1	–
III	6	–

Plasma F_2-isoprostanes levels

In contrast to the results from urine, baseline median total plasma F_2-IsoPs 0.71 ng/ml (0.49–1.09) and esterified F_2-IsoPs 0.68 ng/ml (0.44–1.03) levels prior to starting therapy were not significantly different from baseline median levels of the healthy controls, 0.71 ng/ml (0.18–1.03) and 0.64 ng/ml (0.13–0.89) (Figure 2), respectively. Upon commencement of RT, levels of total F_2-IsoPs and esterified F_2-IsoPs declined, with a significant decrease in levels compared to baseline by week 4 of RT 0.57 ng/ml (0.38–0.70) and 0.52 ng/ml (0.33–0.64), in both cases $p < 0.05$. There was then a significant rise in levels in week 5, when compared to week 4 ($p < 0.05$), followed by a continuous rise until the end of RT to reach the initial levels.

Post-treatment showed a significant decrease in total F_2-IsoPs and esterified F_2-IsoPs in levels to below baseline 0.51 ng/ml (0.32–0.69) and 0.46 ng/ml (0.29–0.62) at week 14 ($p=0.01$) and at week 20 0.45 ng/ml (0.34–0.73) and 0.39 ng/ml (0.30–0.67) ($p < 0.001$). These levels were still significantly lower compared to control levels, $p < 0.05$. There was no significant change in free F_2-IsoPs levels during treatment or upon completion of treatment when compared to baseline levels.

These studies therefore appear to show no rises (in fact falls) on F_2-IsoPs levels during CRT. However, F_2-IsoPs arise from attack of free radicals and other ROS on arachidonic acid residues (AA) in membranes and lipoproteins. As shown in Figure 3 (bottom panel), plasma total arachidonate levels of the patients are significantly lower than control levels ($p < 0.005$) and remained consistently low, with no significant changes throughout the course of treatment.

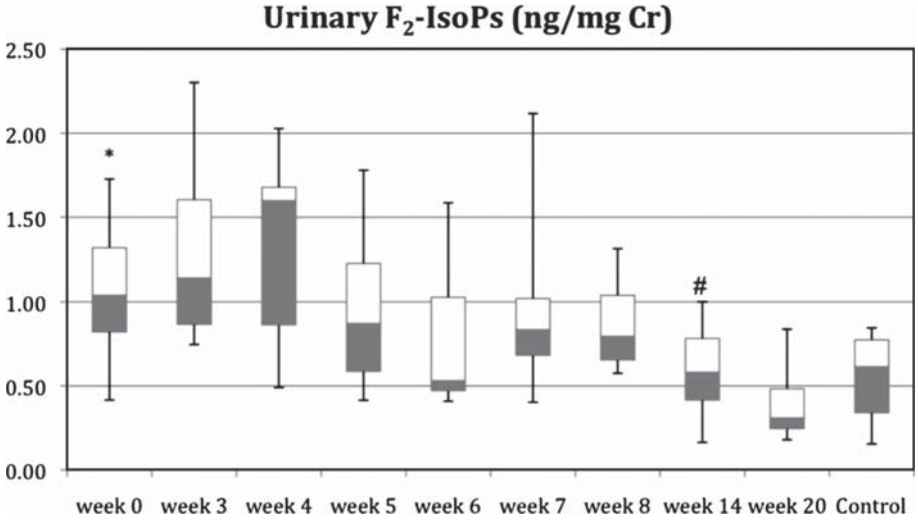

Figure 1. Urinary F_2-IsoPs levels over time in NPC patients. The boxes indicate the 25th and 75th percentile (the lower and upper edge, respectively) and the lines between the shadings within the boxes represent the median. The points at the ends of the whiskers are the greatest or the smallest points. *Indicates the significant elevated baseline levels compared to controls. # Indicates the significant decrease in levels compared to baseline following treatment. R is the ranges in the healthy control subjects.

If we corrected for the lowered arachidonic acid, as shown in Figure 3, median baseline levels of total F_2-IsoPs/AA 16.3 pg/μg (13.4–26.7), esterified F_2-IsoPs/AA 15.0 pg/μg (12.5–25.1), were significantly elevated compared to controls ($p < 0.005$) at all time points, both before and during radiotherapy, but decreased at weeks 14 and 20.

Toxicity

All patients experienced xerostomia, mucositis, dermatitis and weight loss during treatment. Incidence rate by week is shown in Table II. Following completion of treatment, other than xerostomia, all other acute toxicities had resolved. No correlation was observed between levels of urinary or plasma F_2-IsoPs (whether corrected for AA or not) in relation to incidence and severity of acute toxicity.

Patients had a significant loss of weight each week ($p=0.001$) and overall lost an average of 14 kg by completion of treatment. There was no further weight loss after completion of treatment, but patients' weights remained significantly less compared to pre-treatment levels ($p=0.01$). There was no association between weight loss and changes in levels of AA or F_2-IsoPs whether corrected for AA or not (data not shown).

There were no cases of neutropenic sepsis during the course of concurrent chemoradiotherapy. While the absolute neutrophil count of all patients did fluctuate during treatment, there was no significant reduction in level compared to baseline nor was there any correlation found with variations in levels of AA or F_2-IsoPs.

Response

All patients achieved a complete response by 3 months post-treatment (week 20), as confirmed by clinical examination, nasoendoscopy and radiologic imaging. Response to treatment was significantly associated with decrease in the levels of total and esterified F_2-IsoPs. Even after adjusting for arachidonic acid, decreases in mean levels of plasma total and esterified F_2-IsoPs were still significantly associated with response ($p < 0.01$). Similarly, a decrease in level of urinary F_2-IsoPs levels was also significantly associated with response to treatment ($p < 0.001$).

Discussion

In this study to assess the relationship of oxidative lipid damage to ionizing radiation exposure in humans, we decided to focus on patients with nasopharyngeal carcinoma (NPC) only. The rationale for NPC patients is that the 'gold standard' of treatment is RT alone or with low dose chemotherapy (as used here). A randomized trial previously conducted to assess supplementation with antioxidant vitamins in patients with head and neck cancers treated with radiotherapy demonstrated that, while toxicity was reduced, local failure rates were higher as the efficacy of radiation therapy may have been compromised [13]. This emphasizes the uncertainty surrounding the use of antioxidants, a problem which may be amendable to further study, first by establishing if systemic oxidative stress is really increased by radiotherapy, a question examined in our paper.

Our study illustrates that, before treatment, patients with NPC have elevated levels of urinary F_2-IsoPs and, after adjusting for arachidonic acid, total and esterifed F_2-IsoPs. Pre-treatment patients with cancer had significantly lower arachidonic levels than healthy controls and these levels remained low throughout treatment. This could have been due to their reduced

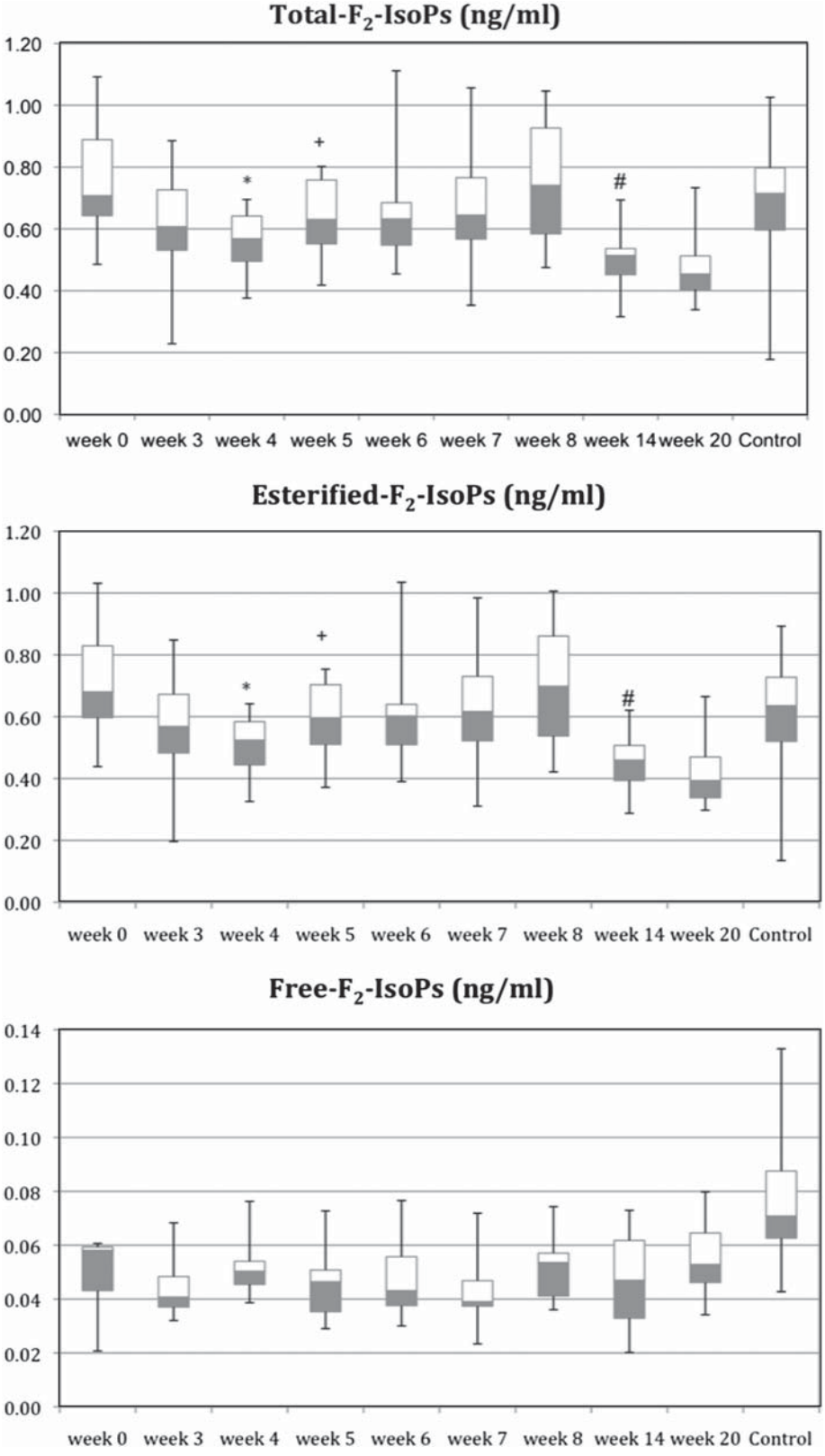

Figure 2. Comparison of control plasma F_2-IsoPs levels over time in NPC patients. The boxes indicate the 25th and 75th percentile (the lower and upper edge, respectively) and the lines between the shading within the boxes represent the median. The points at the ends of the whiskers are the greatest or the smallest points. *Indicates the significant change from baseline levels to the nadir during treatment, + indicates the significant rise from the nadir during treatment and # indicates the significant decrease in levels compared to baseline following treatment.

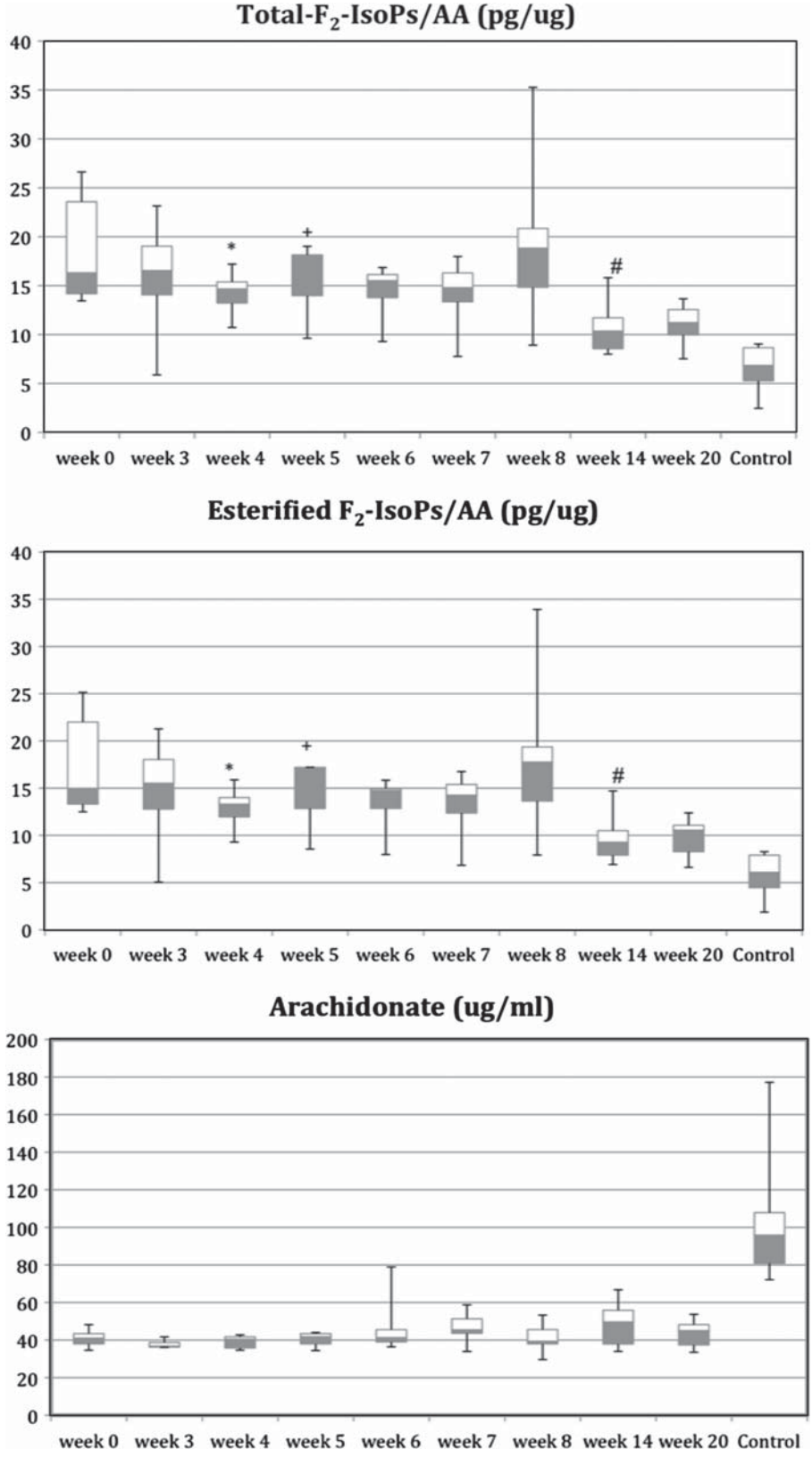

Figure 3. Comparison of F_2-IsoPs levels after accounting for arachidonic acid levels over time in NPC patients. The boxes indicate the 25th and 75th percentile (the lower and upper edge, respectively) and the lines between the colours within the boxes represent the median. The points at the ends of the whiskers are the greatest or the smallest points. *Indicates the significant change from baseline levels to the nadir during treatment, + indicates the significant rise from the nadir during treatment and # indicates the significant decrease in levels compared to baseline following treatment.

Table II. Incidence of acute toxicity and weight loss during the course of treatment.

Incidence of toxicity (week)	0	3	4	5	6	7	8	14	20
Dermatitis (%)	0	0	15	28	43	85	92	0	0
Mucositis (%)	0	0	42	42	57	71	84	0	0
Xerostomia (%)	0	0	84	92	92	100	100	86	71
Average weight (kg)	75	74	73	71	69	68	66	61	60

nutritional state and/or changes in lipid metabolism. Our F_2-IsoPs data suggest that patients are already under substantial oxidative stress, which could be related to the presence of cancer (since cancer may itself cause oxidative stress as well as result from it [8]), to impaired dietary or other antioxidant status (perhaps consequent on poor nutrition) or both.

Impact of radiotherapy on plasma levels of F_2-IsoPs

Perhaps surprisingly, radiotherapy did not increase F_2-IsoPs. We have shown that there is a sequential change in the levels of total and esterified F_2-IsoPs levels in the patients during radiotherapy; a rapid *decline* in the marker levels after start of treatment, followed by a continuous rise mid-treatment and a second drop to below baseline levels after completion of treatment. This sequential change in total and esterified F_2-IsoPs levels was demonstrated even after adjusting for arachidonic acid levels. Following the start of radiotherapy, there was a drop in the F_2-IsoPs compared to pre-treatment levels. If the cancer is causing oxidative stress, this could be due to a reduction in the tumour size following the effects of radiotherapy, resulting in less ROS production by the tumour and/or less tumour associated inflammation [8]. In a study by Barker et al. [18], which investigated the changes in tumour volume in head and neck cancer patients treated with radiotherapy, the gross tumour size was reduced by a median rate of 2 cm^3 per treatment day.

Despite the continuous shrinkage of the tumour during treatment, levels of F_2-IsoPs demonstrated a subsequent significant rise after the 2nd and 3rd weeks of radiotherapy. This seems to coincide with the increase in incidence of acute radiation toxicity and could be due to secondary efforts of the radiotherapy, perhaps inflammation and associated increased ROS production. We were, however, unable to establish an association between the levels of F_2-IsoPs and the incidence and severity of toxicity. This could be due to the study not having sufficient statistical power in this small number of patients. Indeed, one potential criticism of our study is the small number of patients. However, all subjects completed the study and gave samples at the required times, and the samples size was sufficient to reach clear conclusions for the F_2-IsoPs data.

Post-radiotherapy patients showed a significant drop in the total and esterified F_2-IsoPs levels to levels significantly lower than baseline, suggesting that the oxidative stress status (at least as far as can be determined by this biomarker) of the patients had returned to normal. This change in levels was significantly associated with a response to treatment. This is perhaps expected as all patients achieved a complete response to treatment, i.e. the tumour had apparently disappeared and the chemoradiotherapy has ceased. Long-term follow-up will also be required to determine if a regrowth of the cancer will result in a rise in the levels. Potentially, oxidative stress markers might then be used as tools to assess for response to treatment as well as monitor for a relapse.

Sequential changes in urinary F_2-IsoPs levels

Similar to that seen in the AA-corrected plasma levels, baseline levels of urinary F_2-IsoPs were elevated compared to healthy controls, probably due to the effect of the tumour on the patients. In line with the results previously demonstrated by Camphausen et al. [10] in prostate patients undergoing radiotherapy, there was no significant rise in the levels of urinary isoprostanes during treatment nor any correlation with toxicity. However, post-treatment levels of urinary isoprostanes were significantly lower than baseline and correlated with a complete response to treatment.

Anomalies in expressing F_2-IsoPs data

F_2-IsoPs levels in plasma are usually expressed per unit volume. However, in this study (and possibly also in some human intervention studies with pro-oxidants, antioxidants or foodstuffs and in several other diseases), there are substantial changes in plasma total AA levels. When we correct F_2-IsoPs levels for this, different conclusions can result (Figures 2 and 3). This sort of correction is of uncertain validity and was recently discussed in detail [19]. More research is needed to see to what extent variations in the levels of substrates for oxidative damage can alter levels of biomarkers [19], but there is some evidence that they can. For example, a recent study showed that supplementation of animals with DHA decreased F_2-IsoPs levels, apparently by raising DHA and making it a preferred target of oxidation [20]. Infusion of non-esterified fatty acids in obese hypertensive adults increased plasma F_2-IsoPs [21]. Our data add to the list of studies revealing that measurements of F_2-IsoPs on urine and plasma may not always give the same answers in relation to human disease and that it is good to measure both (for a more detailed discussion, please see Halliwell and Lee [19]).

Conclusion

This study demonstrates that the presence of NPC in patients is associated with elevated plasma (after correction for AA) and urinary levels of F_2-IsoPs. This is the first study to demonstrate a sequential change in levels of an oxidative stress marker during radiation treatment and after cessation of radiation as well as a correlation with response with most of the markers. Of course, since concurrent chemotherapy was used (albeit only at a small radiosensitizing dose), we cannot say that radiotherapy alone was responsible for the results. Nevertheless, we can say that the radiation itself did not immediately increase F_2-IsoPs levels. The later rise during treatment may be amendable to amelioration with antioxidants. This will be the subject of further studies, but patients will also need to be followed long-term to determine the predictive value of F_2-IsoPs for chronic radiation toxicity as well as relapse of disease in relation to antioxidant administration.

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