Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial

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Summary

Background The concentration of osteopontin (SPP1) in plasma is associated with tumour hypoxia. The DAHANCA 5 trial found that the hypoxia radiosensitiser nimorazole significantly improved the outcome of radiotherapy for patients with head and neck cancer compared with placebo. However, whether all patients benefit from such modification of hypoxia is unclear. We aimed to assess whether the concentration of plasma osteopontin could predict response to the hypoxia radiosensitiser.

Methods Plasma concentrations of osteopontin were measured by use of ELISA from stored samples of 320 patients randomised in the DAHANCA 5 trial. Samples were grouped into tertiles according to high (167–1382 μg/L), intermediate (69–166 μg/L), or low (0–68 μg/L) concentrations of plasma osteopontin, and analysed for locoregional tumour control and disease-specific survival at 5 years.

Findings Overall, locoregional tumour failure and disease-specific mortality were more frequent in patients assigned placebo than in those assigned nimorazole (relative risk [RR] 0·51 [95% CI 0·32–0·79] and 0·54 [0·35–0·85], respectively). Locoregional tumour failure was more frequent in patients with high concentrations of osteopontin assigned placebo than in those with high concentrations assigned nimorazole (0·19 [0·08–0·44]), as was disease-specific mortality (0·25 [0·11–0·59]). However, neither locoregional tumour failure nor disease-specific mortality differed between groups for patients with low concentrations of plasma osteopontin (0·79 [0·26–1·70]) and (0·69 [0·31–1·51]) or for those with intermediate concentrations (0·90 [0·41–1·98] and 0·89 [0·41–1·96], respectively).

Interpretation High plasma concentrations of osteopontin are associated with a poor outlook after radiotherapy for patients with head and neck cancer, but can be improved by use of nimorazole. High concentrations of osteopontin can predict clinically relevant hypoxia, and might identify patients who will benefit from modification of hypoxia during radiotherapy.

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Introduction

Oxygen needs to be present in cells and tissues to ensure maximum biological damage from the ionising radiation used in conventional radiotherapy. Consequently, hypoxia causes resistance to radiation in cells in an environment of less than 3–4 mm Hg oxygen at the time of radiotherapy. This resistance, expressed as the oxygen enhancement ratio, is usually 2·5–3·0. Thus, hypoxic cells need a dose three times higher than that of non-hypoxic cells.[1] Evidence is strong and accumulating that many solid tumours are hypoxic,[2] and squamous-cell carcinomas of the head and neck and uterine cervix are prime examples.[2]

Several attempts have been made to modify hypoxia during radiotherapy for the the past 50 years, and randomised trials have shown more or less beneficial results.[1,3] A meta-analysis[1] of the effect of hypoxia modification for radiotherapy in squamous-cell carcinomas has confirmed that such modification significantly improves locoregional tumour control and survival if done adequately. The hypoxic environment of the tumour can be changed by use of: methods that increase oxygen delivery to the area (eg, hyperbaric or normobaric oxygen); oxygen mimetic drugs such as nitroimidazoles; or hypoxic cytokines that selectively kill hypoxic cells.[1,3,4] Several large randomised trials are under way to establish the indications for hypoxia-modification treatment, most of which are focusing on squamous-cell carcinoma of the head and neck because patients with this tumour type are likely to benefit from hypoxia modification.

Hypoxic tumours have a poor prognosis[5–7] not only because the lack of oxygen makes them less susceptible to radiotherapy, but also because gene activation induced by hypoxia makes them more aggressive than non-hypoxic tumours.[8–11] Although hypoxia seems to be a feature of many malignant solid tumours, not all tumours are hypoxic and some tumours might be more hypoxic than others;[2] moreover, modification of hypoxia will probably not be needed in the treatment of all tumours.

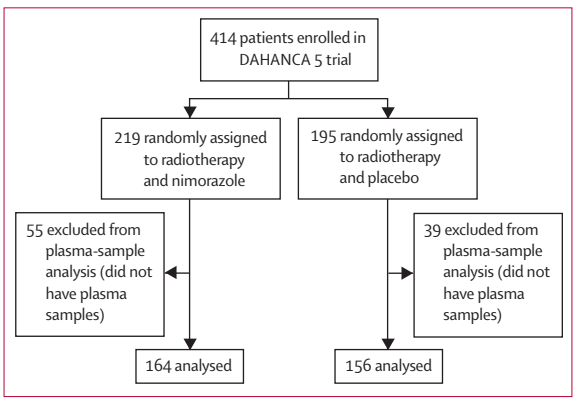

Figure 1: **Study profile**

In head and neck cancer, the presence of hypoxia, its resultant resistance to radiotherapy, and its heterogeneous nature have been established. Thus, markers for hypoxia must be identified that are not only prognostic but also predictive for types of hypoxia that can be modified to improve the effect of radiotherapy. Prospective randomised trials are needed to identify these markers, the findings of which should show that the intervention used to modify the hypoxic environment is beneficial compared with controls. The Danish head and neck cancer study group's DAHANCA 5 trial[12] is a large randomised trial that showed that use of the hypoxia sensitiser nimorazole (Naxogin®, Pfizer [Pharmacia], Italy) significantly improved the outcome of conventional radiotherapy compared with placebo. Both radiotherapy and nimorazole were tolerated well. Compliance with radiotherapy did not differ between groups, and 98% of patients completed the planned radiotherapy. About 75% of patients allocated nimorazole had at least 25 treatments; the rest discontinued treatment, mainly because of nausea. No late nimorazole-related toxic effects were noted.[12] The findings from the trial resulted in the introduction of nimorazole as part of standard treatment for patients with head and neck cancer in several countries, but whether all patients need such adjuvant treatment is unclear.

Three methods can be used to identify hypoxia in tumours: measurement of oxygen partial pressure, which is frequently done with polarographic electrodes such as the Eppendorf histograph[13] and include measurements obtained from necrotic tissue; use of exogenous markers, expression of which is reduced in the absence of oxygen and can be identified by immunohistochemical analysis or imaging techniques such as PET[14–16] (eg, nitroimidazoles that bind to metabolically active hypoxic cells after reduction); and analyses of genes and proteins that might be activated in the presence of hypoxia (eg, those associated with activation of the von Hippel-Lindau cascade such as hypoxia-inducible factor 1α and carbonic anhydrase IX, and other related factors).[17–19]

	Low osteopontin		Intermediate osteopontin		High osteopontin	
	Placebo (n=45)	Nimorazole (n=62)	Placebo (n=58)	Nimorazole (n=47)	Placebo (n=53)	Nimorazole (n=55)
Osteopontin (μg/L)						
Median (range)	48 (12–68)	45 (12–68)	113 (69–156)	113 (69–157)	464 (167–1382)	313 (170–1088)
Age (years)						
Median (range)	58 (36–80)	60 (25–76)	55 (34–79)	62 (28–76)	58 (24–84)	62 (21–84)
Sex						
Women	14	18	13	10	14	17
Men	31	44	45	37	39	38
Tumour location						
Supraglottic larynx	14	17	14	18	17	14
Pharynx	31	45	44	29	36	41
Tumour stage*						
T1–2	29	27	25	22	18	22
T3–4	16	35	33	25	35	33
Nodal status*						
Negative	25	33	27	27	19	21
Positive	20	29	31	20	34	34
Disease stage*						
I–II	14	17	11	13	7	7
III–IV	31	45	47	34	46	48
Tumour differentiation						
Well	26	41	46	29	29	35
Moderate	15	17	10	10	15	14
Poor	4	4	2	8	9	6
Haemoglobin concentration						
High	28	36	36	23	32	36
Low†	17	26	22	24	21	19

*International Union of Cancer Research (UICC) 1982 classification. †<130 g/L in women, <145 g/L in men.

Table 1: **Patient and tumour characteristics**

Several of these hypoxic markers have been associated with outcome after radiotherapy for head and neck cancer,[6,7,20,21] but none have yet been shown to be truly predictive. Le and colleagues[22] investigated potential endogenous markers by screening hypoxic cancer cells, and identified several factors that were associated with changed expression of the von Hippel-Lindau (*VHL*) gene. The most promising marker identified was osteopontin (SPP1), which was inversely associated with *VHL* expression. Previous studies[11,18,22,23] showed that osteopontin seemed to be activated in hypoxia, and, more importantly, its concentration could also be measured easily in plasma. Le and colleagues[22] found an association between concentrations of plasma osteopontin above the median tumour hypoxia, and in a subsequent study[23] we confirmed the relation between plasma osteopontin, hypoxia measurements with the Eppendorf histograph, and outcome after radiotherapy in 63 patients with squamous-cell carcinoma of the head and neck. We divided patients into tertiles on the basis of plasma osteopontin concentration,[23] and noted that patients in the lower or middle tertile had moderately severe tumour hypoxia and a good locoregional control after radiotherapy. By contrast, patients in the upper tertile were characterised by having more severely hypoxic tumours and a significantly worse outcome than did those in the lower or middle tertile (p=0·002). We concluded[23] that patients with high plasma concentrations of osteopontin were a poor-prognosis group that is characterised by having the most severely hypoxic tumours.

On the basis of this study, we hypothesised that high concentrations of plasma osteopontin might identify patients with hypoxic tumours, for whom the effects of radiotherapy could be improved with a hypoxia modifier. We aimed to assess whether patients with high concentrations of osteopontin in plasma samples from patients in the DAHANCA 5 trial (ie, those in the upper tertile) were associated with resistance to radiotherapy, and whether this resistance could be counterbalanced by use of nimorazole as a hypoxia modifier.

Methods

The DAHANCA 5 trial

The DAHANCA 5 study was done between 1986 and 1990, and randomly assigned 414 patients with advanced cancer of the supraglottic larynx or pharynx to conventional radiotherapy and placebo (n=195) or to the same radiotherapy and nimorazole (n=219; figure 1).[12] The treatment protocol has been described previously.[12] Briefly, radiotherapy was applied, in accordance with DAHANCA guidelines, to the primary tumour and involved lymph nodes at a minimum dose of 62–68 Gy (given as five fractions a week at 2 Gy per fraction). The dose depended on tumour size: patients with primary tumour, nodes, or both of 2 cm or less at the widest point received 62 Gy, those with tumours between 2 cm and 4 cm received 64 Gy, and those with tumours 4 cm or greater received 66–68 Gy. These doses were the minimum recommendations, and a higher dose was allowed. Radiation fields included the first non-involved lymph-node station, but were reduced to include only the initially macroscopically known tumour after 50 Gy in 5 weeks. Patients allocated nimorazole were scheduled to receive 1·2 g/m^2 of the drug orally 90 min before radiotherapy for the first 30 fractions. Patients allocated placebo were scheduled to receive gelatine capsules identical in appearance and in lack of taste to nimorazole capsules, and were also to be taken orally 90 min before radiotherapy for the first 30 fractions. All patients received the same radiotherapy protocol, and none received either chemotherapy or surgery as a part of primary treatment. The study was double blind, and clinical investigators have never been informed of the randomisation code. The study was done in accordance with the Helsinki Declaration II and approved by the local ethics committees. Patients gave written informed consent, which included the use of plasma samples for analyses.

	n	Locoregional tumour failure		Disease-specific mortality	
		Event (%)	Relative risk (95% CI)	Event (%)	Relative risk (95% CI)
All patients					
Nimorazole	164	79 (48%)	0·51 (0·32–0·79)	72 (44%)	0·54 (0·35–0·85)
Placebo	156	101 (65%)		92 (59%)	
All patients					
Osteopontin below median 113 μg/L	158	83 (53%)	0·74 (0·48–1·15)	68 (43%)	0·52 (0·33–0·81)
Osteopontin above median 113 μg/L	162	97 (60%)		96 (59%)	
Low osteopontin					
Nimorazole	62	28 (45%)	0·79 (0·26–1·70)	22 (36%)	0·69 (0·31–1·51)
Placebo	45	23 (51%)		20 (44%)	
Intermediate osteopontin					
Nimorazole	47	28 (60%)	0·90 (0·41–1·98)	23 (49%)	0·89 (0·41–1·93)
Placebo	58	36 (62%)		30 (52%)	
High osteopontin					
Nimorazole	55	23 (42%)	0·19 (0·08–0·44)	27 (49%)	0·25 (0·11–0·59)
Placebo	53	42 (79%)		42 (79%)	

Table 2: **Frequency of locoregional tumour failure and disease-specific mortality**

The primary endpoints were locoregional tumour control after radiotherapy and disease-specific survival at 5 years. Locoregional control was defined as complete and permanent disappearance of disease in the primary tumour (T site) and regional lymph nodes (N site) after radiotherapy. Failure was recorded in the event of recurrent tumour, or if the primary tumour did not disappear completely (in which case the tumour was assumed to have failed at the time of randomisation). Disease-specific mortality was defined as death from cancer or with cancer.

Analysis of plasma samples

The study population has been described previously.[12] Of the 414 patients randomised, plasma samples were useful and could be analysed for 320 patients (figure 1).

The samples were intended for assessment of the pharmacokinetics of nimorazole, and because the trial was double blind, plasma was taken from patients assigned active drug and those assigned placebo. Samples were not available for all patients in the trial because of logistical constraints; however, the characteristics of patients and tumours did not differ between those who had plasma samples and those who did not. Plasma samples were taken on the first day of treatment on the trial. They were first stored centrally at –20°C and then transferred to –80°C. We measured osteopontin by use of a commercially available ELISA assay (Human Osteopontin TiterZyme® Immunoassay Kit, Assay Designs, Ann Arbor, MI, USA). The assays were masked until the time of analysis. We used the mean values of highly reproducible duplicate measurements.

Follow-up of patients for plasma osteopontin analysis was completed alongside the trial.[12] All patients were followed up for at least 5 years or until death. Survival status has subsequently been followed up until June 1, 2004.

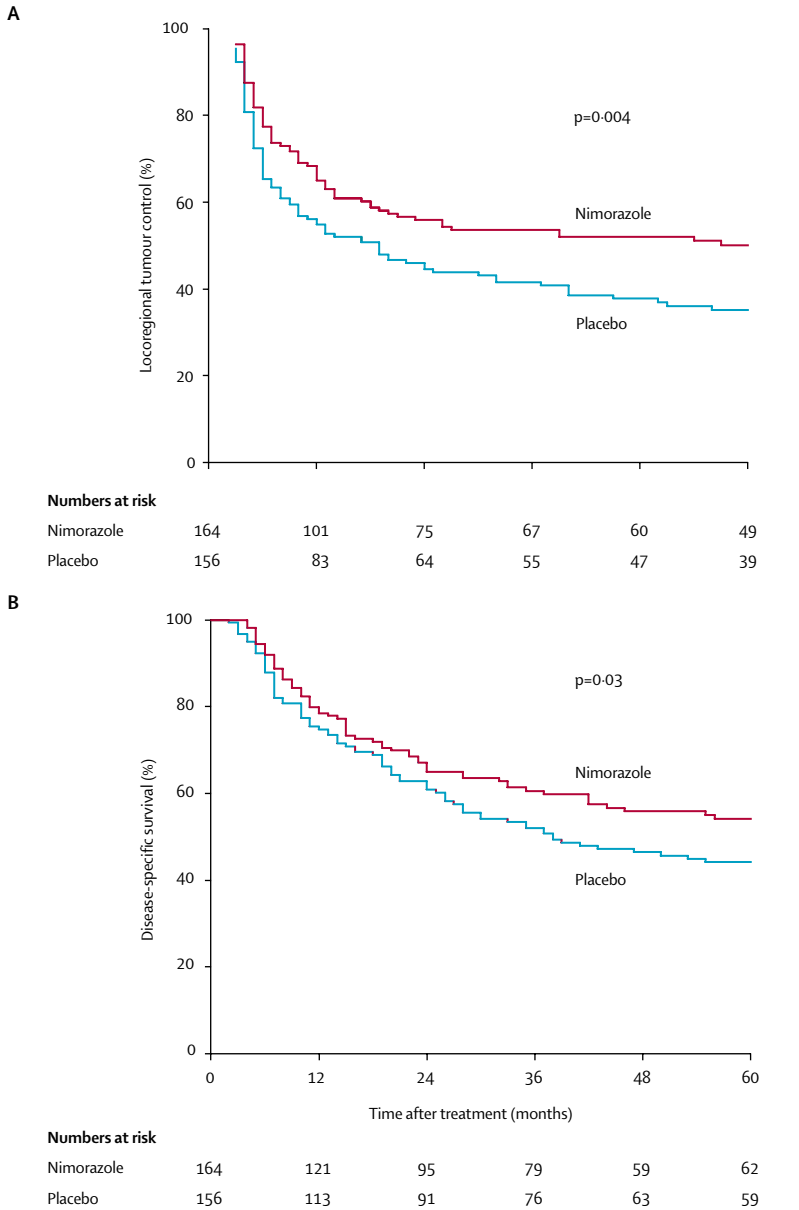

Figure 2: **Primary outcomes by treatment group**
Locoregional tumour control (A) and disease-specific survival (B).

Statistical analysis

Samples were divided into tertiles on the basis of concentration of osteopontin (low: 0–68 μg/L; intermediate: 69–166 μg/L; high: 167–1382 μg/L). Comparison between frequencies in tertiles was by use of the χ^2 test, and actuarial survival curves were assessed as Kaplan-Meier plots and compared by use of the log-rank test. A Cox multivariate proportional-hazards analysis was used to assess the prognostic importance of independent factors. Patients were analysed by the original stratification factors of sex, T stage, nodal involvement, tumour site, and haemoglobin concentration; locoregional failure was the outcome assessed in this analysis. Analyses were done with the BMDP statistical package version 7.0. p values were two sided, and the significance level was 5%.

Role of the funding source

The sponsor of the study had no role in study design; in the collection, analysis, or interpretation of the data; or in the writing of the report. The corresponding author had full access to all data in the study and the final responsibility to submit for publication.

Results

Table 1 shows the characteristics of patients with high, intermediate, and low concentrations of plasma osteopontin. Nodal disease and advanced disease stage were associated with high plasma concentrations of osteopontin. The 320 patients included in this study did not differ in the stratification factors of the original study from those of the overall cohort of 414 patients.[12] Within tertiles, the characteristics of patients and tumours did not differ between those assigned nimorazole and those assigned placebo.

Overall, patients assigned placebo had worse locoregional tumour control and disease-specific survival than did those assigned nimorazole (figure 2 and table 2). Furthermore, patients with a plasma concentration of osteopontin of less than the median 113 μg/L in univariate analysis had significantly improved locoregional control (figure 3) and disease-specific survival compared with those who had a plasma concentration of osteopontin of 113 μg/L or higher (figure 3 and table 2).

Locoregional control and disease-specific survival in patients assigned nimorazole did not differ between tertiles. However, patients assigned placebo who had a high osteopontin concentration had higher locoregional failure (relative risk 2·85 [95% CI 1·32–6·15]) and disease-specific mortality (4·05 [1·88–8·72]) than did those in either of the other tertiles. Patients with high concentrations of osteopontin given placebo had worse locoregional tumour control and disease-specific survival than did those assigned nimorazole (figure 4 and table 2). However, neither locoregional tumour control nor disease-specific survival differed between groups for patients with low concentrations of plasma osteopontin or for those with intermediate concentrations (figure 4 and table 2). Locoregional failure was significantly lower in the nimorazole group than in the placebo group in patients with high concentrations of plasma osteopontin (0·19 [0·08–0·44]) compared with those with intermediate and low concentrations of plasma osteopontin (0·79 [0·46–1·35]; p=0·006), as was disease-specific mortality (0·25 [0·11–0·59] and 0·75 [0·43–1·28], respectively; p=0·03).

Cox proportional hazards analysis showed that T1–2 classification (relative risk 0·63 [95% CI 0·47–0·86]), negative nodes (0·60 [0·44–0·80]), and nimorazole (0·64 [0·48–0·86]) were independently associated with decreased locoregional tumour failure, and with reduced risk of death from cancer (0·62 [0·45–0·86], 0·51 [0·37–0·70], and 0·69 [0·51–0·95], respectively) compared with patients with T3–T4, positive nodes, and those given placebo, respectively; women also had a lower risk of death from head and neck cancer (0·56 [0·38–0·82]) than did men. These results are consistent with the outcome of the original study cohort of 414 patients.[12]

On separate multivariate analysis for the three tertiles, only patients with high plasma concentrations of osteopontin differed by treatment intervention (locoregional failure: 0·46 [0·27–0·76], death from cancer: 0·56 [0·35–0·92]). Plasma concentration of osteopontin was not associated with the outcomes of 5-year locoregional control or disease-specific survival in patients assigned nimorazole, whereas high concentrations of plasma osteopontin were significantly associated with poor outcome in those assigned placebo (p=0·01 for locoregional control and p=0·0004 for disease-specific survival).

We repeated our analyses for all 320 patients, and included an assessment of the interaction between osteopontin concentration and nimorazole. Table 3 shows that the independent prognostic importance of tumour stage and nodal stage remained, and that placebo interacted significantly with high concentrations of plasma osteopontin. Thus, prognosis was significantly worse in patients assigned placebo who had high concentrations of plasma osteopontin compared with any other patient group. Furthermore, this final analysis showed that nimorazole had no significant effect on patients with low or intermediate concentrations of plasma osteopontin.

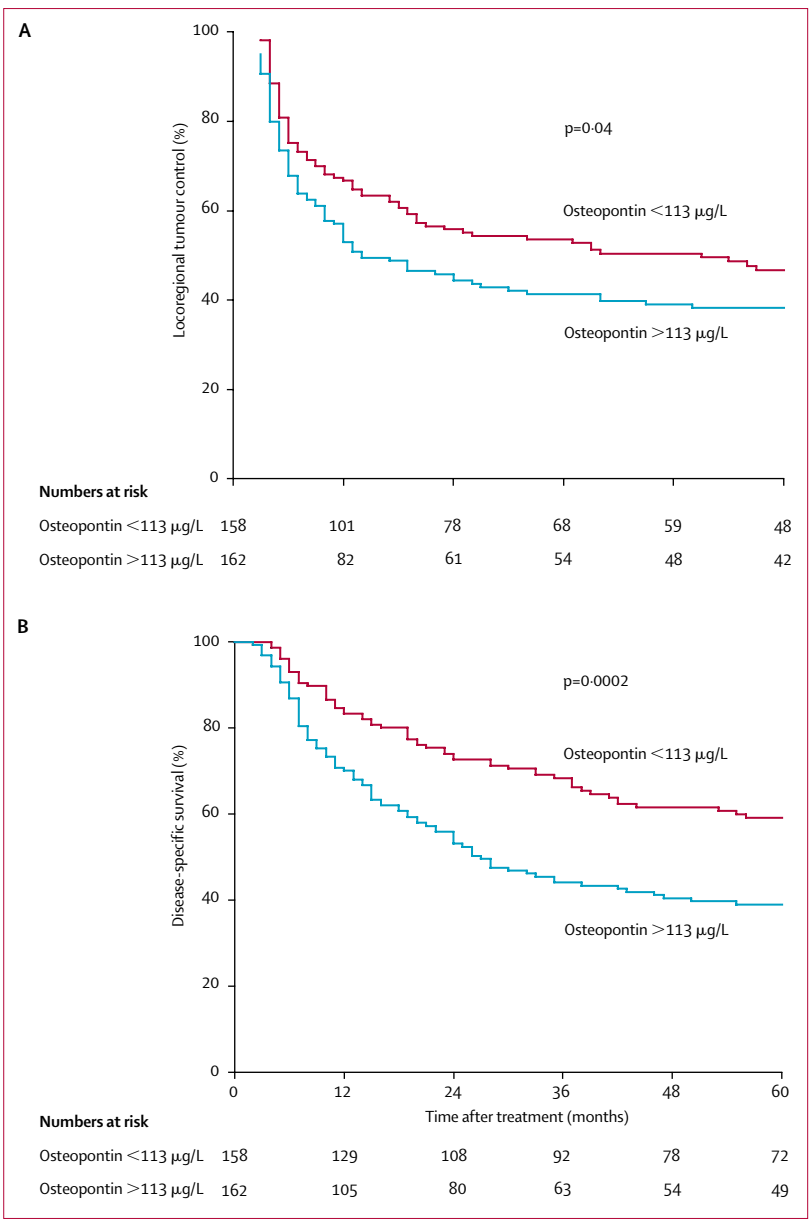

Figure 3: **Primary outcomes as a function of osteopontin concentration above or below median 113 μg/L**
Locoregional tumour control (A) and disease-specific survival (B).

Discussion

We have shown that high plasma concentrations of osteopontin in patients with squamous-cell carcinoma of the head and neck are associated with poor outcome after radiotherapy only, but that the prognosis of these patients can be improved by use of the hypoxia radiosensitiser nimorazole with radiotherapy. Thus, we have confirmed our hypothesis that high plasma

concentrations of osteopontin predicted clinically relevant, modifiable hypoxia-induced resistance to radiotherapy, and this finding might help to identify patients who will benefit from treatment with a hypoxia modifier such as nimorazole during radiotherapy. By contrast, use of nimorazole was not effective in patients with low or intermediate plasma concentrations of osteopontin.

We assume that the plasma concentration of osteopontin is related to production of this protein by the tumour. In vitro, hypoxic squamous-cell carcinoma cells induce up to a 90-times increased expression of

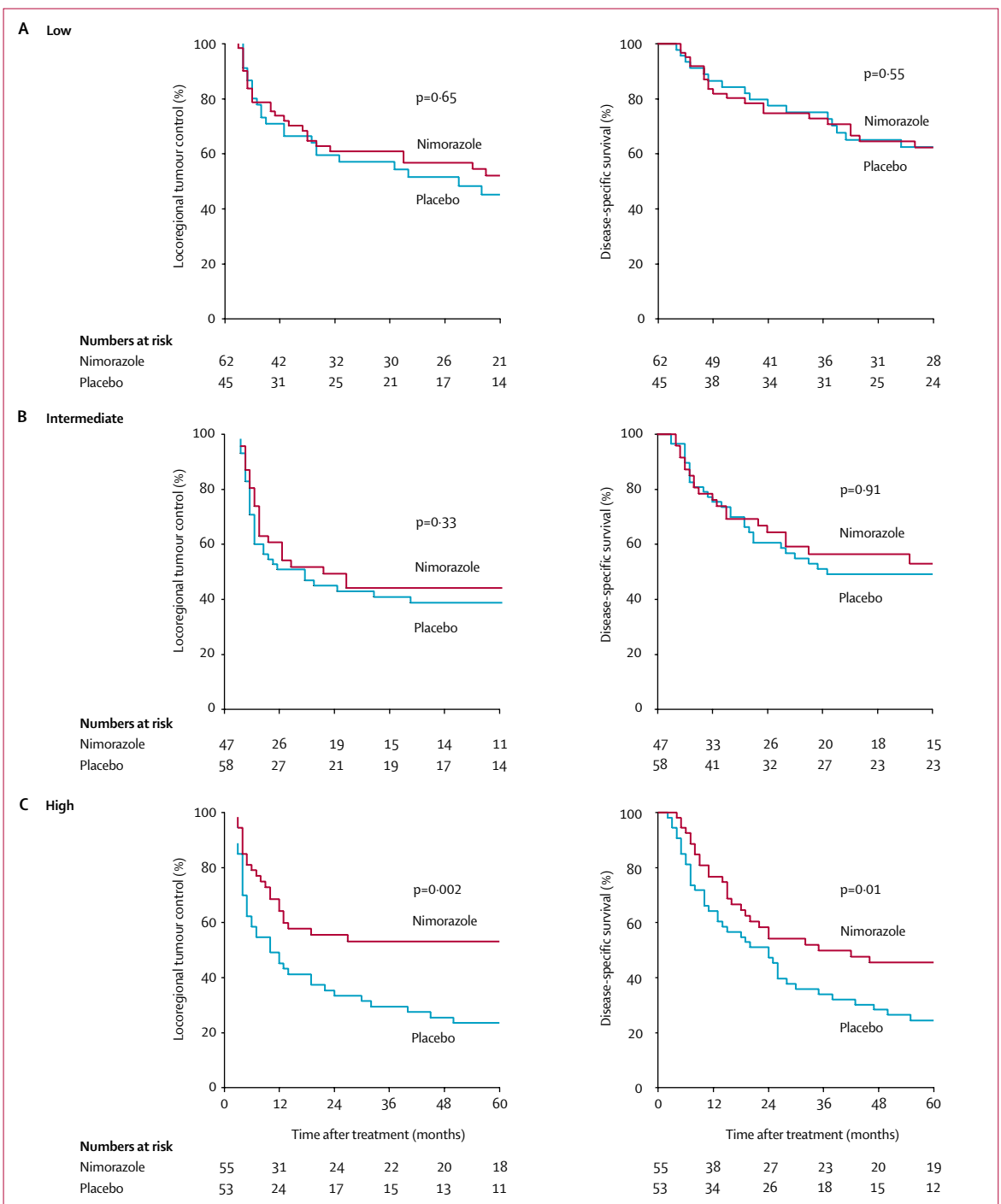

Figure 4: **Primary outcomes by treatment group and concentration of osteopontin**
Low osteopontin (A). Intermediate osteopontin (B). High osteopontin (C).

osteopontin mRNA.[18] However, plasma concentration of osteopontin is an indirect measurement of osteopontin production by the tumour, and several factors might affect the absolute level of hypoxia—eg, variable release of osteopontin by tumours and coexpression of osteopontin in plasma with that induced by other non-cancerous causes such as bone remodelling[24] and by spread of cancer, as noted for breast cancer, colorectal cancer, ovarian cancer, and malignant melanoma.[25,26] Several studies[23,23,27] have suggested that tumour hypoxia can increase the concentration of osteopontin in the plasma of patients with head and neck cancer. Furthermore, in-vitro hypoxic stimulation of cells from squamous-cell-carcinoma cell lines increased osteopontin expression,[18] and in-vivo experiments of tumours have shown that increased tumour hypoxia was associated with a similar increase in concentration of plasma osteopontin (Horsman MR, unpublished data). However, further investigation of the relation between hypoxia, tumour expression of osteopontin, and plasma concentration of osteopontin in human beings is warranted: the expression of osteopontin and that of other endogenous markers of hypoxia such as hypoxia-inducible factor 1α and carbonic anhydrase IX might not be directly correlated because these other factors might be induced after a different extent of hypoxia.[18,23]

Our study suggests that patients with the highest concentrations of osteopontin had hypoxia-induced resistance to radiotherapy that could be modified. However, the absolute concentration of osteopontin needed to predict hypoxia accurately remains to be determined. The plasma samples in our study have been stored for more than 15 years, and thus the amount of osteopontin could have decayed over time; analyses[22,23,27] of fresh plasma samples from patients with similar head and neck tumours suggested that the osteopontin concentrations we measured could have been less than half that of the original value. However, such reduction in osteopontin concentrations should be much the same for all samples and therefore, our conclusion that patients with the highest osteopontin concentrations are those with the most hypoxic tumours, as found in our pilot study,[23] is valid. In this analysis, we separated patients into tertiles, assuming that hypoxic tumours would be distributed equally between the two groups. Our pilot study grouped patients into tertiles to avoid bias in the assessment of a potential predictive factor.[28] Further comparisons between the level of hypoxia, the fresh concentrations of osteopontin in tumours, and plasma concentration of osteopontin are needed to define the absolute concentration of osteopontin that might predict modifiable hypoxia. The importance of our study is therefore not so much the identification of hypoxia itself, but rather the proof of principle that the level of hypoxia varies between patients, and that this variation can be detected and used to identify patients in whom tumour hypoxia can be modified, thereby improving their outcome after radiotherapy. Future studies should investigate other hypoxic markers, both exogenous and endogenous, to further investigate our current findings. Such studies should be done in a randomised setting with an intervention to modify hypoxia. In addition to nimorazole, hypoxic cytotoxins such as tirapzamine and other methods of delivering oxygen to the tumour such as ARCON (carbogen combined with nicotinamide) are being assessed in large international multicentre studies.[29,30] Kanders and colleagues[21] showed in a non-randomised study that the distribution of hypoxia measured with pimonidazole varied between the 38 patients with head and neck carcinomas, and that those with high expression of pimonidazole in their tumours had a better disease-free survival after treatment with ARCON. A similar effect was recorded after treatment for head and neck cancer with chemoradiotherapy and tirapazamine, in which hypoxia was recorded by PET scanning with ^{18}F-misonidazole.[31] Although these data do not show a truly predictive effect, they are consistent with our findings, and suggest that hypoxia can be predicted and consequently lead to more aggressive and effective hypoxia modification in patients who need it.

In conclusion, plasma concentration of osteopontin is an easily obtainable marker for hypoxia, and high concentrations were associated with a poor prognosis after radiotherapy for head and neck cancer. Locoregional tumour control and disease-specific survival were significantly improved in patients with high concentrations of plasma osteopontin who were assigned nimorazole and radiotherapy compared with those with high concentrations of plasma osteopontin who were assigned placebo with radiotherapy. Our study suggests that high plasma concentrations of osteopontin predict the need for hypoxia modification. Accordingly, osteopontin measurements should be included in clinical trials of hypoxia modification to confirm the hypothesis and define the exact plasma concentrations that are important.

	Locoregional failure		Death from cancer	
	Relative risk (95% CI)	p	Relative risk (95% CI)	p
Tumour stage (1–2 *vs* 3–4)	0·68 (0·50–0·92)	0·01	0·67 (0·49–0·92)	0·01
Nodal status (negative *vs* positive)	0·62 (0·46–0·84)	0·002	0·53 (0·39–0·73)	0·0001
Site (supraglottic larynx *vs* pharynx)	Not estimated	0·8	Not estimated	0·10
Women *vs* men	0·71 (0·50–1·00)	0·05	0·55 (0·37–0·81)	0·001
Haemoglobin (high *vs* low)	Not estimated	0·16	Not estimated	0·34
High osteopontin and placebo *vs* all other patients	1·73 (1·22–2·46)	0·003	2·03 (1·43–2·90)	0·0002
Nimorazole *vs* placebo	Not estimated	0·08	Not estimated	0·53
Osteopontin (high *vs* intermediate *vs* low)	Not estimated	0·76	Not estimated	0·08

Table 3: **Cox proportional-hazards analysis**

Contributors
All authors contributed to the design and implementation of the study, and in the generation of data. J Overgaard analysed the data and drafted the paper. All authors took part in the revision of the report and approved the final version.

Conflict of interest
We declare no conflicts of interest.

Acknowledgments
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