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The prognostic and predictive power of redox protein expression for anthracycline-based chemotherapy response in locally advanced breast cancer

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Neoadjuvant chemotherapy has become the standard of care for locally advanced primary breast cancer. Anthracycline-based regimens have proven to be one of the most effective treatments in this setting. As certain cytotoxic antineoplastic agents, such as anthracyclines, generate reactive oxygen species as a by-product of their mechanism of action, we examined whether redox protein expression was involved in the response to anthracycline-based chemotherapy and with clinical outcome. Pre-treatment needle core biopsy and post-anthracycline treatment tumour sections were analysed from 98 cases. In all, 32 individuals had a complete clinical response and 17 had a complete pathological response. Immunohistochemical staining was performed for eight redox proteins: thioredoxin, thioredoxin reductase, thioredoxin interacting protein (TxNIP), glutathione S-transferase (GST) π, θ and α, catalase and manganese superoxide dismutase. GST π ($P=0.05$) and catalase ($P=0.045$) were associated with pathological complete response in pre-chemotherapy samples. TxNIP ($P=0.017$) and thioredoxin reductase ($P=0.022$) were independent prognostic factors for distant metastasis-free survival and TxNIP for overall survival ($P=0.014$). In oestrogen receptor negative patients that are known to have a poor overall survival, a considerably worse prognosis was seen in cases that exhibited low expression of TxNIP ($P=0.000003$), stratifying patients into more defined groups. This study indicates the importance of redox regulation in determining breast cancer response to anthracycline-based chemotherapy and provides ways of further stratifying pre-chemotherapy patients to potentially allow more tailored treatments.

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Neoadjuvant chemotherapy has become the standard of care for locally advanced primary breast cancer patients and aims to reduce tumour burden, to render tumours operable, or facilitate breast conservation and other oncoplastic options. In this setting, anthracycline-based regimens are commonly used. Anthracyclines have a complex mechanism of action including inhibition of enzymes such as topoisomerase II, resulting in DNA double-strand breaks, intercalation into DNA and also generation of reactive oxygen species.[1] The anthracycline drugs have a quinone that undergoes reduction to a semi-quinone-free radical, forming superoxide anions in the presence of oxygen. After dismutation, resultant hydrogen peroxide (H_2O_2) can be converted into the highly damaging hydroxyl radical.[2] The semi-quinone radical can also intercalate and damage DNA. There are currently no markers to determine

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response to neoadjuvant chemotherapy in locally advanced primary breast cancer.

In cancer, the normal redox balance is disrupted due to increased oxidative stress caused by accelerated cell proliferation, constant stimulation of growth promoting signalling pathways and alterations in metabolic activity. Due to this, redox buffering systems such as the thioredoxin and glutathione systems, and antioxidant enzymes such as catalase and superoxide dismutase are often deregulated/overexpressed to compensate.[3–6] These processes can add to the oncogenic transformation and mutation rate in tumours and influence their response to reactive oxygen species generating therapies.[7,8]

The current study investigates expression of a panel of redox proteins representing the key pathways: thioredoxin, thioredoxin reductase, thioredoxin interacting protein (TxNIP), glutathione S-transferase (GST) π, θ, α, catalase and manganese superoxide dismutase (MnSOD), in a well-defined cohort of pre-treatment locally advanced primary breast cancer patients before anthracycline chemotherapy. Their expression was investigated for response to therapy and aiding prognosis. A post-chemotherapy tumour specimen was available from certain patients that allowed a matched comparison of protein expression to be assessed, yielding information on particular redox pathways that are altered in response to chemotherapy.

Materials and methods

Clinical Samples

The study is reported according to REMARK criteria.[9] The consecutive cohort consisted of 98 patients presenting with locally advanced primary breast cancer between December 1996 and December 2009 at Nottingham University Hospitals and treated with neoadjuvant anthracycline-based chemotherapy. The core biopsies from 82 patients were assessed for all redox proteins studied, 16 additional patients were available for assessment of TxNIP only ($n = 98$). A core biopsy was performed before chemotherapy to allow pathological diagnosis and evaluation of biological parameters. Patients were then treated with six cycles of anthracycline-based therapy (5-fluorouracil (5-FU) 500 mg/m^2, epirubicin 75–100 mg/m^2, cyclophosphamide 500 mg/m^2, on day 1 of a 21-day cycle). Patients underwent surgery 4 weeks after the sixth cycle, unless progression after three cycles, in which case taxanes were used. All oestrogen receptor positive cases received adjuvant hormonal treatment.

Assessment of tumour response was undertaken before chemotherapy and after each cycle. The clinical baseline and preoperative measurements were obtained with a calliper by the same clinician or by radiological assessment. Clinical response was recorded according to RECIST criteria.[10] The pathological response was evaluated by histological examination of tumour removed following chemotherapy. For certain cases, whole tumour sections post-chemotherapy were available for redox protein assessment as matched pairs to the pre-chemotherapy samples (see Results section for numbers). Histology was reviewed using the Chevallier classification.[11] Ethical approval was obtained from the Nottingham Research Ethics Committee (C202313).

Immunohistochemistry

Immunohistochemistry was performed on core biopsies and whole tumour sections as previously described.[5] Briefly, microwave antigen retrieval was conducted in 0.01 mol/l sodium citrate buffer (pH 6). Primary antibodies were incubated for 60 min at room temperature (thioredoxin 1:1000 (#705, American Diagnostica, Stamford, USA), thioredoxin reductase 1:1000 (#07-613, Millipore, Billerica, USA), TxNIP 1:3000 (clone JY2, MBL International Corporation, Woburn, USA), catalase 1:5000 (#ab1877, Abcam, Cambridge, UK), MnSOD 1:1000 (#ab13533, Abcam), GST α (clone 2F7) 1:333, GST θ (clone 2E10-1B2) 1:500 (Abnova, Taipei City, Taiwan), GST π 1:2000 (#MSA-102, Assay Designs, Michigan, USA)). Blocking, secondary, ABC and 3,3′-diaminobenzidine (DAB) substrate reagents were supplied in kit form from Vector Labs (Vector Laboratories, Burlingame, USA). Primary antibody was omitted for negative controls. Placenta and breast composite blocks (six stage I breast carcinomas, including grade I–III tumours) were used for antibody optimisations and positive controls, with liver sections used for GST α. All antibodies had been previously assessed for specificity using western blotting on a panel of breast cancer cell lysates.

Assessment of protein staining was conducted by standard semiquantitative immunohistochemistry scoring (H score) conducted independently by two assessors blinded to the study end points. Staining intensity was divided into: none (0), weak (1) moderate (2) and strong (3). H scores were calculated by multiplying the percentage of positive tumour by the staining intensity (range 0–300). Low-*vs* high-expressing tumours were determined using median H scores—thioredoxin 190, thioredoxin reductase 200, TxNIP 160, GST θ 185, GST π 85, catalase 90, MnSOD 175. No tumour staining was observed for GST α.

Statistical Analysis

SPSS version 15.0 software package was used for statistical analysis. Protein expression *vs* clinicopathological criteria was assessed using the Pearson χ^2 test of association or Fisher's exact test when there are less than five cases in a cell. Survival analysis was conducted using Kaplan–Meier and

Table 1 Clinicopathological criteria of locally advanced primary breast cancer patients who underwent neoadjuvant anthracycline-based chemotherapy

	Thioredoxin			*Thioredoxin reductase*			*TxNIP*			*Glutathione S-transferase π*			*Glutathione S-transferase θ*			*Catalase*			*Manganese superoxide*		
	Low (%)	*High (%)*	P-*value*	*Low (%)*	*High (%)*	P-*value*	*Low (%)*	*High (%)*	P-*value*	*Low (%)*	*High (%)*	P-*value*	*Low (%)*	*High (%)*	P-*value*	*Low (%)*	*High (%)*	P-*value*	*Low (%)*	*High (%)*	P-*value*
Age (years)																					
≤40	10 (71)	4 (29)	0.217	7 (47)	8 (53)	0.393	6 (38)	10 (62)	0.352	7 (54)	6 (46)	0.747	8 (67)	4 (33)	0.284	9 (60)	6 (40)	0.790	7 (47)	8 (53)	0.840
41–60	20 (47)	23 (53)		24 (56)	19 (44)		33 (58)	24 (42)		19 (46)	22 (54)		17 (43)	23 (57)		20 (50)	20 (50)		22 (54)	19 (46)	
>60	6 (43)	8 (57)		10 (71)	4 (29)		9 (53)	8 (47)		8 (57)	6 (43)		8 (57)	6 (43)		7 (50)	7 (50)		6 (46)	7 (54)	
Total	36	35		41	31		48	42		34	34		33	33		36	33		35	34	
Histology																					
Ductal NST	35 (53)	31 (47)	0.170*	38 (57)	29 (43)	0.632*	43 (52)	40 (48)	0.276*	33 (52)	30 (48)	0.178*	30 (48)	32 (52)	0.307*	34 (53)	30 (47)	0.458*	33 (52)	31 (48)	0.486*
Lobular	1 (20)	4 (80)		3 (60)	2 (40)		5 (71)	2 (29)		1 (20)	4 (80)		3 (75)	1 (25)		2 (40)	3 (60)		2 (40)	3 (60)	
Total	36	35		41	31					34	34		33	33		36	33		35	34	
TNM stage																					
2	4 (50)	4 (50)	0.593*	3 (33)	6 (67)	0.135*	6 (40)	9 (60)	0.293	5 (63)	3 (37)	0.337*	3 (43)	4 (57)	0.537*	5 (63)	3 (37)	0.408*	5 (63)	3 (37)	0.372*
3	31 (53)	28 (47)		35 (59)	24 (41)		39 (55)	32 (45)		27 (47)	30 (53)		27 (49)	28 (51)		29 (51)	28 (49)		28 (49)	29 (51)	
Total	35	32		38	30		45	41		32	33		30	32		34	31		33	32	
ER status																					
Negative	18 (49)	19 (51)	0.718	20 (53)	18 (47)	0.435	23 (52)	21 (48)	0.844	16 (46)	19 (54)	0.467	15 (43)	20 (57)	0.218	21 (60)	14 (40)	0.187	15 (43)	20 (57)	0.185
Positive	18 (53)	16 (47)		21 (62)	13 (38)		25 (54)	21 (46)		18 (55)	15 (45)		18 (58)	13 (42)		15 (44)	19 (66)		20 (59)	14 (41)	
Total	36	35		41	31		48	42		34	34		33	33		36	33		35	34	
PgR status																					
Negative	20 (54)	17 (46)	0.329	21 (55)	17 (45)	0.464	25 (56)	20 (44)	0.066	17 (49)	18 (51)	0.916	16 (47)	18 (53)	0.316	25 (69)	11 (31)	0.198	18 (50)	18 (50)	0.862
Positive	9 (41)	13 (59)		10 (45)	12 (55)		11 (34)	21 (66)		11 (50)	11 (50)		7 (33)	14 (67)		11 (52)	10 (48)		11 (52)	10 (48)	
Total	29	30		31	29		36	41		28	29		23	32		36	21		29	28	
HER status																					
Negative	17 (43)	23 (57)	0.458	20 (48)	22 (52)	0.174	29 (52)	27 (48)	0.635	18 (45)	22 (55)	0.107	15 (38)	24 (62)	0.116	24 (60)	16 (40)	0.677	21 (54)	18 (46)	0.647
Positive	12 (52)	11 (48)		15 (65)	8 (35)		12 (46)	14 (54)		14 (67)	7 (33)		12 (60)	8 (40)		12 (55)	10 (45)		11 (48)	12 (52)	
Total	29	34		35	30		41	41		32	29		27	32		36	26		32	30	
Pathological complete response																					
CR	7 (54)	6 (46)	0.802	7 (50)	7 (50)	0.559	7 (44)	9 (56)	0.397	3 (25)	9 (75)	**0.050***	6 (46)	7 (54)	0.757	10 (77)	3 (23)	**0.045***	8 (62)	5 (38)	0.387
Non-CR	29 (50)	29 (50)		34 (59)	24 (41)		41 (55)	33 (45)		31 (55)	25 (45)		27 (51)	26 (49)		26 (46)	30 (54)		27 (48)	29 (52)	
Total	36	35		41	31		48	42		34	34		33	33		36	33		35	34	
Clinical complete response																					
CR	15 (56)	12 (44)	0.522	14 (50)	14 (50)	0.342	13 (43)	17 (57)	0.202	16 (64)	9 (36)	0.078	15 (60)	10 (40)	0.205	16 (59)	11 (41)	0.345	16 (59)	11 (41)	0.256
Non-CR	21 (48)	23 (52)		27 (61)	17 (39)		34 (58)	25 (42)		18 (42)	25 (58)		18 (44)	23 (56)		20 (48)	22 (52)		19 (45)	23 (55)	
Total	36	35		41	31		47	42		34	34		33	33		36	33		35	34	

Pearson's χ^2 test of association values stated unless the frequency of observations in a cell was <5; therefore, Fisher's exact stated (indicated by *).
Statistically significant *P*-values are indicated by bold font.

significance was determined using log rank. Cox proportional hazards model was used to test statistical independence and paired *t*-test for the relationship between pre- and post-tumour expression levels. Intraclass correlations were applied to determine the consistency, or conformity, between assessors using the continuous scores that were produced by each individual scoring independently from the same tissue sections. All differences were deemed statistically significant at the level of $P<0.05$.

Results

Redox Proteins Expression and Clinicopathological Characteristics

Table 1 shows the full clinicopathologic characteristics of the patient cohort that were assessable for each protein. In ~80% of cases, the maximum risk of recurrence was within the first 2 years. Only 15 living cases (15%) had a follow-up time ≤2 years. The median age of patients was 51 with a range of 25–76 years. The assessment of protein expression was conducted with a high level of concordance between independent observers: thioredoxin 0.962, thioredoxin reductase 0.976, TxNIP 0.838, GST θ 0.930, GST π 0.973, catalase 0.964, MnSOD 0.916. All proteins were assessed for cytoplasmic staining, with some granularity and heterogeneity between adjacent tumour cells, varying from weak to intense staining. Some occasional nuclear staining was observed for thioredoxin, thioredoxin reductase and GST π but was not assessed. Figures 1 and 2 demonstrate the immunostaining pattern of the proteins. No expression of GST α was observed apart from in the liver sections that were used as positive

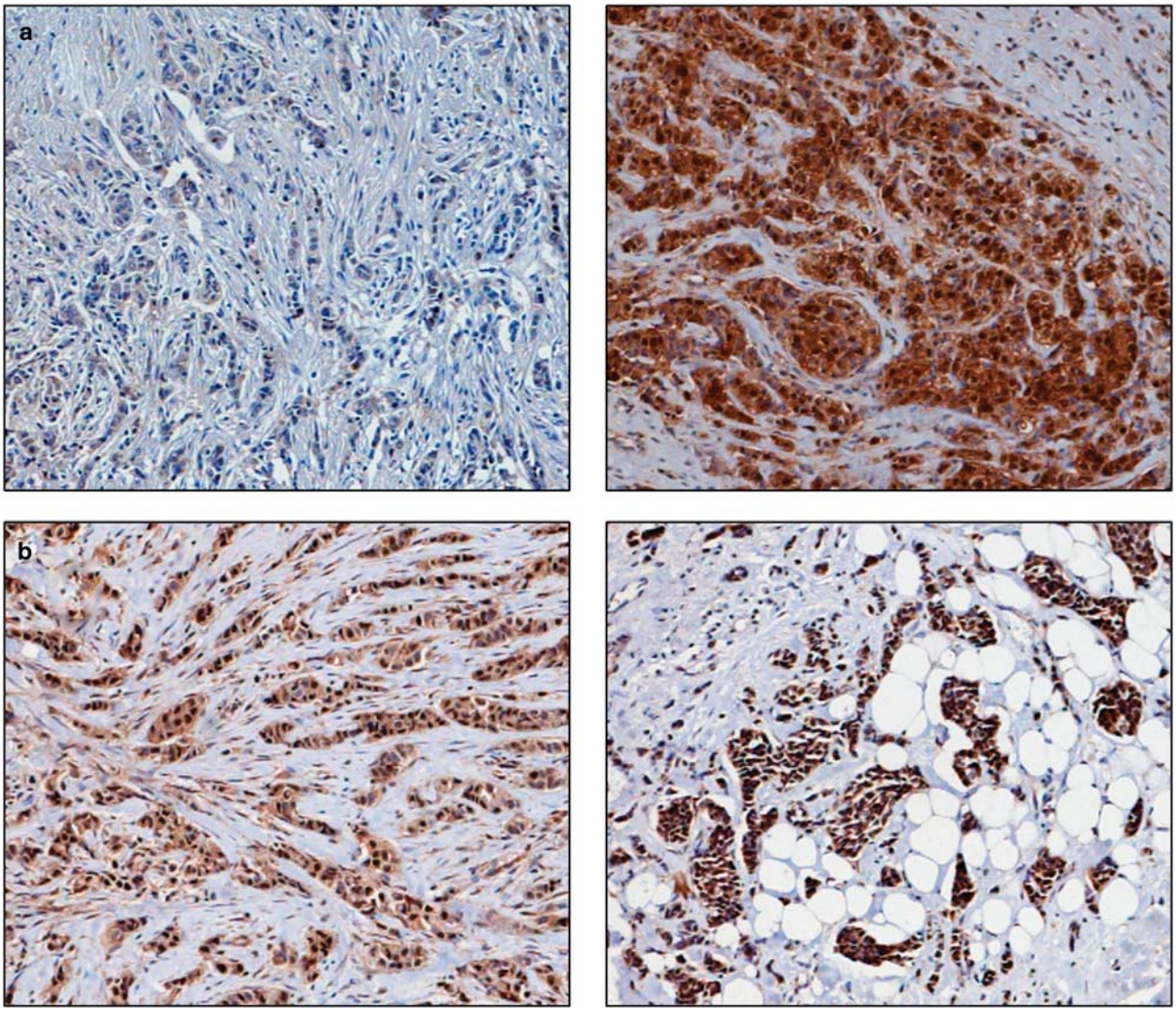

Figure 1 Photomicrographs of low (left panel) and high (right panel) immunohistochemical protein expression at ×10 magnification (**a**) thioredoxin (**b**) thioredoxin reductase (**c**) thioredoxin interacting protein (**d**) manganese superoxide dismutase. Stain is 3,3′-diaminobenzidine counterstained with haematoxylin.

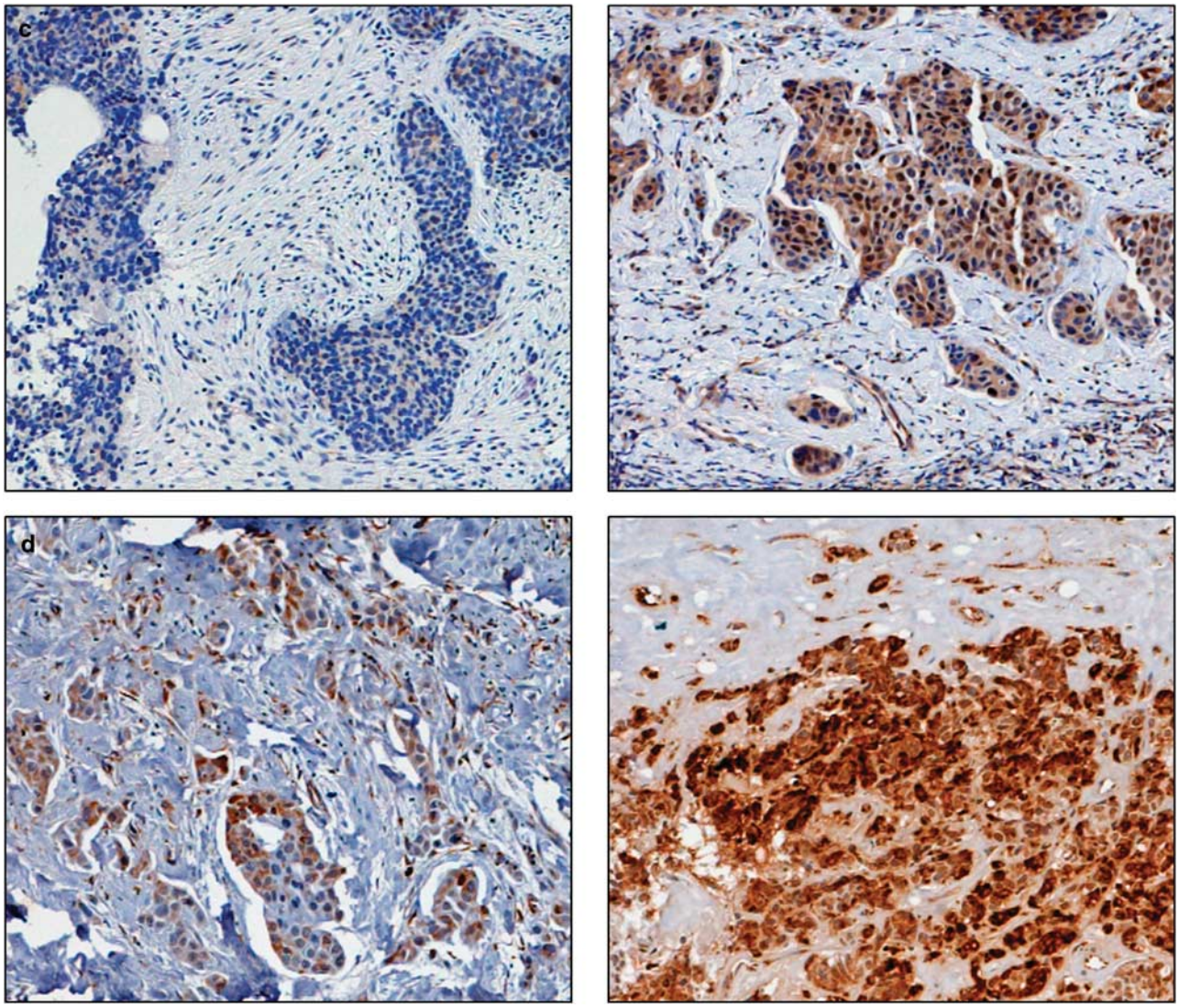

Figure 1 Continued.

controls (Figure 2d). The expression of redox proteins in the pre-chemotherapy samples was not associated with clinicopathologic characteristics, that is, TNM stage, histopathologic subtype, oestrogen receptor, HER2, progesterone receptor status or age (Table 1).

Redox Protein Expression and Response

Pathological complete response is the most reliable end point of response to neoadjuvant treatment. For this study, pathological complete response was analysed *vs* partial response, stable disease and progressed disease that were grouped due to the limited numbers in certain categories. Borderline associations were observed for GST π ($\chi^2 = 3.643$, df = 1, $P = 0.05$) and catalase ($\chi^2 = 3.932$, df = 1, $P = 0.045$) with pathological complete response in the pre-chemotherapy biopsy samples. From the cases that had completely responded, 75% (9/12) had high GST π levels and 77% (10/13) a low expression of catalase. Thioredoxin $P = 0.802$, thioredoxin reductase $P = 0.559$, TxNIP $P = 0.397$, GST θ $P = 0.757$ and MnSOD $P = 0.387$ showed no association with pathological complete response (Table 1).

Oestrogen receptor negative tumours are known to achieve a better pathological complete response[12,13] and in this cohort, oestrogen receptor status was significantly associated with pathological complete response ($\chi^2 = 7.572$, df = 1, $P = 0.005$) with 82% (14/17) of responders being oestrogen receptor negative. Therefore, the combination of GST π or catalase expression with oestrogen receptor status was assessed in the pre-chemotherapy biopsies *vs* pathological complete response. Data were grouped into four combinations, that is, oestrogen receptor negative with low redox protein expression, oestrogen receptor negative with high redox protein expression, oestrogen receptor positive with low redox protein expression

and oestrogen receptor positive with high redox protein expression. In all, 67% (8/12) of complete responders had a high expression of GST π in oestrogen receptor negative tumours ($\chi^2 = 11.024$, df = 3, $P = 0.012$). Analysing this category (ie, oestrogen receptor negative/GST π high) against the other subgroups combined highlighted a strong significant association with pathological complete response ($\chi^2 = 10.853$, df = 1, $P = 0.002$). Catalase/oestrogen receptor combinations showed that the association of a low expression of catalase with oestrogen receptor negative was of interest in the complete responders ($\chi^2 = 4.147$, df = 1, $P = 0.042$, 53.8% cases (7/13)).

Redox protein expression was not associated with clinical complete response—TxNIP $P = 0.202$, thioredoxin $P = 0.522$, thioredoxin reductase $P = 0.342$, GST θ $P = 0.205$, GST π $P = 0.078$, catalase $P = 0.345$, MnSOD $P = 0.256$ (Table 1). Clinical complete response was also analysed as complete response *vs* partial response, stable disease and progressed disease grouped.

Redox Protein Expression and Survival

The age of the patients (≤ 40, 40–60, > 60) was significantly associated with distant metastasis-free survival ($P = 0.032$) with the ≤ 40 age group showing good prognosis. The ≤ 40 group also showed a significantly better overall survival ($P = 0.04$). However, it should be noted that this cohort is a relatively small and homogeneous population.

In the pre-chemotherapy biopsies, Figure 3a demonstrates that a high expression of TxNIP

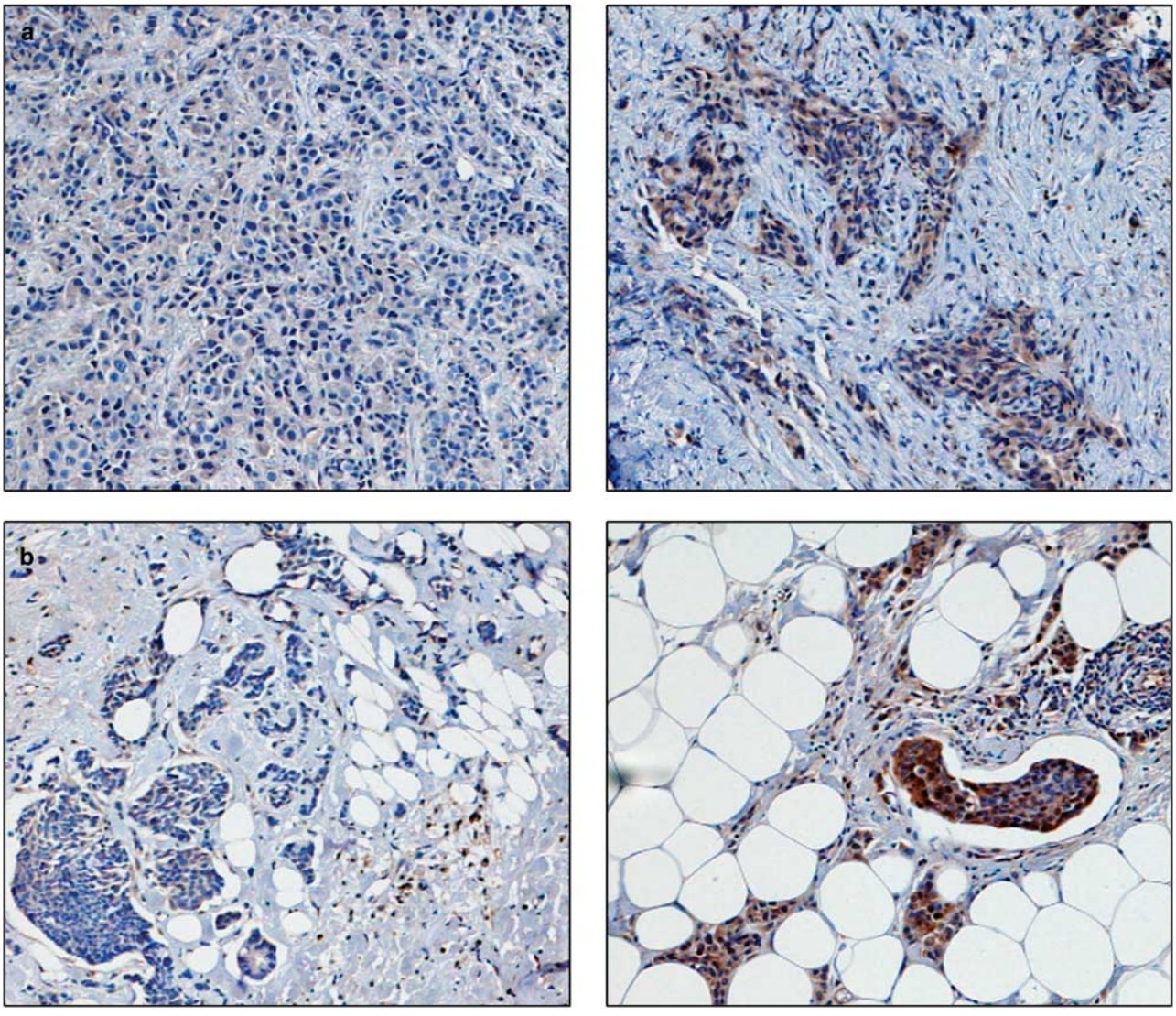

Figure 2 Photomicrographs of low (left panel) and high (right panel) immunohistochemical protein expression at $\times 10$ magnification (**a**) glutathione S-transferase θ (**b**) glutathione S-transferase π (**c**) catalase. Positive control showing glutathione S-transferase α staining on liver sections (**d**) and (**e**) breast composite demonstrating absence of staining. Stain is 3,3′-diaminobenzidine counterstained with haematoxylin.

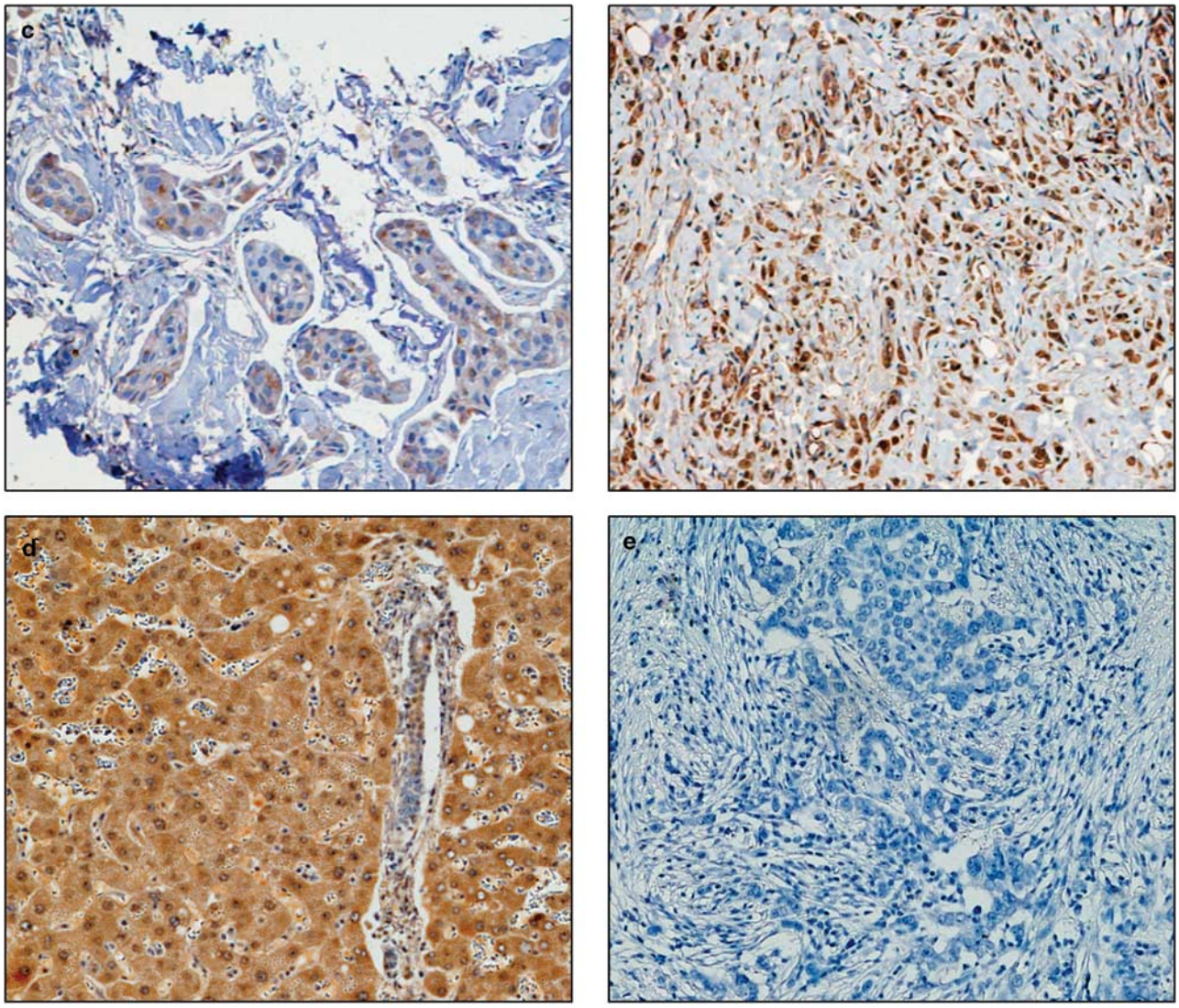

Figure 2 Continued.

($P=0.021$) and Figure 3b demonstrates that a high expression of thioredoxin reductase ($P=0.021$), was associated with distant metastasis-free survival. Multivariate analysis (Table 2) shows that TxNIP ($P=0.017$, hazard ratio (HR) $=0.3$, 95% confidence interval (CI) $=0.1$–0.8) and thioredoxin reductase ($P=0.022$, HR $=0.3$, 95% CI $=0.1$–0.8) are independent factors. Distant metastasis-free survival showed no association with hormone receptor status using Kaplan–Meier/log rank analysis (oestrogen receptor $P=0.180$, HER2 $P=0.585$, progesterone receptor $P=0.892$) and is not known to be of prognostic value in this setting; therefore, no further statistical analysis was conducted incorporating redox protein expression.

In the pre-chemotherapy biopsies, Figure 4a shows that a high expression of TxNIP was associated with a better overall survival ($P=0.037$). Multivariate analysis demonstrated that TxNIP is an independent prognostic factor ($P=0.014$, HR $=0.2$, 95% CI $=0.1$–0.7) and, of note, independent of oestrogen receptor status ($P=0.001$, HR $=0.1$, 95% CI $=0.0$–0.3) (Table 2).

Despite the better pathological complete response from oestrogen receptor negative tumours, surprisingly oestrogen receptor negative patients are known to have a worse overall survival,[12,13] which was also observed in this cohort ($P=0.014$) (Figure 4b). Therefore, TxNIP expression with oestrogen receptor status was analysed, using the same combinations as for pathological complete response. Oestrogen receptor negative/low TxNIP expression had a considerably worse prognosis ($P=0.00006$) (Figure 4c). Analysing this group against the remaining groups combined was highly significant ($P=0.000003$) (Figure 4d) and was an independent prognostic factor ($P=0.0003$, HR $=0.1$, 95% CI $=0.0$–0.4) (Table 2). Oestrogen receptor negative patients can therefore be notably stratified further using TxNIP expression.

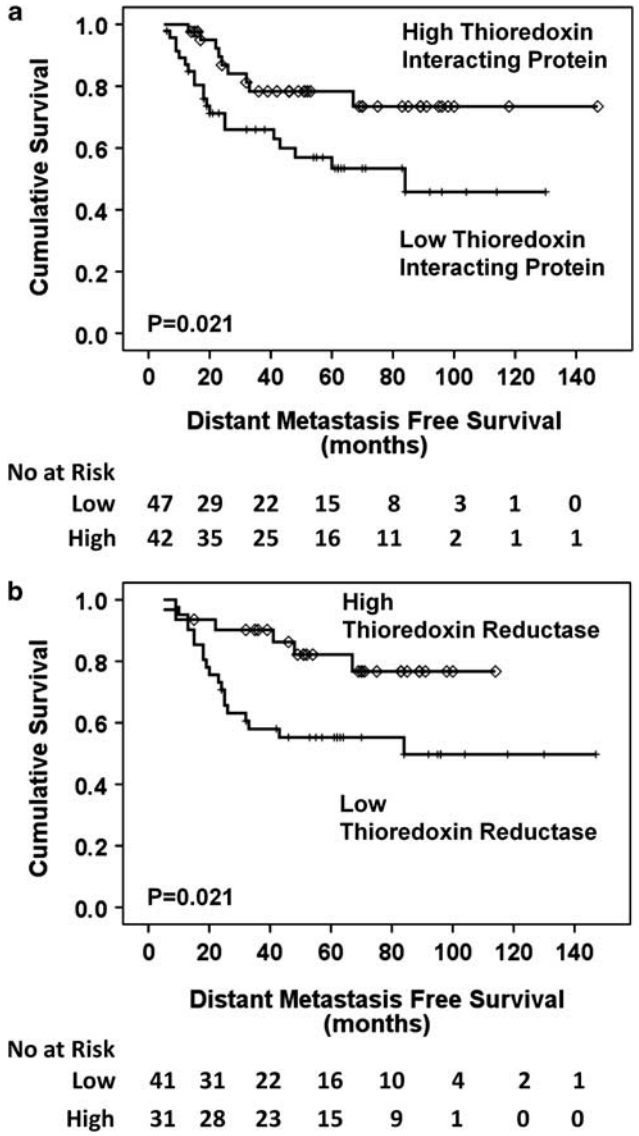

Figure 3 Kaplan–Meier distant metastasis-free survival curves. High thioredoxin interacting protein (**a**) and high thioredoxin reductase (**b**) correlate with a good prognosis ($P=0.021$, $P=0.021$).

Comparison of Redox Expression Pre- and Post-Anthracycline-Based Chemotherapy

A paired set of tumour samples, pre- and post-chemotherapy, were available for certain patients and therefore changes in redox protein expression were evaluated. Thioredoxin ($n=41$), TxNIP ($n=39$), GST θ ($n=43$) and MnSOD ($n=20$) all showed significant increases in expression levels after anthracycline-based chemotherapy ($P=0.00000002$, $P=0.006$, $P=0.023$ and $P=0.048$, respectively) (Figure 5). Although results point towards these pathways being activated by chemotherapy, the level of increase of these four redox proteins was not significant against clinical parameters such as pathological complete response, distant metastasis-free or overall survival. This was not unexpected due to the small sample size involved but this would warrant further investigation in a larger cohort and in serial biopsies after each chemotherapy cycle. Thioredoxin reductase ($n=31$), GST π ($n=46$) and catalase ($n=22$) showed no significant change ($P=0.762$, $P=0.274$ and $P=0.214$, respectively).

Discussion

To our knowledge, this is the first study to examine a panel of redox proteins in a well-defined cohort of locally advanced primary breast cancer cases before receiving neoadjuvant anthracycline-based chemotherapy. Limited studies have utilised pre-biopsy samples and the numbers are generally small due to the nature of the sample type. Results demonstrate that the expression of redox proteins in initial, pre-treatment, tumour specimens may be important prognostically and as determinants of response. The observed absence of expression of GST α was mirrored in two previous studies that reported very low expression in both normal and breast tumour tissues[14] and decreased immunointensity in tumour compared with normal.[15]

High GST π expression associated with a greater probability of pathological complete response and notably further stratified oestrogen receptor negative patients ($P=0.002$). GST π expression has been linked to oestrogen receptor status and also showed an inverse correlation,[16–18] but this is the first report in pre-chemotherapy locally advanced primary breast cancer. Due to the nature of GST π as a detoxification enzyme, studies have connected its high expression to multidrug resistance in various cancers[19,20] but although the literature related to GST π, anthracyclines and breast cancer is relatively consistent *in vitro*, the *in vivo* data are contradictory. In certain studies, a high GST π expression was associated with a shorter distant metastasis-free and overall survival[21,22] whereas others had a better outcome.[23,24] The current study saw no significance with distant metastasis-free or overall survival. In contrast, a low expression of catalase was associated with a greater incidence of pathological complete response ($P=0.045$). Catalase functions to catalyse the decomposition of H_2O_2 to water and oxygen. When catalase expression is low, H_2O_2 formed by anthracyclines may be less effectively processed resulting in hydroxyl radicals and potentially tumour cell death, thus pathological complete response.

High expression of thioredoxin reductase was a prognostic factor for better distant metastasis-free survival ($P=0.022$). Such results may appear to contradict the systems antioxidant function, but it has been shown that endogenous thioredoxin increases the redox cycling of anthracyclines and enhances their apoptotic potential.[25] An increased

Table 2 Multivariate Cox proportional hazards analysis for predictors of distant metastasis-free and overall survival in locally advanced primary breast cancer patients treated with neoadjuvant anthracycline-based chemotherapy

Multivariate analysis for distant metastasis-free survival						
	TxNIP			*Thioredoxin reductase*		
	Hazard ratio	*95% CI*	*Significance*	*Hazard ratio*	*95% CI*	*Significance*
Protein	0.3	0.1–0.8	0.017*	0.3	0.1–0.8	0.022*
ER	0.1	0.0–0.5	0.002*	0.2	0.1–0.7	0.011*
HER2	1.5	0.6–4.2	0.424	1.7	0.6–5.2	0.327
PgR	3.5	1.1–11.7	0.038*	2.1	0.7–6.1	0.166
TNM	1.2	0.3–5.4	0.837	1.7	0.2–14.6	0.642
Age	2.9	1.1–7.3	0.027*	1.8	0.7–4.2	0.206
Multivariate analysis for overall survival						
	TxNIP			*TxNIP with ER status*		
	Hazard ratio	*95% CI*	*Significance*	*Hazard ratio*	*95% CI*	*Significance*
Protein	0.2	0.1–0.7	0.014*	0.1	0.0–0.4	0.0003*
ER	0.1	0.0–0.3	0.001*	—	—	—
HER2	0.4	0.1–2.1	0.294	1.1	0.4–2.8	0.920
PgR	2.1	0.5–8.5	0.288	2.3	0.8–6.7	0.139
TNM	0.4	0.1–2.0	0.254	1.6	0.4–7.1	0.553
Age	3.0	1.1–8.3	0.035*	2.6	1.1–6.2	0.029*

Abbreviation: CI, confidence interval.
*Statistically significant.

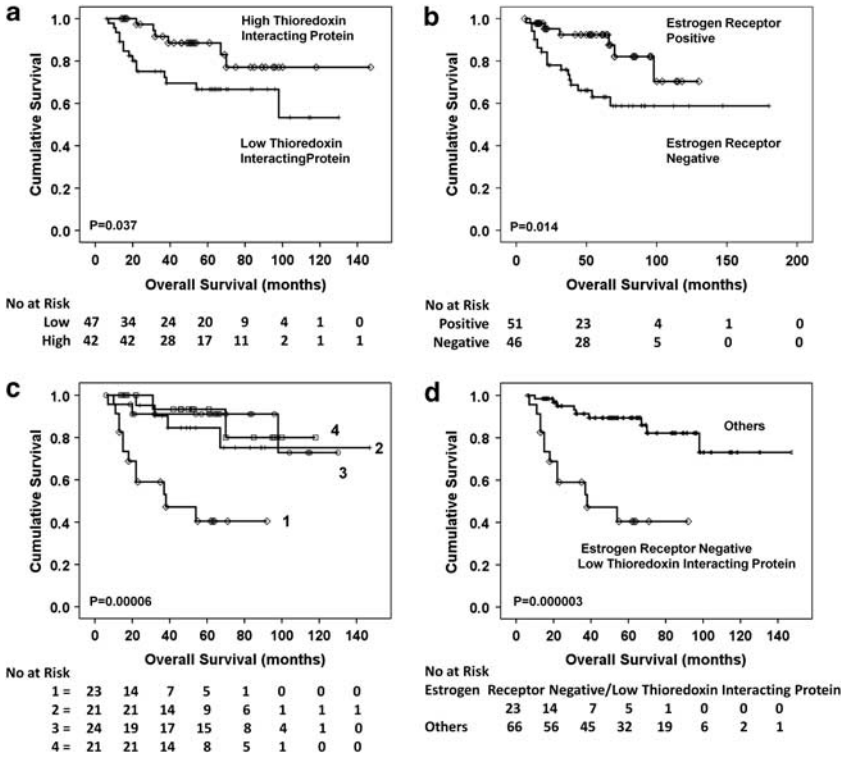

Figure 4 Kaplan–Meier overall survival curves. (**a**) A high expression of thioredoxin interacting protein is significantly associated with a better prognosis ($P=0.037$). (**b**) Oestrogen receptor status is an independent predictive factor ($P=0.014$ in this cohort). Therefore, correlations were made between redox proteins and oestrogen receptor status by grouping patients: 1 = oestrogen receptor negative with low redox expression, 2 = oestrogen receptor negative with high redox expression, 3 = oestrogen receptor positive with low redox expression, 4 = oestrogen receptor positive with high redox expression. (**c**) Oestrogen receptor negative cases with a low thioredoxin interacting protein expression had a considerably worse prognosis ($P=0.00006$) (group 1) and analysing this group against the others combined (**d**) was highly significant ($P=0.000003$).

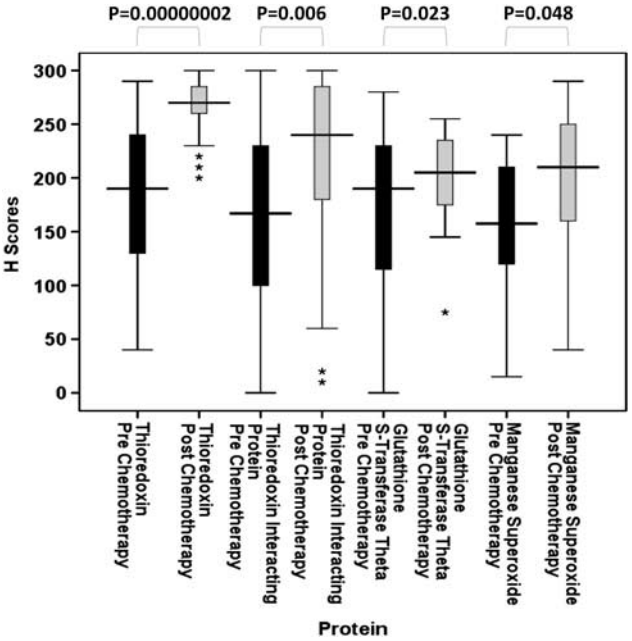

Figure 5 Stem and leaf plots of thioredoxin, thioredoxin interacting protein, glutathione S-transferase θ and manganese superoxide dismutase protein expression pre- and post-anthracycline-based chemotherapy. Figure demonstrates that significant increases in protein expression have occurred after chemotherapy treatment. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

expression of thioredoxin reductase would maintain thioredoxin in its reduced, active state.

In the present study, high expression of TxNIP was also a prognostic factor for a better distant metastasis-free ($P=0.017$) and overall survival ($P=0.014$). Strikingly though, TxNIP combined with oestrogen receptor status subdivided the oestrogen receptor negative cases into those with comparable prognosis to oestrogen receptor positive and those that had a poor outcome for overall survival ($P=0.000003$). To our knowledge, this is the first study to examine the relationship between TxNIP in neoadjuvant locally advanced primary breast cancer cases. One study assessed RNA expression in breast cancer patients that had no treatment and found that a high level of TxNIP was significant for a better metastasis-free survival.[26] TxNIP is a competitive inhibitor of thioredoxin. Overexpression of TxNIP inhibits proliferation of tumour cells,[27] environmental conditions such as H_2O_2 can upregulate its expression[28] and it is downregulated in a number of cancers.[27,29] TxNIP can also function through alternative mechanisms such as Jun activating binding protein affecting its influence on $p27^{kip1}$ [ref. 30] or via suppression of cell invasion/metastasis by association with the β domain of von Hippel-Lindau protein.[31] Importantly, both TxNIP and thioredoxin reductase were independent of oestrogen receptor. Despite reports of oestrogens altering expression of certain redox proteins,[32,33] the authors have not found any reports of this occurring for TxNIP or thioredoxin reductase.

In summary, redox protein expression can determine response to therapy and survival outcomes in patients with locally advanced primary breast cancer subsequently treated with neoadjuvant chemotherapy. The high level of concordance between individual assessors suggests that assessment of redox protein expression may have clinical potential.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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