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# The role of early expression of inducible nitric oxide synthase in human breast cancer

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**Abstract**

Nitric oxide synthases are expressed in breast cancer. To elucidate the clinical role of the inducible NOS (i-NOS) in human breast cancer, 161 primary breast cancer tissues were stained immunohistochemically. Staining patterns for i-NOS were correlated with classical prognostic factors such as lymph node status, age, hormonal receptor status, tumour size and tumour differentiation. With classical prognostic factors such as lymph node status, age, hormonal receptor status, tumour size and tumour differentiation. Patients survival was also analysed. Sixty-one percent of the tumours stained positively for i-NOS. Detection of i-NOS was positively correlated with increasing tumour size and decreasing tumour differentiation ($P = 0.018$ and $P = 0.039$, respectively). However, in the $\leqslant$50 year age group, i-NOS staining also correlated with lymph node status. Patients with i-NOS-positive breast carcinomas had a significantly worse overall survival rate *versus* those with negative stains (5-year survival rate 84.8% *versus* 67.1%; $P = 0.049$; log-rank test). To date, this is the largest analysis of i-NOS expression in breast cancer patients and the only study to assess survival.


*Keywords:* Breast cancer; Nitric oxide synthase; Nitric oxide; Survival

## 1. Introduction

Three isoforms of nitric oxide synthases (NOS) are known; an inducible (i) NOS which is calcium-independent and two calcium-dependent forms: the endothelial (e-NOS) and a neuronal form (n-NOS). Nitric oxide (NO), a diatomic free radical, is highly reactive with other free radicals, molecular oxygen and metal-ions. Intracellular NO is capable of reacting with nitrite and nitrate, S-nithroso-thiols, or peroxynitrite and can cause DNA damage [1]. NO production by endothelial and epithelial cells might be of interest because of its effects on increasing blood flow, inducing angiogenesis, killing tumour cells and reducing tumour cell adhesion [2–5]. NO can protect endothelial cells from tumour necrosis factor alpha (TNF-α)-induced apoptosis. By contrast, cytokine-activated endothelial NO production can kill adherent tumour cells [6]. To understand these conflicting roles of NO in tumour biology, additional studies are required. NO might play a dual role depending on its intra-tumoral concentration.

The role of nitric oxide synthases in breast cancer was first investigated in 1995 [7]. We have previously shown

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that i-NOS and e-NOS are expressed in tumour cells of *in situ* and invasive breast cancer tissues, but not in benign lesions [8]. As determined by an electro-spin trapping (ESR) analysis, we demonstrated that i-NOS and e-NOS in primary breast cancer cells were active enzymes that produce NO [8]. However, only i-NOS was capable of producing NO concentrations in the micromolar range [9].

Several recent studies have investigated the expression of i-NOS in human cancers. An increased expression of i-NOS in tumour cells could be demonstrated for breast, ovarian [10], prostate [11], gastric [12], colorectal [13], and head and neck cancers [14], as well as in non-epithelial malignancies like sarcomas [15].

We conducted the present study using tissue from 161 primary breast cancer patients and correlated the i-NOS expression with classical prognostic factors.

## 2. Patients and methods

### 2.1. Patients

Two hundred patients from the Department of Obstetrics and Gynaecology of the J.-W-Goethe University Hospital were included in the study. 161 patients had a complete follow-up and could be evaluated for tumour recurrence and survival status. Patients with metastatic disease or preoperative chemotherapy were excluded.

### 2.2. Methods

#### 2.2.1. Immunohistochemistry

*i-NOS*. The method has been described previously in detail in Ref. [8], but will be summarised briefly here Tissue blocks were fixed in 4% buffered formalin and embedded in paraffin. Immunohistochemical reactions were performed using antibodies against i-NOS (donated by J. Pfeilschifter, Dept. of Pharmacology, University of Frankfurt, Germany). The polyclonal i-NOS antibody identifies a protein of 130 kDa in lysates of smooth muscle cells. These lysates had been treated with interleukin-1β (100 IE/ml for 24 h) to stimulate the i-NOS (the antibody has been previously described by Nitsch and colleagues in Ref. [16]). Unless otherwise indicated, all other reagents for immunohistochemistry were from Biogenex (San Ramon, USA). The rabbit anti-human i-NOS polyclonal antibody (diluted 1 : 1000, with an incubation time of 60 min at room temperature) was used after the tissue sections had been pre-treated in the microwave oven ($5 \times 3$ min in citrate buffer, 700 W). Thereafter, the alkaline phosphatase – antialkaline phosphatase-method (APAAP) method was applied [17]. The staining procedures were carried out using an automated cell staining system (Optimax plus, Biogenex, San Ramon, USA).

In a semi-quantitative analysis, staining was independently evaluated by two observers using a ranking score of 0 to 4 (0 = negative, 1 = weak positive, 2 = medium positive, 3 = strong positive, 4 = very strong positive). In cases where there were different rankings, a third observer was asked to score them as well and the results were discussed. The cut-off point was the median which was one (all stainings ranked as 1 or above were considered positive).

To control for the specificity of the antibodies used, several control experiments were performed. As cytokines are involved in inflammatory processes, positive control sections were derived from a kidney with interstitial nephritis. Sections from a fibroadenoma of the breast were used as a negative control and were stained in the same way. Tissue sections treated with non-immune rabbit serum in place of the primary antibodies were also used as a second negative control.

#### 2.2.2. Steroid hormone receptor analysis

Oestrogen and progesterone receptor analysis was performed with sections cut from formalin-fixed and paraffin-embedded tissues. After dewaxing through xylol and graded alcohols, the sections were pre-treated in a pressure cooker for 45 sec using ethylene diamine tetra-acetic acid (EDTA)-buffer (pH 8.0). Oestrogen and progesterone receptor expression was examined using mouse-monoclonal antibodies raised against them; oestrogen receptor (NCL-ER-6F11, 1:60), progesterone receptor (NCL-PgR, 1:40) (Novocastra Laboratories Ltd. Newcastle upon Thyne, UK). The incubation time for both antibodies was 30 min. The stainings were developed using the Biogenex Supersensitive MultiLink (detection system, 30 min) (Biogenex, San Ramon, USA) with alkaline phosphatase and fast red as a chromogen (8 min twice). All incubations were performed at room temperature and all staining steps performed using a TECAN robot (Tecan GmbH, Crailsheim, Germany). Sections were washed with TBS (pH 7.6) between each incubation step and were counterstained with Gill's haematoxylin.

#### 2.2.3. Statistical analysis

Associations between variables were analysed using the Spearman–rho test. For univariate analysis of metastatic-free and overall survival, Kaplan–Meier estimation was performed and then compared using Peto's log-rank test. Multivariate Cox proportional hazard analysis was performed in a stepwise backward fashion for lymph node status, grading, tumour size, age, hormonal receptor status, and i-NOS using the Statistical Package for the Social Science (SPSS) software package (SPSS V 9.0, Inc. Chicago, IL). All test decisions were performed at a significance level of $\alpha = 0.05$. Estimated values were given including the 95% Confidence Intervals.

## 3. Results

### 3.1. Patients' characteristics

The patient population consisted of 161 women with invasive breast cancer who had undergone primary surgery. The median age at the time of diagnosis was 54 years (31–88 years). More than one third of the patients (34%) were premenopausal and 62% were postmenopausal. 66% of the patients underwent breast-conserving surgery and 34% had a mastectomy with axillary lymph node dissection. A median number of 15 lymph nodes were excised (range 5–31). Most patients (58%) had involvement of the axillary lymph nodes. Nodal positive patients were divided into different groups. 24% had 1–3 lymph nodes involved, 10% had 4–9 lymph nodes involved and 8% had 10 or more lymph nodes involved. Most patients presented with early stage disease (Table 1). There were no differences for the patients 50 years of age and younger.

All women with breast-conserving surgery received irradiation of the breast. Risk-adapted systemic therapy was given according to age, lymph node involvement, and hormonal receptor status. During the median observation period of 42 months (range 12–84 months), 44 events (local recurrences and distant metastasis) were detected. Twenty-six died during the observation period.

Table 1
Patients' characteristics

| Age (in years) (median) | 54 (31–88) *N* (%) | |
|---|---|---|
| Variables | All ($n$ = 161) | Age ⩽50 years ($n$ = 66) |
| *Menopausal status* | | |
| Premenopausal | 55 (34) | |
| Postmenopausal | 100 (62) | |
| Unknown | 6 (4) | |
| *Tumour stadium (t-stage)* | | |
| 1 | 81 (50) | 33 (50) |
| 2 | 60 (37) | 24 (36) |
| 3 | 10 (6) | 4 (6) |
| 4 | 10 (6) | 5 (8) |
| *Lymph node status* | | |
| Negative | 93 (58) | 40 (61) |
| Positive | 67 (42) | 26 (39) |
| Unknown | 1 (2) | |
| *Grading* | | |
| 1 | 39 (24) | 18 (27) |
| 2 | 93 (58) | 34 (52) |
| 3 | 29 (18) | 14 (21) |
| *Hormone receptor status* | | |
| Positive | 139 (86) | 53 (80) |
| Negative | 22 (14) | 13 (20) |

### 3.2. i-NOS expression and correlation with clinical factors

Overall, 61% of the tumours were positive for i-NOS staining. The staining intensity of most tumours was weak (score 1, 32%). 23% of the analysed tumour specimens had a staining score 2 and only 6% showed strong i-NOS expression (score: 3+ and 4+) (Fig. 1). There were no significant differences in i-NOS expression when patients aged 50 years or less were compared with patients older than 50 years of age – 61% were i-NOS-positive and 39% (26) were i-NOS-negative. None of the tumours showed strong staining for i-NOS (score 4) in the 50 years or less subgroup of patients (Fig. 2).

For the entire population, there was a significant correlation between i-NOS staining intensity and the pathologically-determined tumour size (t-stage) ($P$ = 0.018), as well as the grade of the tumour differentiation ($P$ = 0.039), which was even more significant when categorical variables were used ($P$ = 0.005). The number of i-NOS-positive cases increased from T1: 56% ($n$ = 45/81), T2: 65% ($n$ = 39/60) to T3/T4: 70% ($n$ = 14/20). For grading this increase with increasing grade was even more pronounced (G1: 41%, 16/39; G2: 66%, (61/93; G3:

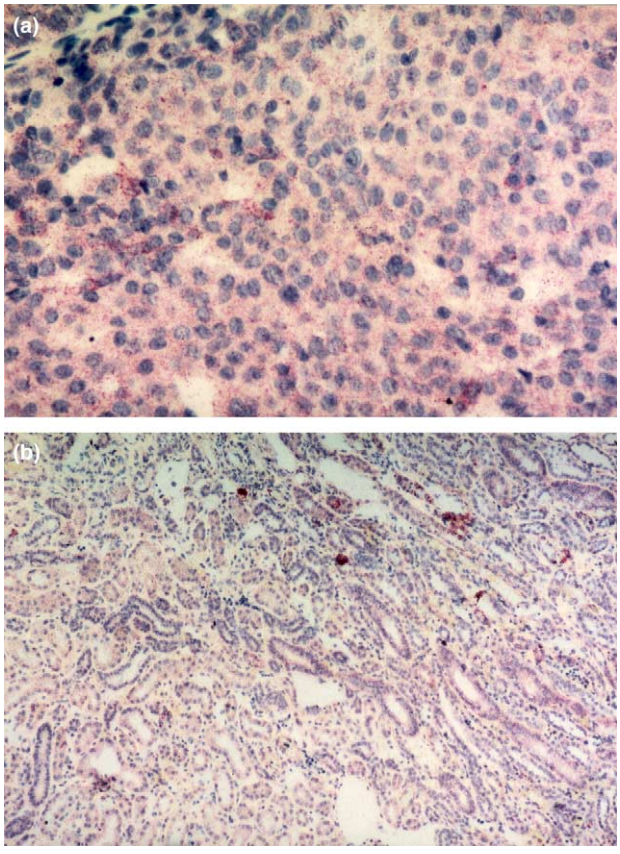


Fig. 1. Immunohistochemical detection of i-NOS in paraffin-embedded breast cancer tissues. (a) Invasive ductal carcinoma, strong staining of tumour cells for i-NOS; Original magnification is 400×. (b) Interstitial nephritis (positive control for i-NOS); Original magnification is 100×.

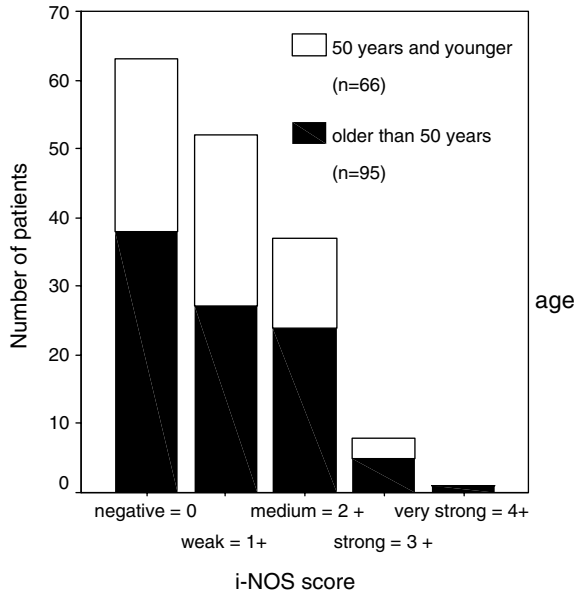


Fig. 2. Diagram demonstrating the i-NOS staining intensity according to age.

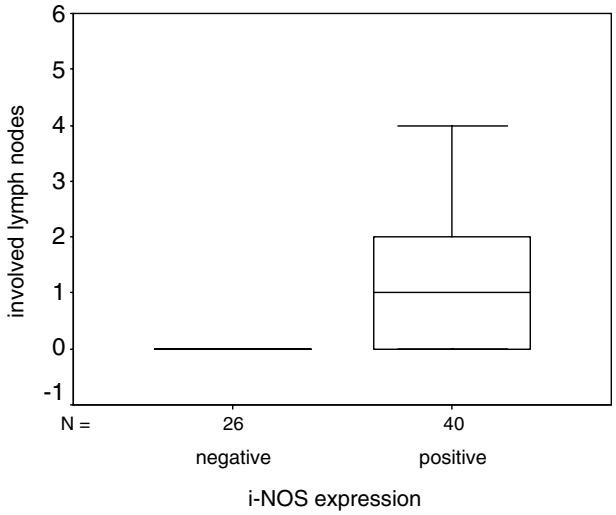


Fig. 3. i-NOS expression and lymph node involvement. The boxplot shows the distribution of lymph node involvement grouped according to i-NOS expression.

72%, 21/29) (Table 2). A significant correlation of i-NOS staining with survival was also observed ($P = 0.015$). There were no correlations between i-NOS status and other classical prognostic factors like lymph node status ($P = 0.13$), age ($P = 0.92$), and hormonal receptor status ($P = 0.30$). However, for patients 50 years of age or younger ($n = 66$), i-NOS expression correlated with the number of involved lymph nodes ($P = 0.011$) and the status of lymph node involvement ($P = 0.005$). None of the patients with i-NOS-negative tumours had an involvement of the lymph nodes, whereas patients with i-NOS-positive tumours showed a median of two positive lymph nodes (Fig. 3).

In the i-NOS-positive group, 24 patients developed metastatic disease, whereas in the i-NOS-negative group, only 11 patients had metastatic disease ($P = 0.21$; log-rank test). Patients with i-NOS-positive tumours had a significantly worse overall survival rate (Fig. 4). During the median observation period of 42 months, only six patients died in the i-NOS-negative group ($n = 63$), whereas 20 patients died in the i-NOS-positive group ($n = 98$) ($P = 0.049$; log-rank test).

### 3.3. Multivariate survival analysis

Multivariate Cox analysis (stepwise backward regression) showed that i-NOS is not an independent prognostic factor for overall survival ($P = 0.077$; Hazard Ratio

Table 2
i-NOS expression and different tumour characteristics

| Characteristics | All patients ($n = 161$) | | | | | ⩽50 years ($n = 66$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | i-NOS score | | | | | i-NOS score | | | | |
| | 0 | 1 | 2 | 3 | 4 | 0 | 1 | 2 | 3 | 4 |
| *T-stadium* | | | | | | | | | | |
| 1 | 36 | 30 | 12 | 3 | 0 | 14 | 13 | 5 | 1 | 0 |
| 2 | 21 | 17 | 18 | 4 | 0 | 9 | 9 | 5 | 1 | 0 |
| 3 | 3 | 1 | 4 | 1 | 1 | 1 | 0 | 2 | 1 | 0 |
| 4 | 3 | 4 | 3 | 0 | 0 | 2 | 2 | 1 | 0 | 0 |
| *Grade* | | | | | | | | | | |
| 1 | 23 | 7 | 7 | 2 | 0 | 10 | 4 | 3 | 1 | 0 |
| 2 | 32 | 32 | 24 | 4 | 1 | 13 | 12 | 8 | 1 | 0 |
| 3 | 8 | 13 | 6 | 2 | 0 | 3 | 8 | 2 | 1 | 0 |
| *Lymph node* | | | | | | | | | | |
| Negative | 39 | 32 | 19 | 2 | 1 | 21 | 12 | 7 | 0 | 0 |
| Positive | 23 | 20 | 18 | 6 | 0 | 5 | 12 | 6 | 3 | 0 |

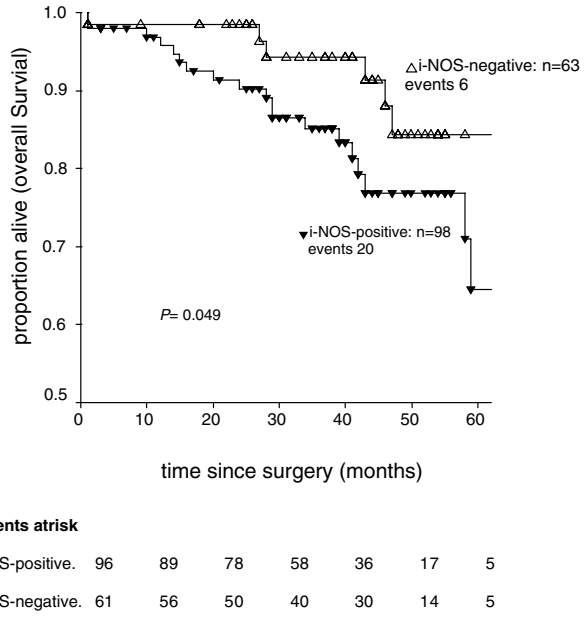


Fig. 4. Kaplan–Meier estimation of breast cancer patients according to i-NOS expression (log-rank test $P = 0.049$). i-NOS overall survival; △, i-NOS-negative: 6/63; ▼, i-NOS-positive: 20/98.

(HR) 2.25 [95% CI: 0.89–5.65]). Only lymph node status, the strongest significant prognostic factor ($P = 0.0015$; HR: 4.89 [95% CI: 1.83–13.07]) and grading ($P = 0.0014$; HR 3.7 [95% CI: 1.61–8.33]) were significant independent prognostic factors for overall survival. Tumour size and the receptor status were only dependent prognostic factors (Table 3).

## 4. Discussion

The precise pathophysiological functions of NO in human breast cancer are not well understood. This is due to the relatively small number of samples analysed, as well as the heterogeneity of human breast cancer. This study was undertaken to investigate the expression of i-NOS in a large group of patients with invasive breast cancer and to correlate the i-NOS expression with standard clinico-pathological parameters. So far, new prognostic factors like urokinase plasminogen activator (uPA) and Plasminogen activator inhibitor-1 (PAI-1), Her-2 neu or cyclin E have not been included as prognostic factors for breast cancer [18]. We therefore compared i-NOS expression and standard clinicopathological factors.

We recently demonstrated that NOS is expressed in invasive breast cancer and in *in situ* lesions of the breast, but not in benign lesions [8]. However, Tschugguel and colleagues observed staining of i-NOS in benign lesions as well [19]. It appears that expression of i-NOS may be early, and probably necessary, event for tumour progression that occurs in parallel with increasing tumour size and decreasing tumour differentiation [19,20]. However, two groups have demonstrated [19,20] that i-NOS expression is higher in grade II than grade III tumours, whereas Vakkala and colleagues [21] and the first investigations by Thomsen and colleagues [7] support our findings of a positive correlation between increasing i-NOS positivity and decreasing tumour differentiation.

Increased expression of i-NOS in human adenocarcinomas has been demonstrated to stimulate tumour growth and metastasis *in vivo* [22]. i-NOS expression has been investigated in several different human carcinomas. Adenocarcinomas like ovarian cancer [10], prostate [11], colorectal [13] and gastric [12] all demonstrate a positive correlation of i-NOS with angiogenesis and tumour progression. Data regarding expression in squamous cell carcinomas, although not extensive, also demonstrates a correlation between i-NOS and tumour progression [14]. By contrast, data derived from patient's lung cancer is conflicting and it seems that i-NOS expression is associated with a favourable prognosis in this group [23].

As described in another study of only 23 breast cancer patients [24], we did not find a correlation between i-NOS expression and axillary lymph node involvement for the entire study population. However, we could demonstrate an association in the younger patient group, supporting the theory that NO synthesis in primary breast cancers favours the development of metastasis [24].

The hypothesis that there is a relationship between i-NOS and angiogenesis in breast cancer had been demonstrated recently in a study that showed the correlation between i-NOS expression and microvessel density [21].

Table 3
Univariate and multivariate analysis of known prognostic factors (Cox-regression) for the overall survival

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | HR (95% Confidence interval) | *P*-value | HR (95% Confidence interval) | *P*-value |
| Grade | 3.67 (1.66–8.15) | 0.0014 | 3.7 (1.61–8.33) | 0.0014 |
| Lymph node | 5.35 (2.01–14.24) | 0.0008 | 4.89 (1.83–13.07) | 0.0015 |
| i-NOS | 1.36 (1.01–3.14) | 0.047 | 2.25 (0.89–5.65) | 0.077 |
| Receptor status | 2.32 (0.97–5.56) | 0.051 | – | – |
| Tumour size | 2.39 (1.00–5.71) | 0.049 | – | – |
| Age | 0.78 (0.35–1.75) | 0.54 | – | – |

HR, Hazard ratio.

Experimental models have shown that solid tumour growth is limited to a maximum diameter of 1–2 mm in the absence of angiogenesis to supply its metabolic needs. Several angiogenic factors are produced either by tumour cells or by tumour-associated cells, such as the surrounding fibroblasts or macrophages [25]. In large tumours, the central area is necrotic and hypoxic. These conditions change the biological behaviour of tumour cells. Hypoxia is a strong stimulus for vascular endothelial growth factor (VEGF), as well as for i-NOS [26–28]. Mammary adenocarcinoma cells (EMT-6) had a significantly higher expression of i-NOS *in vitro* under hypoxic conditions after stimulation with cytokines (interferon gamma and interleukin 1-β) or lipopolysaccharide [29,30]. This indicates that cytokines are capable of stimulating i-NOS to produce NO. As shown by Ziche and colleagues, NO itself plays a direct role in VEGF-mediated angiogenesis [3]. VEGF is correlated with microvessel density and both factors are associated with a poor prognosis in breast cancer patients.

Similar investigations in human colorectal cancers found a significant correlation between i-NOS and VEGF expression; both factors correlated significantly with intra-tumoral microvessel density [13]. Song and colleagues examined the relationship between i-NOS, VEGF and microvessel density in gastric cancers and also found similar results, as well as an impact on survival for i-NOS [12].

We believe these data and our findings indicate that breast cancer patients with i-NOS-positive tumours may have a worse outcome than those with i-NOS-negative tumours, independent of their age.

In conclusion, this is the largest population of primary breast cancer patients to date in which the expression of i-NOS has been examined semi-quantitatively. We demonstrated a correlation between i-NOS expression in patients according to their tumour size, tumour differentiation, as well as a poorer outcome for i-NOS-positive patients. A more profound understanding of the NO pathway may improve our knowledge of mechanisms responsible for tumour angiogenesis and tumour growth and metastasis and may provide new targets for anti-angiogenic drugs.

## Conflicts of interest

None declared.

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