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Extended neoadjuvant chemotherapy in locally advanced breast cancer combined with GM-CSF: effect on tumour-draining lymph node dendritic cells

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Abstract

The effect of long-term administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) on dendritic cell (DC) activation and survival in patients with locally advanced breast cancer (LABC) was studied. To this end, the number of activated DC (i.e. positive for the marker S100) in tumour-draining lymph nodes (TDLN) was determined and compared between LABC patients receiving neoadjuvant chemotherapy with GM-CSF ($n=52$) or without GM-CSF ($n=11$), and a control group of chemo-naïve breast cancer patients ($n=10$). A significantly higher mean percentage of S100+ DC in the TDLN of the GM-CSF-treated patients (9.9%) was found compared with each of the respective control groups (5.3 and 5.1%, $P=0.002$). Moreover, intrapatient comparison before and after treatment showed that the percentage of S100+ DC significantly increased over the course of the GM-CSF treatment ($P=0.018$). In a univariate survival analysis with a median follow-up of 64 months, relatively high percentages of S100+ DC ($\geq 8\%$) were associated with a longer disease-free survival (DFS) ($P=0.078$). In patients with a high tumour load, where immunosuppressed conditions generally prevail, long-term administration of GM-CSF may thus contribute to survival through enhanced DC activation and consequently improved chances of effective antitumour immunity.

Keywords: GM-CSF; Dendritic cells; Lymph nodes; Breast cancer; Neoadjuvant chemotherapy

1. Introduction

In an attempt to improve the overall survival (OS) of patients with locally advanced breast cancer (LABC), we previously performed a phase II study with neo-adjuvant chemotherapy followed by local therapy [1]. We applied four, five or six cycles of moderately high doses of doxorubicin and cyclophosphamide supported by granulocyte-macrophage colony-stimulating factor (GM-CSF). A high response rate of 98% was observed, and patients who received six cycles of treatment showed an improvement in disease-free survival (DFS) and OS [1]. Although this clinical study was not conducted as a randomised trial, the impressive OS of patients receiving six cycles made us speculate as to its cause. An increase in the total dose of chemotherapy seemed unlikely since other studies with dose-intensive chemotherapy failed to significantly improve survival [2,3]. In two aspects this phase II study varied from the conventional approach to LABC. First, in view of its immunomodulatory characteristics, in addition to its haematopoietic effects, we administered GM-CSF instead of the more commonly used granulocyte colony-stimulating factor (G-CSF). Secondly, we applied six cycles (when possible) of neoadjuvant chemotherapy instead of the more commonly used three cycles of chemotherapy followed by local therapy and three additional cycles of adjuvant chemotherapy [4]. As a consequence, the primary tumour and its draining lymph nodes (TDLN) remained longer *in situ*. Conceivably, this may have prevented the outgrowth of possible micrometastases [5], through either one of two

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physiological anti tumour mechanisms. The first mechanism, which will be described elsewhere, involves anti-angiogenic factors produced by the primary tumour that may keep micrometastases dormant. The second mechanism entails the generation of tumour-specific immunity through the release of tumour-specific antigens from the primary tumour and the subsequent priming of specific T cells in the preserved TDLN.

GM-CSF, administered for haematological support in the applied neoadjuvant chemotherapy regimen, is an important cytokine for growth and activation of myeloid DC [6]. It is mostly exploited as an adjuvant in clinical immunotherapeutic strategies. However, data on the immunopotentiating effects of GM-CSF when used as a haematopoietic growth factor in combination with chemotherapy are sparse [7]. DC are the most powerful antigen-presenting cells identified [8]. Recent studies indicate that DC play an important role in tumour immunity. DC are optimally equipped to break immunological tolerance and disrupt active immunosuppression, both conditions that are often associated with malignancy [9]. The primary function of DC is to act as sentinels between the outside world and the body. After antigen uptake, DC migrate rapidly to T-cell areas in the draining lymph nodes where they can activate memory or naive T-cells. As draining lymph nodes are the natural meeting point for DC and circulating T cells and the extent of DC infiltration into draining lymph nodes was previously reported to be correlated with enhanced survival in other tumour types [10,11], we decided to study the presence of DC in the TDLN.

2. Patients and methods

2.1. Patients

324 axillary lymph nodes (ALN) of 73 breast cancer patients were examined in this study. The median number (±standard deviation) of lymph nodes examined and of positive lymph nodes of all patients at the time of surgery was 12 (±5.2) and 2.5 (±3.8), respectively. Three groups of patients were studied.

2.1.1. LABC study group

52 patients with LABC stage IIIA and IIIB (according to the American Joint Committee on Cancer (AJCC) criteria) were treated with neoadjuvant doxorubicin and cyclophosphamide + GM-CSF according to our study protocol as previously described in Ref. [1] (Table 1). Doxorubicin 90 mg/m^2 and cyclophosphamide 1000 mg/m^2 (with a dose reduction of 10% in the second and fourth cycles) were administered by intravenous (i.v.) bolus injection every 21 days, followed by subcutaneous (s.c.) GM-CSF (Leucomax®, Schering-Plough, Madison, NJ, USA) 250 μg/m^2/day administration from days 2 to 11. Local therapy consisted of mastectomy and axillary lymph node (ALN) dissection 4–6 weeks after the last chemotherapy cycle, followed by radiotherapy.

In 21 of these 52 patients, a pretreatment subclavicular lymph node biopsy was performed as a staging procedure. 14 subclavicular lymph nodes contained an insufficient amount of lymphoid tissue due to abundant tumour infiltration. Therefore, only seven pretreatment lymph nodes were available for DC quantitation.

2.1.2. GM-CSF-naïve control group

This control group was chosen to determine the contribution of GM-CSF. 11 patients with LABC stage IIIA and IIIB were treated with comparable dosages of neoadjuvant chemotherapy (three cycles of moderately high dose FEC consisting of 5-fluorouracil 500 mg/m^2, epirubicin 120 mg/ m^2, and cyclophosphamide 500 mg/ m^2 every 3 weeks) without a haematopoietic growth factor [3]. These patients were matched for age, primary tumour size, clinical and pathological response with the patients of the LABC group treated with $\leqslant$4 cycles of chemotherapy.

2.1.3. Chemonaïve control group

Chosen to test the possible effect of neoadjuvant chemotherapy alone on the DC content of the TDLN. Since untreated LABC patients are considered to be non-operable, 10 chemonaïve patients with stage I or II invasive breast cancer were selected who underwent surgery including an ALN dissection as their primary treatment.

2.2. Immunohistochemical staining

For each patient, one or more lymph nodes of each axillary segment (the basal, middle and upper segment, also containing the top nodes) up to a minimum of four lymph nodes were stained by immunohistochemistry (IHC).

The lymph nodes were fixed in neutral buffered formaldehyde and processed to paraffin according to standard procedures. From the paraffin blocks, 4-μm thick sections were cut and mounted on poly-L-lysine coated slides. Endogenous peroxidase was blocked with H_2O_2 in methanol. Enzymatic antigen retrieval was performed with protease II for 16 min at 40 °C according to the Ventana protocol [12]. Staining of the slides was also performed according to the Ventana protocol. Briefly, all of the steps were performed at 40 °C. Incubation with the primary antibody was done for 20 min in a 1:400 dilution. For detection, the slides were incubated with a biotin labelled secondary antibody, followed by incubation with peroxidase labelled streptavidin. Staining was visualised with 0.05% 3,3′-diaminobenzidine

Table 1
Patients' characteristics

	LABC test group	GM-CSF-naïve control group	Chemonaïve control group
Number of patients	52	11	10
Age mean (range) (years)	47 (25–71)	50 (37–59)	58 (34–84)
Clinical stage			
I	0	0	4
IIA	0	0	5
IIB	0	0	1
IIIA	26	6	0
IIIB	26	5	0
Primary tumour			
T1	3	0	7
T2	1	2	3
T3	20	7	0
T4	28	2	0
T5			
Nodal status (clinical)			
N0	13	1	9
N1	17	8	1
N2	22	2	0
Neoadjuvant chemotherapy			
Doxorubicin/cyclophosphamide			
2–4 cycles[a]	8	–	–
5 cycles[a]	13	–	–
6 cycles	31	–	–
5-FU/epirubicin/cyclophosphamide	–	11	–
3 cycles			
GM-CSF			
0 injections[b]	2	11	–
10–29 injections[b]	5	–	–
30–49 injections[b]	8	–	–
50–59 injections[b]	12	–	–
60 injections	25	–	–

GM-CSF, granulocyte-macrophage colony stimulating factor; 5-FU, 5-fluorouracil; G-CSF, granulocyte colony-stimulating factor; LABC, locally advanced breast cancer.

[a] Initially, some patients with a good clinical response received less than six cycles. However, as the study progressed, six cycles were administered whenever possible.

[b] In 11 patients, GM-CSF was not administered according to the protocol. One patient experienced an anaphylactic reaction after the first injection of GM-CSF and therefore G-CSF (Neupogen®, Amgen) was administered as the haematopoietic growth factor during all cycles. One patient was treated with six cycles plus G-CSF by mistake. 9 patients experienced GM-CSF-induced skin toxicity leading to GM-CSF discontinuation.

tetrahydrochloride dihydrate containing 0.02% H_2O_2, followed by a signal enhancing step with copper. Nuclear counterstaining of the slides was done with haematoxylin. The slides were dehydrated in alcohol and mounted in DePeX mounting medium (BDH Laboratory Supplies, UK). In the negative controls, the primary antibody was omitted. Normal tonsil tissue was used as a positive control.

2.3. Quantitation of dendritic cells

Quantitative IHC was performed according to a protocol that was validated as unbiased and reproducible using the Q-Prodit interactive video overlay system (Leica, Cambridge, UK) [13]. In short, following demarcation of the whole lymph node at a low magnification, the software driven scanning stage selected 300–500 fields of vision at random. A six-point Weibel grid was superimposed on the microscopic image on the computer screen, and S100+ or negative cells were registered based on the overlay of the grid points. In this procedure, the typical DC morphology was always considered to avoid false-positive hits. Based on the cumulative data of all fields of vision per lymph node, the area percentage of S100 positivity (further denoted as the percentage of S100+ cells) was calculated for each lymph node. Lymph nodes were assessed by two independent investigators, and mean counts were calculated.

To test the lymphocyte density in TDLN, all lymphocytes in pre- and post-treatment lymph nodes of 7 patients were counted. In brief, following demarcation,

the lymph node was scanned at 40× magnification using the Q-Prodit system (surface area of 1.0×10^3 mm^2 on the video screen). The lymph node was divided into 200 tumour-free fields. In this way, the total number of lymphocytes per square mm could be calculated.

2.4. Statistical analyses

- *For the percentages of DC*: In the 7 patients of whom pretreatment subclavicular lymph nodes were studied, the percentages of S100+ DC before and after treatment were compared with the Wilcoxon Signed Rank test. Percentages of S100+ DC in the different tumour axillary levels in patients from the same treatment groups were compared with the Kruskal–Wallis test. Percentages of S100+ DC in the lymph nodes of the three patient groups were also compared with the Kruskall–Wallis test. To test if there was a trend for more S100+ DC in the lymph nodes with increasing cumulative dose of GM-CSF, the Spearman test was applied. To compare the lymphocyte-density before and after treatment the Wilcoxon-Signed Rank test was used.
- *For survival analysis*: The LABC patients who received GM-CSF were divided into two groups with a low or high percentage of S100+ DC. The cut-off point was the mean percentage of S100+ DC+2× standard deviation of the chemonaïve breast cancer control group. OS was defined as the time between the start of chemotherapy and disease-related death, and DFS as the time between the day of operation and date of last follow-up or recurrence of disease. Kaplan–Meier curves were plotted, and differences between the curves were analysed using the log-rank test. Multivariate survival analysis was performed with the Cox regression model using enter and remove limits of 0.1. Tests were carried out with the Statistical Package for Social Sciences (SPSS) version 9.0 statistical package.

3. Results

We studied the number of S100+ DC in TDLN in three different groups of breast cancer patients. 52 LABC patients received four ($n=8$), five ($n=13$) or six ($n=31$) cycles of neoadjuvant AC chemotherapy with GM-CSF, as published before in Ref. [1]. Due to well-known side-effects [6], 11 patients did not receive the planned dose of GM-CSF although 9 of them received at least 10 injections [1] (Table 1). In the two groups that were retrospectively added to this study, 11 LABC patients received three cycles of neoadjuvant FEC chemotherapy and 10 patients received surgery only (Table 1).

Fig. 1a and b shows examples of TDLN with low and high numbers of S100+ DC, respectively. Typical DC morphology could be observed at a higher magnification (Fig. 1c).

In the 7 patients whose pretreatment subclavicular lymph nodes were studied, the percentage of S100+ DC was significantly increased following neoadjuvant chemotherapy with GM-CSF (with a mean value of 0.5% before and 8.9% after neoadjuvant treatment, $P=0.018$) (Fig. 2).

The TDLN lymphocyte density was the same in the pre- and posttreatment lymph nodes (17.8×10^3/mm^2

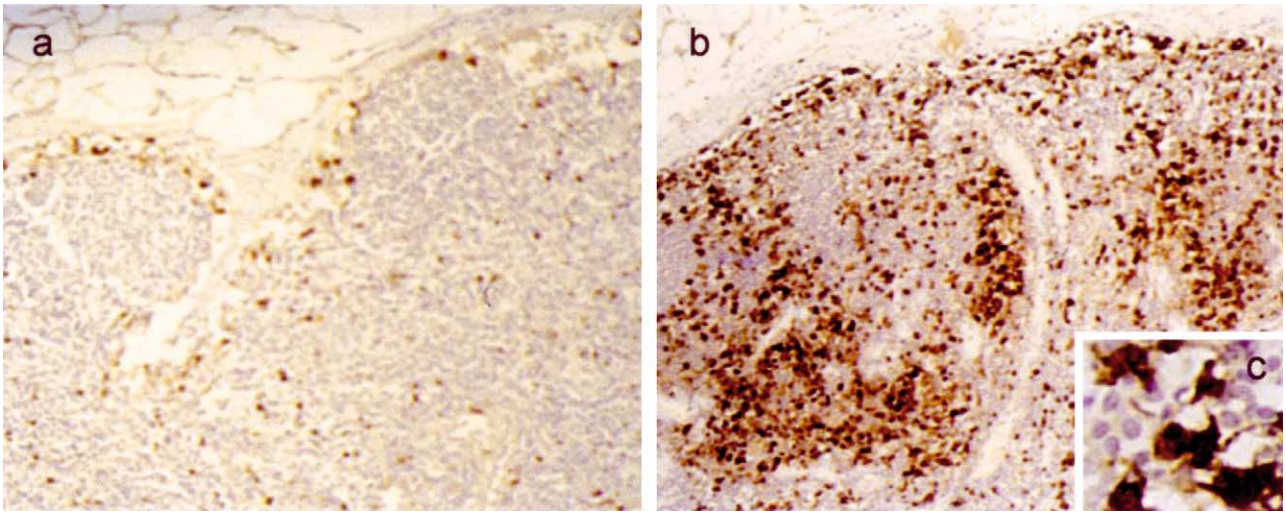

Fig. 1. S100 staining of tumour-draining lymph nodes: (a) low percentage of S100+ dendritic cell (DC) (3.1%) in a lymph node of a locally advanced breast cancer (LABC) patient in control group I, treated with three neoadjuvant chemotherapy cycles without granulocyte-macrophage colony stimulating factor (GM-CSF); (b) high percentage of S100+ DC (20%) in a lymph node of a LABC patient treated with six cycles of neoadjuvant chemotherapy combined with GM-CSF; (c) at a higher magnification, the typical DC morphology is visualised.

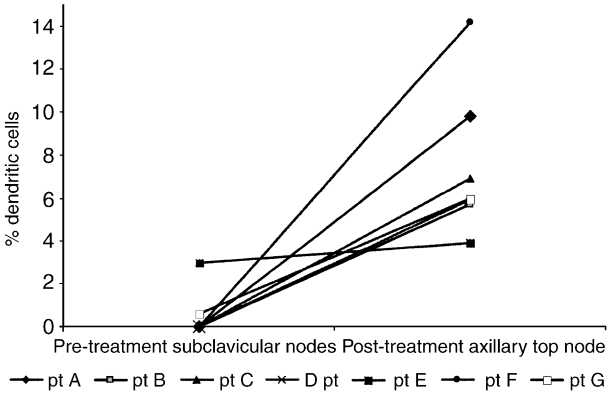

Fig. 2. Percentage of axillary S100+ dendritic cell (DC) before and after treatment within the same patient ($n=7$). pt, patient.

and $17.1\times10^3/mm^2$), indicating that the observed increase in DC percentages is an absolute and not a relative increase (e.g. due to a cyclophosphamide-induced decrease in the number of lymphocytes [14]).

Percentages of S100+ DC did not differ between the different tumour draining levels in patients from the same treatment groups (data not shown), indicating that the distance to the tumour had no effect on the percentage of S100+ DC.

No difference in percentages of S100+ DC was found between the chemonaïve and GM-CSF-naïve control groups (5.1% versus 5.3%), but there was a significant increase in the LABC test group treated with GM-CSF (9.9%, $P=0.002$, Fig. 3) compared with the other groups separately. Moreover, the percentage of S100+ DC in patients of the LABC group treated with $\leqslant4$ cycles of chemotherapy in combination with GM-CSF was higher than in GM-CSF-naïve control group (8.6% versus 5.3%; $P=0.03$), which received the same number of treatment cycles.

There was a trend for higher percentages of S100+ DC with increasing duration of GM-CSF administration ($P=0.05$) (Fig. 4).

Several prognostic factors, divided into 'novel' DC-related and 'traditional' pathological and clinical factors

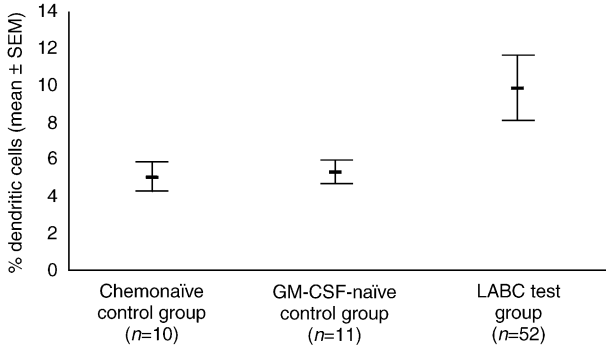

Fig. 3. Percentage S100+ dendritic cell (DC)±standard error of the mean (SEM) according to the different patient groups (see text).

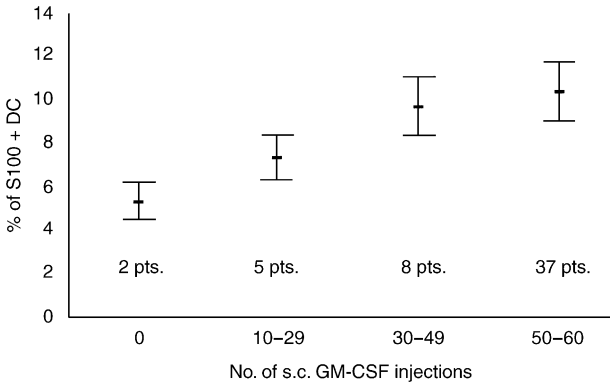

Fig. 4. Percentage of S100+ dendritic cell (DC)±standard error of the mean (SEM) according to the duration of GM-CSF administration. pts., patients; s.c., subcutaneous.

(Table 2) were analysed at a median follow-up time of 64 months from the start of chemotherapy (range 10—114 months). Patients with high percentages of S100+ DC ($\geqslant8\%$) in the axillary lymph nodes tended to have a better DFS ($P=0.078$) (Fig. 5).

Table 2
Characteristics of the prognostic variables of 52 locally advanced breast cancer patients, treated with neoadjuvant doxorubicin, cyclophosphamide and GM-CSF[a]

	Number of patients (%)
a. % of axillary DC	
Low (<8%)	29 (56)
High (≥8%)	23 (44)
Number of GM-CSF injections	
0–9	2 (4)
10–29	5 (10)
30–49	8 (15)
50–60	37 (71)
b. Tumour size	
T1	3 (6)
T2	1 (2)
T3	20 (38)
T4	28 (54)
Number of tumour-containing axillary lymph nodes at surgery	
0	16 (31%)
>1	36 (69%)
Number of chemotherapy cycles	
<6	21 (40%)
6	31 (60%)
Clinical response	
Stable disease	1 (2%)
Partial response	27 (52%)
Complete response	24 (46%)

[a] These variables were used for the survival analysis (see Table 3) at a medium follow-up of 61 months (range 10–93 months): a: 'new dendritic cell (DC)-related' prognostic factors; b: 'traditional' pathological and clinical risk factors.

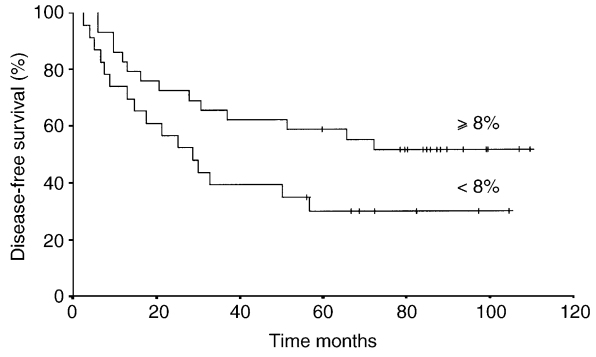

Fig. 5. Disease-free survival (DFS) of the locally advanced breast cancer (LABC) group ($n=52$) divided according to a high ($\geq 8\%$) or low ($<8\%$) percentage of tumour draining lymph node S100+ dendritic cell (DC).

Univariate survival analysis data of all 52 LABC patients treated with doxorubicin and cyclophosphamide are shown in Table 3. The number of chemotherapy cycles, the number of GM-CSF injections and the absence of tumour-containing lymph nodes at the time of operation were associated with DFS (Table 3). For OS, the number of chemotherapy cycles, the absence of tumour-containing lymph nodes at the time of operation and the number of GM-CSF injections were significantly associated. Overall, the LABC group showed a 5-year OS of 63%, and the subgroup of patients treated with six cycles of chemotherapy a 5-year OS of 74%, which compares favourably with historical controls [2,4].

In the multivariate analysis performed with the Cox regression model, the number of chemotherapy cycles and the absence of positive lymph nodes at surgery were prognostic variables for DFS. For OS, no prognostic variables were significant in the multivariate analysis.

4. Discussion

Immunohistochemical analysis of S100 positive DC revealed that the administration of GM-CSF in combination with prolonged neoadjuvant chemotherapy results in an increased number of activated DC in the TDLN of LABC patients. The higher percentage of DC in TDLN may reflect a favourable immunotherapeutic effect of this treatment in addition to the direct tumoricidal effect of the chemotherapy [15]. Indeed, there was a trend for higher percentages of S100+ DC with increasing duration of GM-CSF administration in the presence of the primary tumour and draining lymph nodes.

The applied neoadjuvant treatment resulted in a clinical complete or partial remission in 98% of the LABC patients, and in 30% of these patients in a pathological complete remission of the axillary lymph nodes [1]. The post treatment axillary top nodes were free of tumour or contained only microscopic amounts of tumour.

It is our hypothesis that these clinical results may be partly the result of an enhanced tumour-specific immunity related to the chemo-immunotherapeutic approach. Effective immunity against solid tumours is often hampered on several levels. We postulate that the therapeutic approach described in this study may help to overcome obstacles at several of these levels [15].

Tumour-derived factors hamper DC differentiation *in vitro* [16] and reduced DC numbers and a deficient DC function and differentiation has been reported in patients with advanced breast cancer [17,18]. This is consistent with the strikingly low percentage of DC in pretreatment subclavicular LN in comparison to the posttreatment LN, particularly since more than 50% of these pretreatment LN were infiltrated by tumour cells. Treatment with GM-CSF may counteract tumour-associated DC-inhibitory effects through DC precursor

Table 3
Survival analysis with the proportional hazards model of disease-free survival (DFS) and overall survival (OS) in 52 LABC patients treated with neoadjuvant chemotherapy and GM-CSF

Factor	Hazard ratio	95% Confidence Interval	*P* value
Disease-free survival			
Univariate analysis			
Number of chemotherapy cycles	0.48	0.33–0.72	0.0004
Number of GM-CSF injections	0.60	0.40–0.89	0.0122
Positive axillary lymph nodes at surgery	3.83	1.33–11.03	0.0129
Multivariate analysis			
Number of chemotherapy cycles	0.51	0.29–0.91	0.0234
Positive axillary lymph nodes at surgery	3.16	1.06–9.48	0.0397
Overall survival			
Univariate analysis			
Number of chemotherapy cycles	0.44	0.26–0.73	0.0016
Positive axillary lymph nodes at surgery	4.39	1.30–14.83	0.0172
Number of GM-CSF injections	0.60	0.39–0.92	0.0194
Clinical response	2.38	1.02–5.53	0.0437

mobilisation from the bone marrow and through enhanced DC maturation and activation. Other studies have shown that the isolated and contained growth patterns of solid tumours can hamper immune responses, both in their induction and effector phase [19]. By the induction of both necrotic and apoptotic events in the tumour cells, the applied neoadjuvant chemotherapy can affect the release of tumour antigens at the tumour site. Subsequently, as a result of both the prolonged conservation of the TDLN and the co-administration of GM-CSF, tumour-specific cytotoxic T lymphocyte (CTL) activation by DC may be optimised [19]. The compromised structural integrity of the primary tumour may further facilitate its infiltration by effector CTL, that home there after their dissemination in the peripheral blood.

In addition to these immunological effects, the longer presence of the primary tumour may have contributed to the encouraging results through inhibiting effects on angiogenesis in micrometastases.

In conclusion, based on the present data, we hypothesise that the combination of prolonged preoperative chemo- and immunotherapy may have many putative synergistic effects that could result in an improved outcome for LABC patients. A randomised bifactorial multicentre trial for LABC patients comparing six cycles of neoadjuvant chemotherapy with three neoadjuvant and three adjuvant cycles combined with either GM-CSF or G-CSF has therefore been initiated. Lymph nodes and primary breast cancer tissue, as well as peripheral blood, will be analysed, e.g. for DC numbers and function and CTL responses. Through its bifactorial design, this prospective study should be able to confirm our hypothesis that the combination of prolonged neoadjuvant chemotherapy with GM-CSF may contribute to an improved survival in LABC patients. If so, the present approach would be applicable in various locally advanced tumours, i.e. bladder, stomach and oesophageal cancers.

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