Original paper

SMAC protein expression as a potent favorable prognostic factor in locally advanced breast cancer

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Preoperative systemic therapy including neoadjuvant chemotherapy (NCT) is standard treatment in locally advanced breast cancer (LABC), the aim of which is to enable a radical surgery and to reduce the risk of local and distant recurrence. It has been established that NCT in LABC may effectively induce apoptosis. The study objective was to assess the role of a proapoptotic second mitochondria-derived activator of apoptosis (SMAC) in LABC.

The study group comprised 56 patients with advanced non-metastatic breast cancer (stage IIB –node positive and III), who received NCT followed by surgery and adjuvant treatment. Expression of SMAC protein was analysed using the immunohistochemistry technique in core biopsies sampled from the patients' breasts before NCT and in surgical specimens collected after completion of NCT. Expression of SMAC was significantly higher in the breast cancer specimens after NCT ($p < 0.01$). High expression of SMAC in the core biopsy before NCT correlated with a pathological complete remission (pCR, $p < 0.01$). The patients with a high expression of SMAC in the surgical specimens after NCT had longer DFS.

Our study proves a potential role of SMAC expression in LABC as a novel favourable prognostic factor in LABC for pCR and disease-free survival (DFS).

Key words: locally advanced breast cancer, chemotherapy, SMAC protein.

Introduction

Breast cancer, the most common malignancy in the female population, is a disease with a relatively good prognosis. Screening programs for early breast cancer detection and a multimodal therapy result in an almost 90% five-year survival rate in breast cancer patients [1]. However, the range of population-based mammography programs is limited, since 3% to even 25% of patients disclose the locally advanced breast cancer – a disease with a poorer prognosis, a large tumour, skin/muscle involvement, fixed metastatic axillary lymph nodes, or extra-axillary lymph nodes metastases (IIB or III stage) [2, 3]. Administration of the preoperative systemic therapy including neoadjuvant chemotherapy (NCT), which is a standard form of treatment in this group of patients, has to enable a radical surgery and reduce the risk of local and distant recurrence [4, 5, 6].

One of the crucial goals of chemotherapy is to induce apoptosis in tumour cells. Chemotherapeutic drugs activate a mitochondrial pathway of cell apoptosis via oligomerisation BAX and BAK proteins, belonging to the Bcl-2 family [3, 7]. Activat-

ed BAX and BAK form pores in the mitochondrial membrane, which leads to the release of cytochrome c and SMAC (second mitochondrial-derived activator of caspase; also known as DIABLO: direct IAP binding protein with low PI) from mitochondrion to the cytosol. In the cytosol cytochrome c binds to APAF1 (apoptotic protease activating factor 1) and procaspase 9 forming an active multi-protein complex apoptosome, which activates effector caspases 3 and 7 ultimately leading to the cell death. The caspases activation is regulated negatively by several proteins including inhibitors of apoptosis (IAP protein family). Conversly, active SMAC (DIABLO) promotes apoptosis by binding to IAPs and preventing them from inhibiting caspases [8, 9].

The substantial regulatory function of SMAC in apoptosis raises interest concerning the correlation between its expression, clinico-pathological factors, and prognosis in different cancers. It has been demonstrated that the expression of SMAC in the tumour cells varies depending on the type of cancer. Yoo *et al.* reported that SMAC was not present in prostate, lung, and soft tissue cancer, which may reflect an impaired apoptosis of these tumour cells [10]. In contrast, in gastric, colorectal, and ovarian cancer cells and in haematological malignancies, a high mitochondrial expression of SMAC has been shown [10, 11]. Using the flow cytometry technique, we have previously demonstrated that in breast cancer patients SMAC protein expression correlated inversely with tumour stage, which indicated a potent role of this protein in breast cancer development [12].

The potent therapeutic role of SMAC expression in breast cancer encouraged us to perform assessment of its expression by the use of the standard immunohistochemistry technique. In the present study we investigated expression of SMAC protein in advanced breast cancer tissues collected from patients before and after chemotherapy, and evaluated its potential effect on the response to chemotherapy and survival.

Material and methods

Patients

Our study encompassed data on 56 patients, who were diagnosed with advanced non-metastatic breast cancer (stage IIB- node positive and III) and received NCT followed by surgery and adjuvant treatment. NCT was administered in the Department of General Oncology, and the patients were operated on in the Department of Surgical Oncology, Medical University, Copernicus Memorial Hospital in Lodz between January 2006 and December 2010. The median follow-up was 70 months (range 10-113 months). The Research Ethics Committee of the Medical University of Lodz approved this study. All the patients signed a written agreement to participate in the study.

The mean age of the study participants was 55 years (range 29-74 years). In all the patients a core-needle biopsy was performed before the treatment. Pathological inclusion criteria included: invasive carcinoma of no special type (NST) (previously named "invasive ductal carcinoma, not otherwise specified") and infiltrating lobular carcinoma. All the patients with other pathological types of breast cancer and the patients who received preoperative hormonal therapy were excluded from the study. The staging was assessed in accordance with the 2010 pTNM AJCC/UICC classification [13].

All the patients received the anthracycline-based chemotherapy (4 to 6 cycles; doxorubicin 60 mg/m^2; cyclophosphamide 600 mg/m^2 every three weeks or doxorubicin 50 mg/m^2, docetaxel 75 mg/m^2 every three weeks). When NCT was complete all the patients underwent a modified radical mastectomy. Adjuvant hormonotherapy or trastuzumab were administered if required. All the patients in the study group were clinically node-positive, and therefore they received postsurgical radiotherapy.

A complete pathological tumour response (pCR) for chemotherapy was defined as an absence of invasive cancer in the breast and axillary nodes, irrespective of ductal carcinoma in situ and nodal involvement (ypT0/is) [5]. The remaining cases (partial or no pathological response) were regarded as non-pCR. The examples of microscopic images of pCR and non-pCR after NCT are presented in Fig. 1.

Immunohistochemistry

Paraffin blocks of core biopsies and postoperative specimens that best represented the tumour were selected for the immunohistochemistry (IHC). All IHC expressions of SMAC were independently evaluated by two experienced pathologists (DJK and RK) who had no knowledge on clinical details.

Monoclonal IgG1kappa Anti-Smac/Diablo Antibody, clone 3A9.1 (Merck KGaA Darmstadt, Germany), dilution 1:50, pH 9.0 was used. Paraffin-embedded sections were deparaffinised with xylene (2 × 5 min) and dehydrated with ethanol (1 × 1 min 100% ethanol, 1 × 1 min 96% ethanol). Subsequently, the slides were rinsed with peroxide. For antigen retrieval, the HIER (heat-induced epitope retrieval) method was used according to the manufacturer's protocol (PT Module Deparaffinisation and Heat-Induced Epitope Retrieval Solutions, Thermo Scientific, USA). Primary mouse monoclonal antibody was incubated for one hour at room temperature. Then, the sections were rinsed with buffer (Tris Buffered & saline Tween 20, Dako, Denmark). For detection and visualisation the EnVision Detection Systems-HRP Peroxidase/DAB, Rabbit/Mouse was used following the criteria recommended by the manufacturer. All the sections were counterstained with Harris haematoxylin (Instant

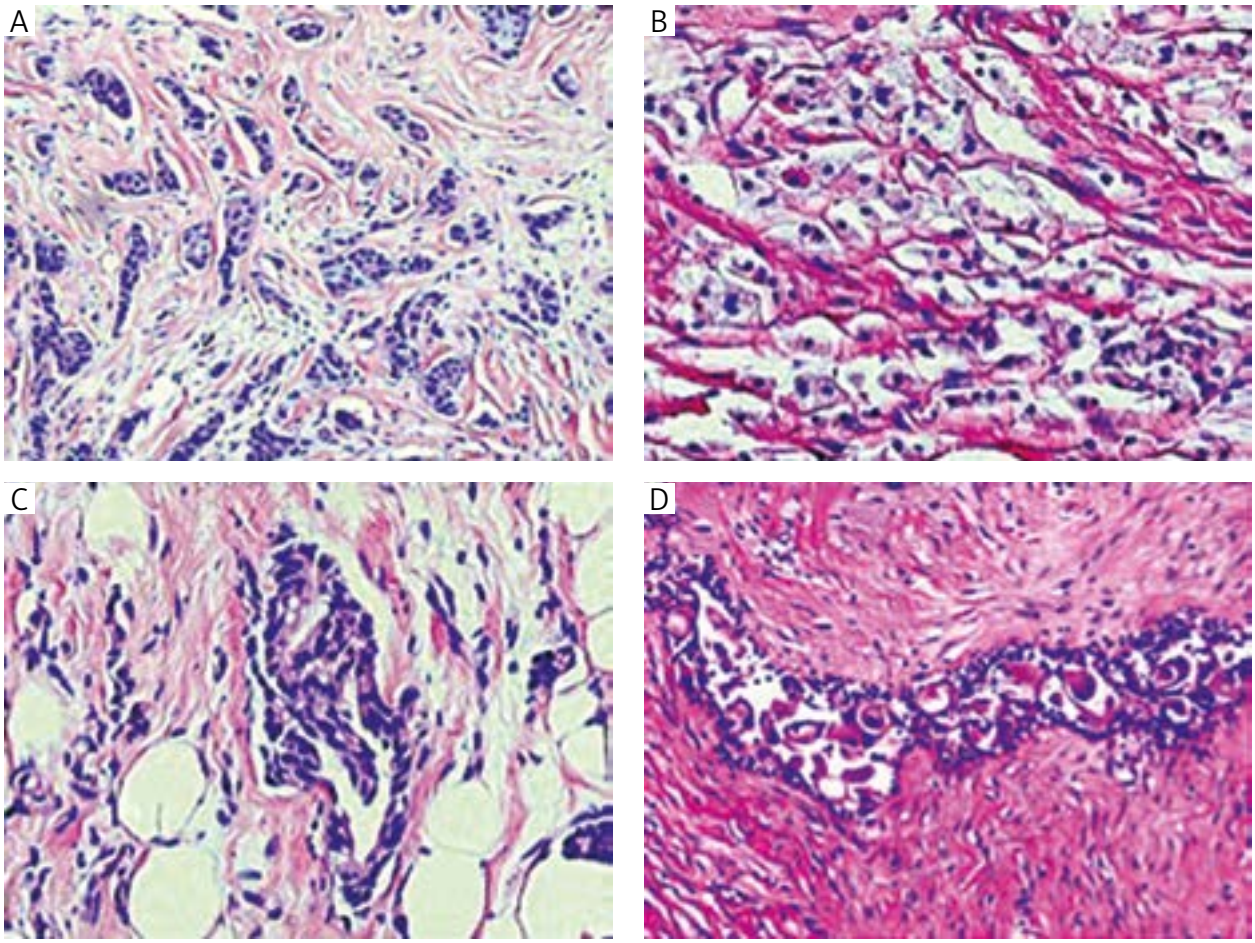

Fig. 1. Representative photomicrographs of the core biopsy with breast carcinoma NST G2 before NCT (A, B) and the same cases after NCT showing pCR with the absence of carcinoma cells and the infiltration of reactive foamy macrophages, (C) and non-pCR with the presence of invasive carcinoma with morphologically altered cells after chemotherapy (D) (haematoxylin/eosin staining × 200)

NST – invasive carcinoma of no special type; NCT – neoadjuvant chemotherapy; pCR – pathological complete response

Haematoxylin, Bio-Optica, Italy). The microscopic evaluation (Olympus, BX43) was performed at 200× and 400× magnification. Positive controls were performed on the formalin-fixed, paraffin-embedded specific tissue samples on the renal tubule tissue. Positive cytoplasmic staining was observed in the renal tubule cells. Negative control stainings were evaluated using mouse isotype antibody Ready-to-Use FLEX Negative Control Mouse (Cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM, IR750, DAKO, Denmark).

Expression of SMAC in the core biopsies and post-surgical specimens was assessed using the scoring system described by Perone *et al*., which is based on a combination of scores for the percentage of positive (stained) cells and intensity of staining [14]. The percentage of immunopositive cells was scored as follows: score 0 for < 1%; score 1 for 1-20%; score 2 for 21-50%; score 3 for 51-80%; and score 4 for > 80%. The staining intensity was scored as 0 for weak; 1 for moderate; and 2 for strong. The immunopositivity score and staining intensity score were then added, resulting in the final expression score, interpreted as follows: low for the total value of 3; intermediate for values between 3 to 5; and high for values higher than 7. Figure 2 illustrates examples of SMAC expression evaluated according to the IHC scoring system.

Expressions of oestrogen receptor (ER) and progesterone receptor (PR) were evaluated by IHC, using the Envision System (Dako North America, Inc., Carpinteria, CA, USA) and Dako Auto-Stainer Plus (Dako North America, Inc.) with the use of mouse monoclonal antibodies: Oestrogen Receptor α, Clone 1D5 and Progesterone Receptor Clone 636, according to the instruction provided by the manufacturer. Staining was assessed according to the Allred method as a total score (TS) for staining, which was determined by calculating the sum of the proportion of positive cells (IP) and the intensity of staining (IS) scores as follows: TS 0-2 was regarded as negative, and samples exhibiting scores between 3 and 8 were considered as positive [15]. In detail, the IP was scored as 0 (no positive cells), 1 (≤ 1% positive cells), 2 (≤ 10% positive cells), 3 (≤ 33% positive cells), 4 (≤ 66% positive cells), and 5 (> 66% positive cells).

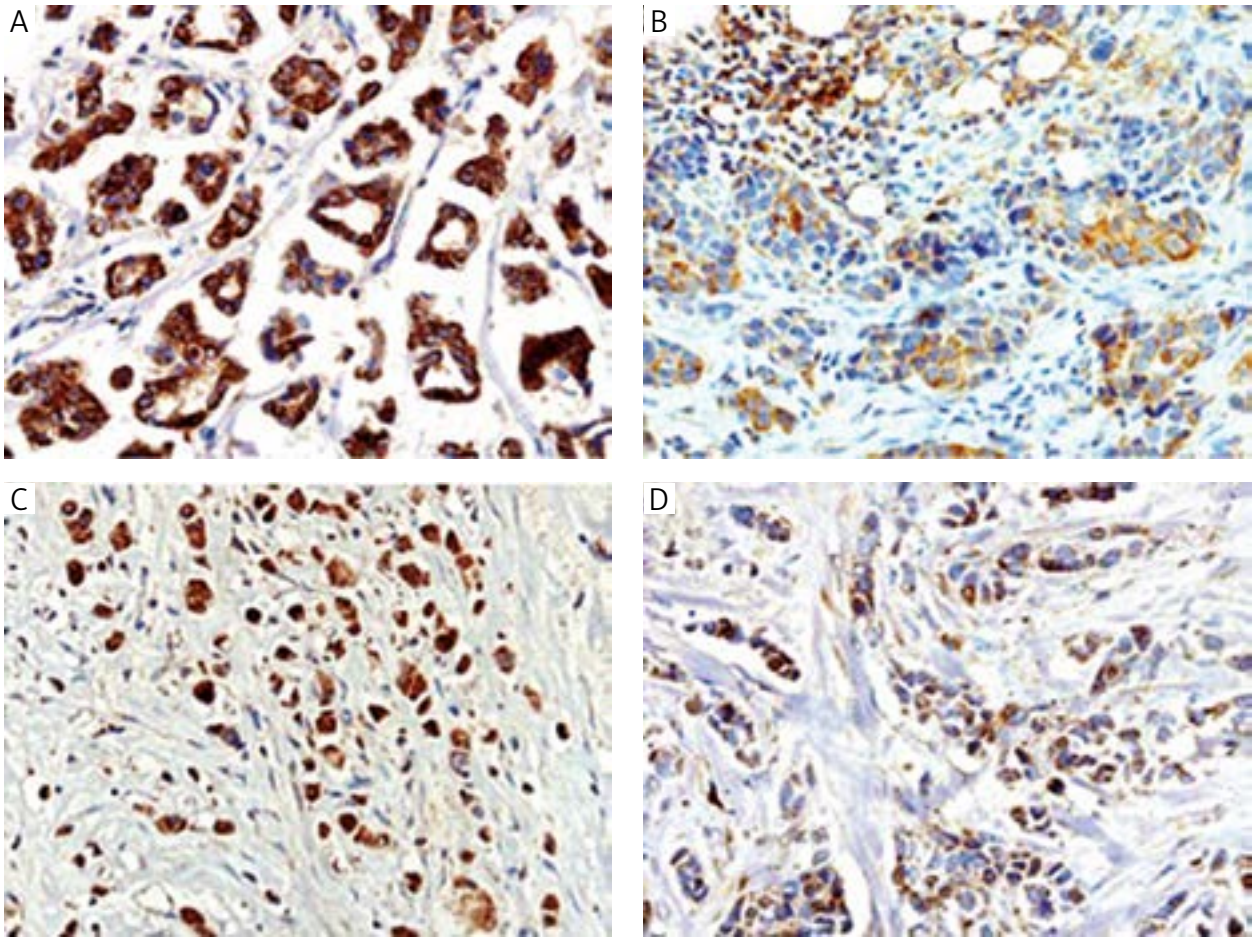

Fig. 2. Examples of a high and intermediate/low immunohistochemical expression of SMAC protein assessed according to the scoring system (200×) in the before NCT (core biopsies) samples and the after NCT (surgical specimens) samples. A) High SMAC expression in the core biopsy (100% of immunopositive cells: score 4 + strong staining: score 3 = total score 7: high expression). B) Low expression of SMAC protein in the core biopsy (20% of immunopositive cells: score 1; weak staining: score 1; total score 2 = low expression). C) High SMAC expression in the surgical specimen (80% of immunopositive cells: score 3; strong staining: score 3 = total score 6: high expression). D) Intermediate SMAC expression in the surgical specimen (30% of immunopositive cells: score 2; weak staining: score 1 = total score 3: intermediate expression)
SMAC – second mitochondria-derived activator of caspases; NCT – neoadjuvant chemotherapy

The IS was assessed on a scale from 0 (no staining) to 3 (a strong reaction).

IHC expression of human epidermal growth factor receptor 2 protein (HER-2) was detected using an iView DAB Detection kit (Ventana Medical Systems, Inc., Tucson, AZ, USA) and a BenchMark BX automated slide-staining instrument (Ventana Medical Systems, Inc.). HER-2/neu membrane staining was evaluated according to the manufacturer's instructions by a qualified pathologist in accordance with the recommendations for HER-2 Testing in Breast Cancer: ASCO/CAP Guideline Update [16]. Expression of HER-2 was scored as follows: 0, negative (no membrane staining); 1+, negative (faint, partial staining of the membrane in any proportion of the cancer cells); 2+, equivocal (weak to moderate complete staining of the membrane in >10% of cancer cells); and 3+, positive (strong, complete staining of the membrane in >30% of cancer cells).

In HER-2 IHC score of 2+, *HER-2* gene status was determined using the PathVysion® HER -2 DNA Probe and Paraffin Pretreatment kits (Abbott Laboratories, Abbott Park, IL, USA) according to the manufacturer's instructions. The scoring method was described in detail in our previous work [17].

In summary, the samples were considered ER/PR negative if < 1% of the tumour cells were immunoreactive. The samples were considered HER-2 negative with IHC 1+ staining or with a score of 2+ and no *HER-2* gene amplification when assessed using the fluorescence *in situ* hybridisation of gene amplification (FISH).

Statistical analysis

The statistical analysis was performed with Statistica 12.0 (Tulusa, OK, USA) software. SMAC expression in the core biopsies and postoperative specimens

was compared with the Wilcoxon signed-rank test. Disease-free survival (DFS) and overall survival (OS) were assessed using the Kaplan-Meier method. The correlation between the clinico-pathological factors and SMAC was calculated by means of the chi-square test with the corresponding corrections to the numbers for dichotomic variables and the U Mann-Whitney test for the continuous variables. The Kendall rank correlation coefficient was calculated using the χ^2 test. Evaluation of the correlation coefficient was assessed on the basis of the ranges established by Cohen: correlation coefficient greater than 0.6 is a strong correlation, correlation coefficient between 0.3-0.6 is a moderate correlation, while correlation coefficient of less than 0.3 is a poor correlation [18]. Comparisons between the examined parameters were considered significant when $p < 0.05$.

DFS is defined as a type of survival rate that measures the length of time from the surgery to the disease progression. OS is defined as a type of survival rate that measures the length of time after the surgery until death or until the date of the last contact with a patient.

Results

SMAC protein expression before and after NCT

In the core biopsy (before NCT) specimens, SMAC expression was scored as low in 10 cases (18%), as intermediate in 33 cases (59%), and as high in 13 cases (23%). In the surgical (after NCT) specimens low, intermediate, and high expressions of SMAC were found in 0 (0%), 26 (46%), and 30 (54%) of cases, respectively. Expression of SMAC was significantly higher in the breast cancer specimens after NCT ($p < 0.01$; Fig. 3).

The relationship between expression of SMAC protein and clinico-pathological characteristics of the study group

In the pre-treatment specimens SMAC expression was higher in grade 1 (G1) tumours compared to G2 and G3 tumours ($p < 0.01$). Additionally, in the cancer tissues collected before the chemotherapy in the subgroup of patients under 50 years old, there was a trend toward a higher SMAC expression, in contrast to the older patients ($p = 0.059$).

Expression of SMAC protein and its correlation with clinico-pathological characteristics is summarised in Table I.

SMAC protein expression and response to the treatment

We found a correlation between a high SMAC expression and pathological complete response (pCR) achievement in the pre-treatment samples ($p < 0.01$). No such relationship in the post-treatment samples was observed (Table I).

The pCR was achieved in nine of 56 patients (16%). In the univariate logistic regression, pCR achievement was correlated with age below 50 years, a high SMAC expression, and lack of triple-negative ($p = 0.046$, $p = 0.015$, and $p = 0.004$, respectively). None of the other clinico-pathological factors influenced achievement of pCR (Table II).

SMAC protein expression survival

Kaplan-Meier estimates were calculated for the subgroups of patients with low/intermediate expressions vs. high expression of SMAC proteins. The impact of SMAC expression for DFS and OS is shown in Fig. 4.

During the medium follow-up period, a high expression of SMAC in the postsurgical specimens was found to be a favourable prognostic factor to achieve longer DFS ($p = 0.001$). The OS was not influenced by SMAC.

Discussion

Preoperative chemotherapy in breast cancer tumours may effectively induce apoptosis, which has been indicated *in vivo* by the use of 18F-FDG PET (fluorine-18 fluorodeoxyglucose positron emission tomography) [19]. In preclinical studies, breast cancer cells with the overexpression of SMAC or treated with

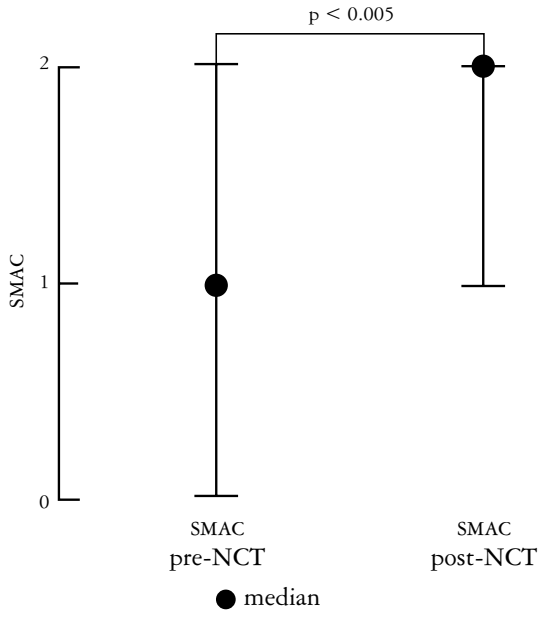

Fig. 3. Descriptive statistics for expression of SMAC protein before (SMAC pre-NCT) and after chemotherapy (SMAC post-NCT) in locally advanced breast cancer patients. A significant increase of SMAC expression was observed after administration of NCT ($p = 0.00003$)

SMAC – second mitochondria-derived activator of caspases; NCT – neoadjuvant chemotherapy

Table I. Correlation between SMAC protein expression and clinico-pathological characteristics of the patients

Parameters	Number of patients in row	SMAC pre – NCT			SMAC post – NCT		
		low and medium	high	p-value χ^2	low and medium	high	p-value χ^2
Age (years)							
< 50	20	12	8	0.059	9	11	0.905
≥ 50	36	31	5		17	19	
cT							
T1/T2	26	21	5	0.734	12	14	0.818
T3/T4	30	22	8		14	16	
Histological type							
NST	50	39	11	0.615	24	26	0.804
lobular	6	4	2		2	4	
Histological grade							
G1	6	0	6	0.000	0	6	0.052
G2	24	18	6		13	11	
G3	26	25	1		13	13	
Stage							
IIB	6	5	1	1.000	2	4	0.805
III	50	38	12		24	26	
Oestrogen receptor							
negative	25	20	5	0.754	12	13	0.954
positive	31	23	8		14	17	
Progesterone receptor							
negative	27	21	6	0.8831	10	17	0.275
positive	29	22	7		16	13	
HER-2 receptor							
negative	32	22	10	0.122	11	21	0.069
positive	24	21	3		15	9	
Triple negative							
no	45	33	12	0.426	21	24	0.791
yes	11	10	1		5	6	
Pathological response							
non-pCR	45	40	5	0.000	23	22	0.275
pCR	11	3	8		3	8	

SMAC – second mitochondria-derived activator of caspases; NCT – neoadjuvant chemotherapy; NST – invasive carcinoma of no special type; HER-2 – human epidermal growth factor receptor 2; cT – clinical tumour stage; pCR – pathological complete response

SMAC peptide have shown an enhanced apoptosis-inducing potential of chemotherapy and irradiation [20]. In our study, we observed that administration of NCT increases the expression of proapoptotic SMAC protein in breast cancer cells. Moreover, a pathological complete response to NCT was more frequent in the high SMAC expression specimens collected prior to NCT. This observation is concordant with the study conducted by Zhao *et al*. [21]. They observed that in a group of 98 patients with locally advanced breast cancer, treated with anthracycline-based chemotherapy, expression of SMAC protein in tumour

Table II. The univariate logistic regression model for the achievement of pathological complete response of tumour after preoperative systemic therapy

Parameters	p-value	OR	CI 95%	
Histological type (NST vs. lobular)	0.997	0.00	0.00	0.00
Histological grade (G2 vs. G3)	0.106	1.99	0.86	4.60
Oestrogen receptor (positive vs. negative)	0,086	0.48	0.21	1.11
Progesterone receptor (positive vs. negative)	0.106	0.50	0.22	1.16
HER-2 receptor (positive vs. negative)	0.996	0.00	0.00	0.00
Triple negative (no vs. yes)	0.004	3.60	1.52	8.50
cT (T1/T2 vs. T3/T4)	0.551	0.80	0.39	1.65
Stage (IIB vs. III)	0.966	0.98	0.31	3.05
Age (< 50 years vs. ≥ 50 years)	0.046	0.46	0.22	0.99
SMAC expression before NCT (low/medium vs. high)	0.015	5.52	1.40	21.84
SMAC expression after NCT (low/medium vs. high)	0.395	0.72	0.34	1.53

SMAC – second mitochondria-derived activator of caspases; NCT – neoadjuvant chemotherapy; NST – invasive carcinoma of no special type; HER-2 – human epidermal growth factor receptor 2; cT – clinical tumour stage; pCR – pathological complete response

tissues was also increased after NCT, and that a high expression of SMAC in pre-chemotherapy samples of their patients correlated with a pathological complete response. This confirmed the suggestion that SMAC protein may play an important role in an apoptosis-related response to neoadjuvant chemotherapy in locally advanced breast cancer patients. Taking into consideration the fact that SMAC expression in both studies has been assessed using the standard IHC technique, which is relatively inexpensive and fast, it is likely that the SMAC protein expression level in pretreatment specimens may serve as a predictive factor of NCT response in LABC.

The analysis of correlation between SMAC protein expression and conventional prognostic factors of breast cancer indicated that a high expression of SMAC proteins in the pretreatment specimens was present significantly more frequently in G1 tumours, comparing to G2/3. It may suggest, that the low-grade tumours have a greater possibility to induce apoptosis. In our previous study performed in early breast cancer patients we did not observe a correlation between any conventional prognostic factors of breast cancer, except pT (pT1 breast cancer patients expression of SMAC protein was higher than in those with pT2-3) and diffuse cancer infiltration, which significantly correlated with a lower expression of SMAC [12]. Based on these results, we may suppose that a higher SMAC expression in breast cancer is related to a better prognosis, due to a higher possibility of apoptosis in these tumour cells. We also observed a trend towards a high expression of SMAC in patients younger than 50 years old. This is partly concordant with our previous study, which was conducted in early breast cancer patients, where the expression of SMAC protein was higher in the patients younger than 50 years of age [12]. Additionally, younger age of the patients was one of the factors that predicted pCR in our study. It indicates that in younger patients the mechanism of apoptosis including the role of proapoptotic SMAC protein is more efficient. However, other studies have not shown any correlations between SMAC expression and clinico-pathological characteristics of LABC [21].

Prognostic improvements have been observed steadily in ER positive and HER-2-positive patients due to novel endocrine and HER-2-targeting therapies [22]. In triple-negative breast cancer, the absence of well-defined molecular targets makes cytotoxic chemotherapy the only treatment option. A pathological response to systemic chemotherapy reflects the extent of clinical response [23]. In our study we observed that pCR was more likely in tumours with the absence of a triple-negative receptor status. This might result from a high proportion of Her-2-positive patients in our cohort (45%; 24/56), compared to other studies where HER-2-positive tumours accounted for about 25-30% of all breast cancer cases [24]. In a recently published study, Erbes *et al*. reported that HER-2-positive receptor status but not triple-negative tumour predicts pCR after NCT in breast cancer patients [25]. However, triple-negative and HER-2-positive/ER-negative breast cancer cases are recognised as having higher sensitivity to the anthracycline-based chemotherapy with higher rates of pCR compared to ER-positive tumours [26].

In our study we reported a significant correlation between a high expression of SMAC in the core biopsy and DFS, which proves the role of this protein in breast cancer cell death. The impact of SMAC protein expression on the OS in our study was not observed, which is likely to reflect the relatively good

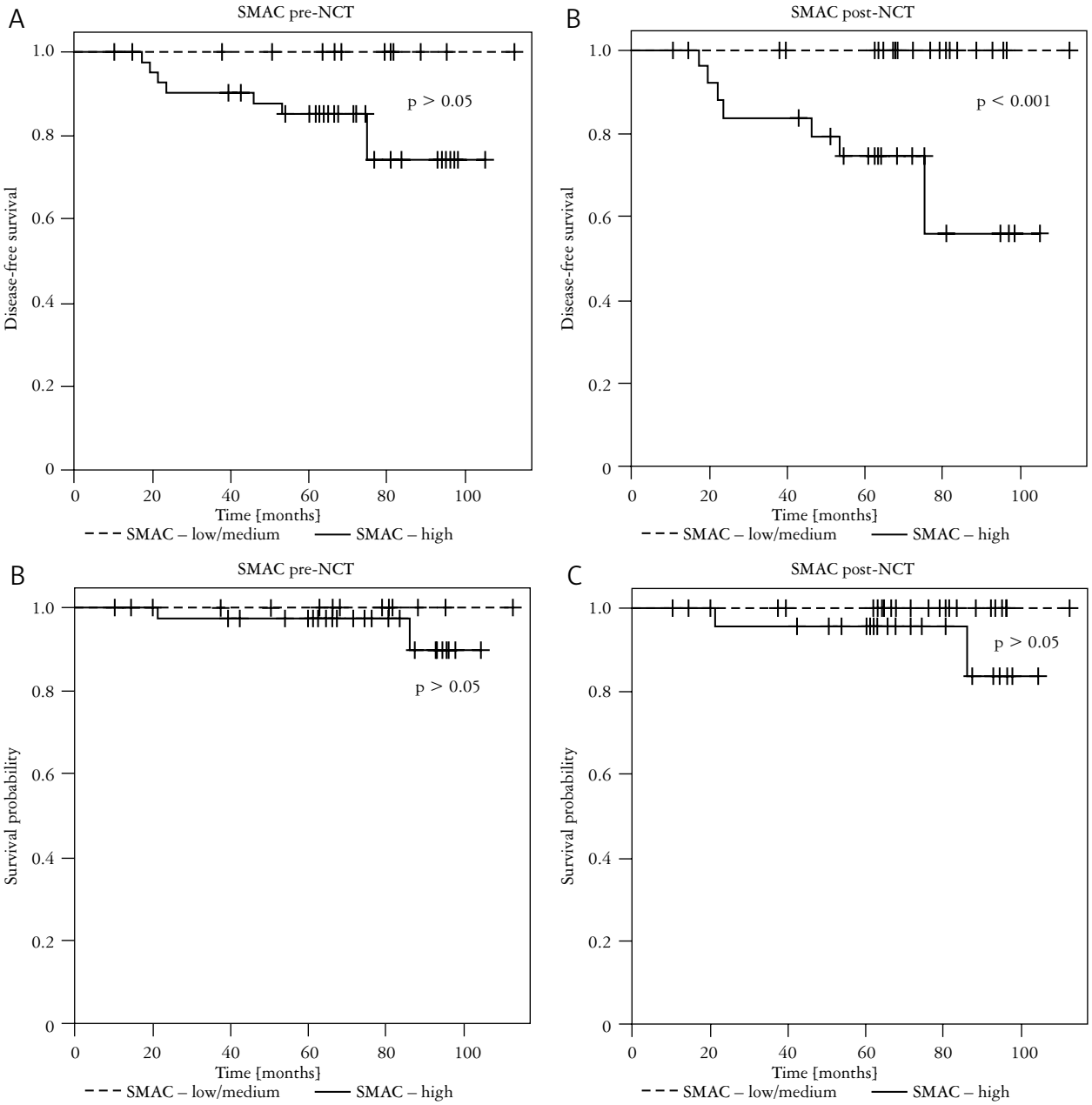

Fig. 4. Kaplan-Meier estimates of disease-free survival (A, B) and overall survival (C, D) in LABC according to the SMAC protein expression (low/intermediate vs. high) before and after chemotherapy

SMAC – second mitochondria-derived activator of caspases; NCT – neoadjuvant chemotherapy; DFS – disease-free survival; OS – overall survival; LABC – locally advanced breast cancer

five-year-survival rates in LABC, which surpass 80% [1]. However, in a larger cohort of LABC, a significant correlation between a high SMAC expression and better DFS and OS was revealed [21].

Our study confirmed the potent role of SMAC as a predictive and prognostic factor in the locally advanced breast cancer. It is important to take into consideration the fact that intense research has been conducted with several small-molecule SMAC mimetics in experimental and clinical trials for cancer treatment [7]. In breast cancer cell lines Jin et al. have recently demonstrated that LCL 161, which is a designed SMAC mimetic, induced multiple forms of programmed cell death including apoptosis and necroptosis [27]. Another preclinical report has indicated that SMAC mimetic (TL32711) displayed antitumour activity alone and in combination with multiple chemotherapies [28]. Initial clinical trials have shown that SMAC mimetics are active and well-tolerated antitumour agents in patients with advanced solid cancer and haematological malignancies [29].

The current study has a number of limitations. The statistical analysis was performed in a small and heterogeneous group of patients (56 patients, 12% lobular breast cancer) with a high percentage of HER-2-positive patients (45%). Additionally, we

did not compare the expression of SMAC in the tumour and in the vicinity of normal tissue as it is in the case of healthy specimens. Such an approach would require additional oncologically unjustified core biopsies of the breasts. Finally, the expression of SMAC was assessed only by the use of the standard IHC in breast cancer. We realise that further studies in a larger group and by the use of a different technique are necessary to verify the study results.

In conclusion, our results suggest that SMAC expression might play a role in LABC as a potential favourable prognostic factor in LABC. Additionally, we suggest that the use of IHC assessment of the SMAC expression in LABC, which is a feasible and accessible method, may contribute to deployment of SMAC mimetic to breast cancer treatment in the future.

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The authors declare no conflict of interest.

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