

## Cyclooxygenase-2 and Bcl-2 expression in patients with triple-negative breast cancer

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**Introduction.** Triple-negative breast cancer (TNBC) is a rare type of breast cancer associated with lack of expression of estrogen and progesterone receptors and the HER2 protein. It is characterized by a poor outcome and chemotherapy resistance. Cyclooxygenase-2 (COX-2) is a constitutional enzyme responsible for prostaglandin synthesis, present in neoplastic cells and premalignant lesions. The B-cell lymphoma 2 (Bcl-2) protein is considered one of the most potent apoptosis-regulating agents, assuring body homeostasis.

**Material and methods.** The aim of the present study was to evaluate the immunohistochemical (IHC) profile of COX-2 and Bcl-2 expression in patients suffering from TNBC in order to obtain more detailed data on additional factors negatively influencing TNBC outcome. The IHC evaluation of COX-2 and Bcl-2 expression among 21 women with diagnosis of TNBC was performed.

**Results.** The most common histological subtype was invasive ductal cancer of no special type. COX-2 was present in all examined samples with moderate to strong expression detected in 20 of 21 cases. There was a positive correlation between histological grade (G) and COX-2 expression ( $p = 0.002$ ). Bcl-2 was present in all examined samples. The analysis showed that tumours presenting highly positive expression of Bcl-2 accounted for the majority of examined cases (57.2%).

**Conclusions.** The achieved results might lead to a conclusion that COX-2 and Bcl-2 high expression in TNBC may be linked to tumour aggressiveness and poor overall survival. However, before their consideration as additional markers to be used in routine histological examinations and breast cancer grading, it will be necessary to undertake further studies.

**Key words:** Bcl-2, cyclooxygenase-2, immunohistochemistry, prognostic factors, triple-negative breast cancer

### Introduction

Breast cancer is the most common type of cancer among women worldwide. This heterogeneous group of malignant neoplasms represents 22.2% of newly diagnosed cancer cases and 13.3% of cancer-related deaths. Unfortunately, its incidence is constantly on the rise [1]. Triple-negative breast cancer (TNBC) is a rare histological type of breast cancer characterized by a lack of presence of estrogen and progesterone receptors

and the HER2 protein. A wide range of studies have shown its association with a poor outcome, low 5-year overall survival rate, chemotherapy resistance and co-existence with younger patients age [2].

Cyclooxygenase is a constitutional enzyme responsible for prostaglandin and thromboxane synthesis, occurring in two isoforms. Cyclooxygenase-2 (COX-2) is present in inflamed tissues, neoplastic cells and premalignant le-

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sions. It enhances cellular proliferation, tissue invasion and angiogenesis, in addition to its anti-apoptotic effect [3], it subsequently provides prime conditions for developing a tumour. Epidemiological studies showed a relation between COX-inhibiting drugs (nonsteroidal anti-inflammatory drugs – NSAIDs) and reduced cancer risk of the gastrointestinal tract [3]. Studies conducted over the years showed that medications inhibiting COX-2 might act as possible chemopreventive agents in breast cancer, since increased expression of COX-2 in tumour samples was often observed [4, 5]. As a result of those findings, cyclooxygenase was also considered as a biochemical marker of poor prognosis.

Another fundamental aspect of neoplastic processes is evasion of programmed cell death. B-cell lymphoma 2 (Bcl-2) protein is a product of the *BCL-2* gene and is considered one of the most potent apoptosis-regulating agents, assuring body homeostasis. This protein prevents apoptosis by deterring cytochrome C and apoptosis inducing factor (AIF) in mitochondria, thus inhibiting the caspase-dependent apoptosis pathway [6]. Overexpression of Bcl-2 was observed in number of cancers; also in case of breast cancer. Moreover, expression of Bcl-2 was established as an independent risk factor of poorer breast cancer prognosis [6, 13, 30].

The aim of our study was to evaluate the immunohistochemical profile of COX-2 and Bcl-2 expression in patients suffering from TNBC in order to obtain more detailed data on additional factors negatively influencing TNBC outcome (fig. 1).

## Material and methods

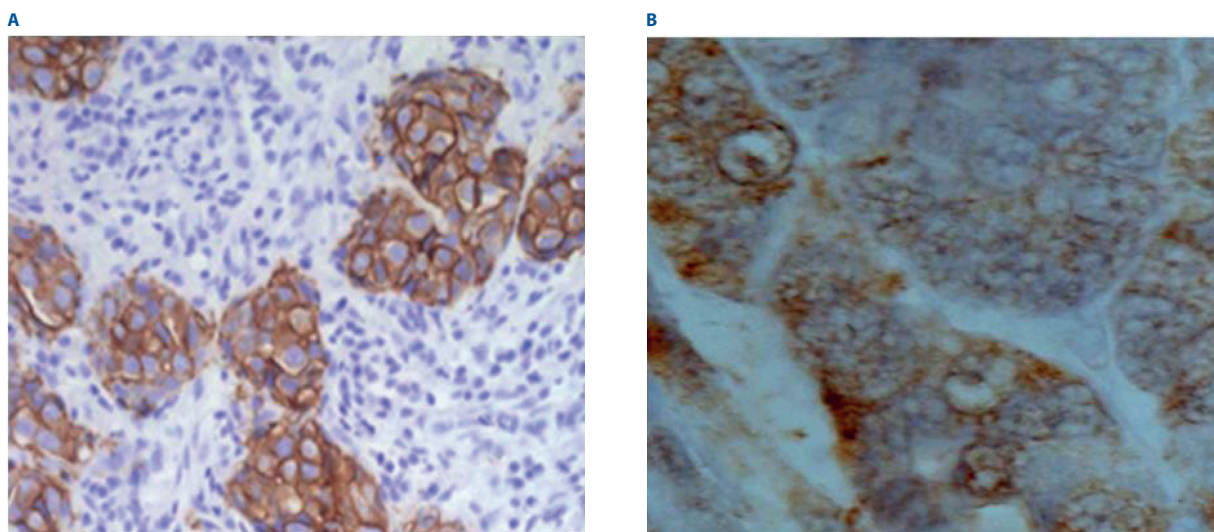
### Patients

The patient population comprised 21 women with a diagnosis of triple-negative breast cancer. The material came from biopsies, excisional biopsies and modified radical mastectomies. They were fixed in 10% buffered formalin phosphate, dehydrated by a set of alcohols of increasing concentrations,

embedded in paraffin and cut into serial sections of 4 µm thick. Then, the samples were rehydrated and stained with haematoxylin and eosine, allowing to classify them according to the WHO classification. Moreover, the samples allowed for an evaluation of the histological grade (G), tumour grade (T) and lymph node involvement (N) of the given tumours. Additionally, the expression of receptors for estrogen (ER), progesterone (PR) and HER2 receptors was assessed by means of immunohistochemical staining, using mouse monoclonal antibodies (DAKO: IR654, IR068 and K5204) and the DAKO EnVision™ system for visualisation of results. Stain intensity was assessed by a computed image analysis of a number of stained nuclei per 1000 neoplastic cells.

### Detection of COX-2

Cyclooxygenase expression was determined using the Monoclonal Mouse Anti-Human COX-2 antibody. First, the samples were dewaxed using a set of alcohols of decreasing concentrations. Then, they were put into pH 6 buffer and put into a water bath for 30 minutes in 90°C for antigen retrieval. Subsequently, the preparations were left at room temperature for 20 minutes. Then, samples were rinsed twice in distilled water and then incubated with 3% hydrogen peroxide for 5 minutes in order to quench endogenous peroxidase activity. After that, they were washed in TRIS (Tris-Buffered Saline, pH 8, SIGMA) and then incubated with a primary antibody in a humidity chamber for 60 minutes at room temperature. In the next stage, samples were again washed in TRIS for 10 minutes and incubated with visualisation reagent for 30 minutes. Next, after being washed in TRIS, were incubated with 3,3-diaminobenzidine (DAB) for a visualisation of staining results. The time of incubation was controlled in order to obtain the desired stain intensity. At the end of the procedure, preparations were counter-stained with haematoxylin. Stain intensity was assessed by computed image analysis of



**Figure 1.** A histopathological image of invasive triple negative breast cancer (TNBC) (H&E): left (A) – positive immunohistochemical staining for Bcl-2 (original magnification 200x); right (B) – positive immunohistochemical staining for Cox-2 (original magnification 1000x)

a number of stained cytoplasm per 1000 neoplastic cells. The following score was adapted, similarly to Nam et al. and others' research [7, 18, 35]:

- none – less than 10% positively stained cells,
- weak – 10% positively stained cells,
- medium – from 10% to 30% positively stained cells,
- strong – over 30% positively stained cells.

### Detection of Bcl-2

Bcl-2 expression was assessed using the monoclonal mouse anti-human Bcl-2 antibody. After dewaxing, the samples were incubated for 10 minutes in 1% hydrogen peroxide diluted in phosphate buffer saline (PBS) to quench endogenous peroxidase activity. Then, they were washed in PBS twice for 5 minutes. Next, they were incubated with 1.5% blocking serum in PBS for one hour at room temperature. Then they were incubated with a primary antibody diluted (1:50) in 1.5% blocking serum in PBS for 30 minutes at room temperature, and then washed thrice with PBS. Thereafter, sections were incubated for 30 minutes with a AB enzyme reagent (avidin + biotinylated horseradish peroxidase [HRP]) and then washed with three changes of PBS for 5 minutes each. At the end, the samples were incubated with 3 drops of peroxidase substrate for 5 minutes, until the desired stain intensity developed. The process concluded with a counterstain with haematoxylin. Stain intensity was assessed by computed image analysis of a number of stained cytoplasm per 1000 neoplastic cells. The scoring method was modified from a score used by van Slooten et al. [8] and others [32–34] in their research, to emphasise different levels of stain intensity, and adapted as followed:

- none – less than 10% positively stained cells,
- weak – from 10% to 50% positively stained cells,
- medium – from 50% to 80% positively stained cells,
- strong – from 80% to 100% positively stained cells.

### Statistical analysis

All the results were obtained using SPSS v. 12.0 PL Windows and the Statistica 13.1. Chi-square test and Fisher exact test were performed. Statistical significance was set at  $p = 0.05$ , however, for some of the calculations  $p$  was set at 0.008 (0.05/6) because Bonferroni correction was used to counteract the problem of multiple comparisons. In order to establish relations between COX-2 levels, Bcl-2 levels and patient age, the Spearman rang test was performed. An R value lesser than 0.2 is considered as without correlation.

### Results

A pathological examination was performed on total of 21 female patients with confirmed diagnosis of TNBC. In the present study, we observed and analysed the expression and relationship of COX-2 and Bcl-2 with means of immunohistochemistry (tab. I). 14 out of 21 patients (66.7%) were above

**Table I.** Clinicopathological characteristics of the patients included in the study

Characteristics	Number of patients (n = 21), (%)
<b>mean age</b>	55.5
under 50 y.o.	7 (33.3%)
above 50 y.o.	14 (66.7%)
<b>histological type</b>	
IDC	6 (28.6%)
IDC-NST	13 (61.9%)
metaplastic	2 (9.5%)
<b>tumour size (T)</b>	
T1	4 (19.1%)
T2	12 (57.1%)
T3	3 (14.3%)
T4	2 (9.5%)
<b>lymph node involvement (N)</b>	
N0	8 (38.1%)
N1	11 (52.3%)
N2	1 (4.8%)
N3	1 (4.8%)
<b>histological grade (G)</b>	
G1	1 (4.8%)
G2	12 (57.1%)
G3	8 (38.1%)
<b>COX-2 expression</b>	
0	0
1	1 (4.8%)
2	9 (42.8%)
3	11 (52.4%)
<b>Bcl-2 expression</b>	
0	0
1	0
2	9 (42.8%)
3	6 (28.6%)
4	6 (28.6%)

Bcl-2 – B-cell lymphoma 2; COX-2 – cyclooxygenase 2; IDC – invasive ductal carcinoma, IDC-NST – invasive ductal carcinoma – no special type; y.o. – years old

50 years of age at the time of diagnosis (mean age 55.5 years old). The most common histological subtype was invasive ductal cancer of no special type (IDC-NST – 61.9%). The majority of samples were assessed as pT2 (57.1%). The lymph node involvement examination showed the dominance of pN1 stage, with 11 cases out of 21 (52.4%), followed by N0 (38.1%). Detailed pathological characteristics are included

in table I. The presence of distant metastases was not evaluated in the study.

The vast majority of examined tumours were assessed as moderately differentiated G2 (57.1%) and poorly differentiated G3 (38.1%), leaving only one sample with well differentiated cell architecture. Correlations between the histological grade, tumour size and lymph node status were examined, with no statistically significant relations.

As shown in table II, COX-2 was present in all examined samples with moderate to strong expression detected in 20 of 21 cases (staining intensity of 2 and higher).

**Table II.** IHC staining of COX-2 in studied samples

Percentage and degree of positively stained cells			TNBC samples with positive reaction
>10%	1	none	0
10%	2	weak	1 (4.8%)
>10–30%	3	medium	9 (42.9%)
>30%	4	strong	11 (52.3%)

COX-2 – cyclooxygenase 2; IHC – immunohistochemical; TNBC – triple negative breast cancer

**Table III.** Relations between degree of COX-2 staining and clinicopathological features

	Degree of immunohistochemical expression of COX-2 in TNBC samples				p
	None	Weak	Medium	Strong	
<b>histological type</b>					
IDC-NST	0	1 (4.8%)	6 (28.6%)	6 (28.6%)	0.889
IDC	0	0	3 (14.3%)	3 (14.3%)	
metaplastic	0	0	1 (4.8%)	1 (4.8%)	
<b>histological grade (G)</b>					
G1	0	1 (4.8%)	0	0	0.002
G2	0	0	7 (33.5%)	5 (23.8%)	
G3	0	0	6 (28.6%)	2 (9.5%)	
<b>tumour size (T)</b>					
T1	0	0	2 (9.5%)	2 (9.5%)	0.828
T2	0	1 (4.9%)	5 (23.8%)	6 (28.6%)	
T3	0	0	2 (9.5%)	1 (4.8%)	
T4	0	0	0	2 (9.5%)	
<b>lymph node involvement (N)</b>					
N0	0	1 (4.8%)	1 (4.8%)	6 (28.6%)	0.130
N1	0	0	6 (28.6%)	5 (23.8%)	
N2	0	0	1 (4.8%)	0	
N3	0	0	1 (4.8%)	0	

COX-2 – cyclooxygenase 2; IDC – invasive ductal carcinoma; IDC-NST – invasive ductal carcinoma – no special type; TNBC – triple negative breast cancer

There was a positive correlation between the histological grade (G) and COX-2 expression ( $p = 0.002$ ). However, there was no statistically significant relationship between COX-2 presence, lymph node involvement (N) and the type of neoplasms. The relation between the patient's age and COX-2 levels was also not significant ( $R = 0.00$ ). Considering the COX-2 expression, tumours were more likely to be identified as IDC-NST (tab. III).

Bcl-2 was present in all examined samples (tab. IV), demonstrating a moderate and higher level of cytoplasmic expression in nearly half of them (a staining intensity of 3 and higher – 12/21 of the analysed specimens). No correlation was found between the tumour stage, histological grade, lymph node involvement and the level of expression of Bcl-2 (tab. V). We identified no association between Bcl-2 expression and patients age ( $R = 0.167$ ). Analysis has shown that tumours presenting a positive expression of Bcl-2 (of staining intensity of 3 and higher) accounted for the majority of examined cases (57.2%) and were more likely to be assessed as T2, N1 and G2.

## Discussion

Breast cancer is one of the most frequently diagnosed neoplasms in developed countries, resulting in almost 15% of cancer-related deaths amongst women [1]. Triple-negative breast cancer is a very rare subtype of this type of cancer,

**Table IV.** IHC staining of Bcl-2 in studied samples

Percentage and degree of positively stained cells			TNBC samples with positive reaction
<10%	1	none	0
10–50%	2	weak	9 (42.8%)
50–80%	3	medium	6 (28.6%)
>80%	4	strong	6 (28.6%)

Bcl-2 – B-cell lymphoma 2; IHC – immunohistochemical; TNBC – triple negative breast cancer

**Table V.** Relations between the degree of Bcl-2 staining and clinicopathological features

	Grade of IHC expression of Bcl-2 in TNBC samples				p
	None	Weak	Medium	Strong	
<b>histologic type</b>					
IDC-NST	0	4 (19.0%)	4 (19.0%)	5 (23.8%)	0.522
IDC	0	4 (19.0%)	1 (4.8%)	1 (4.8%)	
metaplastic	0	1 (4.8%)	1 (4.8%)	0	
<b>histological grade (G)</b>					
G1	0	0	0	0	1.0
G2	1 (4.8%)	5 (23.8%)	3 (14.3%)	4 (19.0%)	
G3	0	3 (14.3%)	3 (14.3%)	2 (9.5%)	
<b>tumour size (T)</b>					
T1	0	3 (14.3%)	0	1 (4.8%)	0.828
T2	0	4 (19.0%)	5 (23.8%)	3 (14.3%)	
T3	0	1 (4.8%)	1 (4.8%)	1 (4.8%)	
T4	0	1 (4.8%)	0	1 (4.8%)	
<b>lymph node involvement (N)</b>					
N0	0	5 (23.8%)	2 (9.5%)	1 (4.8%)	0.610
N1	0	4 (19.0%)	3 (14.3%)	4 (19.0%)	
N2	0	0	1 (4.8%)	0	
N3	0	0	0	1 (4.8%)	

Bcl-2 – B-cell lymphoma 2; IDC – invasive ductal carcinoma; IDC-NST – invasive ductal carcinoma – no special type; IHC – immunohistochemical; TNBC – triple negative breast cancer

characterized by the lack of expression of ER, PR and HER2, accounting for 15–20% of cases. Previous studies have shown that TNBC diagnosis is a negative prognostic factor in breast cancer [9, 10], as well as high COX-2 expression [11, 12] and Bcl-2 expression [13, 14]. Considering all of the above, we aimed to obtain more detailed data on additional factors that negatively influence TNBC outcome. The goal of the present study was to evaluate the immunohistochemical profile of COX-2 and Bcl-2 expression in patients suffering from TNBC.

COX-2 is known for its association with poor prognosis in breast cancer patients. In 2015, Xu et al. [14] conducted

a meta-analysis including twenty-one studies with 6739 patients trying to evaluate the prognostic value of COX-2 and its association with clinicopathological characteristics. Their study proved that the expression of COX-2 predicts greater tumour size and presence of lymph node metastasis, whereas they indicated no significant correlation between ER, PR and HER2 status and COX-2 expression. The mechanism of detected association remained unclear and the role of COX-2 in TNBC was not widely discussed and examined.

In the present study there was no statistically significant relation between COX-2 presence and lymph node involvement, nevertheless this correlation was found in many previ-

ously conducted studies [14]. Some researchers try to explain the mechanism of this correlation. In 2017, Krishnamachary et al. [15] investigated the role of COX-2 expression by TNBC cells in shaping the structure and function of the tumour extra-cellular matrix (ECM), which may affect metastasis forming. In their study, COX-2 downregulation impacted the ECM structure by reducing collagen I (Col1) fiber volume, which then resulted in a reduced ability of TNBC cells to metastasize to lymph nodes. Col1 fiber density and orientation were previously linked to breast cancer metastasis in 2012 by Kakkad et al. as their pilot study [16] revealed statistically significant increases of Col1 fiber density in breast cancers with lymph node involvement.

Our results showed that the vast majority of TNBC cases were characterised by a highly positive expression rate of COX-2 (95.2% of cases). In a study performed by Chikman et al. [17], only 57.4% of patients were classified as COX-2-positive. They found a prognostic significance of COX-2 for TNBC – the 5-year disease-free survival rate reached 83.9% in COX-2-negative patients, whereas it was only 58.3% in COX-2-positive TNBC patients. No prognostic significance of COX-2 expression was proved for other types of breast cancer.

Molsapuria et al. investigated a cohort with similar clinicopathological characteristics (dominant T2, 31 TNBC cases), with positive association between COX-2 expression and both TNBC and high tumour grade, whereas in the present study the correlation was positive only with the histological grade [18]. However, Zhau et al. [19] showed no correlation between any of the clinicopathological characteristics. Similarly, Basudhar et al. [20] showed no correlation between COX-2 levels and the histological grade. Chickman et al. [17] presented a lack of correlation between any hormonal receptor status and COX-2 expression, and our results are in accordance to those findings. On the other hand, Ristmaki et al. [21] showed positive correlation between COX-2 expression levels and negative hormone status, a large tumour size, high histological grade, high proliferation rate (identified by Ki-67), high p53 expression, ductal type and axillary lymph node involvement, which is a well-known independent risk factor for poorer outcomes [22]. In the present study, positive nodal involvement was common, the majority of which assessed as N1, with no statistical significance.

Simonsson et al. carried out one of the largest studies evaluating COX-2 expression in breast cancer, where non-TNBC cancers were associated with a high COX-2 expression, lower, less aggressive tumour characteristics and increased age [23]. Moreover, in their study, TNBC correlated negatively with high COX-2 expression. In the present study, the results did not indicate any relation between age and moderate tumour malignancy.

Members of the Bcl-2 family belong to a group of pivotal arbiters of mitochondria-mediated apoptosis, consisting of anti-apoptotic and pro-apoptotic members. The role of Bcl-2 in apoptosis regulation seems to be well established, how-

ever its role in tumorigenesis remains unclear. Changes in the genome that lead to the overexpression of anti-apoptotic proteins like Bcl-2 or Bcl-xl are reported in a wide range of malignancies, including breast cancer [24]. Paradoxically, Bcl-2 protein expression in breast cancer is associated with a favourable phenotype of low-grade, ER-positive, that has more slowly proliferating breast tumours and better prognosis [25]. What it more, Bcl-2 was established as a marker that could improve the prognostic power of the Nottingham Prognostic Index [26].

One study found a correlation between increased COX-2 expression and Bcl-2 expression both in TNBC and non-TNBC patients [27]. The potential role of Bcl-2 as a prognostic factor for breast cancer has been examined in previous studies; nevertheless, its role in pathogenesis and the course of TNBC needs further research.

The frequency of Bcl-2 overexpression in TNBC varies significantly. In the present study, all examined samples presented strong Bcl-2 expression (of score 2 and higher), whereas Escórcio-Dourado et al. observed it in 40% of the 30 studied cases [28]. In 2013, Abdel-Fatah et al. described Bcl-2 as an independent prognostic marker of TNBC [29]. They observed a positive expression of Bcl-2 in 29.8% of the examined samples. Moreover, it was significantly associated with a high expression of p27, MDM4 and SPAG5. Taking into consideration only the Bcl-2-positive group, they found that G2 and G3 made up the largest percentage of cases – similarly to the present study. As far as tumour size is concerned, they observed T2 stage in 44.1% of cases comparable to 57.1% of studied cases; in both studies T2 tumours accounted for the largest group. Their study proved that loss of Bcl-2 considerably escalates the risk of both death and recurrence in TNBC.

In a study conducted by Abd El-Hafez et al. on a similar group of patients with TNBC, they observed Bcl-2 positive staining in 85% of invasive ductal carcinomas [30]. It is worth mentioning that they reported opposite results concerning patients' age and grading of the tumours. In the present study, 66.7% of patients were above 50 years old at the time of diagnosis, whereas Abd El-Hafez et al. reported that Bcl-2 was more frequently expressed in younger patients, accounting for 81.3% of all cases. Moreover, they correlated Bcl-2 expression with lower grading, whereas in the present research we did not observe the group of G0. These contradictory statements lead us to the conclusion that the role of Bcl-2 and its prognostic value in TNBC still seems unclear and needs further research on a wider group of patients.

## Conclusions

All the aforementioned details may lead to the conclusion that COX-2 and Bcl-2 high expression in triple-negative breast cancer may be an interesting asset in routine histological examination and the grading of breast cancer, however further studies with a larger group is necessary. Moreover, as they are usually present in higher graded neoplasms, COX-2 and Bcl-2 may also serve as potential new targets for systemic treat-

ment. This approach could potentially reveal new methods in the therapy of triple-negative breast cancer. This is crucial, as hormone therapy and HER2 targeting remain unavailable for those patients. The described association should be investigated further, as the group of patients was small, even though representing a rare histological subtype of breast cancer.

**Conflict of interest:** none declared

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