# *Effect of selected pre-analytic factors on immunohistochemical analysis of estrogen receptors, progesterone receptors and cell proliferation marker Ki-67 in breast carcinomas*

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**The goal of the work was to study the effects of pre-analytic factors such as freezing, delayed and prolonged fixation on the immunohistochemical expression of following biomarkers in breast carcinoma tissue: estrogen receptors, progesterone receptors and cell proliferation marker Ki-67. Then we compared the data with immunohistochemical results found in standard processed samples of the same biological material. Research was performed on a set of 50 tissue excisions of mammary carcinomas. The outputs were compared with analogous parameters of similar samples processed by standard histotechnology methodology. Proven statistically, significant deviations from the standard were as follows: after the tissue freezing, we observed a significant reduction in the quantitative and qualitative immunohistochemical expression of all three antibodies. In the tissue samples with the 2-hour delayed fixation, we saw a significant decrease only in the intensity of the staining (expression quality) of all three biomarkers. In the case of the 7-day prolonged fixation, only the quality of the staining (expression) of the cell proliferation marker was significantly reduced. Some of our findings appear to have significant clinical effects in some specific situations.**
**Keywords**: **freezing, delayed fixation, prolonged fixation, immunohistochemical analysis, biomarkers, breast carcinoma**

***Vplyv vybraných predanalytických faktorov na imunohistochemickú analýzu estrogénových receptorov, progesterónových receptorov a markeru bunkovej proliferácie Ki-67 v karcinómoch prsníka***
**Cieľom práce bolo sledovať vplyvy predanalytických faktorov ako zmrazenie, oneskorená a predĺžená fixácia na imunohistochemickú expresiu biomarkerov estrogénových receptorov, progesterónových receptorov a markeru bunkovej proliferácie Ki-67 v tkanivách karcinómov prsníka a následne získané údaje objektívne porovnať so štandardne spracovanými vzorkami rovnakého biologického materiálu. Výskum sme realizovali na súbore 50 tkanivových excízií karcinómov mliečnej žľazy, výstupy sme následne porovnali s analogickými parametrami podobných vzoriek spracovaných štandardnou histotechnologickou metodikou. Preukázané štatisticky významné odchýlky od štandardu boli nasledovné. Vplyvom mrazu sme zaznamenali signifikantné zníženie kvantitatívnej aj kvalitatívnej imunohistochemickej expresie všetkých troch protilátok. V tkanivových vzorkách exponovaných 2-hodinovej oneskorenej fixácii sme preukázali signifikantný pokles iba intenzity farbenia (kvality expresie) všetkých troch biomarkerov. V prípade 7-dňovej predĺženej fixácie sa významne znížila len kvalita farbenia (expresie) protilátkou proti antigénu Ki-67. Niektoré naše zistenia sa javia, že by v niektorých špecifických situáciách mohli mať aj významné klinické dôsledky.**
**Kľúčové slová:** **zmrazenie, oneskorená fixácia, predĺžená fixácia, imunohistochemická analýza, biomarkery, mamárny karcinóm**

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## Introduction

Breast cancer is generally regarded as the most worldwide common oncological disease with a high mortality rate. In the past the disease occurred mostly in older women aged around 60. In recent 20 years, the occurrence of breast cancer has risen in younger women aged 25-45, which respresents an increase of more than 30%. This issue also includes the male population, but the incidence in males is so low that we may consider it to be still rare. Despite the facts presented, diagnostics and especially the treatment of this disease has progressed over the last decade. In an effort to provide patients with the longest survival and highest quality of life, it is desirable to continually gain new insights and innovations in this issue, which we then successfully incorporate into practice. Early disease detection is the main task of screening programs for better prognosis and, ultimately, for lower mortality[1].Immunohistochemistry (IHC) is a laboratory test method that allows us to detect different antigens in the examined tissues, more precisely in cells, on the basis of the specific binding of the antigen to the antibody[2,3].

Such an analysis helps to accurately define the type of tumor of the mammary gland and consequently it allows the oncologist to indicate a targeted therapy. The key test for breast cancer treatment is the analysis of estrogen receptor (ER), progesterone receptor (PgR) and cell proliferation marker (Ki-67). These immunohistochemical stains must be standardized precisely, and therefore even slight deviations from the standard pre-analytical manipulation with a breast tissue may have a negative impact on the results of the monitored parameters[4].

The object of our interest was to monitor and assess differences in the immunohistochemical expression of ER, PgR and Ki-67 in pre-analytically processed breast cancer tissues compared to breast carcinoma tissues that were treated with well-defined deviations from standard pre-analytical treatment. These deviation from standard included freezing, delayed and prolonged fixation.

## Material and methods

The series included 50 female patients with breast cancer, who were clinically indicated and subsequently examined by frozen section (FS) intraoperative method in Medicyt, s. r. o. surgical pathology laboratory Trenčín from 6th December 2017 to 31th May 2018. The age range of the patients was 32-81 years. The processing of all monitored parameters in all patients was anonymous.

From a surgical point of view, the procedures included partial excisions or mastectomies and they were performed at the gynecological and surgical clinic of the Faculty Hospital in Trenčín. For our examinations, we used carcinoma samples obtained from received fresh unfixed mammary surgical tissue.

Four tissue samples (4x2 mm) were obtained from each tumor, and they were subsequently processed by the methods listed below.

The first tissue sample was dedicated for standard histological processing (STD) and biopsy examination. After excision we placed immediately in 10% formalin. After 24 hours of fixation, it was included in conventional histotechnological tissue processing followed by IHC analysis.

The second tissue sample was exposed to frost. Immediately after its excision, we applied it to a cryogel-covered target and we placed it in the cryostat at -25 °C for 30 min. After removal from the cryostat, the sample was allowed to thaw at a room temperature and then it was fixed in 10% formalin. After a 24-hour fixation, we included the sample into standard tissue processing followed by IHC analysis.

The third sample was used to examine the delayed fixation. After excision, the sample was left at room temperature for 2 hours and then we placed it in 10% formalin. After 24 hours fixation, the sample was included into standard processing and IHC analysis.

The fourth tissue sample was exposed an extended fixation. After it was excised by us, we immediately placed it in 10% formalin for 7 days. After the fixation, we included it into the standard tissue processing and IHC analysis as in the abovementioned cases.

The processing was performed as follows. We made 3 tissue sections from a sample, processed by standard histotechnological method, and placed on 3 xylanized slides each with a control. Subsequently, the sections were stained immunohistochemically for ER, PgR and Ki-67 (one marker per slide). We also produced 3 tissue sections from each frozen, prolonged and delayed fixation sample that were applied to 3 pre-prepared xylanized slides. There were 4 sections on each slide (one from the frozen tissue, the second from the late fixation tissue, the third from the extended fixation tissue and the fourth was the control) ***(Figure 1)***. IHC analysis of ER, PgR and Ki-67 (one marker per slide) was also performed on individual slides. Monoclonal antibodies ER (clone EP1, 1:100, Santa Clara, USA, Dako), PgR (clone PgR636, 1:25, Santa Clara, USA, Dako) and Ki-67 (clone MIB-1, FLEX, Ready-to-Use, Santa Clara, USA, Dako) were used. The positive controls included tissue of the uterine cervix (ER and PgR) and appendix vermiformis (Ki-67) as recommended by NordiQC ***(Figure 2)***.

IHC analysis was performed in the automated VENTANA system. For each excision, conventional hematoxylin eosin (HE) stained section was also made ***(Figure 3)***. All sections were examined by surgical pathologist who used the template protocol for evaluating the results of tested biomarkers in breast cancer patients (the protocol developed by the College of American Pathologists)[5]. When a situation arose that the carcinoma was not captured in the section (e.g., the carcinoma tissue was exhausted), the sample was evaluated as non-diagnostic.

For the statistical analysis, in accordance with our aim to test the hypothesis of the influence of the pre-analytical factors on the expression of the biomarkers which we studied, we selected a pair test because it is a set of the same samples that have been subjected to several different conditions. We expressed the degree of expression both quantitatively and qualitatively. We have substituted the categorical variables for the nominal ones with the purpose to quantify the change in the degree of expression due to a specific pre-analytic factor. Using Shapiro-Wilk's test, we verified the normality of the data distribution according to Gaussian's normal distribution. Since none of the data sets showed normal distribution, we selected a nonparametric Wilcoxon pair test with a p-value of ≤ 0.05 for hypothesis testing.

***Figure 1.*** *Scheme for the preparation of tissue for IHC analysis*

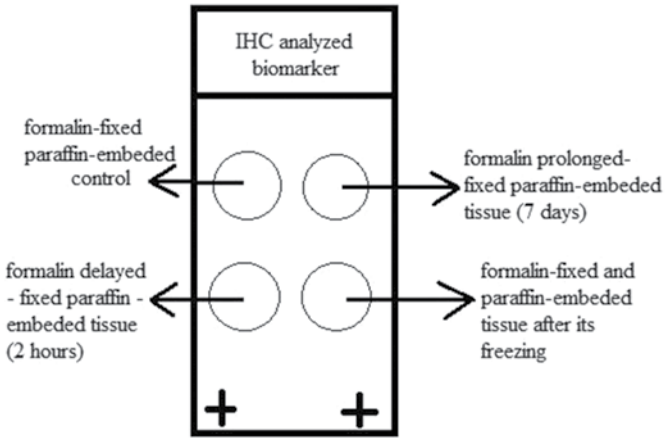

***Figure 2.*** *Immunohistochemical analysis of monitored biomarkers ER, PgR and Ki-67 (magnification 630x)*

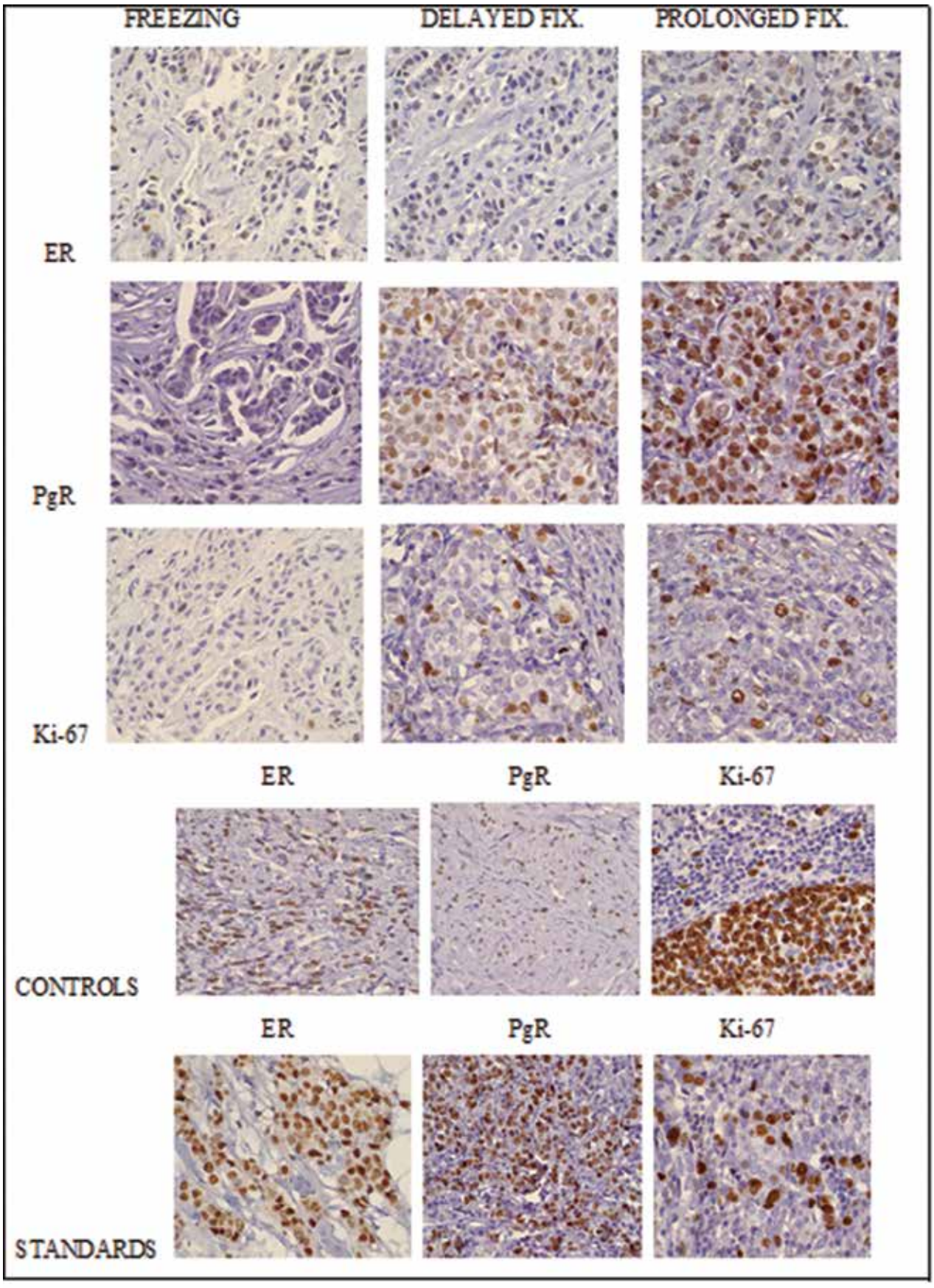

*Figure 3. The mounted slides with stained sections*

## Results

Our statistical analysis showed following results:

- Significant decrease in quantitative and qualitative expression of ER (quantity: p = 0.0001, quality: p = 0.0000), PgR (quantity: p = 0.0036, quality: p = 0.0002) and Ki-67 (quantity: p = 0.0024; quality: p = 0.0002) due to freezing.
- With delayed fixation, we observed a significant reduction in only the qualitative expression in all biomarkers studied (ER: p = 0.0000; PgR: p = 0.0102; Ki-67: p = 0.0128). Quantitative expression showed a non-significant decrease in ER (p = 0.0801) and PgR (p = 0,8415), Ki-67 in this group did not differ from the standard, i.e., it was without change (p = 0.8337).
- Significant effect of prolonged 7-day fixation was observed only in a form of decreased qualitative expression of Ki-67 (p = 0.0030). An increased quantitative expression of ER (p = 0.4009), PgR (p = 0.4179) and Ki-67 (p = 0.3125) was also detected, but it was not significant. In the quality of ER expression (p = 0.0688), we also noticed a significant decrease. The quality of PgR expression (p = 0.5755) did not differ from the standard ***(Table 1)***.

## Discussion

Current knowledge of an effect of freezing on the IHC expression of ER, PgR and Ki-67 is still scarce, which stimulated our effort put into this study. Previously, Argon et al. (2014) studied the effect of freezing on the quantitative and qualitative immunoprofile of ER, PgR and HER2 biomarkers in breast cancer. Their study included 53 cases of breast cancer with intraoperative histologic consultation followed by processing of the same tissue for conventional histology and for IHC examination of ER, PgR and HER2. The sample used for comparison was also taken from the same tissue, subjected to standard processing, and analyzed by the same IHC method. Pre-analytical influence of freezing was not studied. The change in the quantity of ER expression in the investigated samples compared to the standard was not demonstrated in 42 cases. On the contrary, in 10 cases it was lower and in one case it was higher. The quality of ER expression was the same in 46 cases, 6 cases showed lower expression and in one case the expression was higher. Changes in the quantity of PgR expression were not present in 44 cases, but in 9 samples the authors observed a decreased expression. The quality of PgR expression in 47 patients was unchanged in comparison with the standard and 6 cases showed lower expression. The differences in the expressions of both biomarkers between frozen and non-frozen tissue samples were evaluated as statistically significant for both quantity and quality[6]. In our study, we recorded a 2.5-fold increase of cases with reduced expression (25 cases), a 6-fold rise of cases with quantitatively increased expression (6 cases), and a 2.4-fold decrease of cases without change (19 cases). Regarding quality of expression, our series showed a 5.6-fold increase of cases with decreased expression quality (34 cases), and also 2 times more cases with increased expression quality (2 cases) and up to 15 times fewer cases without change (3 cases). When compared to our outcomes, when determining the quantity of PgR expression, we have detected 1.5 times more cases with reduced expression (14 cases), and 1.4 times less cases without expression change (14 cases). As Argon et al. observed no case with an increase in PgR expression, we do not have a correlation to our 5 cases. In terms of quality, we detected a 3.5-fold increase of cases with reduced PgR expression (21 cases) and a 9.4-fold decrease in cases without changing the quality expression (5 cases). They also did not record any case with an increased quality of PgR expression due to freezing, in contrast with 2 recorded cases in our study[6]. In a further comparison with Argon et al. study we saw significant differences

*Table 1. Results of statistical analysis of the influence of selected pre-analytic factors on the quantitative and qualitative expression of ER, PgR and Ki-67*

| | ER | | PgR | | Ki-67 | |
|---|---|---|---|---|---|---|
| | Quantity | Quality | Quantity | Quality | Quantity | Quality |
| **FREEZE** | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| **DELAYED FIXATION** | (non-signif. ↓) | ↓ | (non-signif. ↓) | ↓ | (no change ↓) | ↓ |
| **7-DAY FIXATION** | (non-signif. ↑) | (non-signif. ↓) | (non-signif. ↑) | (no change ↑) | (non-signif. ↑) | ↓ |

*Blue and red arrows mean increase and decrease, respectively*

in the results especially in the number of cases with reduced ER and PgR expression. It is difficult to explain these differences exactly. One reason could be the effect of the different cryogel composition that was in direct contact with tissues during the rapid intraoperative examinations. Because they did not report the exact size of tissue in the study (the thickness of tissue excisions determined by rapid intraoperative biopsy - RIOB), we can suppose that our investigated excisions affected by frost were thicker. Larger thickness of our tissue excisions could result in slower/insufficient penetration of the fixative in the already thawed sample and thus autolytic processes could start. This process could cause alteration of ER and PgR antigenicity of our material. The authors also did not report the exact time exposure of frost to the tissue. We assumed that tissue sent to RIOB must be examined within 15 minutes. If it was immediately thawed after FS, the time exposure of frost to tissue was about half the time shorter compared to us. An indirect factor to be taken into account is the stagnation of pre-prepared sections prior to the IHC analysis itself. Equipment of our laboratory at the time of the research did not allow a prompt IHC analysis of the research specimens. Our pre-prepared sections were often analysed more than a week after set up of tissue-cut slides. Routine (standard) IHC examinations were preferred for reason of quick diagnosis in the patients. Also, Downsett et al. (2011) reported in their study as one of the factors negatively impacting on expression of Ki-67 the extended exposure of the sections on air at room temperature, before the IHC analysis[7]. In spite of the different biomarkers we observed, we could, to a certain extent, accept this factor as having a possible negative effect on the expression of antibodies. Another potential factor could be the histological heterogeneity of the tumor tissue itself in individual tissue excisions.

Mengel et al. (2002) performed an extensive inter-laboratory study of 1-minute exposure to freezing (liquid nitrogen) of various tissue types inserted into the cryogel for expression of Ki-67. 172 laboratories were integrated with 5,160 samples analysed from 30 different tissue sample types. The proliferation index of frozen tissue samples that were subsequently thawed for 30 minutes and immersed into non-buffered formalin was significantly lower compared to the same type of tissue sample fixed immediately in un-buffered formalin without freezing. The complex output of frost effect monitoring on the quantitative and qualitative expression of ER, PgR and Ki-67 represents a significant shift in all monitored parameters towards the STD[8]. The influence of frost on the tumor tissue itself in the cryodestruction therapy process was described by Babaian et al. (2008). The consequences can be applied to all three studied biomarkers. They describe the formation of ice extracellular crystals with the induction of a hyperosmotic environment, which leads to the transfer of water from the cell, its shrinkage and the destruction of the intracellular matrix. At temperatures below -15 °C, intracellular ice is also formed, which expands, destroying the cell surface as well as individual cellular structures, including lysosomes, which is associated with the release of the enzymes present. Damage also occurs in the process of defrosting of the tissue. In the body, the damaged cells are removed using the immune syste[9]. Considering these facts, we suppose that in our study the damage of cellular structures, including lysosomes, followed by releasing of enzymes, could lead to autolysis and thus to the change/loss of the antigenicity.

According to the American Society of Clinical Oncology / College of American Pathologists, delay of the tissue fixation dedicated for the IHC analysis of ER and PgR should not exceed more than 1 hour to avoid its antigenic degradation[5]. For the Ki-67 antigen, we did not find any study which mentions such time-limits for delayed fixation. However, there are several research papers that followed the effects of delayed fixation on ER and PgR expression. Khoury et al. (2009) observed the effect of delayed fixation on the quantitative and qualitative expression of ER and PgR with a delay of 10 min, 30 min, 1 h, 2h, 4h and 8h in comparison with STD. Since the resected breast tissue from the operating room was transported to the pathology department, 2-5 minutes was taken into account. They found that ER and PgR expression declined with progressive ischemia at room temperature. ER expression analyzed by the clone of the primary antibody 1DE began to decrease after 2 hours and the PgR detected by the PgR636 clone at 1-hour fixation delay, but is statistically insignificant[10]. Quite similar results are also reported by Qui et al. (2010). They also compared the ER and PgR expression of the examined samples affected by delayed fixation and STD with the same time lags and at room temperature. They observed that the quantitative and qualitative expression of the SP1 clone of ER decreases with a 1-hour delay in fixation as well as expression of the PgR636 antibody clone, but are non-significant in both. They also observed the effect of delayed fixation with the same conditions on 3 different clones of the primary ER antibody (SP1, 1D5 and 6F11). They found that despite of the fact that expression of the ER clone SP1 already declined with a 1-hour delayed fixation, the overall effect is ultimately lower compared to the other two anti-ER antibody clones. This clone also presented more intense nuclear staining, weaker backgrounds and no cytoplasmic staining compared to clones 1D5 and 6F11[11]. The results of a study conducted by Kim, et al. (2015) also bring interesting findings. They observed the effects of delayed fixation (cold ischemic time CIT) ≤ 3 hours at room temperature and delayed fixation >12 hours at a controlled temperature of 4 °C on the immunohistochemical expression of ER, PgR and Ki-67. The results showed a significant increase in ER expression in ER-positive tumors, and the increased proliferation index in tumors with original Ki-67 expression < 10% in the set of tissue with delayed fixation longer than 12 hours at 4 °C. These findings were not observed in other studies. On the contrary, they show a decrease in expressions due to delayed fixation. This can be explained by the fact that long CIT (> 12 h) samples were placed in a refrigerator at 4 °C with a short exposure to room temperature, while short CIT (≤ 3) specimens were exposed to room temperature for the entire delayed fixation[12]. Compared to our observations, where we recorded a significant decrease only in the quality of ER, PgR and Ki-67 expressions, we can state that the results are similar in some parameters. Discrepancies can be attributed to the before mentioned stagnation of the prepared sections before IHC analysis with a time difference of more than 7 days, or to the fact that the tumor tissue volume

could show some histological variability. However, differences in the compared studies could also have been due to the different composition of fixative. In the both mentioned studies of the effect of delayed fixation on IHC expression of ER and PgR, the authors used as a fixative solution a 10% buffered formalin. We also used 10% formalin but it was un-buffered. We can assume that a significant decrease in the quality of expression of all three biomarkers studied could be due to the synergistic effect of delayed fixation (and the resulting degradation of antigens or epitopes), together with fixation in 10% un-buffered formalin where the pH is not constant. The negative effect on the quality of expression of 10% un-buffered formalin in this case could rest in its decreasing pH after immersing the tissue in which the degradation of the antigens has already been started due to cold ischemia (delayed fixation). A significant decrease in the quality of expression of individual markers is anticipated as a consequence of degradation at epitope level, not at the level of whole cell. Therefore, we noticed a significant reduction in only the quality of individual expressions. However, we can't exclude the effects of proteolytic degradation with the consequent lowering of pH that could degrade antigenic epitopes.

Prolonged fixation is considered by Clinical Oncology / College of American Pathologists as a factor influencing the decrease of immunoreactivity of ER and PgR expression, but it was not mentioned an exact length of prolonged fixation[5]. We have noticed a relative lack of studies in the available literature in the case of the effect of the prolonged fixation on expression of Ki-67, similarly as in the case of delayed fixation. Yildiz-Aktas et al. (2012) investigated the effect of 96-hour fixation on IHC expression of ER and PgR in mammary carcinoma tumor cells. They compared the results from tumor excisions with results from core-cut biopsies of specific cases. Of the 47 cases, they observed 1 quantitative variation of the result in terms of the appearance of positive ER expression in a 96-hour fixed sample against a standard that did not show any ER expression. In general, only minor non-significant changes in the qualitative and quantitative expression of ER and PgR have been recorded. The authors attributed these changes to the histological heterogeneity of the tumor or intra-observative variability[13]. Against this background, we can consider IHC analysis of samples fixed for 96 hours as valid and equivalent to IHC analysis of tissue samples processed by standard method.

In another study, Arima et al. (2015) observed a non-significant decrease in quantitative and qualitative ER expression in samples fixed for 2 days compared to standard, while the effect of prolonged fixation on Ki-67 expression was shown to be significant (quantitatively decreasing expression with prolonged fixation time)[14]. Compared to the results of our study, we can point to some concordance in the findings. Due to the 7-day prolonged fixation, we also noticed a statistically non-significant shift in quantitative and qualitative ER expression both in terms of decreasing quality and increasing quantity. In the occurrence of qualitative expression of PgR, the results of our examinations were unchanged on average and we only noticed a non-significant increase in the quantitative expression of the biomarker. The minimum deviations in our results could again be attributed to histological heterogeneity of tumors or intra-observative variability. A statistically significant decrease in quality and a non-significant increase in quantity of Ki-67 expression can be attributed to the protein „cross-link" bonds formed by the effect of the chemical properties. Thus, pre-analytically affected samples could mean in practice, in some (rare) cases, a false decrease in the proliferation index below the critical value of 20% expression, with possible consequences for the patient's treatment. Mengel et al. (2002) also dealt with the effect of 6-hour, 24-hour, 3-day and 7-day prolonged fixation for Ki-67 expression in its extensive inter-laboratory (172 laboratories) study. They performed a total of 5,160 analyzes separately for each tissue specimen in different fixative solutions. Their analyzes show a mild decrease of Ki-67 proliferation index in samples fixed in un-buffered formalin as compared to buffered formalin at 6-hour (29% vs. 30%) and 24-hour fixation (24% vs. 28%). However, after a 3-day fixation, they noticed an increase of 34% vs. 29%. They performed the 7-day fixation only on tissue samples in un-buffered formalin, but they observed again the drop to 30%. They conclude that for a common practice a better choice for analysis of Ki-67 is when the tissue is included in a processing after at least 24-hour fixation in buffered formalin, because the results showed a 4% higher proliferation index Ki-67[8]. In comparison with the results of our study, some discrepancies can be attributed to a significant difference in the histological types of examined tissue. We focused only on the tumor tissue of the mammary gland. Mengel et al. in their studies observed 30 various types of tissues[8] It should also be kept in mind that our series of 50 samples is substantially smaller than their series with 5,160 analyses. Thus, except of the Ki-67 cell proliferation marker quality expression, we could consider the prolonged fixation of 7 days as clinically insignificant. The results of the IHC analysis of the three markers (ER, PgR and Ki-67) in the tissues pre-analytically affected by prolonged exposure to 10% formalin at room temperature are comparable to those of the same IHC analysis in the standard tissues.

## Conclusion

Our results show that freezing has a significant negative impact on the quantity and quality of expression of all three antibodies. The most altered antigen by freezing was ER (EP1), followed by PgR (PgR636) and Ki-67 (MIB1). These findings indicate that the frozen breast cancer tissue is not suitable for further immunohistochemical analysis. This is important for indication of intraoperative examinations in cases with small tumor size. Regarding delayed fixation, we observed a significant decrease in only the qualitative expression (color intensity) of all three biomarkers. Even in the case of delayed fixation, ER appears to be the most alterable marker. The PgR and Ki-67 antibodies were less altered. We conclude that after the delayed fixation the quantitative expression of all three antibodies do not show significant changes and therefore delayed fixation has not a significant impact on managements and treatment of the patients. A significant decrease in only the quality of expression of all three biomarkers suggests that delayed fixation of non-frozen tissue degrades antigens at epitope level. Significant changes in prolonged fixation were noticed only in reduced quality (staining intensity) of the antibody against

the Ki-67 antigen. The changes in immunohistochemical expression in tissues due to prolonged fixation are comparable to standard specimens and therefore we consider prolonged fixation clinically non-significant. We also have to note that in all of the tested biomarkers we observed sporadic variations which were statistically non-significant. However, we think that in rare cases, they could have negative impacts on the treatment of individual patients in real clinical practice. Based on these facts, we believe that correctly defining, standardizing and observing pre-analytical and analytical laboratory procedures in biopsy diagnostics is necessary to constantly emphasize and improve.

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