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# Prognostic value of the Ki-67 proliferation index in patients with triple negative breast carcinoma. Preliminary report.

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**Key words:** breast carcinoma; proliferation index; Ki-67.

**Abstract.** The Ki-67 index is a biomarker that indicates the proliferation of cancer cells and is considered an effective prognostic factor for breast cancer. However, a standard cut-off point has not yet been established for the Ki-67 index in triple negative breast carcinomas. Therefore, the objective of this retrospective study was to determine an optimal cut-off point to establish it as a more accurate prognostic factor in the triple negative molecular subtype. The immunohistochemical analysis of the Ki-67 index was performed in 98 patients with breast cancer. The survival study using the Kaplan-Meier method was used to analyze the factors related to overall survival. The cut-off points (20 and 25%) were selected from the univariate analysis because they had the highest Hazard ratio to perform the multivariate analysis. With statistical significance ($p<0.001$), the analysis revealed that in this series the optimal cut-off point of Ki-67 is 25%, with an independent value regarding the clinicopathological variables considered in the study. These data suggest that the optimal cut-off point at 25% is a more effective prognostic factor for triple negative phenotype breast cancer. Due to the importance of these findings, it is recommended to verify the prognostic value of Ki-67 25% in series with a greater number of patients.

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## Valor pronóstico del índice de proliferación Ki-67 en pacientes con carcinoma de mama triple negativo. Reporte preliminar.

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**Palabras clave: c**arcinoma de mama; índice de proliferación; Ki-67.

**Resumen**. El índice Ki-67 es un biomarcador que indica la proliferación de células cancerosas y se considera un factor pronóstico eficaz para el cáncer de mama. Sin embargo, todavía no se ha establecido un punto de corte estándar para el índice Ki-67 en carcinomas de mama triple negativo. Por lo tanto, el objetivo de este estudio retrospectivo fue determinar un punto de corte óptimo para establecerlo como un factor pronóstico más preciso en el subtipo molecular triple negativo. El análisis inmunohistoquímico del índice Ki-67 se realizó en 98 pacientes con cáncer de mama. Se utilizó el estudio de supervivencia mediante el método de Kaplan-Meier para el análisis de los factores relacionados con la supervivencia global. Los puntos de corte (20 y 25%) fueron seleccionados del análisis univariado por tener el Hazard ratio más alto para realizar el análisis multivariado. Con significancia estadística ($p<0,001$), el análisis reveló que en esta serie el punto de corte óptimo de Ki-67 es 25%, con valor independiente respecto a las variables clínico-patológicas consideradas en el estudio. Estos datos sugieren que el punto de corte óptimo en 25% es un factor pronóstico más efectivo para el cáncer de mama con fenotipo triple negativo. Por la importancia de estos hallazgos, es recomendable verificar el valor pronóstico de Ki-67 25% en series con un mayor número de pacientes.

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### INTRODUCTION

Uncontrolled proliferation is one of the characteristics of the malignant disease, which can be evaluated by some methods, including mitotic counting and immunohistochemical determination of antigens associated with cell proliferation (1). The mitotic count is a proliferation measure widely used in tumor classification systems, however, it is subject to factors associated with the fixation of the sample, which could lead to erroneous conclusions about the biology of the tumor (1-3).

On the other hand, of all biomarkers associated with cell proliferation, the immunohistochemical evaluation of Ki-67 is the one that is frequently used to evaluate the proliferative characteristics of tumor cells (4,5). Except in the resting phase (G0), Ki-67 is detected in all proliferative stages of the cell cycle (G1, S, G2 and M). Today it is considered the "gold standard" against which other proliferation methods, such as the expression of the proliferating cell nuclear antigen and the SP6 peptide, must be compared (6,7).

It should be noted that breast cancer is a clinically heterogeneous disease, which has been classified into four main molecular subtypes through studies of microarray profiles of complementary deoxyribonucleic acid (Luminal A, Luminal B, HER2+ and triple negative). These are associated with significantly different clinical results and poor prognosis in the two subtypes with negative hormonal receptors (triple negative and

with overexpression of HER2), compared to the positive hormone receptor subtypes (Luminal A and Luminal B). Regarding the latter, the 2015 St. Gallen International Experts Consensus found that the Ki-67 proliferation index allows discriminating tumors of the Luminal A subtype against Luminal B, based on the Ki-67 cut-off point in 20% (8).

However, despite the large number of studies of the Ki-67 expression index, there is still no consensus on the biomarker cut-off points in the other subtypes of breast carcinoma. Among all the molecular subtypes, the triple negative (TN) is the one that has generated the most interest, due to the lack of expression of the estrogen, progesterone and HER2 receptors, and its association with an unfavorable prognosis (9-11).

Finally, considering that the Ki-67 expression pattern helps to predict the tumor response to adjuvant treatments, such as chemotherapy, which is currently the only systemic therapy modality available for TN tumors, in this study we evaluated the point of optimal cut of Ki-67 with prognostic significance in women with breast carcinoma TN.

## PATIENTS AND METHODS

The present study was conducted in women with follow-up at the Institute of Oncology "Dr. Miguel Perez Carreño" (IOMPC) from Valencia, Venezuela, between 2011 and 2016. With the approval of the Ethics Committee and the IOMPC Research Commission, a non-random, intentional series was formed, with 98 patients diagnosed of triple negative breast carcinoma. Due to the retrospective nature and at the time of review of the medical records, some patients had died, it was not possible to obtain informed consent; however, the confidentiality of the data of the women under study was maintained. The data of interest for the investigation were taken from those contained in the clinical history of each patient, established by the IOMPC Breast Pathology Service. For overall survival (OS) in months, a follow-up of 60 months, with a minimum of 36 months, was considered as cut-off point. Only the OS was evaluated, establishing the survival time as the time elapsed from the diagnosis to the date of death if it occurred before 60 months.

**Tissue matrix construction.** Tissue samples were fixed in formalin and included in paraffin following conventional methods. From the paraffin blocks, histological sections of 4 $\mu$m thickness were obtained and subsequently stained with hematoxylin-eosin. Histological preparations were reviewed and areas with tumor were carefully selected, marking those same areas on the paraffin block, in order to construct the tissue matrices as described in the literature (12).

**Immunohistochemistry.** The deparaffination of the histological sections, their incubation with the primary antibody (Ki-67, clone MIB-1, Dako) and subsequent processing of the samples, were performed according to what was established in previous investigations (12,13). For the quantification of Ki-67, four photomicrographs were taken from each case, two from each cylinder, in a Zeiss Axiostar plus microscope, with a Canon camera incorporated and connected to a computer with the Axiovision program. Then the positive and negative nuclei in each image were counted using the Bronze program, prepared by the engineer Víctor Barrios of the University of Carabobo. The figures of the four counts were added and the proliferation index was obtained as an average of the percentage of positivity for each case. Finally, different cut-off points of the biomarker expression were established (20, 25, 30, 35, 40, 45 and 50%). The 10 and 15% cut-off points were excluded due to the low number of tumors with Ki-67 expression <15%, which prevented the statistical analysis.

**Statistic analysis.** The analysis of the data collected was performed using the statistical package SPSS (Statistical Package for Social Sciences, version 22). The survival study was performed using the Kaplan-Meier method and tested using the log-rank test. Uni- and multivariate analyzes were per-

formed using the Cox proportional hazard model. Significant values of $p<0.05$ were considered.

## RESULTS

The average age of the patients at the time of diagnosis was 48.7 years. The most frequent clinical stage was III and histologically, the tumors were mostly undifferentiated. Most of the patients died during the follow-up. The main clinical-pathological data of the patients included in this study are detailed in Table I.

A univariate analysis was carried out considering the OS where significant relationships were evidenced with all the cut-off points evaluated (Table II). The cut-off points (20 and 25%) were selected from the univariate analysis because they had the highest Hazard ratio to perform the multivariate analysis. With statistical significance ($p=0.018$), the analysis revealed that the optimal cut-off point for Ki-67 is 25% (Table III), with an independent value regarding the clinical-pathological variables considered in the study (Table IV).

Cumulative rates of OS in patients with triple negative tumors were calculated using a Ki-67 cut-off point of 25%. The OS of patients with Ki-67 values <25% were significantly higher than those patients with Ki-67 values >25%, with $p<0.001$ (Fig. 1). Finally, Fig. 2 shows representative examples of Ki-67 immunohistochemical expression.

## DISCUSSION

Several studies on breast cancer have reported that increased expression levels of Ki-67 are associated with poorly differentiated tumors, larger tumor size, presence of axillary lymph node metastases and worse prognosis (4,11). In addition, Ki-67 is one of the chemosensitivity markers in breast carcinomas, but the correlation between its expression and chemosensitivity in the TN phenotype is unclear, probably due to the heterogeneous characteristics of these types of tumors (4,6,11).

**TABLE I**
SERIAL CLINICAL-PATHOLOGICAL CHARACTERISTICS.

| Variable | | |
|---|---|---|
| Age (years): mean (range) | --- | 48.7 (31-80) |
| | | n (%) |
| Age groups | ≤50 | 59 (60.2) |
| | >50 | 39 (39.8) |
| Clinical stage | I | 1 (1.0) |
| | II | 28 (28.6) |
| | III | 65 (66.3) |
| | IV | 4 (4.1) |
| Histological grade | I | 3 (3.1) |
| | II | 36 (36.7) |
| | III | 59 (60.2) |
| Overall survival (average in months) | --- | 35.3 |
| Condition | Deceased | 35 (35.7) |
| | Live | 63 (64.3) |
| Ki-67 (average of total cases) | --- | 42.9 |

**TABLE II**
UNIVARIATE ANALYSIS FOR OVERALL SURVIVAL USING DIFFERENT Ki-67 CUT-OFF POINTS.

| Cut-off point (%) | Hazard ratio(CI 95%) | p |
|---|---|---|
| 20 | 5.090 (1.591-16.286) | 0.006 |
| 25 | 3.875 (1.901-7.897) | <0.001 |
| 30 | 2.756 (1.555-4.885) | 0.001 |
| 35 | 2.897 (1.723-4.870) | <0.001 |
| 40 | 2.920 (1.752-4.867) | <0.001 |
| 45 | 3.116 (1.871-5.189) | <0.001 |
| 50 | 2.866 (1.708-4.808) | <0.001 |

CI: Confidence interval.

**TABLE III**
MULTIVARIATE ANALYSIS FOR OVERALL SURVIVAL ACCORDING TO THE SELECTED Ki-67 CUT-OFF POINT.

| Cut-off point (%) | Hazard ratio (CI 95%) | p |
|---|---|---|
| 20 | 2.190 (0.547-8.761) | 0.268 |
| 25 | 2.778 (1.191-6.481) | 0.018 |

CI: Confidence interval.

**TABLE IV**
MULTIVARIAte ANALYSIS OF Ki-67 AND THE CLINICAL-PATHOLOGICAL FACTORS.

| Variables | p | Hazard ratio (CI 95%) |
|---|---|---|
| Ki-67 25% | <0.001 | 4.215 (1.991-8.920) |
| Age | 0.038 | 0.565 (0.330-0.969) |
| Histological grade | 0.289 | 1.305 (0.797-2.137) |
| Clinical stage | 0.013 | 2.051 (1.162-3.620) |

CI: Confidence interval.

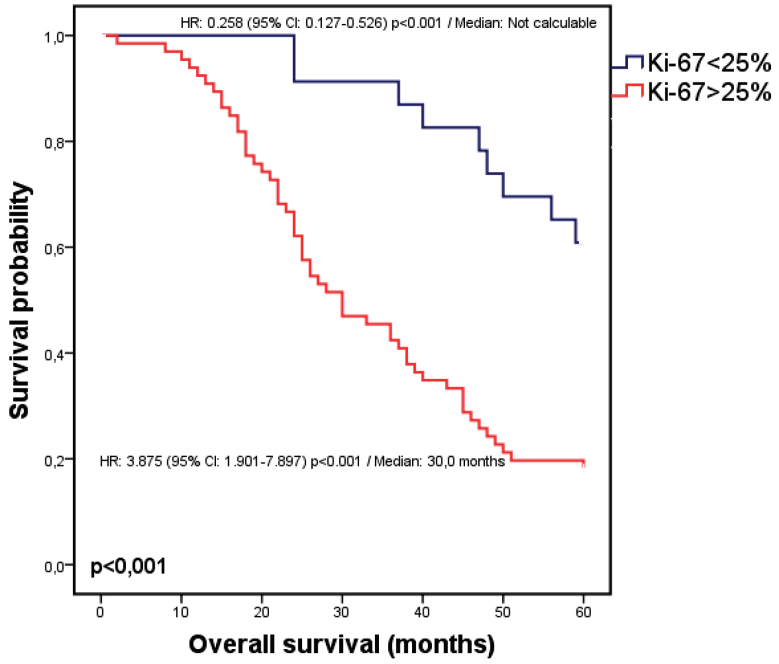


**Fig. 1.** Overall survival based on the Ki-67 cut off point of 25% in negative triple breast carcinoma.

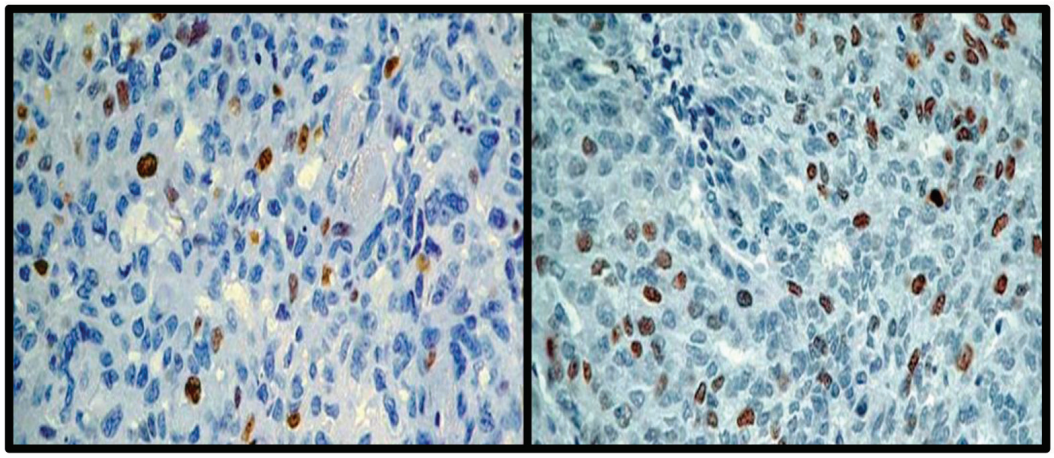

**Fig. 2.** Expression of Ki-67 studied by immunohistochemistry in tissue matrices. (A) Ki-67 with low proliferative index (<25%). (B) Ki-67 With high proliferative index (>25%).

The main objective of this study was to identify the optimal cut-off point for the Ki-67 index that could be used as an optimal prognostic factor for triple negative breast cancer. Regarding the expression of Ki-67, the average was 42.9%, much higher than that of the other molecular subtypes, as indicated by other studies (14,15). Statistical analysis revealed that a wide range of cut-off points are significant for the OS of the series. These findings suggest that dividing patients according to the Ki-67 index using cut-off points 20, 25, 30, 35, 40, 45 and 50%, are clinically significant because they have prognostic value. This range was considered because 20% is the average used in Luminal tumors and 50% represents a high proliferative potential, characteristic of TN tumors. The univariate analysis showed that the highest Hazard ratio (HR) was obtained with the Ki-67 index in 20%, however, in the multivariate analysis, the cut-off point in 25% had the highest HR, with independent statistical significance ($p<0.001$). Similarly, significant differences were observed in the OS of the series, considering a Ki-67 with a 25% cut-off point.

In the literature, references were found that established similar findings, with a cut-off point that ranges between 20 and 30% with prognostic value in triple negative carcinomas (16-22). However, the recommendation for cutting the level of Ki-67 expression that affects the prognosis is controversial internationally. In a study on the clinical implication of the limit value of Ki-67, it is established that the choice of the cut-off point depends on the clinical objective, that is, if the expression of the biomarker is used to exclude patients with tumors with slow proliferation of chemotherapy protocols, a threshold of 10% would help avoid over-treatment. On the contrary, if the expression of Ki-67 is used to identify tumors that are sensitive to chemotherapy, it is preferable to set the cut-off point at 25% (23).

Other authors have established that the cut-off points used for the differentiation of luminal tumors could have limited eligibility for other molecular subtypes of breast carcinoma, since the initial values of Ki-67 for triple negative and HER2 positive tumors are much higher than for luminaires (10). In carcinomas TN, Miyashita et al. described similar results, but with the cut-off point set at 40% as the optimal value (24). In another series, the optimal cut-off value in TN was 61% and Cox regression analysis revealed that Ki-67 has an independent prognostic value (10). Even authors such as Aleskanda-

rany et al. reported that the optimized Ki-67 limit in TN is 70% (25).

These diverse findings may be due to the selection criteria of the patients included in the studies, the sample size and/or the different chemotherapeutic regimens used (5). In addition, it could be related to the limit established for the positivity of hormonal receptors and HER2, which has changed in recent years (reduced from 10 to 1% in the case of hormonal receptors, and from 30 to 10% in the case of HER2) (26). Therefore, new studies are needed to determine how these factors could influence the definition of the Ki-67 cut-off point in carcinomas with TN phenotype.

In summary, because TN tumors are characterized by a high proliferation rate, it is not clear in the literature what the cut-off point is to consider a high or low Ki-67, which can vary between 10 to 60%. In addition, the Ki-67 value seems to vary in the prognosis according to age (26). These results should be confirmed in subsequent studies so that in the future, patients with TN can be separated into risk groups according to their age and Ki-67 value, to determine those that require more aggressive treatments. Due to the importance of these findings, it is recommended to verify the prognostic value of Ki-67 25% in series with a greater number of patients.

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