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Is it possible to identify subpopulations of triple negative breast cancer?

Summary

Introduction: Numerous publications have individually evaluated the expression of the p53 protein, the presence of androgen receptors and the magnitude of the lymphocyte infiltrate TILs in triple negative tumors. The presence or absence of these variables could help identify subgroups in triple negative breast cancer (TNBC).

Objectives: The objective of this study is to evaluate in triple negative tumors feasibility of using lymphocyte infiltration, the expression of p53 protein and androgen receptors as prognostic markers (overall survival and disease-free) .and collaborate in the identification of biomarkers for the development of specific target treatments.

Material and Methods: We intend to analyze those patients diagnosed with TNBC treated at the German Hospital of Buenos Aires and at the Mater Dei Sanatorium from December 2002 to December 2014.

Results: Thirty-five patients with TNBC were analyzed. The prevalence in the p53 mutation was 57% in the general population, 64,7% in relapses and 69% in deceased patients. 22.8% of the patients studied showed expression of androgenic receptors. The average lymphocytic tumor infiltration (TILs) was 20.6% (5%-70%), being lower when the population of patients who relapsed was only evaluated (19.6%) and even lower when evaluating the population of deceased patients (17.1%).

Discussion: The mutation in p53 in our population has a RR of 1.6 for survival with a p = 0.596. The presence of ILV together with the mutation in p53 constitutes a mortality risk factor with p = 0.0147. The expression of androgenic receptors has a RR of 1.5 as a mortality risk factor and a p: 0.974. The presence of TILS greater than 20% is predictive of mortality and recurrence with a p: 0.0269. This is maintained with values of TILs of 18% and 15% with p = 0.0131 and p = 0.0032 respectively.

Conclusion: The determination of the TILs together with the evaluation of the mutation of the p53, and its evaluation in combination with other prognostic factors (ILV and Ki67) is useful for predicting prognosis in the TNBC. We think that the presence of mutation of p53 and the degree of TILs are determinations whose evaluation should be standardized in patients with TNBC and be duly recorded in the pathology report.

Keywords: triple negative, p53, androgenic receptors, TILs

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Introduction

Breast cancer is the main cause of mortality from neoplasia in women over 25 years of age. It causes approximately 500 thousand deaths per year. 70% of cases are diagnosed in developing countries, with mortality being higher in underdeveloped countries as a result of less access to health services. In 2010, the standardized mortality rate was 18.7 per 100,000 women aged 25 and over, which represents an increase of 49.5% in the last 20 years. From a statistical point of view, 1 in 8 women who live to the age of 80 will suffer from breast cancer.[1–3]

Due to their negativity for hormone receptors and HER-2, triple-negative tumors do not benefit from antihormonal or anti-HER2 treatments. It has no specific treatment and chemotherapy continues to be the only systemic treatment modality[4–8] together with the recent approval of neoadjuvant immunotherapy and for some cases in adjuvant therapy.

Just as for a long time a heterogeneity of behaviors within breast cancers was observed without finding a theoretical explanation for said variation, today we know that Perou's classification is still incomplete. Within each of the subgroups it is possible to identify subpopulations with different characteristics, behaviors and responses.[3]

Triple negative breast cancer (TNBC) is a heterogeneous group of cancers of variable aggressiveness characterized by the aforementioned absence of hormone receptors and HER2, high expression of cytokeratins 5/6 and 17 (myoepithelial), laminin and binding protein 7 to fatty acids. They are also called basal-like tumors because of their genomic expression profile similar to that of normal basal cell epithelium and normal mammary myoepithelial cells. It also shares histologic features with normal breast basal epithelial cells, showing a high proliferation rate, central necrosis, and infiltrative margin, as well as sparse stroma, frequent apoptotic cells, and a lymphocytic stromal response.[9] Despite these coincidences, not all triple negative breast cancers (according to immunohistochemistry) are basal (according to the microarray technique), nor are all basal cancers triple negative. About 77% of basal tumors are triple negative, while 71-91% of triple negative tumors are basal type.[10] Both affect young patients more predisposedly, are of high histological grade, clinically aggressive and have a poor prognosis, but not all basal tumors are negative for estrogen, progesterone and HER2 receptors, nor do they have the same response to neoadjuvant chemotherapy as other triple negative tumors.[4]

The biological behavior of triple negative tumors is usually more aggressive and with a greater tendency to present distant metastases compared to other subtypes of breast cancer.

In 2012, *"Comprehensive molecular portraits of human breast tumors"* was published in the journal Nature,[11] which reported the results of *The Cancer Genome Atlas (TCGA)* in breast cancer. Determinations were made on the DNA, RNA and proteins of tumor samples and adjacent tissue from 825 patients. It was shown that there is a wide variety of very low frequency mutations in breast cancer. The most common were P53 (37%), PIK3CA (36%) and GATAS (11%). In the subpopulation of triple negative tumors, the incidence of mutation in P53 was even higher (80%). What is specific is that the absence of molecular alterations present with a high incidence and the limited number of known biomarkers are responsible for the limited development of specific therapeutic strategies in triple negative breast cancer.

The fundamental role of the immune system is to maintain tissue homeostasis through continuous surveillance and the subsequent correct initiation of the inflammatory cascade with the adequate activation of the cells responsible for the immune response.[12]

Neoplastic transformation alters the structural order of tissues and induces an immune response that often manages to eliminate incipient tumors. When elimination is incomplete, neoplastic cells manage to escape immunological control. This malignant progression is described in the immunological theory of cancer, which divides it into three stages: elimination, equilibrium, and escape. While most cancer patients are diagnosed during this last stage, the relationship between the tumor and the immune system continues to evolve and often the magnitude of cancer progression depends on this interaction, with its consequent impact on survival.[12]

Many recent studies have demonstrated the prognostic and predictive value of tumor lymphocytic infiltration (TILs) in breast cancer, which led to the development of "The International TILs Working Group" that established specific parameters for its proper assessment. and interpretation in breast cancer.[12] The determination of the degree of lymphocyte infiltration carried out in tumor blocks stained with Hematoxylin-Eosin has been shown to have prognostic and predictive value in positive TNBC and Her 2.[13,14] This was first reported in 297 patients with TNBC in the BIG 2-98 study[15] and then prospectively and randomized in 481 patients with TNBC in the *United States Eastern Cooperative Oncology Group (ECOG)* studies.") 2197 and 1199.[14] These studies showed that the greater the lymphocytic stromal infiltration at the time of diagnosis, the better the outcome after anthracycline-based chemotherapy.

TP53 functions as a checkpoint in the cell cycle, triggering responses to DNA damage, including repair and apoptosis. It detects abnormalities in DNA replication, inhibiting cell proliferation until the damage is repaired and if it cannot be resolved, it triggers apoptosis or programmed suicide. Unlike other subtypes, triple negatives have a high frequency (82%) of mutations in TP53 (in tyrosine) and p53 protein expression.[9] Biganzoli managed to divide patients with CMTN into 2 subpopulations, with different overall survival and disease-free survival, through the determination of p53 protein expression. Concluding that those who expressed said protein had a worse prognosis.[16] The mutated p53 oncogene increases the incidence of recurrence 3-fold, reducing overall and disease-free survival.

The expression of androgen receptors in triple negative tumors is associated with higher rates of overall survival and is considered a promising therapeutic alternative, mainly in the subgroup of non-basal triple negative tumors.[17] Its expression has a lower incidence in triple negatives than in the rest of the tumor population (24.8% vs 81.6%). Lehmann proposes a subclassification of the CMTN into 6 subgroups in order to facilitate the identification of target therapies, one of these subgroups is the so-called Luminal AR *(androgen receptor)* that would be sensitive to androgen antagonists (bicalutamide) and inhibitors of the PI3K pathway.[18]

From a purely histological perspective, CMTN brings together a subgroup of tumors with different biology, most of them aggressive, and others of lesser prevalence, such as secretory tumors or cystic adenoids, which, despite being triple negative, are less aggressive.

Similar to what happens with Her2-positive tumors, but with less evidence, there is consensus on the need to treat with chemotherapy those NSCs larger than 0.5 cm, but it is difficult to determine whether or not it is necessary in cases smaller than 0.5cm.

Numerous publications have individually evaluated the expression of p53 protein, the presence of androgen receptors, and the magnitude of the lymphocytic infiltrate in triple-negative tumors. The presence or absence of these variables, individually or in combination, could help identify TNBC subgroups with potential prognostic and therapeutic implications.[2,4,19–22]

Objectives

The objective of this work is to evaluate in triple negative tumors the feasibility of using lymphocyte infiltration, p53 protein expression and androgen receptors as prognostic markers (overall survival and disease-free period) and collaborate in the identification of biomarkers for the development of specific target treatments.

Operational objectives:

a) To retrospectively evaluate lymphocyte infiltration, p53 protein expression and androgen receptors in paraffin blocks from a population of patients with triple negative breast cancer from Sanatorio Mater Dei and Hospital Alemán in Buenos Aires.

b) Retrospectively evaluate the rates of overall survival (OS) and disease-free period (DFP).

c) To compare and analyze the rates of overall survival and progression-free survival in different populations, according to the percentage of lymphocyte infiltration, expression of p53 protein and androgen receptors.

Material and method

We analyzed those patients with a diagnosis of TNBC treated at the Hospital Alemán of Buenos de Aires and at the Sanatorio Mater Dei from January 2002 to January 2014.

Authorization was requested from the Ethics Committees and the Teaching and Research Departments of both centers prior to sending the corresponding research protocol.

Inclusion criteria:

i. Patients with triple negative breast cancer.

ii. Patients whose histological diagnosis has been made in the Pathological Anatomy Services of the Hospital Aleman or the Sanatorio Mater Dei.

iii. Patients whose clinical history is available.

Exclusion criteria:

Those patients for whom it is not possible to have access to the paraffin blocks to carry out the desired determinations.

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Methodology

The research design is a retrospective prognostic cohort. The work was carried out in three stages. In the first, the evolution of the patients was retrospectively evaluated from the moment of diagnosis, obtaining data from the computerized Clinical History in the Hospital Aleman group and from the Clinical History of the office in those patients from the Sanatorio Mater Dei. The second stage was carried out by pathologists from both services, who evaluated the aforementioned markers in the paraffin blocks of patients with triple negative breast cancer from the Hospital Aleman and the Sanatorio Mater Dei. The third stage consisted of the processing and analysis of said information.

Demographic (age, personal and family history) and clinical data (onset clinical stage, pathological characteristics of the tumors, treatment and oncological follow-up) were collected. Patient staging was performed according to the American Joint Committee on Cancer (AJCC) seventh edition. The treatments performed included local procedures, such as surgery and radiotherapy, and systemic chemotherapy (both neoadjuvant and adjuvant).

Pathological characteristics of the tumors were analyzed. Triple Negative histological subtype cancers were considered to be those tumors that met the criteria of the ASCO/CAP guidelines corresponding to the time of diagnosis by means of immunohistochemical techniques.[23,24]

The determinations were carried out using the immunohistochemical technique, in sections of the blocks included in paraffin. They were performed in Ventana® and Bond Leica® automatic colorants.

Lymphocyte infiltration was determined and quantified as established by "The International TILs Working Group".[12]

The p53 protein mutation was determined to be present when it expressed nuclear positivity. Androgen receptor expression was considered positive when nuclear positivity was expressed in at least 1% of tumor cells.

Ki67 (cell proliferation marker) was assessed by counting stained tumor nuclei (mib1, Dako).

The follow-up was carried out with six-monthly clinical control, annual mammography and breast ultrasound every 6 months for the first 5 years.

Disease progression or relapse was considered to be those that presented lesions compatible with locoregional or secondary recurrence during follow-up, evidenced both in the physical examination and in complementary studies and/or pathological anatomy. Disease-free period (DFP) was defined as the time in months from the date of histological diagnosis to relapse or death, and overall survival (OS) from the date of diagnosis to death. Relapse or death of patients were determined as events during follow-up.

Statistics

The continuous and nominal variables were expressed as means and percentages with their respective distribution measures. Overall survival (OS) and progression-free survival (PFS) were evaluated using Kaplan Meier survival curves. Survival between the different groups was compared using the Log-rank test, considering $p<0.05$ significant.

Results

Forty-three patients with TNBC were studied, of which 8 were excluded because they did not meet the inclusion criteria.

Retrospective evaluation of lymphocyte infiltration, p53 protein expression, and androgen receptor expression was performed in paraffin wafers from 35 patients with triple-negative breast cancer according to the criteria established in the protocol.

The clinical and histopathological characteristics of the patients are shown in Table 1. The age of the patients ranged from 28 to 89 years, with a mean of 58 years and a median of 60 years. 74% of the patients were menopausal at the time of diagnosis (n=26). 31% of the patients had a family history of breast cancer (n=9) and 9% had a personal history of previous breast cancer (n=3).

Conservative treatment was performed in 71.4% of the patients (n=25), 8 patients underwent mastectomy + axillary lymphadenectomy and another 2 patients underwent lumpectomy and mastectomy as the only treatment due to advanced age and comorbidities (both without examination). Axillary or subsequent adjuvant treatments). Table 1 shows 26 patients with quadrantectomy since to the 25 patients with conservative treatment we add the patient who underwent lumpectomy only. Of the 9 patients who underwent mastectomy, 4 underwent immediate reconstruction with an expander. Axillary evaluation was performed in 94.3% of the patients (n=33), 23 of them using the sentinel node technique and the remaining 10 with direct axillary lymphadenectomy.

74.3% of the patients (n=26) had NST tumors, 1 patient had a ductal-lobular carcinoma, 5 patients had lobular carcinomas, 1 patient a papillary carcinoma, 1 patient a small cell carcinoma and finally 1 patient had adenoid cystic carcinoma. The average tumor size was 2.8 cm with a range of 0.5 cm to 7.5 cm. In line with their triple negative status, 71.4% (n=25) were histological grade (GH) 3, while the remaining 28.6% (n=10) were GH 2.

The mean Ki67 was 33% with a range of 3% to 80%. Lymphovascular invasion was present in 14 of the 35 cases (40%).

Of the 33 patients who underwent axillary evaluation, 17 of them had axillary involvement, with an overall incidence of 48.6%, 16 patients did not have axillary involvement, 8 patients had only 1 involved node, and 9 patients had 2 or more involved nodes, one of them was a stage 4 at the time of diagnosis. Within the group that underwent direct lymphadenectomy (n=10), 90% had axillary involvement. Axillary lymphadenectomy was performed in 18 patients (10 directly and another 8 after the sentinel lymph node), with an average of 18,6 lymph nodes resected.

37.1% of the patients were stage I at the time of diagnosis (n=13), 28.6% were stage II (n=10), equally distributed between stages II A and B. 14.3% had stage IIIA disease at diagnosis (n=5) and 17.1% of patients had stage IIIC. Only one patient had metastatic disease at diagnosis (Table 1).

Regarding adjuvant treatment, 26 patients received chemotherapy, 2 of them prior to surgery. The patients who underwent neoadjuvant treatment received Docetaxel/Adriamycin/Cyclophosphamide (TAC) with a partial pathological response, with a residual tumor of 2.2cm and 11/13 positive nodes, and the other Adriamycin/Cyclophosphamide x 4 cycles followed by Taxanes x 12 weeks (AC/Tax) with a partial pathological response and a residual tumor of 1.2 cm and 2/16 positive nodes. The most used scheme (n=15) was AC/Tax, 5 patients received Cyclophosphamide/Methotrexate/5-Fluorouracil (CMF), 2 patients received Adriamycin/Cyclophosphamide (AC), 1 patient received Docetaxel/Cyclophosphamide (DC) and another patient received Carboplatin/Taxanes. There were 9 patients who did not receive chemotherapy, 3 of them, despite having an indication, did not do so due to associated comorbidity factors, another was stage

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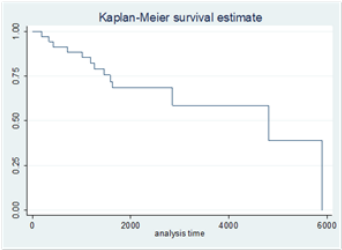
IV at the time of diagnosis, and with respect to the remaining 5, 2 presented relapses, one local and another at a distance at 65 and 45 months (Table 1).

Table 1 Clinical and histopathological characteristics

n= 35		
Age in years Media (SD)	58,4 (15,06)	CI 95% 53,4 – 63,4
Menopausal (%)	26 (74%)	
Family Background (%)	11 (31,4 %)	
Personal History (%)	3 (8,6 %)	
Histology		
Tumor cm Media (SD)	2,8 (1,97)	CI 95% 2,1 – 3,4
Invasive NST Cancer	26 (74,3%)	
Histological grade 3	25 (71,4%)	
Ki67	33 (3-80)	
LVI	14 (40%)	
Stage (%)		
I	13 (37,1%)	
IIA	5 (14,3%)	
IIB	5 (14,3%)	
IIIA	5 (14,3%)	
IIIC	6 (17,1%)	
IV	1 (2,9%)	
Neoadyuvant Chemotherapy (%)	2 (5.7%)	
Tyoe of Surgery		
Mastectomy	9 (25,7%)	
Lumpectomy	26 (74,3%)	
Negative Sentinel Node (%)	15 (65,2%)	
Axillary Lymphadenectomy	18 (51,4%)	
Adjuvant Chemotherapy (%)	24 (68,6%)	
Radiotherapy (%)	26 (74,3%)	
Events (%)		
Deaths	13 (37,1%)	
Local Relapses	6 (17,4%)	
Distant Relapses	11 (31,4%)	
Follow-up in months Mediana (min-max)	64 (5 - 194)	

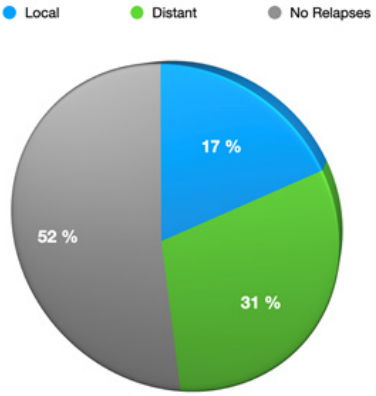
Two-year mortality in our population was 11.43% (95% CI: 3.20-26.74) and five-year mortality was 28.57% (95% CI: 14.64-46.30).

Total mortality in our population was 37.1% (13 of 35 patients died), with a median survival of 4,809 days or 160 months (Graph 1). The average follow-up was 67 months and a median of 64 months with a range that went from 194 months to 5 months, this last case was that of an elderly patient with multiple comorbidities who underwent a lumpectomy as the only treatment and after two months of surgery he died.

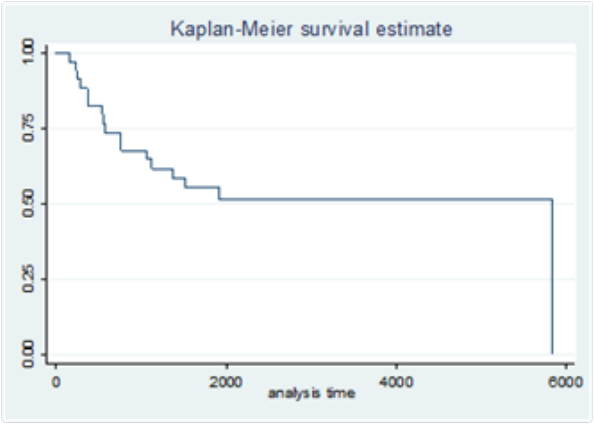


Graph 1 Overall survival curve.

In Graph 2, we can see that 17 of the 35 patients studied relapsed (48.6%), of which 35.3% (n = 6) did so locally (none at the axillary level) and 64.7% remaining (n=11) did so at a distance (3 of them in the CNS). Median progression-free survival was 5,844 days or 194.8 months (Graph 3).



Graph 2 Relapse distribution.



Graph 3 Progression-free survival curve.

The two-year relapse rate was 25.71% (95% CI: 12.49-43.26) and the five-year relapse rate was 42.86% (95% CI: 26.32-60.65).

57% (n=20) of the patients studied presented a mutation in the p53 protein (Graph 4). 64.7% of the patients who relapsed (11 of 17 patients) were carriers of this mutation (Graph 5). When analyzing the patients who died, we found that 69% of them (9 of 13 patients) had this mutation. There were 9 patients who did not receive chemotherapy, 3 of them, despite having an indication, did not do so due to associated comorbidity factors and another was stage IV at the time of diagnosis. Of the remaining 5 patients, 2 relapsed, one locally and the other distantly at 65 and 45 months, respectively, the latter having a p53 mutation. If we analyze the patients who underwent chemotherapy (n=26), there were 15 patients who had p53 mutation and 11 patients who did not. In the subgroup that received chemotherapy and had p53 mutation (n=15), 53% of the patients relapsed (n=8) and the remaining 47% did not relapse (n=7). Of the 11 patients who did not have a p53 mutation and received chemotherapy, 7 patients (64%) did not relapse while 4 patients (36%) did, 2 locally and 2 distantly.

22.8% (n=8) of the studied patients presented androgen receptor expression, with an average expression of 39.6% (2-90%) (Graph 6). 23.5% of patients who relapsed (4 of 17 patients) expressed androgen receptors (Graph 7). When analyzing the patients who died, we found that 30.8% of them (4 of 13 patients) were positive for androgen receptors. Of the 5 patients who did not receive chemotherapy

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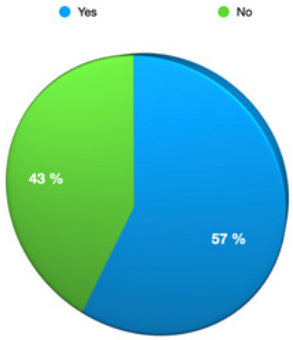
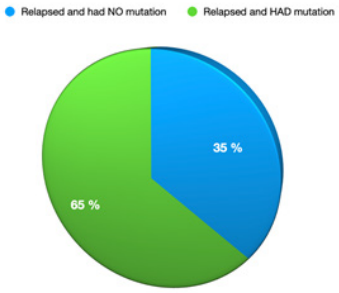
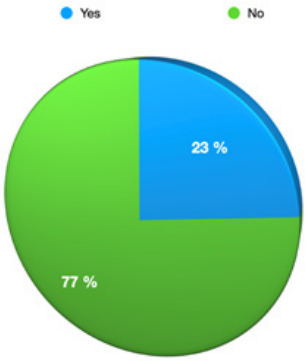
because it was not indicated, only 2 relapsed, of which one expressed androgen receptors in the tumor membrane (AR: positive, 90%), the relapse was distant at 45 months.



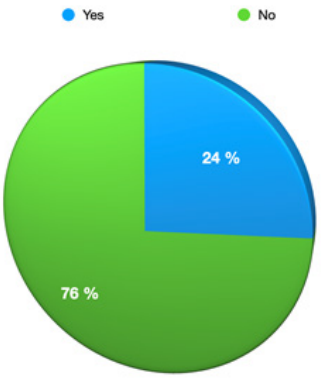
Graph 4 Prevalence of p53 mutation.



Graph 5 Prevalence of p53 mutation in relapse patients.

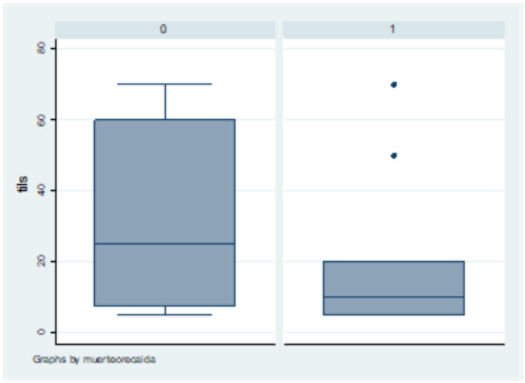


Graph 6 Prevalence of androgen receptor expression.



Graph 7 Prevalence of androgen receptor expression in relapse patients.

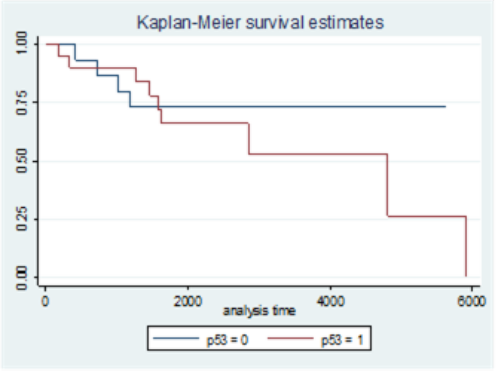
The mean tumor lymphocytic infiltration rate (TILs) in the entire population was 20.6% (5%-70%). In the group of patients who relapsed (n=17) this average was 19.6% and when analyzing only the patients who died (n=13) we found that the average TILs was 17.1%. There were 14 patients who received chemotherapy and did not relapse, in this group the average TILs was 31.4%, while the average TILs in the group of patients who received chemotherapy and did relapse (n=12) was 19.41% (Graph 8).



Graph 8 Comparison of the median TILs between the group that received chemotherapy and then died or relapsed (Ctp + event) and the group that received chemotherapy and neither died nor relapsed (disease-free Ctp). (0: Ctp free of disease, 1: Ctp + event, the central blue line of each rectangle expresses the median of TILs of each group, which is 25 for the non-relapse group and 10 for the relapse group) The statistical test Wilcoxon rank sum test compares both medians (ie 25 vs 10) obtaining a p of 0.062.

Discussion

According to the international literature, TNBC have a high frequency (80%-50%) of mutations in TP53 (in tyrosine) and p53 protein expression,[9] in our population the prevalence was slightly lower (57%). Coinciding with the publication by Biganzoli,[16] this prevalence increases when analyzing the population of relapsed patients (64.7%) and even more so in the population of deceased patients (69%). The same occurs when exclusively analyzing patients who underwent chemotherapy, where the recurrence rate is higher in those who had p53 mutation (53% vs 36%). In accordance with the international bibliography and in line with our working hypothesis, the p53 mutation seems to be a poor prognostic factor in TNBC that is associated with a lower response to systemic therapy.[2] In our population, its presence had a RR of 1.6 for survival with p = 0.596, being statistically non-significant in our population (Graph 9). It is likely that its lack of statistical significance is associated with the insufficient number of patients studied.



Graph 9 Survival curve according to p53 expression (0: they do NOT express p53, 1: they DO express p53).

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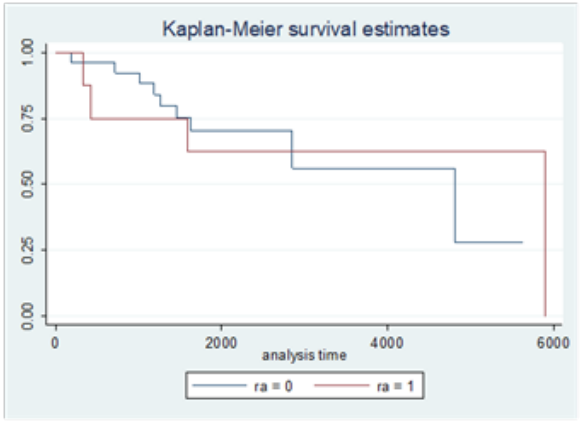
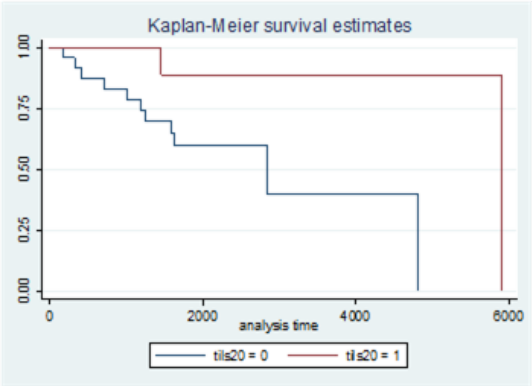
22.8% of the patients studied presented expression of androgen receptors in agreement with the published medical literature.[17,18] The expression of androgen receptors is reported in most studies as a good prognostic factor, especially in cases of triple negative tumors that are negative for CK5. In our population, these data not only could not be confirmed, but even seemed to show the opposite, since the deceased patients presented an average incidence of expression of androgen receptors higher than that of our study population (30.8% vs 22.8%). The small number of patients who express androgen receptors in our population (n=8) could justify this contradiction. The expression of androgen receptors has a RR of 1.5 as a risk factor for mortality and a p: 0.974, being statistically non-significant in our population (Graph 10).



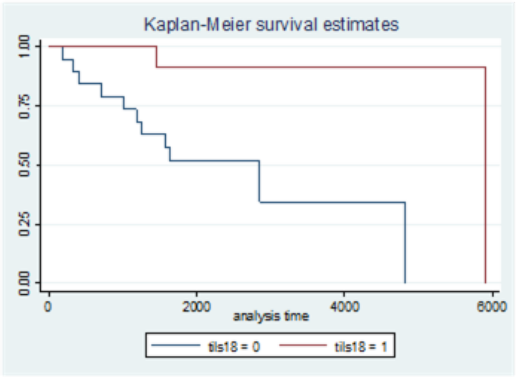
Graph 10 Survival curve according to AR expression (0: They do not express AR, 1: They do express AR).

The evaluation of androgen receptors will probably become the future target of a specific therapy that contributes to improving overall and disease-free survival in patients who express it. As we already mentioned, Lehmann was the first to propose a Basal-type breast cancer subclassification, suggesting dividing it into 6 subgroups, in order to facilitate the identification of target therapies, one of these subgroups is the so-called Luminal AR (androgen receptor) that would have sensitivity to androgenic antagonists (bicalutamide) and inhibitors of the PI3K pathway.[18] The difficulty in analyzing this subgroup lies in its low prevalence, which makes it difficult to better evaluate it. If we take into account that the rate of TNBC is between 10 to 20% of all breast cancers[25–27] and that the Luminal AR subgroup is only 11% of TNBC or 2% of all cancers of breast,[18] the need for a very large population for its correct evaluation becomes evident.

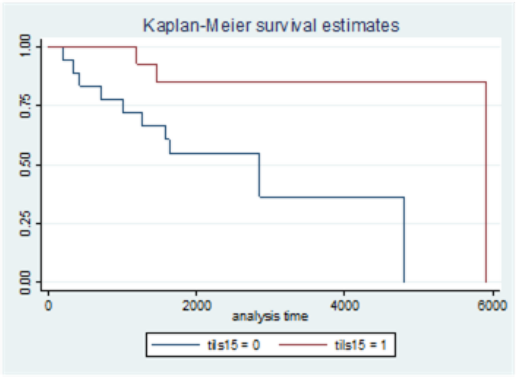
Of the three determinations carried out in our work, the determination of the degree of lymphocyte infiltration (TILs), carried out in tumor blocks stained with Hematoxylin-Eosin, is the determination that has the greatest scientific evidence, demonstrating its prognostic and predictive value in TNBC.[12–14] As we have already said, this was first reported in 297 patients with TNBC in the BIG 2-98 study[15] and then prospectively and randomized in 481 patients with TNBC in the ECOG studies (for its acronym in English "United States of America"). States Eastern Cooperative Oncology Group") 2197 and 1199.[14] In our population, the average tumor lymphocytic infiltration (TILs) was 20.6% (5%-70%), being lower when evaluating exclusively the population of patients who relapsed (19.6%) and even lower when evaluating the population of deceased patients (17.1%). There were 14 patients who received chemotherapy and did not relapse, in this group the average TILs was 31.4%. These data coincide with international reports. In line with the aforementioned publications, we take a cut-off value for the evaluation of TILS of 20%, with this value its presence is a predictor of mortality and recurrence with a p: 0.0269, being statistically significant in our population (Graph 11). This statistical significance is maintained with TILs values of 18% and 15% with p=0.0131 and p = 0.0032 respectively (Graph 12) (Graph 13).



Graph 11 Survival curve according to TILS greater or less than 20% (0: TILs <20, 1: TILs >20).



Graph 12 Survival curve according to TILS greater or less than 18% (0: TILs <18, 1: TILs >18).



Graph 13 Survival curve according to TILS greater or less than 15% (0: TILs <15, 1: TILs >15).

We believe that the determination of tumor lymphocyte infiltration together with the evaluation of p53 protein expression will be useful tools when evaluating the prognosis of these patients, since both are associated with increased overall survival and disease-free survival illness. When analyzing the association between the absence of mutation in p53 and the presence of TILs greater than 20%, we found that the presence of both factors tends to be of good prognosis, although it is not statistically significant, with a p=0.0570 . It is probable that if we could increase the number of patients in our population this association would become significant (Graph 14).

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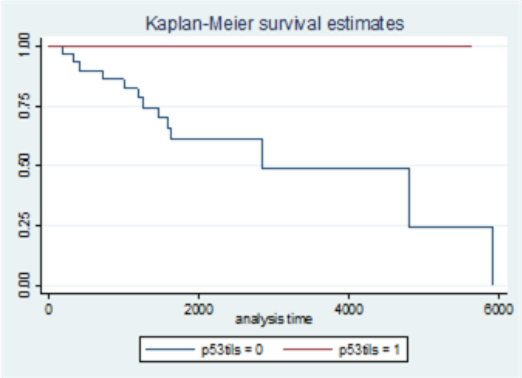
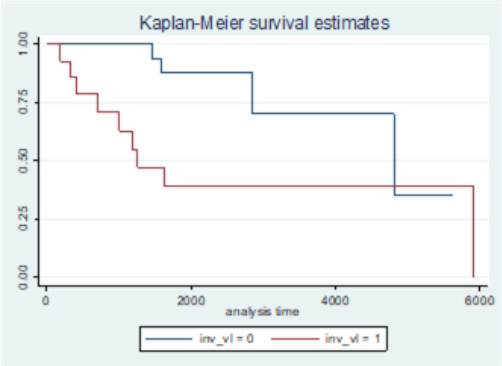
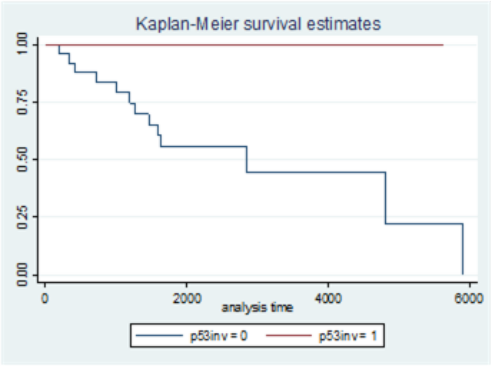


Graph 14 Survival curve of patients with TILS greater than 20% and absence of the p53 mutation (1:TILs >20% and p53 negative, 0:Those who do not meet both criteria).

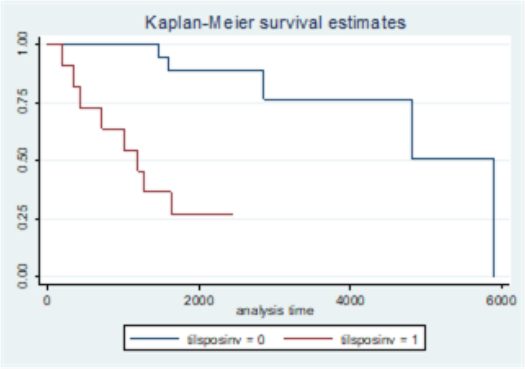
Lymphovascular invasion (LVI) is a known prognostic factor in breast cancer.[28] It is described in 40% of the patients under study (14 of 35 patients). In our population, its presence is associated with higher mortality with a p=0.0093 (Graph 15). When we jointly analyze the presence of LVI and the mutation in p53, we find that both constitute a risk factor for mortality with a p=0.0147 (Graph 16). The same occurs when we jointly analyze the presence of LVI and TILs <20%, finding that both are associated with a higher risk of mortality with a p=0.001 (Graph 17). If we analyze the three variables together, positive p53 + TILs < 20% + positive LVI, we see that this association also constitutes a risk factor for mortality and recurrence with p = 0.021 (Graph 18).



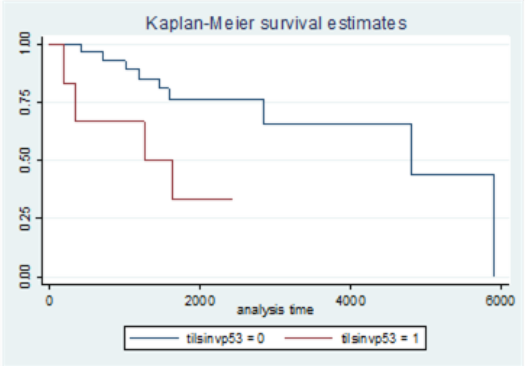
Graph 15 Survival curve according to the presence of LVI (0: LVI absent, 1: LVI present).



Graph 16 Survival curve according to the presence of LVI and p53 expression (1: LVI present and p53 positive, 0:Those that do not meet both criteria)



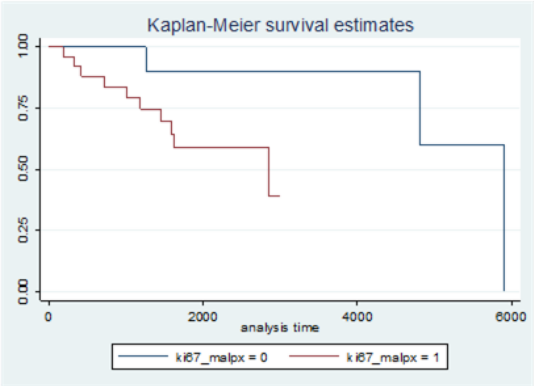
Graph 17 Survival curve according to the presence of LVI and TILs <20% (1: LVI present and TILs <20%, 0: Those that do not meet both criteria).



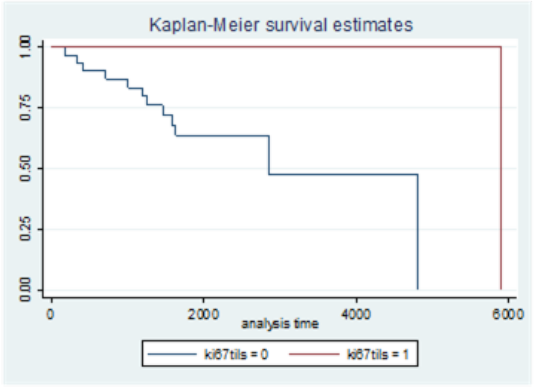
Graph 18 Survival curve according to the presence of LVI, TILs <20% and p53 expression (1: LVI present + TILs <20% + p53 positive, 0: Those that do not meet all the criteria).

As described by the intersociety consensus of "prognostic and predictive factors in breast cancer", cell proliferation has always played a role in tumor classification and therefore is part of the prognostic and predictive factors. It is included within the histological tumor grade, taking into account the mitoses of the tumor. Ki67 is a protein that has been shown to play an important role in cell cycle regulation, is absent in cells without replication, and reaches maximum expression levels during mitosis. The Ki67 gene is located on the long arm of human chromosome number 10. In addition, it is one of the phenotypic characteristics that differentiate genetic subtypes of breast cancer. Ki67 determination is performed using immunohistochemical techniques. Currently the use of Ki67 as a prognostic or predictive factor is controversial. This is mainly due to a lack of consensus on the cut-off points for the evaluation of Ki67, since it is based on a method that generates a great deal of variability in interpretation between laboratories and observers. The definition of low and high expression and what is the best methodology for optimal evaluation (sector of the tumor to be evaluated, hot spots, type of antibodies, clones, etc.) have not yet been determined. However, studies have recognized its value as a predictive factor, demonstrating its usefulness in measuring the response to a given adjuvant treatment. In addition, high levels of Ki67 predict a better response to chemotherapy treatment. Regarding its role as a prognostic factor, high levels of expression are associated with a higher probability of relapse in early-stage cancer, regardless of axillary involvement. In 2009, the first Ki67 cut-off point was published to differentiate Luminal A carcinomas (less than 14%) from Luminal B (greater than 14%). In 2011, recommendation guidelines for breast cancer screening were published, covering pre-analytical, analytical, and interpretation variables. In any case, there are still

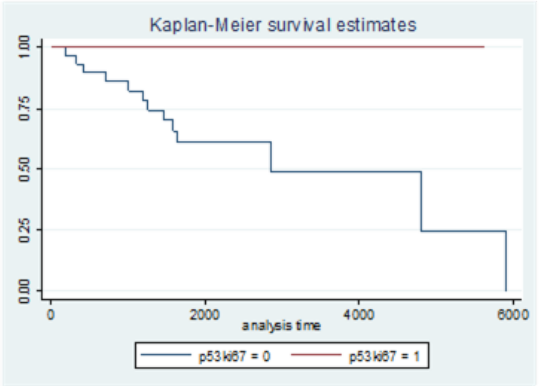
difficulties in unifying criteria in the measurement of Ki67, as well as in defining its high or low cut-off points. However, the same consensus reaffirms the importance of its use despite the difficulties described.[29] In our population, the patients presented an average Ki67 of 33%. Using a cut-off value of 14%, we obtained a p=0.056, being statistically insignificant as a risk factor for mortality and relapse (Graph 19). When we analyzed those patients with Ki 67 < 14% and TILs > 20%, we obtained a p = 0.0491 as a marker of lower risk of death or relapse (Graph 20). When analyzing patients with Ki67 <14% and who did not express p53 mutation, we obtained p=0.0596, their joint evaluation being statistically non-significant (Graph 21).



Graph 19 Survival curve according to Ki67 (0: Ki67 > 14%, 1: Ki67 < 14%).



Graph 20 Survival curve according to Ki67 and TILs (1: Ki67 < 14% and TILs > 20%, 0: Those that do not meet both criteria).



Graph 21 Survival curve according to Ki67 and p53 expression (1: Ki67 < 14% and no p53 expression, 0: Those that do not meet both criteria).

Conclusion

The numerous tools and factors that we use every day to analyze breast cancer patients are still insufficient to unequivocally determine the evolution of each one of them and to predict which ones will benefit. Of one or another treatment. Since the appearance of genetic studies, a path has been opened that seems to be the answer to this problem, but there is still a long way to go.

While we find the definitive tools that allow us to identify the real prognosis of each patient and determine their benefit with each treatment, we must continue to search for objective parameters that temporarily help us to take action.

In accordance with our working hypothesis, the determination of tumor lymphocyte infiltration together with the evaluation of the p53 protein mutation are useful tools when it comes to predicting prognosis in TNBC, since both are associated with an increase in overall survival and survival. Disease-free survival, although in our population it was not statistically significant for p53 in isolation. Its evaluation in combination with other known prognostic factors (LVI and Ki67) is useful to predict prognosis, and potentially to determine therapeutic conduct, although its usefulness as a predictive factor has not been evaluated in this work. That is why we think that the presence of p53 mutation and the degree of TILs are determinations whose evaluation should be standardized in patients with TNBC and duly recorded in the pathology report. The evaluation of androgen receptors will probably become the future target of a specific therapy that contributes to improving overall and disease-free survival in patients who express it.

New studies are necessary to confirm these assumptions and continue to advance in the identification of new prognostic factors that allow acting as molecular targets for the development of targeted therapies in TNBC.

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Conflicts of interest

There are no conflicts of interest.

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