

# Expression and Clinical Significance of Claudin-7, PDL-1, PTEN, c-Kit, c-Met, c-Myc, ALK, CK5/6, CK17, p53, EGFR, Ki67, p63 in Triple-negative Breast Cancer— A Single Centre Prospective Observational Study

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**Abstract.** *Background/Aim:* To explore the relationship between p53, p63, c-kit, Ki67, cMet, claudin7, CK5/6, CK17, AR, PTEN, EGFR, ALK, PDL-1 and c-MYC expression with the clinicopathological features of triple-negative breast cancer. *Materials and Methods:* Immunohistochemistry was performed in 84 triple-negative breast cancer samples. *Results:* A statistically significant relationship between tumour grade and claudin-7 ( $p=0.004$ ) and between protein p53 and positive lymph nodes ( $p=0.015$ ) was found. High expression of claudin-7 ( $OR=65.8$ ,  $95\%CI=4.35-995.19$ ,  $p-value=0.003$ ) and low expression of c-kit ( $OR=0.14$ ,  $95\%CI=0.025-0.793$ ,  $p-value=0.026$ ) and protein p63 ( $OR=0.18$ ,  $95\%CI=0.035-0.978$ ,  $p-value=0.047$ ) was associated with higher tumour grade. Higher AR expression ( $OR=13.44$ ,  $95\%CI=1.28-141.56$ ,  $p-value=0.031$ ) and lower expression of CK5/6 cytokeratins was found in patients with positive lymphovascular invasion (LVI) ( $OR=0.072$ ,  $95\%CI=0.007-0.732$ ,  $p-value=0.026$ ). Only the cell proliferation index (Ki67) has been proven to be statistically significant for disease-free survival ( $p-value=0.0378$ ), and overall survival ( $p-value=0.0186$ ). *Conclusion:* High expression of claudin-7 and low expression of c-kit and protein p63 are

associated with higher tumour grade. AR and CK5/6 expression seem to be important in LVI.

With over 1 million women affected worldwide, breast cancer (BC) is currently the commonest malignancy in females and the second leading cause of cancer related death (1). Triple negative breast cancer (TNBC) immunohistochemically characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2), is responsible for a disproportionate share of mortality owing to its aggressive clinical behavior, poor prognosis and lack of targeted therapies (2).

Recent studies have classified TNBC into 4 major subtypes: basal-like (BLBCs), mesenchymal, luminal androgen receptor and immune enriched. Of the total cases of TNBC, 70-80% are Basal like Breast Cancers sharing numerous clinical and pathological features and these terms are often used interchangeably by clinicians although they are not synonymous. New therapeutic approaches have been proposed based on different factors, however they are not currently used to stratify patients for decisions about clinical therapy. In addition, there are no predictive markers that have been widely accepted and proven to be significant across all different studies.

The aim of the study was to explore the relationship between p53, p63, c-kit, Ki67, cMet, claudin 7, CK 5/6, CK17, AR, PTEN, EGFR, ALK, PDL-1 and c-MYC with tumor grade, number of positive lymph nodes, lymphovascular invasion (LVI), local recurrence, distant recurrence, disease-free survival and overall survival in TNBC.

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*Key Words:* Triple-negative breast cancer, p53, p63, c-kit, Ki67, cMet, claudin 7, CK 5/6, CK17, AR, PTEN, EGFR, ALK, PDL-1 and c-MYC.

## Materials and Methods

The study was performed in Hygeia General Hospital, Athens Greece from 2003-2011. A total of 84 TNBC cases were included from a prospectively collected database. Samples with an absent or incomplete immunohistochemistry report for the respective pathology were excluded. Tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax. The tumors were classified and graded according to the suggested criteria of World Health Organisation (WHO) 2014 and Tumor Node Metastasis status/American Joint Committee on Cancer (TNM/AJCC) 2009 system. Suitable selected paraffin blocks containing representative tumor areas were identified on corresponding hematoxylin-eosin – stained sections. Areas of interest were identified and marked on the source block. The source block was cored and a 1.5 mm core was transferred to the recipient “master block” using the Tissue Microarray (MTA-1, Beecher Instruments, Sun Prairie, WI, USA). Two, representative tumor cores were arrayed per specimen as cores of normal tissue from breast, endometrium, colon and spleen and used as reliability indicators (control). Immunohistochemical staining was performed for 14 antigens: p53, p63, c-kit, Ki67, cMet, claudin 7, CK 5/6, CK17, AR, PTEN, EGFR, ALK, PDL-1 and c-MYC.

*Statistical analysis.* Univariate analysis was performed to explore the association of biomarkers’ expression with all the dependent variables of interest: tumor grade, number of positive lymph nodes, LVI, local recurrence (LR) and distant metastasis. Categorical variables were reported as counts and percentages and compared with Fisher’s Exact test.

Multivariate logistic regression models were used for modelling the effect of biomarkers to the dependent variables tumor grade, lymph nodes and LVI. The backward elimination method with removal criterion  $p=5\%$  has been used, resulting to models with statistically significant effects. Odds ratios (OR), 95%CI and Likelihood ratio test values were reported for each analysis.

Cox proportional-hazards regression models, with backward elimination with  $p=10\%$ , are provided for the disease-free survival (DFS) and overall survival (OS). We also performed the Log-rank test to test the equality of survival functions.

All comparisons were two tailed and a  $p$ -value of  $<0.05$  was considered statistically significant. Data analysis was performed using STATA statistical software version 12.0.

## Results

A total of 84 patients were included in the analysis. All patients were females with median age 49 years (range=25-79 years). Seventeen (20.2%) were treated with mastectomy and 67 (79.8%) with lumpectomy and radiotherapy. All patients received adjuvant chemotherapy and all histological types were invasive ductal carcinoma. The association of biomarkers with tumor grade is summarized in Table I. The analysis of the tumor grade was conducted using the degree of malignancy as a dependent variable. Fisher’s exact test resulted in a statistically significant relationship between the degree of malignancy and binding of protein claudin 7 for the two different values of the biomarker, negative and weak low to strong high. Independent predictors of the grade of cancer

were determined in a multivariate logistic regression model with stepwise backward elimination of variables with a  $p$ -value=0.05. The expression of claudin 7 appeared to be much higher in patients with higher tumor grade (OR=65.8, 95%CI=4.35-995.19,  $p$ -value=0.003). According to the logistic regression model for the degree of malignancy with 71 observations, there is also a statistically significant relationship with c-kit ( $p$ -value=0.026), and protein p63 ( $p$ -value=0.047). Their expression appeared to be lower in patients with higher tumor grade with values of odds ratio OR=0.14, 95%CI=0.025-0.793,  $p$ -value=0.026 and OR=0.18 95%CI=0.035-0.978,  $p$ -value=0.047, respectively.

The association of lymph nodes status with the biomarkers under study is summarized in Table II. There is a statistically significant relationship between the protein p53 biomarker and the lymph nodes status according to Fisher’s exact test and the multivariate logistic regression model with 71 observations, where we examined the stepwise backward elimination of variables with a  $p$ -value=0.05. The expression of protein p53 was higher in patients with positive lymph nodes (OR=3.84, 95%CI=1.22-12.07,  $p$ -value=0.021).

Independent predictors for LVI were determined in a multivariate logistic regression model with 69 observations and stepwise backward elimination of variables with a  $p$ -value=0.05. The expression of androgen receptor (AR) appeared to be much higher in those patients with positive LVI process (OR=13.44, 95%CI=1.28-141.56,  $p$ -value=0.031). Also, the expression of ck5/6 cytokeratins appeared to be lower in patients with positive LVI (OR=0.072, 95%CI=0.007-0.732,  $p$ -value=0.026).

*Disease free survival and overall survival.* DFS and OS status between groups of patients with different levels of each biomarker have been compared using log-rank test with a 5% level of statistical significance. Only the cell proliferation index (Ki67) has been proven to be statistically significant for DFS ( $p$ -value=0.0378), and for OS ( $p$ -value=0.0186), which means that the survival function is differentiated for the two levels of the biomarker. The significance of this biomarker has also been confirmed by the Cox regression model for DFS with backward elimination (HR=0.39, 95%CI=0.153-0.994,  $p$ -value=0.048). We run the same Cox regression model with backward elimination for OS and it concluded that biomarker Ki67 is an important predictor for the overall survival of patients (HR=0.277, 95%CI=0.087-0.886,  $p$ -value=0.03). Table III presents the results for the Log-rank test for equality of survival functions in 2 different groups of each biomarker. Kaplan-Meier graphs are provided for both OS (Figure 1), and DFS status (Figure 2).

## Discussion

TNBC has unfavourable prognosis characterized by larger size and features of aggressive behavior. Many published studies

Table I. Association of degree of malignancy with the biomarkers under study.

| Biomarkers                    | Degree of malignancy             |                     |       | Fisher's exact p-Value |
|-------------------------------|----------------------------------|---------------------|-------|------------------------|
|                               | Poorly-moderately differentiated | Well differentiated | Total |                        |
| Claudin 7 n (%)               |                                  |                     |       |                        |
| Negative                      | 4 (26.7%)                        | 1 (1.6%)            | 5     | 0.004                  |
| Weak low to strong high       | 11 (73.3%)                       | 61 (98.4%)          | 72    |                        |
| Total                         | 15                               | 62                  | 77    |                        |
| PTEN n (%)                    |                                  |                     |       |                        |
| Negative                      | 13 (76.5%)                       | 56 (83.6%)          | 69    | 0.492                  |
| Weak positive to positive     | 4 (23.5%)                        | 11 (16.4%)          | 15    |                        |
| Total                         | 17                               | 67                  | 84    |                        |
| c-met n (%)                   |                                  |                     |       |                        |
| Negative                      | 6 (35.3%)                        | 33 (50%)            | 39    | 0.414                  |
| Weak to strong positive       | 11 (64.7%)                       | 33 (50%)            | 44    |                        |
| Total                         | 17                               | 66                  | 83    |                        |
| CMYC n (%)                    |                                  |                     |       |                        |
| Positive cells (<1%, 1-25%)   | 8 (50%)                          | 26 (38.8%)          | 34    | 0.572                  |
| Positive cells (26-50%, ≥51%) | 8 (50%)                          | 41 (61.2%)          | 49    |                        |
| Total                         | 16                               | 67                  | 83    |                        |
| ckit n (%)                    |                                  |                     |       |                        |
| Negative                      | 5 (35.7%)                        | 41 (62.1%)          | 46    | 0.082                  |
| Positive                      | 9 (64.3%)                        | 25 (37.9%)          | 34    |                        |
| Total                         | 14                               | 66                  | 80    |                        |
| PDL1 n (%)                    |                                  |                     |       |                        |
| Negative                      | 14 (82.4%)                       | 61 (91%)            | 75    | 0.378                  |
| Positive                      | 3 (17.6%)                        | 6 (9%)              | 9     |                        |
| Total                         | 17                               | 67                  | 84    |                        |
| ALK n (%)                     |                                  |                     |       |                        |
| Negative                      | 15 (88.2%)                       | 66 (98.5%)          | 81    | 0.103                  |
| Positive                      | 2 (11.8%)                        | 1 (1.5%)            | 3     |                        |
| Total                         | 17                               | 67                  | 84    |                        |
| CK17 n (%)                    |                                  |                     |       |                        |
| Negative                      | 9 (56.3%)                        | 36 (53.7%)          | 45    | 1.000                  |
| Positive                      | 7 (43.7%)                        | 31 (46.3%)          | 38    |                        |
| Total                         | 16                               | 67                  | 83    |                        |
| CK5/6 n (%)                   |                                  |                     |       |                        |
| Negative                      | 5 (33.3%)                        | 30 (44.8%)          | 35    | 0.566                  |
| Positive                      | 10 (66.7%)                       | 37 (55.2%)          | 47    |                        |
| Total                         | 15                               | 67                  | 82    |                        |
| AR n (%)                      |                                  |                     |       |                        |
| Negative                      | 14 (87.5%)                       | 55 (82.1%)          | 69    | 1.000                  |
| Positive                      | 2 (12.5%)                        | 12 (17.9%)          | 14    |                        |
| Total                         | 16                               | 67                  | 83    |                        |
| Protein P63 n (%)             |                                  |                     |       |                        |
| Negative                      | 12 (75%)                         | 54 (80.6%)          | 66    | 0.731                  |
| Positive                      | 4 (25%)                          | 13 (19.4%)          | 17    |                        |
| Total                         | 16                               | 67                  | 83    |                        |
| Protein p53 n (%)             |                                  |                     |       |                        |
| Negative                      | 4 (26.7%)                        | 20 (30.8%)          | 24    | 1.000                  |
| Positive                      | 11 (73.3%)                       | 45 (69.2%)          | 56    |                        |
| Total                         | 15                               | 65                  | 80    |                        |
| EGFR n (%)                    |                                  |                     |       |                        |
| Negative                      | 5 (31.3%)                        | 23 (34.3%)          | 28    | 1.000                  |
| Low to high score             | 11 (68.7%)                       | 44 (65.7%)          | 55    |                        |
| Total                         | 16                               | 67                  | 83    |                        |
| KI67 n (%)                    |                                  |                     |       |                        |
| Positive cells ≤ 30%          | 6 (46.2%)                        | 29 (43.9%)          | 35    | 1.000                  |
| Positive cells >30%           | 7 (53.8%)                        | 37 (56.1%)          | 44    |                        |
| Total                         | 13                               | 66                  | 79    |                        |

Table II. Association of lymph nodes status with the biomarkers under study.

| Biomarkers                    | Lymph nodes status |            |       | Fisher's exact p-Value |
|-------------------------------|--------------------|------------|-------|------------------------|
|                               | Negative           | Positive   | Total |                        |
| Claudin 7 n (%)               |                    |            |       |                        |
| Negative                      | 3 (7.1%)           | 2 (5.7%)   | 5     | 1.000                  |
| Weak low to strong high       | 39 (92.9%)         | 33 (94.3%) | 72    |                        |
| Total                         | 42                 | 35         | 77    |                        |
| PTEN n (%)                    |                    |            |       |                        |
| Negative                      | 36 (78.3%)         | 33 (86.8%) | 69    | 0.396                  |
| Weak positive to positive     | 10 (21.7%)         | 5 (13.2%)  | 15    |                        |
| Total                         | 46                 | 38         | 84    |                        |
| C-MET n (%)                   |                    |            |       |                        |
| Negative                      | 19 (41.3%)         | 20 (54.1%) | 39    | 0.275                  |
| Weak to strong positive       | 27 (58.7%)         | 17 (45.9%) | 44    |                        |
| Total                         | 46                 | 37         | 83    |                        |
| CMYC n (%)                    |                    |            |       |                        |
| Positive cells (<1%, 1-25%)   | 15 (32.6%)         | 19 (51.4%) | 34    | 0.116                  |
| Positive cells (26-50%, ≥51%) | 31 (67.4%)         | 18 (48.6%) | 49    |                        |
| Total                         | 46                 | 37         | 83    |                        |
| c-kit n (%)                   |                    |            |       |                        |
| Negative                      | 24 (54.5%)         | 22 (61.1%) | 46    | 0.651                  |
| Positive                      | 20 (45.5%)         | 14 (38.9%) | 34    |                        |
| Total                         | 44                 | 36         | 80    |                        |
| PDL1 n (%)                    |                    |            |       |                        |
| Negative                      | 41 (89.1%)         | 34 (89.5%) | 75    | 1.000                  |
| Positive                      | 5 (10.9%)          | 4 (10.5%)  | 9     |                        |
| Total                         | 46                 | 38         | 84    |                        |
| ALK n (%)                     |                    |            |       |                        |
| Negative                      | 45 (97.8%)         | 36 (94.7%) | 81    | 0.587                  |
| Positive                      | 1 (2.2%)           | 2 (5.3%)   | 3     |                        |
| Total                         | 46                 | 38         | 84    |                        |
| CK17 n (%)                    |                    |            |       |                        |
| Negative                      | 22 (48.9%)         | 23 (60.5%) | 45    | 0.377                  |
| Positive                      | 23 (51.1%)         | 15 (39.5%) | 38    |                        |
| Total                         | 45                 | 38         | 83    |                        |
| CK5/6 n (%)                   |                    |            |       |                        |
| Negative                      | 19 (42.2%)         | 16 (43.2%) | 35    | 1.000                  |
| Positive                      | 26 (57.8%)         | 21 (56.8%) | 47    |                        |
| Total                         | 45                 | 37         | 82    |                        |
| AR n (%)                      |                    |            |       |                        |
| Negative                      | 39 (86.7%)         | 30 (78.9%) | 69    | 0.390                  |
| Positive                      | 6 (13.3%)          | 8 (21.1%)  | 14    |                        |
| Total                         | 45                 | 38         | 83    |                        |
| Protein P63 n (%)             |                    |            |       |                        |
| Negative                      | 36 (80%)           | 30 (78.9%) | 66    | 1.000                  |
| Positive                      | 9 (20%)            | 8 (21.1%)  | 17    |                        |
| Total                         | 45                 | 38         | 83    |                        |
| Protein P53 n (%)             |                    |            |       |                        |
| Negative                      | 18 (41.9%)         | 6 (16.2%)  | 24    | 0.015                  |
| Positive                      | 25 (58.1%)         | 31 (83.8%) | 56    |                        |
| Total                         | 43                 | 37         | 80    |                        |
| EGFR n (%)                    |                    |            |       |                        |
| Negative                      | 14 (31.1%)         | 14 (36.8%) | 28    | 0.645                  |
| Low to high score             | 31 (68.9%)         | 24 (63.2%) | 55    |                        |
| Total                         | 45                 | 38         | 83    |                        |
| KI67 n (%)                    |                    |            |       |                        |
| Positive Cells ≤30%           | 18 (40.9%)         | 17 (48.6%) | 35    | 0.649                  |
| Positive Cells >30%           | 26 (59.1%)         | 18 (51.4%) | 44    |                        |
| Total                         | 44                 | 35         | 79    |                        |

Table III. Log-rank test for equality of survival functions in 2 different groups of each biomarker.

| Biomarkers                    | Disease free survival (DFS) |                |         |         | Overall survival (OS) |                |         |         |
|-------------------------------|-----------------------------|----------------|---------|---------|-----------------------|----------------|---------|---------|
|                               | Event observed              | Event expected | chi2(1) | p-Value | Event observed        | Event expected | chi2(1) | p-Value |
| c-kit                         |                             |                |         |         |                       |                |         |         |
| Positive                      | 8                           | 8.32           | 0.02    | 0.8765  | 5                     | 6.06           | 0.36    | 0.5482  |
| Negative                      | 10                          | 9.68           |         |         | 8                     | 6.94           |         |         |
| Total                         | 18                          | 18.00          |         |         | 13                    | 13.00          |         |         |
| Androgen receptor             |                             |                |         |         |                       |                |         |         |
| Positive                      | 4                           | 2.54           | 0.99    | 0.3205  | 3                     | 2.04           | 0.53    | 0.4676  |
| Negative                      | 17                          | 18.46          |         |         | 13                    | 13.96          |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| Cytokeratins Ck5/6            |                             |                |         |         |                       |                |         |         |
| Positive                      | 12                          | 11.81          | 0.01    | 0.9307  | 8                     | 9.06           | 0.30    | 0.5853  |
| Negative                      | 9                           | 9.19           |         |         | 8                     | 6.94           |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| Cytokeratins Ck17             |                             |                |         |         |                       |                |         |         |
| Positive                      | 7                           | 9.13           | 0.91    | 0.3390  | 5                     | 6.89           | 0.94    | 0.3320  |
| Negative                      | 14                          | 11.87          |         |         | 11                    | 9.11           |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| Claudin 7                     |                             |                |         |         |                       |                |         |         |
| Moderate to strong high       | 15                          | 14.73          | 0.03    | 0.8672  | 12                    | 11.47          | 0.14    | 0.7090  |
| Negative to weak low          | 3                           | 3.27           |         |         | 2                     | 2.53           |         |         |
| Total                         | 18                          | 18.00          |         |         | 14                    | 14.00          |         |         |
| Phosphatase - PTEN            |                             |                |         |         |                       |                |         |         |
| Positive                      | 4                           | 3.86           | 0.01    | 0.9380  | 4                     | 2.93           | 0.49    | 0.4830  |
| Negative                      | 17                          | 17.14          |         |         | 12                    | 13.07          |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| Protein P63                   |                             |                |         |         |                       |                |         |         |
| Positive                      | 2                           | 3.76           | 1.04    | 0.3073  | 0                     | 2.84           | 3.56    | 0.0592  |
| Negative                      | 19                          | 17.24          |         |         | 16                    | 13.16          |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| Protein p53                   |                             |                |         |         |                       |                |         |         |
| Positive                      | 13                          | 13.64          | 0.10    | 0.7558  | 11                    | 10.16          | 0.22    | 0.6358  |
| Negative                      | 7                           | 6.36           |         |         | 4                     | 4.84           |         |         |
| Total                         | 20                          | 20.00          |         |         | 15                    | 15.00          |         |         |
| ALK                           |                             |                |         |         |                       |                |         |         |
| Positive                      | 1                           | 0.94           | 0.00    | 0.9491  | 1                     | 0.72           | 0.12    | 0.7279  |
| Negative                      | 20                          | 20.06          |         |         | 15                    | 15.28          |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| PDL1                          |                             |                |         |         |                       |                |         |         |
| Positive                      | 2                           | 2.15           | 0.01    | 0.9117  | 3                     | 1.58           | 1.46    | 0.2269  |
| Negative                      | 19                          | 18.85          |         |         | 13                    | 14.42          |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| C-MET                         |                             |                |         |         |                       |                |         |         |
| Positive                      | 13                          | 10.59          | 1.15    | 0.2838  | 11                    | 8.24           | 1.97    | 0.1609  |
| Negative                      | 8                           | 10.41          |         |         | 5                     | 7.76           |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| KI67                          |                             |                |         |         |                       |                |         |         |
| Positive cells > 30%          | 7                           | 11.36          | 4.32    | 0.0378  | 4                     | 8.25           | 5.54    | 0.0186  |
| Positive cells ≤ 30%          | 12                          | 7.64           |         |         | 10                    | 5.75           |         |         |
| Total                         | 19                          | 19.00          |         |         | 14                    | 14.00          |         |         |
| EGFR                          |                             |                |         |         |                       |                |         |         |
| Low to high score             | 14                          | 13.38          | 0.08    | 0.7760  | 10                    | 10.46          | 0.06    | 0.8071  |
| Negative                      | 7                           | 7.62           |         |         | 6                     | 5.54           |         |         |
| Total                         | 21                          | 21.00          |         |         | 16                    | 16.00          |         |         |
| CMYC                          |                             |                |         |         |                       |                |         |         |
| Positive cells (26-50%, ≥51%) | 9                           | 12.62          | 2.93    | 0.0869  | 6                     | 9.39           | 3.40    | 0.0653  |
| Positive cells (<1%, 1-25%)   | 11                          | 7.38           |         |         | 9                     | 5.61           |         |         |
| Total                         | 20                          | 20.00          |         |         | 15                    | 15.00          |         |         |

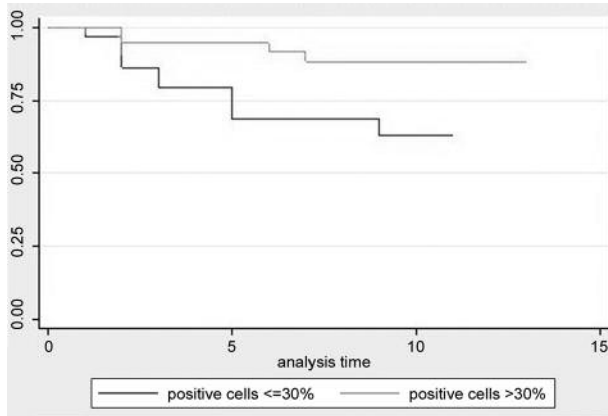


Figure 1. Kaplan-Meir overall survival estimates for cell proliferation index ki67.

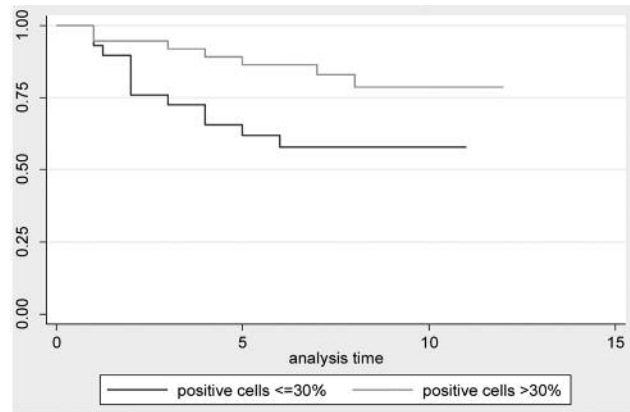


Figure 2. Kaplan-Meir disease free survival estimates for cell proliferation index ki67.

have attempted to identify new biomarkers to sub-classify TNBC into different prognostic groups and to select patients who are candidates for more aggressive targeted therapy regimens.

Claudins are transmembrane proteins which have a major role on the regulation of cell adhesion, proliferation and differentiation, in controlling paracellular permeability and the maintenance of epithelial cell polarity (3-6). TNBC is often characterized by low claudin expression, especially claudin -3, -4, -7 (7). However, in our study, higher expression of claudin 7 was significantly correlated with higher tumor grade (OR=65.8, 95%CI=4.35-995.19,  $p$ -value=0.003). Although this observation seems to contradict the hypothesis that claudin low cells are associated with poor outcome, it is consistent with other recent studies, as the Bernardi *et al.* study, who did not find any association between claudin-7 and different subtypes and supported that claudin-7 expression in invasive ductal carcinoma was associated with a shorter time of recurrence, suggesting a contribution of this marker to the aggressiveness of breast cancer (8).

C-Met is a transmembrane tyrosine kinase receptor that plays a vital role in cell-cell detachment, invasiveness, tumour angiogenesis, proliferation, metastasis and survival (9). The evidence of the influence of c-Met expression on survival outcomes is inconclusive. In the present study, there was no significant correlation of c-Met expression with tumour grade, LVI and number of positive lymph nodes. However, Fisher's exact test resulted in a statistically significant relationship between AR ( $p$ -value=0.041) and was also significantly correlated ( $p$ -value=0.002) with cell proliferation index Ki67 behaviour. In a recent meta-analysis by Yan *et al.* (10), it was shown that c-Met overexpression was associated with 1.41-fold increased risk of recurrence in the hormone positive group compared to 2.31-fold in TNBC, suggesting that c-Met could be a therapeutic target for TNBC.

Additional high-quality data is needed in order to draw more reliable conclusions.

PTEN is a tumour suppressor 200 kb gene, located on chromosome 10q23 that regulates many cellular functions including cell growth, proliferation and migration. It is lost or mutated in many types of cancer including breast, prostate, and lung cancer (11). *PTEN* losses have been observed in up to 37-74% of TNBCs (12-14), suggesting a rationale to evaluate mTOR inhibitors in patients with TNBC as there is no alternative targeted treatment. Inanc *et al.* detected 44.3% *PTEN* loss among 99 TNBC tumors, which was also associated with shorter DFS, increased recurrence and mortality risk compared to patients without a *PTEN* loss. No relationship was found between *PTEN* loss and other clinical and pathological parameters, only that it was higher among patients with LVI (15). In the present study, no statistically significant relationship was found between *PTEN* loss and tumor grade, LVI, and the number of positive lymph nodes.

C-MYC is a basic helix-loop-helix zipper motif transcription factor. Its amplification is one of the most frequent aberrations in BC that has been detected in 1-94% of patients in different studies and found to be associated with the basal subtype. Bouchalova *et al.* in a study of 187 patients with TNBC, c-MYC protein expression was found in the majority of the TNBCs (164 out of 187 patients) and was associated with worse survival (16). Horiuchi *et al.* investigated the biology of TNBC and identified that MYC signalling is elevated in these tumours and the expression of multiple MAX (another basic helix-loop-helix zipper protein) binding partners, which regulate MYC activity, was altered in TNBC and may therefore contribute to increased MYC pathway activity and worse patient outcome (17). In the present study, no statistically significant relationship was found between c-MYC protein expression and a role in

regulation of cell proliferation, tumor grade, LVI, and the number of positive lymph node.

C-kit is a transmembrane tyrosine kinase receptor present on the surface of hematopoietic stem cells and also of other cells. It induces apoptosis and also increases the invasiveness of cancer cells (12). In this study the expression of c-kit appeared to be lower in patients with higher tumor grade (OR=0.14, 95%CI=0.025-0.793,  $p$ -value=0.026). Fisher's exact test resulted in a statistically significant relationship between c-kit ( $p$ -value=0.022) and cytokeratins ck5/6 ( $p$ -value=0.022). Furthermore, there was a statistically significant association between c-kit, and EGFR ( $p$ -value=0.045).

Similarly Tsuitsui *et al.* found that loss of c-kit expression was associated with lymph node metastases, and worse prognosis as it was associated with an advanced stage of breast cancer (18). Opposite results were presented by Diallo *et al.* who underlined that c-kit expression represents an independent negative prognostic marker in high-risk breast cancer (19). Zhu *et al.* evaluated the expression of c-kit protein and the mutations of the *c-kit* gene in triple negative breast cancers; 41.7% of the TNBCs positive for c-kit might benefit from tyrosine kinase inhibitors (20). Kanapathy Pillai *et al.* reported c-kit expression in 89% of TNBCs and this was also associated with EGFR, CK5/6 and high Ki67 proliferation index (21). The significance of c-kit expression is controversial and the rate of positive c-kit varies from 1 to 82%, which is likely attributable to the different methods of determination of c-kit expression.

PD-L1, programmed cell death 1 ligand 1, is a 40kDa transmembrane protein, expressed on the surface of activated cytotoxic T cells, causing inhibition of IL-2 production and T cell migration and proliferation (22). Few studies have investigated PD-L1 expression in BC, however the results varied regarding its expression rate and prognostic value. The present study demonstrated PD-L1 expression in 10.4% of patients while 69 out of 77 (89.6%) patients did not express PD-L1 however, no statistically significant relationship was found between PD-L1 and tumor grade, LIV, and the number of the positive lymph nodes. Similarly, Beckers *et al.* in a study of 161 primary TNBCs, PD-L1 expression was very common in TNBC but was not an independent prognostic marker as there were differences in the outcome depending on which cellular compartment PDL-1 was expressed (tumour cell membrane, cytoplasm, and stromal cellular compartment) (23). Qin *et al.* has shown high PD-L1 expression associated with significantly decreased survival, higher tumor grade (24).

ALK is a tyrosine receptor kinase whose pathway, has been identified by Lehmann *et al.* as an important signalling pathway that is common in TNBCs (25). In the present study only two women with TNBC expressed this receptor and 75 out of 77 women did not.

Cytokeratins 5/6 and 17 are important markers for the identification of the basaloid group and are correlated with poor patient outcome in TNBC (26-29). In our study 57.3% of women were found positive for CK5/6 while 35 out of 82 were negative. The expression of CK5/6 cytokeratins appeared to be lower in patients with LVI (OR=0.072, 95%CI=0.007-0.732,  $p$ -value=0.026). Also, a significant association was found between CK5/6 and EGFR ( $p$ -value=0.00417) and CK17 and Ki67 ( $p$ -value=0.041). These results are contradictory to many results in the literature where high expression of CK5/6 is significantly associated with worse clinicopathological features in TNBC (30)-(31). CK5/6 positive expression rates vary from 24 to 72 % in the literature as, there are different scoring systems used in IHC studies for CK5/6 and no precise cutoff value exists (32).

Androgen receptor is a member of the steroid hormone receptor family, which functions as a classic ligand-activated intracellular transcription factor. AR expression in TNBC is reported lower than estrogen receptor positive breast cancers, and the prognostic value of this is still unclear. In the present study, 82.9% of women were found positive for AR while 14 out of 82 were negative. The expression of AR appeared to be much higher in patients with positive LVI (OR=13.44, 95%CI=1.28-141.56,  $p$ -value=0.031). Hu *et al.* who analyzed AR expression in 211 TNBCs, found that patients with AR positive tumours had an 83% increase in overall mortality compared with the AR-negative group (33). McGhan *et al.* found AR positive TNBCs to correlate with higher grade and LN metastases (34). Millis *et al.* in the largest to date cohort study were 6341 breast cancers were evaluated (2,111 TNBC and 4,230 non-TNBC) showed that higher AR expression in TNBC was associated with lower ki-67 levels suggesting that androgens might have an antiproliferative effect (35). These differences in the prognostic role of AR expression are due to the variability in antibodies, scoring systems and cut-offs used to define AR positivity but also on the complexity of this pathway.

*TP53* is a tumour suppressor gene that encodes a nuclear phosphoprotein. Mutation in *TP53* results in loss of the usual wt-p53 tumour suppressor functions and it often exerts opposing effects. 20-35% of all breast cancers have a *TP53* mutation; in TNBC *TP53* mutation is present in almost all of them (25, 36). Previous studies have shown that p53 expression is higher in TNBC and that may play a role in the worse prognosis of TNBC (37). It has also been shown that breast tumours with mutant p53 were generally TNBC and were associated with decreased survival (38, 39). Similarly, in the present study, the expression of p53 was higher in patients with positive lymph nodes (OR=3.84, 95%CI=1.22-12.07,  $p$ -value=0.021) suggesting worse prognosis of these patients.

Epidermal growth factor receptor (EGFR), is one of the most notable cancer molecular targets. In breast cancer, EGFR expression level or gene mutation status is increasingly being used to select patients for selective treatments. In our study,

no statistical significant correlation was found between EGFR tumor grade, lymphovascular invasion, and the number of the positive LNs. A statistically significant association according to a Fisher's exact test was found between EGFR and c-kit ( $p$ -value=0.045) and CK5/6 and EGFR ( $p$ -value= 0.004). The uncertainty in establishing EGFR as a prognostic and predictive factor in breast cancer is mostly due to the different methods used for the detection of EGFR dysregulation.

Ki67 immune expression is closely associated with the cell cycle and can be used as a prognostic and predictive marker (40, 41). In TNBC patients, high expression of Ki67 has a direct correlation with tumour size, grade and higher levels (>35% staining) have been linked with an increased risk of death. Ki67 levels were significantly increased in ductal TNBC compared to other histologic types (80% in TNBC vs. 10-30% in other types) (42). Li *et al.* investigated the expression of Ki67 in TNBC and found that it was significantly correlated with tumour size and lymph node metastases, no correlation was observed with age and clinical stage; suggesting that Ki67 may be an indicator of poor prognosis in TNBC patients (43). Niikura *et al.* on the other hand showed that Ki67 was not associated with survival in the hormone receptor (HR)-negative group of 716 patients (44).

In contrast to the established predictive and prognostic value of Ki67 expression in patients with HR-positive tumours (45-47), there is only little evidence to support Ki67 as a predictive marker for chemotherapeutic efficacy and defining good prognosis in HR-negative breast cancers after neoadjuvant chemotherapy. Sueta *et al.* reported that Ki67 had no predictive value for pathologic Complete Response (pCR) in HER2 and triple-negative subtypes with neoadjuvant chemotherapy (NAC) (45). Similarly, Jones *et al.* also reported that Ki67 had no predictive value for pCR in triple-negative subtypes, as a greater chemotherapy sensitivity was generally observed in these tumours (48).

However, one clinical trial with 552 breast cancer patients showed that Ki67 independently improved the prediction of treatment response in a group of luminal tumours as well as triple-negative tumours post neoadjuvant chemotherapy (49). Tan *et al.* examined Ki67 expression as a predictor of pCR after anthracycline and/or taxane-containing neoadjuvant chemotherapy in a total 183 HR-negative patients, 61 of which were TNBC. Ki67 labelling index was a predictive marker for pathologic complete response and higher Ki67 expression was associated with HER2 status, tumor size, lymph node status, LVI and tumor grade. Also, high Ki67 expression in residual tumours was strongly correlated with poor disease-free, but not overall survival (50). How *et al.* has concluded that lower Ki67 has poor prognosis relevance in TNBC patients diagnosed at  $\leq 50$  years-old in a study of 571 patients (51).

In our study, Fisher's exact test resulted in a statistically significant relationship ( $p$ -value=0.041) between cell

proliferation index Ki67, which was positive in 44 out of the 79 patients, and cytokeratin 17 which was positive in 38 cases out of the 79.

DFS and OS between groups of patients with different levels of each biomarker have been compared using log-rank test with a 5% level of statistical significance. According to the Log-rank test, only the cell proliferation index (Ki67) has been proven to be statistically significant for the DFS ( $p$ -value=0.0378), and for the OS ( $p$ -value=0.0186), which means that the survival function is differentiated for the two levels of the biomarker. The significance of this biomarker has also been confirmed by the Cox regression model for DFS with backward elimination (HR=0.39, 95%CI=0.153-0.994,  $p$ -value=0.048). We run the same Cox regression model with backward elimination for OS and it concluded that biomarker Ki67 is an important predictor for the overall survival of patients (HR=0.277, 95%CI=0.087-0.886,  $p$ -value=0.03).

Further studies are required to assess the benefit of Ki67 assessment in TNBC.

P63 is a transcription factor member of the p53 gene family. Recent data suggest a complex role for p63 in breast cancer with certain studies suggesting an oncogenic role for  $\Delta Np63$ , and others a tumor suppressor role (52, 53). In our study, the expression of protein p63 appeared to be lower in patients with higher tumor grade (OR=0.18, 95%CI=0.035-0.978,  $p$ -value=0.047).

## Conclusion

High expression of claudin-7 and low expression of c-kit and protein p63 are associated with higher tumour grade. AR and CK5/6 expression seem to be important in LVI. These findings suggest that these biomarkers may be useful as prognostic or predictive indicators, as well as possible markers for novel therapies.

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