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Evaluation of Prognostic Factors in Triple Negative Breast Cancer Patients with Emphasis on Angiogenesis

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Abstract

Developing new therapeutic methods and exploring other possible future strategies in Triple Negative Breast Cancer (TNBC) is an area of interest. Angiogenesis is one such sought for area that can even serve as a targeted therapy in this subset of breast cancer. The objective of the study was to evaluate the prognostic factors in TNBC patients with emphasis on angiogenesis. Prospectively 120 patients with histologically confirmed breast cancer from June 2015 to July 2019 were included. The Colour Doppler evaluation of breast lump and axilla in terms of RI, PI and Vmax was done. Patients were categorized as having 'high' or 'low' RI, PI and Vmax on the basis of their mean value. Immunohistochemistry (IHC) was performed on viable tumour blocks obtained from mastectomy specimen using CD31 vascular endothelial staining. The idea was to obtain Microvascular Density (MVD) by counting all immunostained vessels at magnification of 400x. The association between TNBC and non TNBC with well-known Doppler parameters, tumour size, clinical lymph node status, number of positive lymph nodes, tumour grade, stage of disease and hormonal receptor status was investigated. Also the association between high and low MVD with these prognostic parameters were evaluated. A total of 120 patients were included in the study with the mean age of 42.43±7.73 years (range 30-65 years). The mean RI, PI and Vmax were 0.92±0.26, 2.19±1.84 and 16.52±10.70 respectively. The comparison between TNBC and non-TNBC with prognostic parameters showed significant association with age of patients, duration of disease, use of oral contraceptive pills (>1 year), tumour size, histological grade, RI and MVD (p=0.041, p=0.011, p=0.002, p=0.029, p=0.026, p=0.014 and p=0.007 respectively). The MVD value >13.17 (high) was found in 45 (37.5%) patients while 75 (62.5%) patients had low MVD value (<13.17). The high MVD (>16.52) was significantly associated with tumour size (p<0.001), axillary lymph node (p=0.022), clinical stage (p=0.015), histological grade (p<0.001), RI (p<0.001), ER status (p<0.001) and HER2 over expression (p=0005). TNBC is a subset of breast cancer showing aggressive biological behaviour as seen by presence of poor prognostic marker and increased vascularity.

Keywords: Colour Doppler, CD31, Micro-vessel Density, Triple Negative Breast Cancer

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1. Introduction

Breast Cancer (BC) has proven to be a heterogeneous disease with varying presentations, outcome, molecular characteristics, and chemotherapeutic response[1]. Various prognostic factors are identified in an effort to predict patient's fate in BC as a whole. The important factors among these are age of presentation, size of tumour, clinical or pathological stage, histology, tumour grade, lympho-vascular invasion, lymph node/distant spread and neoangiogenesis and/or lymphangiogenesis.

Tumours without expression of hormone receptors (Estrogen and Progesterone receptors) and human epidermal growth factor receptor 2 (HER2) are called triple negative breast cancer (TNBC). In terms of disease-free and overall survival they have a poor prognosis[2,3]. TNBC constitutes less than one fifth of all BC and is distinguished by its aggressive nature and insensitivity to targeted treatment approaches associated with endocrine and anti-HER2 therapies[4,5]. While TNBC is sensitive to chemotherapy, early recurrence with metastatic disease is usual, with poor prognosis[6]. It is therefore important to establish new treatment approaches and to research other putative targets in TNBC, such as angiogenesis[7,8].

Angiogenesis is important for BC growth and can be assessed by micro vessel density (MVD) measurement[9]. The potential of angiogenesis assessment also lies with its use for novel agents with antiangiogenic properties alone or in combination[10,11].

In the study done by Kumar *et al.*[12,13] preoperative Doppler ultrasound findings were shown to be helpful for the evaluation of intra-tumoural blood flow and was associated with histological grade and extent of breast cancer pathology. There is a clear evidence of significantly higher values of Resistivity Index (RI), Pulsatility Index (PI), and Maximum Flow Velocity (Vmax) in malignant breast lump compared to benign ones[14].

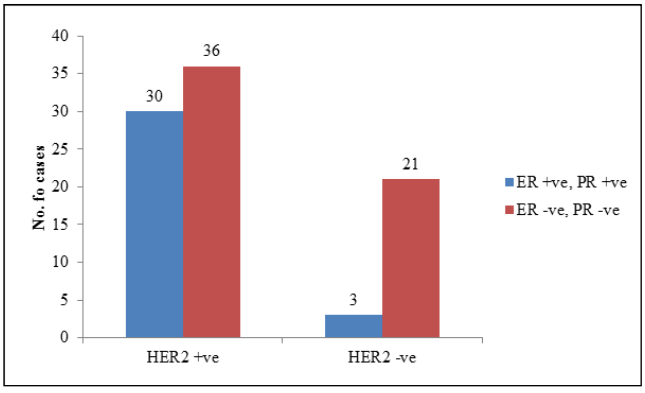


Figure 1. Distribution according to receptor positive and triple negative status.

The aim of this study is to define the prevalence of triple negative status in this part of the country among BC patients coming to a tertiary care University hospital. The difference of established prognostic markers with special emphasis on vascularity as assessed by Colour Doppler and CD-31 Immunohistochemistry was investigated.

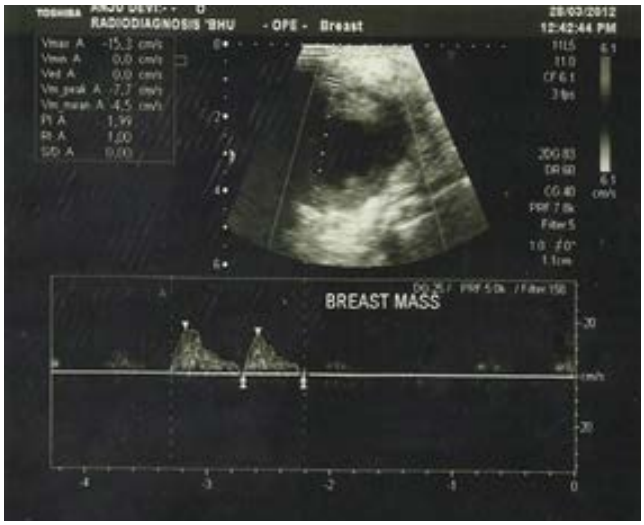
2. Methods

This was a prospective study undertaken on 120 patients of advanced breast carcinoma, who were operated primarily. Informed consent was taken from all the patients included in the study conducted in one surgical unit of the Department of General Surgery, Institute of Medical Sciences from June 2015 to July 2019.

The present work was approved by the Institute's Ethics Committee [No. Dean/2011-12/382], and all patients had received informed consent prior to the surgery. Patients who received any prior treatment in the form of chemotherapy, radiotherapy or breast surgery were excluded. Biopsy was taken from tumour and surrounding breast tissue using a 16 G Tru-Cut needle. Tissue specimen obtained was approximately 17 mm × 1 mm; 3-6 such pieces of tissues were collected. Preoperative demographic and clinical data, relevant investigations findings, Doppler study, details of pathological diagnosis, treatment details and follow-up period were collected prospectively in all the patients.

Colour Doppler of lump and axilla of breast was examined by an experienced radiologist using a 7.5 MHz Doppler (Xario Toshiba) probe to look for Resistivity Index (RI), Pulsatility Index (PI) and Maximum flow velocity (Vmax). Patients were categorized as having 'high' or 'low' RI, PI and Vmax depending on whether their individual RI, PI or Vmax was higher or lower than the mean RI, PI or Vmax value (Figure 2a, b).

Mastectomy specimen were cut into slices and fixed in 10% buffered formalin. The formalin fixed specimens were cut into 3μm section and stained with Hematoxylin and Eosin (H & E). After H & E staining these sections were evaluated under light microscopy for histopathological details – size, type and grade of tumour, number of lymph nodes dissected and number of positive lymph nodes, lymphovascular invasion and receptor status (ER, PR and HER2).



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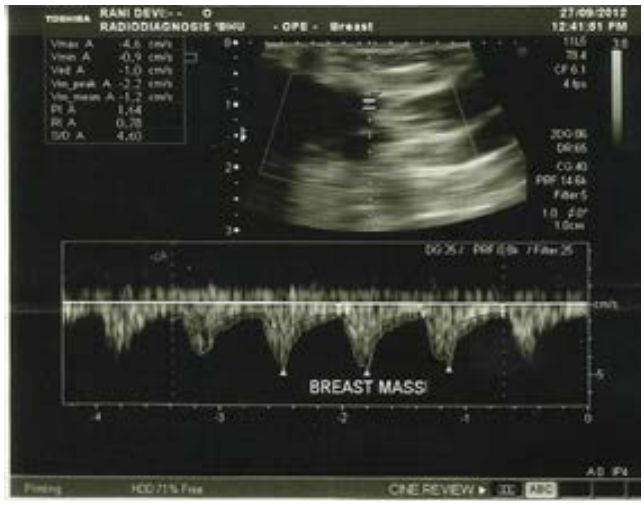

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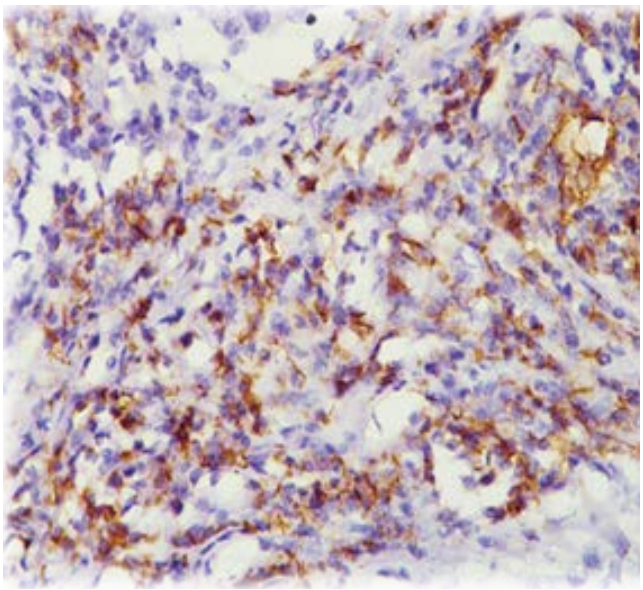
Figure 2. (a) Colour Doppler of breast showing high RI value (b) Colour Doppler Study of breast showing relatively low RI, PI and Vmax.

Tumour representative blocks were selected for Immunohistochemistry (IHC) by staining with CD31. The primary antibody used was a monoclonal mouse anti human CD31 antibody from BIOGENEX, Netherlands and biotinylated goat anti-mouse antibody was used as the secondary antibody.

The 4 µm sections were taken on 1% Poly L-lysine coated slides. De-waxing was done by dipping the slides in Xylene-1 and then in Xylene-2 for five minutes and then rehydrated in absolute alcohol (95%) for 1-2 minutes. Antigen retrieval was done in microwave using citrate buffer (pH 6.0) at 95^{0}C and second at 97^{0}C for 10 minutes each. Slides were washed in TRIS buffer (pH 7.6) followed by endogenous peroxidase blocking by 3% H_2O_2. Sections were finally incubated in primary antibody solution and washed. Diaminobenzidine dihydrochloride (DAB) chromogen was applied and then treated with secondary antibody. After final blotting and drying, slides were observed under microscope.

For determining the micro vessel density, Weidner criteria was utilized[15]. A vessel was defined if wall had endothelium with immunopositivity with CD31 and a vascular lumen. In the hotspot areas, usually at the periphery of the slides, highest stained areas were identified at low magnification (40X). Three such hotspots were selected at low magnification followed by counting of all CD31 immunostained vessels at 400x magnification to determine the MVD (Figure 3a, b). The mean value of MVD was observed by two investigators in each patient and in the case of interobserver differences of more than 30% in micro vessel count, the respective slides were reinvestigated by both observers using a discussion microscope. The mean of all values were taken and patients having higher MVD than mean value were considered as high and with values below the mean as low MVD.

The data analysis was carried out using the Social Sciences Statistical Package (SPSS), version 16.0. For continuous variables Student t test was used and Chi square and Fisher exact test were used for categorical variables. P-value < 0.05 was considered to be significant.



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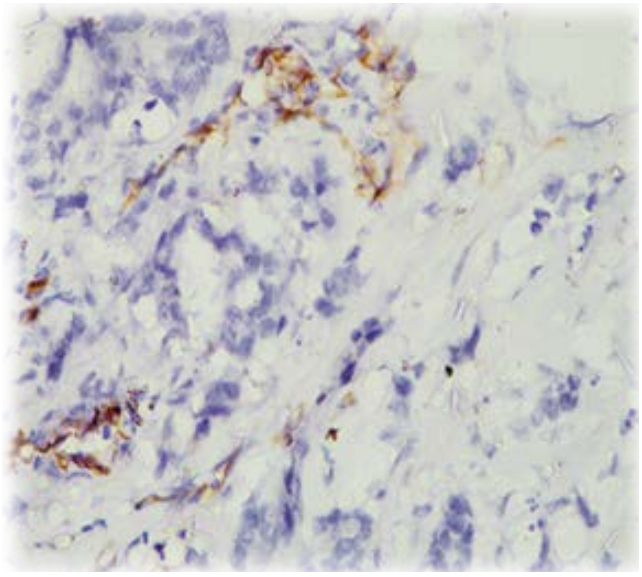

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Figure 3. (a) Anti Human CD31 Antibody staining showing very high neovascularisation (b) Anti Human CD31 Ab staining showing relatively low neovascularisation.

3. Results

A total of 120 patients were included in the study with the mean age of 42.43±7.73 years (range 30-65 years). Majority of patients (65%) were pre-menopausal and rest were post-menopausal (35%). At the time of the presentation, lump was present in all the patients. The clinical and pathological characteristics of patients are presented in (Table 1). The Doppler parameters were classified according to their mean values. The mean RI, PI and Vmax were 0.92±0.26, 2.19±1.84 and 16.52±10.70 respectively. ER was positive in 39(32.5%) patients, PR in 57(47.5%) and HER2 was positive in 93(77.5%) patients. Thirty three (27.5%) patients were positive for both ER and PR. Overexpression of HER2-neu was found in 93(77.5%) patients. Fifty seven (47.5%) patients were both ER and PR negative (Figure 1).

Out of 120 patients, 21(17.5%) patients were triple negative. The comparison of various prognostic parameters between TNBC and non-TNBC is shown in (Table 2). Significant association were found in age of patients, duration of disease, use of oral contraceptive pills (>1 year), tumour size, histological grade, RI and MVD (p=0.041, p=0.011, p=0.002, p=0.029, p=0.026, p=0.014 and p=0.007 respectively) but no significant association were found in axillary lymph node, clinical

Table 1. Patient characteristics

Characteristics	
Age (years) <40 >40 Mean ± SD	45 (37.5) 75 (62.5) 42.43±7.73
Menopausal Status Premenopausal Postmenopausal	78 (65.0) 42 (35.0)
Duration of disease (months) <6 >6	51 (42.5) 69 (57.5)
Use of oral contraceptive pills (>1 year) Yes No	21 (17.5) 99 (82.5)
Tumour size (cm) < 6 cm > 6 cm Mean ± SD	48 (40.0) 72 (60.0) 6.32±2.36
Axillary node Positive Negative	90 (75.0) 30 (25.0)
Clinical stage Early (IIa+IIb) Advanced (III+IV)	48 (40.0) 72 (60.0)
Histological grade I + II III	78 (65.0) 42 (35.0)
Positive lymph nodes Negative Low (<6 lymph nodes) High (>6 lymph nodes)	21 (17.5) 18 (15.0) 81 (67.5)
ER Positive Negative	39 (32.5) 81 (67.5)
PR Positive Negative	57 (47.5) 63 (52.5)
HER2 Positive Negative	93 (77.5) 27 (22.5)
Resistivity Index High (>0.92) Low (<0.92)	69 (57.5) 51 (42.5)
Pulsatility Index High (>2.19) Low (<2.19)	39 (32.5) 81 (67.5)
Maximum flow velocity High (>16.52) Low (<16.52)	54 (45.0) 66 (55.0)

Microvessel density High (>13.17) Low (<13.17)	45 (37.5) 75 (62.5)
Breast cancer subtype TNBC NON-TNBC	21 (17.5) 99 (82.5)

Table 2. Comparison of Prognostic parameters between TNBC and non TNBC

Prognostic parameters	TNBC (n=21)	Non TNBC (n=99)	P value
Age (years) <40 >40	15 6	30 69	**0.041**
Duration of disease (months) <6 >6	18 3	33 66	**0.011**
Use of oral contraceptive pills (>1 year) Yes No	12 9	9 90	**0.002**
Tumour size (cm) <6 >6	0 21	48 51	**0.029**
Axillary node Positive Negative	18 3	72 27	0.656
Clinical stage Early (IIa+IIb) Advanced (III+IV)	6 15	39 60	0.591
Histological grade I-II III	6 15	72 27	**0.026**
Positive lymphnodes Negative High Low	3 15 3	18 66 15	0.964
Resistivity Index High (>0.92) Low (<0.92)	21 0	48 51	**0.014**
Pulsatility Index High (>2.19) Low (<2.19)	9 12	30 69	0.519
Maximum flow velocity High (>16.52) Low (<16.52)	6 15	48 51	0.336
Microvessel density High Low	18 3	27 72	**0.007**

stage, positive lymph nodes, PI, Vmax, ER, PR and HER2 overexpression.

The MVD was categorized (high/low) on the basis of their mean value. The mean MVD of 120 patients was 13.17. The MVD value >13.17 (high) was found in 45(37.5%) patients while 75(62.5%) patients had low MVD value (<13.17). The association between MVD (high and low) and clinic-pathological prognostic markers are shown in (Table 3). The high MVD (>16.52) was significantly associated with tumour size (p<0.001), axillary lymph node (p=0.022), clinical stage (p=0.015), histological grade (p<0.001), RI (p<0.001), ER status (p<0.001) and HER2 overexpression (p=0005).

Table 3. Comparison of Prognostic parameters between high and low MVD

Prognostic parameters	High MVD (n=45)	Low MVD (n=75)	P value
Age (years) <40 >40	13 32	32 43	0.131
Duration of disease (months) <6 >6	21 24	30 45	0.474
Use of oral contraceptive pills (>1 year) Yes 21 No 99	8 37	13 62	0.950
Tumour size (cm) <6 >6	9 36	39 36	**<0.001**
Axillary node Positive Negative	39 6	51 24	**0.022**
Clinical stage Early (IIa+IIb) Advanced (III+IV)	11 34	35 40	**0.015**
Positive lymphnodes Negative High Low	7 29 9	14 52 9	0.483
Histological grade I-II III	16 29	62 13	**<0.001**
Resistivity Index High (>0.92) Low (<0.92)	37 8	32 43	**<0.001**

Pulsatility Index High (>2.19) Low (<2.19)	17 28	22 53	0.339
Maximum flow velocity High (>16.52) Low (<16.52)	17 28	37 34	0.218
ER Positive Negative	27 18	12 63	**<0.001**
PR Positive Negative	23 22	34 41	0.539
HER2 Positive Negative	41 4	52 23	**0.005**

4. Discussion

TNBC represent a consistent subgroup of breast cancer with heterogeneous clinical presentation, clinical behaviour, histology and response to therapy. The etiological profile of TNBC that is associated with high mortality and insufficient therapeutic choices is little understood.

TNBC accounts for 10-20% of all BCs[3,6] worldwide and nearly 30% among Asian women population[16]. From India, Patil *et al.*[17] observed 19.9% to be TNBC in a cohort of 683 BC patients. Similarly, Ghosh *et al.*[18] reported TNBC status in 29.8% patients in 2008. In the present study, 32.5% patients were ER-positive, 47.5% patients were PR-positive, 93 (77.5%) patients were HER2 positive and 21 (17.5%) patients were found to be triple negative.

Studies have shown an increased incidence of TNBC in younger and black skinned women[6]. In their research with Asian women, Tan *et al.*[19] observed TNBC to more likely to present in patients with less than 40 years of age. Surprisingly, Indian published data showed large numbers of patients were even younger, i.e. <35 years. In the present study, 71.4% of patients in the triple negative group were also under 40 years of age, compared to just 30.3% in the non-triple-negative group (p=0.041). The mean age of presentation among the triple negative patients was 39.85 years.

Presentation as a lump in breast is common to both TNBC and non-TNBC alike. In western studies where mammographic screening is routine, TNBC is more likely to be identified by clinical examination than through serial imaging[20,21]. The reason for this paradox being a faster growth seen in TNBC which may miss tumour detection in fixed screening protocols. The possible reasons apart from missing tumour in between mammograms could be the variations in breast tissue density among TNBC phenotype making it more difficult to distinguish them on conventional mammography. We also found that a clinically detectable lump as presenting complain was seen in both TNBC and non-TNBC patients. The reasons may be the less use of mammography screening programs in this region of the country. On the other hand we found significantly larger proportion of TNBC patients presenting with shorter history of less than 6 months compared to non TNBC patients (85.7% versus 33.3% respectively, p=0.011). The association of breast feeding with occurrence of TNBC has also been studied but the results lack any conclusive statement. Studies vary from safety of occurrence of TNBC with breast feeding beyond 6 months[22,23] to no correlation of it to TNBC[24]. Parity and history of breast feeding was not correlated with the occurrence of TNBC and non TNBC in our analysis.

Extensive research has been carried out on the relationship between the use of oral contraceptives and BC risk but to a meagre number compared to other personal attributes like family history, early menarche, nulliparity and lack of breast feeding[25,26]. Oral contraceptive pills have shown to increase the chance of having BC as shown in a meta-analysis by Kahlenborn *et al*[27]. Studies have shown that the risk of developing cancer is more in young premenopausal women[28,29]. Duration of oral contraceptive use more than a year increases the risk of TNBC with a relative risk of 2.7[30]. In the present study, 9(9.1%) non-TNBC patients compared to 12(57.1%) TNBC patients used oral contraceptive pill for more than 1 year. This difference is significant but the small sample size limits drawing such conclusion from the present study.

The tumour size is ranked after the axillary lymph node status for deciding the prognosis in BC. This becomes single most important consideration if lymph nodes are not involved. Size is directly linked to an increased vascularity as well as the likelihood of having regional metastasis[31]. Dent *et al.,* found that TNBC patients presented with larger lump size than non TNBC(7.77% T3 lesion compared to 1.12% T3 lesions in non-TNBC patients)[20]. We found that all patients with TNBC had a larger tumour size (> 6 cm) compared to only 51.5% of patients with non-TNBC (p=0.029).

The axillary lymph node status remains the single most important factors indicating the systemic spread and therefore distant metastasis[32]. However, the association

between TNBC status and lymph node spread is not well established[20,33–35]. Eighteen out of 21 TNBC patients (85.7%) had positive axillary lymph nodes compared to 72.7% of non-TNBC patients in the present study, but the association was not statistically significant (p=0.656). The lack of variation in our research may be explained by selection of the early stage of patients in which primary surgery was performed.

The grade of tumour has a direct link with TNBC as shown in studies[3,6,36]. Dent *et al.*[20] found that TNBC group are likely to have grade 3 tumours (66% versus 28%; P<0.0001). Study from India also observed that the TNBC was associated with a higher histological and nuclear grade compared to non-TNBC (p=0.001 and p=0.001)[19]. In present study, 15 (71.4%) of patients with TNBC had a high histological grade compared to 27 (27.3%) non-TNBC patients (p=0.026).

It has been reported that the use of colour Doppler ultrasonography in BC patients is useful in differentiating the tumour being benign or malignant[37]. The surrogate representation of Doppler for neovascularisation has been utilized[37,38]. The higher RI, PI and Vmax values of colour Doppler in more vascular lesions such as malignancy has been established[14]. Patients with malignant lesions usually exhibit enhanced colour Doppler signals due to neovascularisation of the tumour[37,39]. High values of these indices can successfully predict malignancy in a breast lump and the resistance caused due to presence of tumour itself or its emboli can cause an increased resistance affecting all the three variables[40,41]. Study published by Wang *et al.*[41] demonstrated a relationship between the power Doppler index and the levels of VEGF protein emphasizing the role of pre-operative colour Doppler assessment. High-grade and negative hormone receptors are shown to be associated with presence of marked vascularity[42]. While analysis by Kojima *et al.,* found that the TNBC patients had fewer colour spots or vessel structures and rarely show significant vascularization (12.5%)[43]. Similarly, Lacroix *et al.*[44] found no association between the parameters of the colour Doppler and the TNBC. The RI, PI, and Vmax were obtained in all patients in the present study. When comparison was made between patients with TNBC and non-TNBC, RI was found to be high (>0.92) in patients with TNBC (p=0.014), however when PI and Vmax could not be related.

Angiogenesis and lymphangiogenesis are essential to the development, invasion, and metastasis of tumours[45]. Recent researches have shown that intratumoural MVD is a significant predictor of BC survival and for predicting systemic metastasis risk[46]. Neovascularisation assessment can also be used as a valuable method in the preoperative prognosis of BC patients. In their study, Mohammed *et al.*[9] found 57 out of 99 TNBC cases with high MVD, compared with 108 out of 334 non-TNBC cases (p<0.001). In the present study, CD31 antibody has examined histological slides for angiogenesis assessment. The mean MVD was 13.17±4.13. Eighteen (85.7%) TNBC patients had high score as compared to 27 (27.3%) of non TNBC patients (p=0.007).

In this study, we also have determined angiogenesis in all the BC patients by counting micro vessels using anti-CD-31 antibody and compared MVD that we have obtained from each patient with prognostic factors. We found a statistically significant association between tumour size and MVD (p<0.001). This is consistent with some published studies[46–49] while some studies found no significant association between MVD and tumour size, and some studies reported an inverse association among them[50–52].When comparing MVD with involvement of axillary lymph nodes, we observed that MVD was higher in patients with positive axillary lymph nodes relative to patients without axillary lymph nodes (p=0.022). In similarity to our findings, several studies have found that high MVD is associated with metastases of the axillary lymph nodes[48,49,53,54]. Nonetheless, some other studies have also found no association between MVD and metastases of the axillary lymph node[50,55].

We found a statistically significant relationship between advanced tumour stage (III+IV) and MVD stage (p=0.015) but no statistically significant relationship between lymph node positivity (high) and MVD (p=0.483) was observed. Comparing MVD to histological grade, we observed that MVD was higher in patients with a higher histological grade (grade III) compared to those with grade I+II (p<0.001). Strong literature studies[50,54,56] are available, and some research[57] find no significant association between MVD and histological grade.

We found a statistically significant association between high RI and MVD (p<0.001). When comparing MVD with PI and Vmax, no significant association was observed (p=0.339 and p=0.218).

We also found a statistically significant relationship between ER positives and HER2 overexpression and MVD (p<0.001 and p=0.005 respectively) in BC patients. But between PR and MVD (p=0.539) there was no statistically significant association observed. Several

studies have found a significant association between high MVD and oestrogen receptor[50,51] and overexpression with HER2[58]. However, some studies have reported that there is no association between MVD and ER, PR and HER2/neu receptor status[56].

We found no association in our analysis between MVD and the age of the patient, duration of the disease, use of oral contraceptive pills (> 1 year), positive lymph nodes, PI, Vmax and PR. Some studies have reported, in the literature, that there is no relationship between MVD and patient age[49,54,56] and PR[49,56].

In studies published in literature the correlation between MVD and prognostic parameters is variable. One explanation for this may be the use of various antibodies to highlight the microvessels[53,59,60]. We used the monoclonal antibody anti-CD31 to calculate the MVD and no other antibody hence we are not sure whether that selection influenced our tests. The MVD method of measurement may be another explanation for getting different results. The approach employed by Weidner is used in many microvessel count studies[50,57,61]. Some writers counted a single area under x200 or x250, while others counted one area under x400 magnification[62–64]. In this analysis, we have used Weidner's system of microvessel counting.

Different cut-off values used to identify patients according to their MVD may be another explanation for the different findings between studies. Many studies define the cutoff value as the average number of microvessels[56,65], while in other research, the cut off value is the median number of microvessels[66,67]. Also certain other studies accept absolute values as the cut-off value[62,68,69]. We accepted the mean number of micro vessels as the cut-off value in this study. By influencing the p value, all these various cut-off values may be the principal cause of different outcomes.

5. Conclusion

In conclusion, TNBC is not unusual as revealed in the present analysis. Triple negative cases have high vascularity and are aggressive. In comparison to non-triple negative cases, TNBC have increased presence of poor prognostic markers. The authors also conclude the need for a multicentric study with larger number of patients and a long follow-up to confidently label TNBC status in comparison to its Non-TNBC counterpart.

6. Funding

None

7. Conflict of Interest

None

8. References

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