

## Original Article

# Distribution of Myofibroblast Cells and Microvessels around Invasive Ductal Carcinoma of the Breast and Comparing with the Adjacent Range of their Normal-to-DCIS Zones

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## Abstract

**Background:** This study seeks to determine the relationships between manifestation of myofibroblasts in the stroma tissue of hyperplastic pre-invasive breast lesions to invasive cancer by investigating clinicopathological data of patients, their effect on steroid receptor expression and HER2, and angiogenesis according to CD34 antigen expression.

**Methods:** Hundred cases of invasive ductal carcinoma were immunohistochemically investigated for the presence of smooth muscle actin (SMA), ER/PR, HER2, anti-CD34 antibody and microvessel count (MVC). Patients were scored in four different zones of invasive areas: invasive cancer, DCIS, fibrocystic disease ± ductal intraepithelial neoplasia (FCD ± DIN), and normal tissue.

**Results:** There was a significant difference in stromal myofibroblasts between all areas except for the stroma of DCIS and FCD ± DIN ( $P < 0.001$ ). We observed positive significant correlations between stromal myofibroblasts, HER2 expression, and the numbers of involved lymph nodes in invasive cancer, DCIS, and FCD ± DIN ( $P < 0.001$ ). More myofibroblasts were present in grade III cases, with the least frequent observed among grade I cases in the stroma of those with invasive disease, DCIS, and FCD ± DIN ( $P < 0.001$ ).

MVC was inversely related to stromal myofibroblasts in invasive cancer ( $P < 0.001$ ) and DCIS ( $P < 0.001$ ), whereas there was a positive correlation in the stroma of FCD ± DIN ( $P = 0.002$ ) and normal areas ( $P = 0.054$ ). There was a significant difference in MVC observed in all areas except for DCIS and FCD ± DIN ( $P < 0.001$ ). We noted significant inverse correlations between MVC, HER2 expression, and the numbers of involved lymph nodes in invasive cancer and DCIS ( $P < 0.001$ ). Most MVC were present in grade I, with the least frequent observed in grade III cases in the stroma of invasive cancer, DCIS and FCD ± DIN ( $P < 0.001$ ).

**Conclusion:** Angiogenesis can be observed before any significant myofibroblastic changes in the pre-invasive breast lesions. The elevated content of myofibroblasts in stroma of tumor; probably may be a worse prognostic factor and the steps from atypical epithelial hyperplasia to DCIS and then to the invasive carcinoma do not appear to be always part of a linear progression.

**Keywords:** Angiogenesis, CD34, ER/PR, HER2, myofibroblast

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## Introduction

The recent trend toward improvement in breast cancer mortality rate is largely due to the increased diagnosis of early stage disease, while our therapeutic options for the advanced stage breast carcinomas are still fairly limited. Thus, there is a need to better understand the cellular and molecular basis of breast cancer initiation and progression and to use this knowledge for the design of targeted, molecular based therapy.<sup>1</sup>

The importance of stromal interaction with epithelial cells is well established in the embryonic development and tumorigenesis.<sup>2-4</sup>

Myofibroblasts through the secretion of chemokines, cytokines, growth factors, inflammatory mediators, as well as extracellular matrix proteins and proteases, play an important role in organogenesis, oncogenesis, inflammation, repair and fibrosis in most organs and tissues.<sup>5</sup>

CD34+ fibrocytes/fibroblasts are derived from myeloid precursors, invade sites of tissue damage and are capable of connective

tissue matrix synthesis. Besides its function as a matrix-production cell, the CD34+ fibrocyte/fibroblast is a potent antigen-presenting cell and therefore it has been claimed that CD34+ may play a role in host response to tissue damage.<sup>6,7</sup>

Some molecular markers are associated with the prognosis,<sup>8</sup> of which the most common, well-known molecular markers are ER, PR and HER2 (also known as c-erbB-2 or neu). CD10+ myofibroblast has also been shown to be a potentially negative prognostic factor.<sup>9</sup>

Amplification of the HER2 gene plays an important role in the pathogenesis of breast cancer.<sup>10,11</sup> In general hormone receptor status (both estrogen and progesterone receptors) correlate with a positive outcome whereas HER2 overexpression has been associated with a poor outcome.<sup>12</sup> The growth, invasion, and metastasis of many cancers depend on angiogenesis. Many observational studies (prospective or retrospective) have concluded that angiogenesis is a prognostic factor in the invasive breast cancer, however others disagree.<sup>13</sup>

Finding the different markers associated with angiogenesis may assist with the identification of those patients at increased risk for recurrence and metastasis who require more aggressive therapy and closer surveillance.<sup>14</sup> In addition, the quantification of tumor microvasculature is a candidate target for antiangiogenic therapy.<sup>15</sup>

Antibodies against CD34 which have been recently used, react not only with newly formed vessels but also normal vessels

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trapped within tumor tissues and are thus referred to as pan-endothelial markers.<sup>14</sup>

In this study we assessed the microvessels count (MVC), as well as the number of the myofibroblasts in the stroma of various areas of invasive cancer, DCIS, fibrocystic disease (FCD) with or without ductal intraepithelial neoplasia (DIN) changes, and normal tissue by a pan-endothelial marker, CD34 and an anti-SMA antibody. In addition, the relationship between the presence of the myofibroblasts and the number of stromal microvessels with principal pathological parameters, as well as the expressions of steroid receptors and HER2 proteins, and patients' clinicopathologic characteristics were evaluated.

## Materials and Methods

We performed immunohistochemistry analyses on 100 cases of invasive ductal carcinoma. Patients included in this study were from hospitals in the city of Kerman (Afzalipour, Shahid Bahonar) and private laboratories during 1380 to 1390. Included were all women between the ages of 25 to 77 years. Specimens obtained from open surgery or excisional biopsy were fixed in 10% buffered formalin for 48 hr, and then embedded in paraffin blocks by standard histological techniques. Formalin-fixed paraffin blocks of tumor and non-tumor tissue sections from each patient were re-examined by pathologists and classified according to the Nottingham combined system histological grading scheme which included mitotic rate, nuclear and structural properties. Specimens were classified as grades 1, 2, and 3. The block from each tumor sample with areas of invasive cancer, DCIS, atypical ductal hyperplasia and flat epithelial atypia, (DIN Ia, Ib), fibrocystic changes and normal areas were selected for further analyses. If these were not located on one block, the specific areas of the different blocks were cut and placed on one slide. Serial sections of 5  $\mu$ m were obtained from each block and mounted on silanized slides for immunohistochemical staining.

### Immunohistochemistry

Dehydrated, deparaffinized sections along with retrieval buffer were microwaved for 20 min (3 min at 850 watts; 17 min at 180 watts), then blocked for 10 min with 0/5 % H<sub>2</sub>O<sub>2</sub>. Sections were incubated for 1 hr at room temperature with the monoclonal antibodies as follows: ER (1:50; DAKO, Clone 1D5); PR (1:100; DAKO, Clone PgR 636); HER2-neu (1:100; DAKO), CD34 (1:100; DAKO, Clone QBEnd 10); CD34 (1:100; Novocastra, Clone QBEnd 10); and SMA (DAKO, Clone 1A4): Ready to use. Slides were rinsed with wash buffer for 5 min, which was repeated twice, between all main stages. Envision polymer (30 min) was added using 3, 3'-diaminobenzidine (DAB) as the chromogen (10 min) followed by staining with hematoxylin for 2 min, dehydration and mounting the slides.

### Immunohistochemistry scores

For microvessel count (MVC) the areas that contained the great-

est numbers of microvessels (vascular hot-spots) were selected by scanning the stained sections at low magnification with a light microscope. Any brown-stained endothelial cell with a visible nucleus, which was clearly separate from adjacent microvessels, tumor cells, and other stromal elements, was considered a single, countable microvessel, with no requirement for a lumen or the presence of erythrocytes.<sup>16</sup>

We evaluated the number of stained microvessels per field in the adjacent stroma of each chosen area at high power magnification ( $\times 400$ ).

For ER and PR analyses, at least 10% of the tumor cells with positive nuclear reactions, regardless of their intensity, were considered positive. HER2 expression was scored as follows: 0 (negative or <10% of cells stained); 1+ (partial membrane); 2+ (complete weak membrane), and 3+ (complete strong membrane). SMA was evaluated based on semiquantitative measurement of SMA-positive cells (myofibroblasts) compared with the population of tumor stromal cells in five microscopic fields at  $\times 400$  magnification. For all analyses, samples were separately read and classified by two pathologists, who were blinded to the results.

### Statistical analysis

The student's *t*-test and ANOVA with *post hoc* (Bonferroni) were used to compare quantitative variable groups with normal distribution.  $P < 0.05$  was considered significant in the study. The correlations between MVC, myofibroblast numbers, and other parameters were measured by Pearson's correlation coefficient. The data were presented as Mean  $\pm$  Standard Error (SE).

## Results

### Patients

We included 100 females diagnosed with the invasive ductal carcinoma of the breast; with a mean age of  $49.1 \pm 1.1$  years and mean tumor size of  $3.2 \pm 0.2$  cm. The mean number of the involved axillary lymph nodes was  $8.1 \pm 0.8$  and the following tumor grades were reported: grade I (35%), grade II (42%), and grade III (23%) as listed in Table 1.

**Table 1.** Patient and tumor characteristics.

		No (%)
Age (years)	$\leq 50$	60 (60)
	$> 50$	40 (40)
Tumor size (cm)	$< 2$	17 (17)
	2–5	70 (70)
	$> 5$	13 (13)
Lymph nodes	$\leq 3$	13 (28.9)
	$> 3$	32 (71.1)
Tumor grade	1	35 (35)
	2	42 (42)
	3	23 (23)

Presence of myofibroblasts in the invasive cancer, DCIS, FCD $\pm$ DIN and normal areas

According to post hoc ANOVA, there was a significant difference in stromal myofibroblasts in all areas with the exception of DCIS and FCD  $\pm$  DIN ( $P < 0.001$ ; Table 2).

**Table 2.** Distribution of CD34 and SMA.

	Invasive	DCIS <sup>1</sup>	FCD $\pm$ DIN <sup>2</sup>	Normal	P-value
CD34	$39.9 \pm 1.3$	$53.2 \pm 2.2$	$49.2 \pm 2.5$	$87.1 \pm 2.2$	0.0001
SMA	$29 \pm 1.2$	$13.2 \pm 1.7$	$10.5 \pm 1.2$	$3.6 \pm 0.2$	0.0001

Data are presented as mean  $\pm$  SEM of 100 cases. <sup>1</sup>Did not show significant difference; Areas: DCIS = Ductal carcinoma in situ; FCD  $\pm$  DIN = Fibrocystic disease  $\pm$  ductal intraepithelial neoplasia.

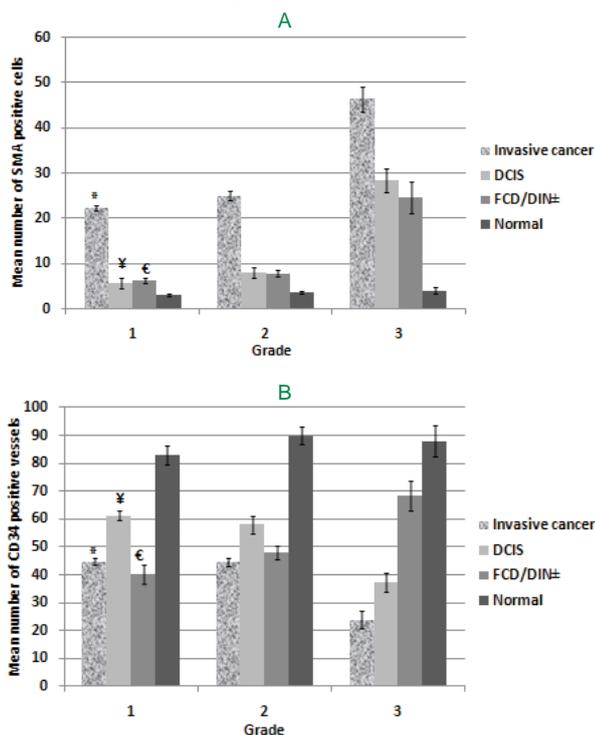
**Table 3.** Correlations of SMA and CD34 with other parameters.

		<i>P</i> -value			
		Invasive	DCIS	FCD±DIN	Normal
SMA	CD34	0.0001*	0.0001*	0.002	0.054
	HER2	0.0001	0.0001	0.0001	—
	Grade	0.0001	0.0001	0.03	0.19
	Lymph node	0.0001	0.0001	0.0001	0.61
CD34	HER2	0.0001*	0.0001*	0.008	—
	Grade	0.0001*	0.0001*	0.0001	0.31
	Lymph node	0.001*	0.18*	0.02	0.22

\*: Negative correlation. Areas: DCIS = Ductal carcinoma in situ; FCD±DIN = Fibrocystic disease ± ductal intraepithelial neoplasia.

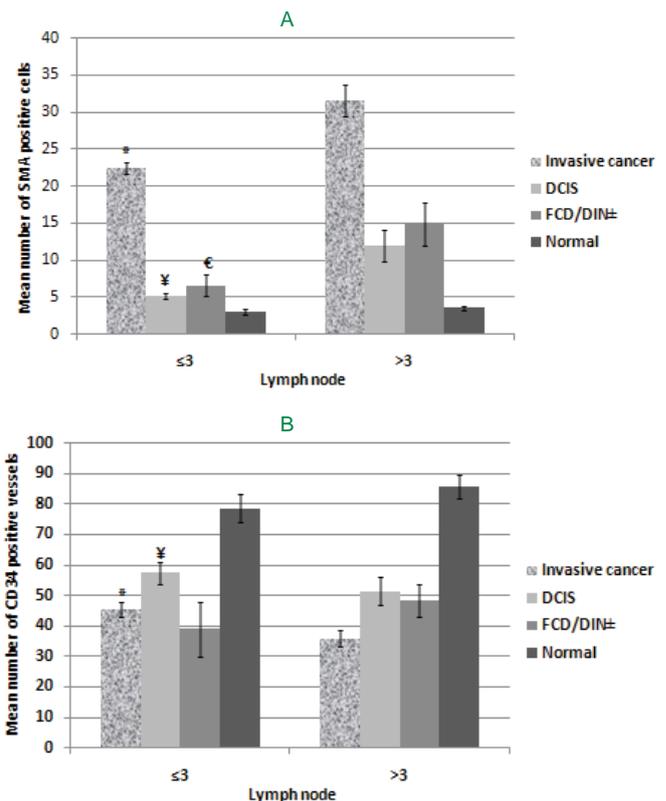
### Myfibroblasts and the clinicopathological parameters

The ANOVA and student's *t*-test revealed no significant differences in the stromal myfibroblasts based on tumor size or age in the stroma of invasive cancer, DCIS and FCD ± DIN. However, there was a significant positive correlation between tumor grade and stromal myfibroblasts ( $P < 0.001$ ; Table 3). According to ANOVA a significant difference existed in stromal myfibroblasts based on tumor grades in the invasive cancer, DCIS and FCD ± DIN areas ( $P < 0.001$ ; Figure 1).



**Figure 1.** Myfibroblasts (SMA) and microvessel count (MVC) as CD34 in tumor stroma according to different tumor grades. **A)** Myfibroblasts show an increasing trend in invasive cancer, DCIS and FCD ± DIN stroma based on tumor grade. **B)** MVC shows a decreasing trend in invasive cancer and DCIS stroma. \*:  $P < 0.001$ , significant difference among invasive cancer stroma; †:  $P < 0.001$ , significant difference among DCIS stroma. ‡:  $P < 0.001$ , significant difference among FCD ± DIN stroma. €:  $P < 0.001$ , significant difference among FCD ± DIN stroma.

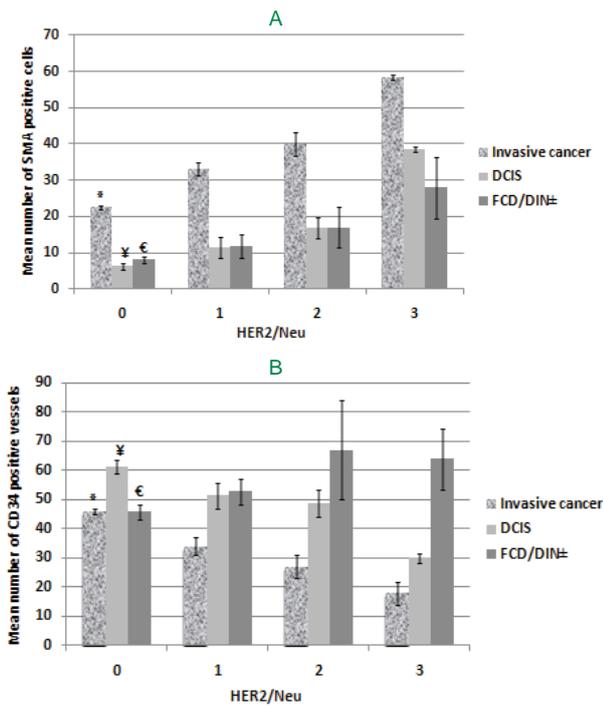
As noted in Figure 2, the student-*t* test showed a significant difference in stromal myfibroblasts based on the number of involved lymph nodes in the stroma of invasive cancer ( $P < 0.001$ ), DCIS ( $P < 0.001$ ) and FCD ± DIN ( $P = 0.05$ ). There was a significant, positive correlation between stromal myfibroblasts and involved lymph nodes in these areas ( $P < 0.001$ ; Table 3).



**Figure 2.** Myfibroblasts (SMA) and microvessel count (MVC) as CD34 in tumor stroma according to number of involved lymph nodes. **A)** Myfibroblasts show an increasing trend in the stroma of invasive cancer, DCIS and FCD ± DIN between lymph node groups. \*:  $P < 0.001$ , significant difference among invasive cancer stroma; †:  $P < 0.001$ , significant difference among DCIS stroma. ‡:  $P < 0.001$ , significant difference among DCIS stroma. €:  $P = 0.05$ , significant difference among FCD ± DIN stroma. **B)** MVC show a decreasing trend in the stroma of invasive cancer, DCIS and FCD ± DIN. \*:  $P = 0.03$ , significant difference among invasive cancer stroma; †:  $P = 0.02$ , significant difference among DCIS stroma.

### Myfibroblasts, steroid receptors, and HER2/neu expression

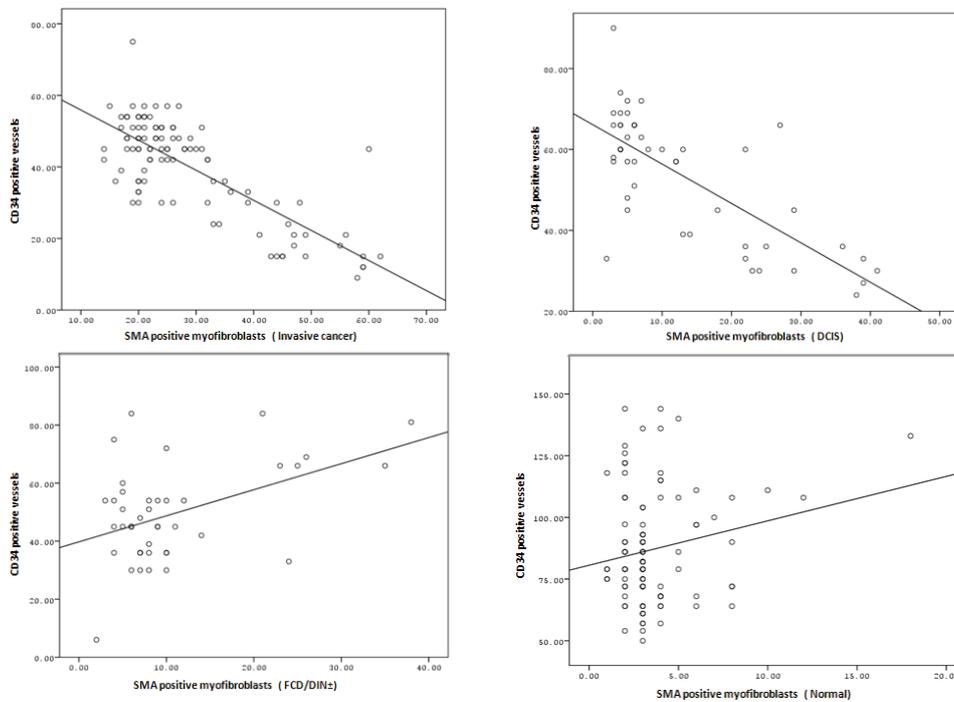
According to the student's *t*-test, stromal myfibroblasts had no significant difference between ER/PR groups in any of the included areas. However, in the ANOVA test we observed a significant difference in the stromal myfibroblasts based on HER2/neu expression ( $P < 0.001$ ; Figure 3). There was also a significant positive correlation between stromal myfibroblasts and HER2/neu expression in invasive cancer, DCIS, and FCD ± DIN stroma ( $P < 0.001$ ; Table 3).



**Figure 3.** Myfibroblasts (SMA) and microvessel count (MVC) as CD34 in tumor stroma according to HER2 receptor expression. **A)** Cases with higher expression of HER2 have a higher content of myfibroblasts, and **B)** lower content of MVC in the stroma of invasive cancer and DCIS. \*:  $P < 0.001$ , significant difference among invasive cancer stroma; ¥:  $P < 0.001$ , significant difference among DCIS stroma. €  $P < 0.001$ , significant difference among FCD ± DIN stroma.

Microvessel number in invasive cancer, DCIS, FCD ± DIN and normal areas

In post hoc ANOVA testing we observed a significant difference in the number of microvessels between the stroma of all areas, with the exception of DCIS and FCD ± DIN ( $P < 0.001$ ; Table 2).



**Figure 4.** Correlation analysis between SMA (myfibroblasts) and CD34 (microvessel count, MVC). A significantly negative correlation between SMA staining and microvascular count is present in invasive cancer and DCIS stroma ( $P < 0.001$ ). In FCD ± DIN and normal stroma, there is a positive correlation, which was only significant in the FCD ± DIN area.

Microvessel number and clinicopathological parameters

In the ANOVA and student's *t*-test there was no significant difference in the microvessel numbers based on tumor size or age in the stroma from the invasive cancer, DCIS, and FCD ± DIN. According to the student's *t*-test there was a significant difference in microvessel numbers based on involved lymph nodes in the invasive cancer ( $P = 0.03$ ) and DCIS ( $P = 0.02$ ) stroma (Figure 2). There was also negative significant correlation between microvessel number and the involved lymph nodes in the stroma of invasive cancer ( $P < 0.001$ ; Table 3). Tumor grade showed a significant inverse correlation with microvessel number in the stroma of invasive cancer and DCIS ( $P < 0.001$ ; Table 3). The ANOVA results showed a significant difference of microvessel number based on tumor grade in invasive cancer, DCIS, and FCD ± DIN areas ( $P < 0.001$ ; Figure 1).

Microvessel number, steroid receptors, and HER2/neu expression

Results from the student *t*-test showed no significant difference in the microvessel number between the ER/PR groups in any of the included areas. According to the ANOVA test we observed a significant difference in microvessel number based on HER2/neu expression in the stroma of invasive cancer and DCIS ( $P < 0.001$ ; Figure 3). There was also an inverse significant correlation between microvessel number and HER2/neu expression in the stroma of the invasive cancer and DCIS ( $P < 0.001$ ; Table 3).

Myfibroblasts and microvessel number

The number of microvessels had a significant inverse relationship with the stromal myfibroblasts in invasive cancer and DCIS ( $P < 0.001$ ); however, a significant positive correlation was observed with the FCD ± DIN stroma ( $P = 0.002$ ; Table 3). In the normal area we also observed a positive correlation between stromal myfibroblasts and microvessel number, however this was not significant ( $P = 0.054$ ; Figure 4).

Figure 5 shows a comparison of the immunohistochemical findings between SMA and CD34 in the four different stromal areas.

## Discussion

Evidence from numerous studies have shown that the stroma surrounding the tumor has not only a passive element in creating an immune response to reject the tumor, but also is an active structure in the process of tumor development.<sup>3,17-20</sup>

It has been suggested that carcinogenesis does not result from mutations in epithelial or stromal cells alone, but rather from disrupting the interaction of stromal – epithelial cell components.<sup>21,22</sup>

Recent results suggest that tumor stroma may facilitate the metastatic spread of tumor by inducing reversible changes in cancer cell phenotype.<sup>23</sup> The main cells involved in the development of reactive stroma caused by the tumors appear to be myofibroblasts.<sup>24,25</sup> It has been shown that cancer cells have the capability to induce normal fibroblasts to change into reactive phenotypic myofibroblasts.<sup>26</sup>

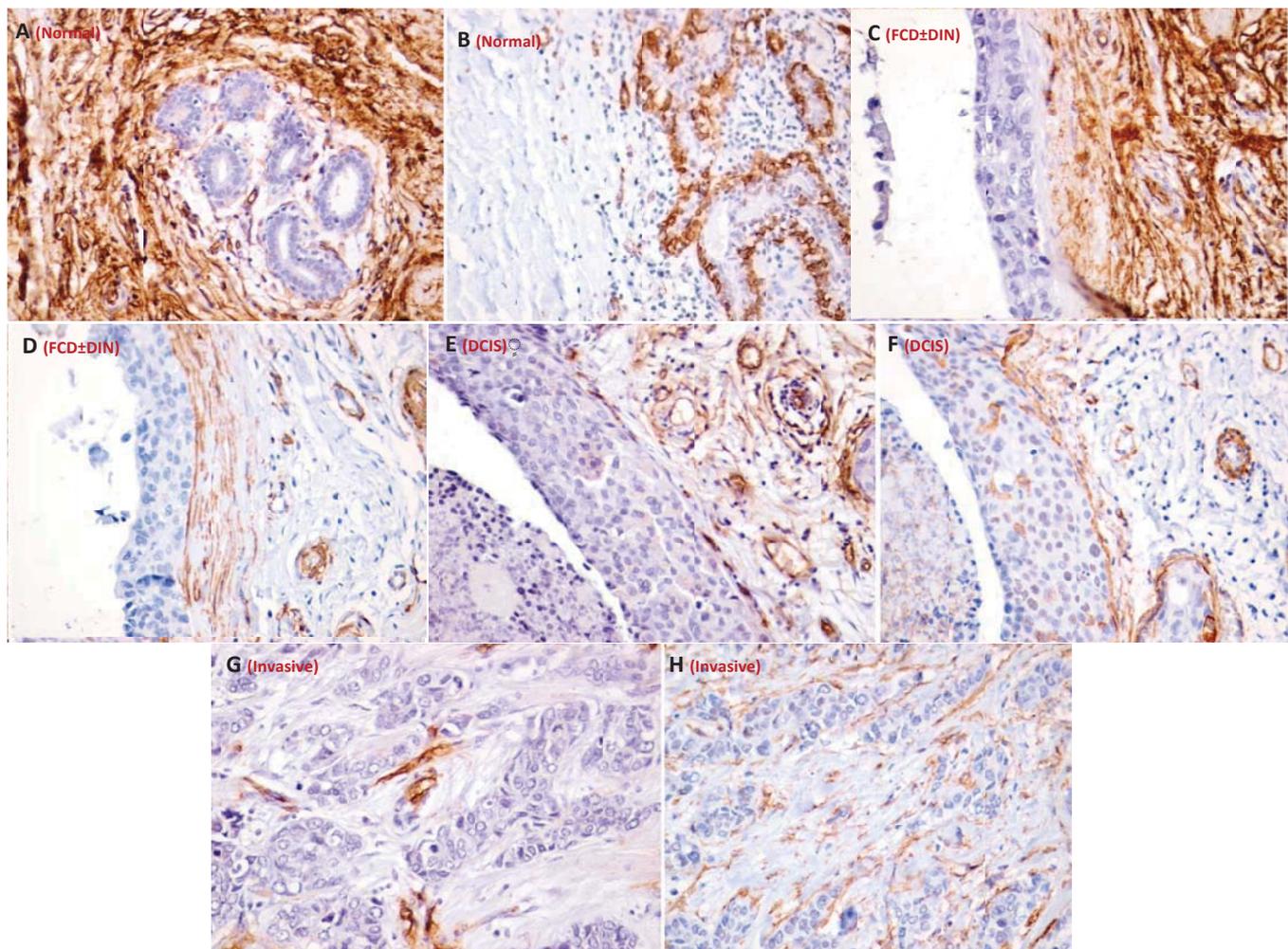
Many substances are produced by myofibroblasts such as collagen I and II, fibronectin isoforms, tenascin, versican and proteases. Metalloproteinases (MMPs), urokinase plasminogen activator and fibroblast activating factor (FAP), induced a remodeling of

the extracellular matrix (ECM) that could stimulate cancer growth and migration. Myofibroblasts secrete growth factors such as the connecting tissue growth factor (CTGF) and the transforming growth factor beta-1 (TGF beta-1) which have potent angiogenic activities.<sup>27</sup>

During synthesis of breast cancer stroma there is downregulation of some stromal genes, such as CD34 and upregulation of other genes indicative of myofibroblastic differentiation, such as smooth muscle actin (SMA).<sup>28</sup>

In this study we investigated the occurrence of SMA-reactive stromal myofibroblasts with the clinico-pathological and other common prognostic variables in relation to the type of underlying breast disease. In invasive breast cancer the stroma showed focal accumulations of SMA-positive myofibroblasts which decreased gradually in precancerous (DCIS), atypical epitheliosis and normal stroma. This accumulation of SMA-reactive myofibroblasts is not specific for invasive cancer stroma and can be observed in benign as well as in the malignant lesions of the breast that supported by other similar studies.<sup>28</sup>

We have shown that higher grade cases had higher numbers of the myofibroblasts in the tumor stroma, which was similar to results noted by Surowiak et al.<sup>29</sup> Interestingly, this positive correlation also existed in our precancerous and FCD areas.



**Figure 5.** A) Normal breast ducts show packed CD34+ vascular structures; B and D) less numbers of SMA positive myofibroblasts; C) Ductal hyperplasia and fibrocystic disease (FCD) with a similar pattern; E) In stroma adjacent to DCIS and G) invasive cancer the number of microvessels (CD34) were decreased. F and H) Increased myofibroblast numbers (SMA). Magnification: x400.

In our study the relationship between manifestation of the myofibroblasts and age was not significant as noted in the Surowiak et al. study<sup>29</sup>; however, patient over the age of 50 had less SMA-positive myofibroblasts in the invasive cancer and DCIS stroma.

As mentioned in numerous previous researches,<sup>8</sup> the number of involved lymph nodes is a known prognostic factor. In this study we have shown a direct relationship between the presences of the myofibroblasts in the stroma of the invasive cancer to axillary lymph node involvement. Since the stroma around the precancerous area also had high myofibroblast content we were unable to suggest that this was a specific prognostic factor.

We have also examined potential relationships between the manifestation of the myofibroblasts in tumor stroma, the expression of steroid receptors and HER2 in breast cancer and precancerous cells. None of the examined areas had a significant relationship between the presence of SMA-positive cells and the incidence of steroid receptors.

In the present study, however, cases that had a higher content of the myofibroblasts in the invasive cancer stroma were found to contain lower proportions of cancer cells that had steroid receptor expression. We have suggested that the myofibroblast-released factors might prevent expression of steroid receptors in breast cancer cells.

In this study we have also found that cases with higher numbers of myofibroblasts showed more intense expression of HER2 in the breast cancer cells. As mentioned in other investigations, myofibroblasts may secrete insulin-like growth factor-2 (IGF-2) and hepatocyte growth factor (HGF)<sup>30</sup> which facilitate expression of HER2 in breast cancer cells. HER2 represents one of the best recognized negative prognostic factors.<sup>31</sup> According to Surowiak et al.<sup>29</sup> we can suggest that the increased numbers of the myofibroblasts in the tumor stroma might be linked to an unfavorable prognosis in the breast cancer patients. Additionally this effect on epithelial cells could be induced by the myofibroblasts in the stroma surrounding precancerous areas.

Another key step in growth and proliferation of tumor cells, as well as their metastatic dissemination, has been shown to be preceded and facilitated by the formation of new blood vessels from preexisting vasculatures.<sup>32</sup> This phenomenon is induced by angiogenic chemokines that are produced by the neoplastic cells.<sup>33</sup> Angiogenesis has been considered an independent prognostic factor.<sup>32</sup> Higher levels of angiogenic marker molecules seem to be associated with poor prognosis.<sup>34</sup>

The current study also examined the distribution of CD34+ vessels in the stroma of the invasive and precancerous areas and its relationship with stromal myofibroblasts, common prognostic factors, and clinicopathologic variables. The vascular parameters measured (VC), using antibodies directed against CD34, were significantly decreased in malignant breast lesions compared to normal specimens. These findings have contrasted results from a study by Teo et al.<sup>35</sup> possibly, the CD34 antibody is not a specific marker for neoplastic vessels and stains both normal and newly formed vessels.

Wang et al.<sup>36</sup> have found that monoclonal antibodies to CD105 reacted strongly with the endothelium in various tumor tissues but only weakly in normal tissues, which could be used as a powerful marker of neovascularization. This was not researched in the current study.

Pavlakakis et al.<sup>26</sup> studied angiogenesis by measuring microvessel density (MVD) and observed stromal microvessels before any

stromal changes. We found a high MVC in all in situ ductal carcinomas and to a lesser extent around glands that harbored atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH), whereas the myofibroblastic stromal changes were mostly observed around ducts with DCIS.

However, in our study we observed an inverse correlation between the presence of SMA-positive stromal cells and CD34+ vessels in the stroma of the invasive cancer area, whereas our data from the precancerous area support the idea of Pavlakakis et al.<sup>26</sup> who have reported that angiogenesis is present prior to prominent invasive stromal changes.

We also did not find any significant relationship between distribution of microvessels in stroma of invasive cancer and precancerous areas to age or tumor size. However, patients over the age of 50 years who had with tumor sizes less than 5 cm showed higher numbers of CD34+ microvessels in DCIS stroma.

Estrogen deprivation has been shown to result in a marked reduction in angiogenesis, which returns to pretreatment levels following estrogen replacement.<sup>32,37</sup> The number of microvessels was significantly greater in the estrogen receptor-negative tumors in a study by Parentes-Vieira et al.<sup>37</sup> In the current study, there was no significant difference observed between mean MVC values in estrogen and progesterone receptor-positive and negative tumors. Paraffin blocks kept in storage for a long time may result in loss of antigenicity for some markers.<sup>39</sup>

According to Ludovini et al.<sup>40</sup> there were significant positive correlations observed between MVD evaluated by CD34 antibody and HER2 over expression and the number of axillary lymph nodes involved. In our study we found an inverse significant relationship; patients with higher HER2 expression or involvement in greater than three lymph nodes had lower microvessel numbers in the invasive cancer and precancerous areas.

We have shown that cases with higher tumor grade had lower numbers of microvessels in the tumor stroma, which contrasted the results found by Erdoğan et al.<sup>41</sup> who observed no relationship between these two variables.

However, these conflicting results can be the result of the methodological problems such as inter- and intra-observer variability, tumor heterogeneity, and selection of an area with the most intense neovascularization. Absolute vessel counts are also influenced by total magnification and selection of the examination area; the skill and experience of the investigator are additional contributing factors.

We can suggest that for evaluation of MVC it is preferable to stain with specific vessel markers such as CD31, CD105, and VWF along with CD34.

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