ORIGINAL PAPER

Angiogenesis and progesterone receptor status in primary breast cancer patients: an analysis of 158 needle core biopsies

S. VAMEŞU

Department of Histology,
Faculty of Medicine, "Ovidius" University, Constanta

Abstract

Formation of new blood vessels from a preexisting vascular bed (angiogenesis) is a complex multistep process, which may also permit metastasis. Progesterone receptor is a surrogate marker for ER activity and has been used as an additional predictive factor for hormonal therapy in breast cancer. To investigate how tumor angiogenesis correlates with progesterone receptor (PR) status in breast carcinoma diagnosed on core biopsy, microvessels were counted (and graded the density of microvessels) within the initial invasive carcinomas of 158 patients. Using light microscopy, the number of microvessels was counted manually in a subjectively selected hot spot (in the most active areas of neovascularization per 400× field), and their values were separated as above or below median (low and high), without knowledge of the outcome in the patient or any other pertinent variable. When the mean values of MVD of the various groups defined by PR status were compared, significant difference was noted (P = 0.008557). When tumors were classified as high or low MVD, based on a cut-off value (30.70175 microvessels/mm^2), cases with high MVD were significantly more numerous. MVD did show a relationship with groups defined by PR status (P = 2.03076E-05). The correlation of angiogenesis with PR status may be a potential therapeutic target for the treatment and prevention of breast cancer, using antiangiogenic molecules.

Keywords: angiogenesis, breast carcinoma, PR status, needle breast core biopsy.

⬚ Introduction

The steroid hormone progesterone is a key component in the complex regulation of normal female reproductive function. Broadly speaking, the major physiological roles of progesterone in the mammal are [1] in the uterus and ovary: release of mature oocytes, facilitation of implantation, and maintenance of pregnancy, by promotion of uterine growth and suppression of myometrial contractility; [2] in the mammary gland: lobular-alveolar development in preparation for milk secretion and suppression of milk protein synthesis before parturition; and [3] in the brain: mediation of signals required for sexually responsive behavior.

Recent evidence also supports a role for progesterone in modulation of bone mass. The recent description of a mouse model carrying a null mutation of the progesterone receptor (PR) gene [1] has served to answer many of the complex questions of progesterone action *in vivo* and has confirmed the importance and diversity of roles of progesterone in normal female development and reproduction.

It has been well documented that estrogen and progesterone are also important in breast tumorigenesis [2–4], and their effects on the breast are mediated by their respective receptors, the estrogen receptor (ER) and the progesterone receptor (PR) [5–8].

Like ER, PR has been measured in breast cancer patients as a "standard" biomarker for a long time, initially by ligand–binding assay and for over a decade by immunohistochemistry. Panels of experts for both the College of American Pathologists and the American Society of Clinical Oncology have recommended that both ER and PR must be measured in all primary breast cancers to select patients for therapeutic and adjuvant hormonal treatment [9, 10].

Both are relatively weak *prognostic* factors, but strong *predictive* factors for response to adjuvant and therapeutic hormonal therapy. The primary reason for measuring these biomarkers today is the latter.

However, not all patients with ER-positive breast cancers derive benefit from endocrine therapy. Therefore, additional markers for response to endocrine therapy have been sought. Since ER induces progesterone receptor (PR) expression, it has been studied as a surrogate marker for ER activity and has been used as an additional predictive factor for hormonal therapy in breast cancer. The results of overview analyses of randomized clinical trials in early breast cancer have shown that PR may add to the power of ER for predicting response to endocrine therapy [11]. PR also predicts response to endocrine therapy in metastatic breast cancer [12].

Angiogenesis is the growth of new vessels from existing vasculature that occurs during development and in vascular remodeling in the adult [13, 14]. The microvasculature is a dynamic system that plays an important role in a variety of physiological and pathological processes and it switches between quiescent and activated states. New vessels can arise by several processes: angiogenesis, vascular remodeling,

and the recruitment of endothelial precursor cells from bone marrow and blood vessels [15].

Angiogenesis is a multistep process that depends upon cooperation and interaction between a variety of cells, growth factors, and components of the extracellular matrix. It requires the destabilization of existing vessels, increased permeability with extravasation of plasma proteins and enzymes into the surrounding stroma, changes in endothelial cell adhesion with endothelial cell migration, proliferation, survival, and stabilization of newly formed vascular channels. Vascular remodeling describes new vessel formation by the insertion of interstitial tissue columns into the vessel lumen, with subsequent growth of these columns and partitioning of the vessel. This process of intussusception occurs in normal developing organs and has been shown to occur in some carcinoma [16].

Co-option of endothelial progenitor cells from the circulation into new vessels is known to occur in development, but may also occur in adults. Angiogenesis is recognized as a key factor in the progression of invasive tumors, as enunciated in the "angiogenesis progression" hypothesis [17].

The pathways controlling the switch to an angiogenic phenotype in tumors are complex and poorly characterized but include hypoxia, genetic mutation, and stromal and inflammatory cell responses. There is evidence that changes in oncogene and tumor suppressor gene expression influence new vessel growth during tumor progression [18].

In many tumors, including breast cancer, areas of increased tumor cell proliferation are associated with areas of increased microvessel density ("hot spots") [19]. Not only do new vessels supply oxygen and nutrients to metabolically active tumor cells, but also there is strong evidence for the presence of reciprocal interactions between tumor cells and endothelial cells.

Stereotactic core needle biopsy (SCNB) is a faster, less invasive, and less expensive alternative to surgical biopsy for the diagnosis of breast lesions, and its results have high concordance (87–96%) with those of histopathologic findings at surgery [20–24].

Purpose

This retrospective study was to evaluate the correlations between intratumoral microvessel density (MVD) and PR status, in order to identify those tumors with a prominent angiogenic phenotype. It would be important advances if high MVD could be used to help in predicting the prognosis of patients, particularly in high-risk individuals.

⊟ Patients and methods

Selection of cases

The histological slides of non-palpable, mammographically detected lesions, in which percutaneous stereotactic biopsy was performed from January 2004 until December 2004 in SAPAG Hautepierre, Strasbourg, France, were retrospectively reviewed.

Lesions were defined as non-palpable when patients, surgeons, and the SCNB examiner (a radiologist) could not palpate any breast lesion during physical examination.

For all cases, mammography and ultrasonography reports and films were collected for review. In addition, medical charts were reviewed to verify that none of the patients included in the study had clinical evidence of malignancy or a history of ipsilateral breast carcinoma and to collect clinical information, such as age, family history of breast carcinoma, parity, hormone replacement therapy received, and history of contralateral breast carcinoma.

To be eligible for this retrospective study, women had to have undergone a SNCB of a primary breast cancer. The criteria of inclusion in this study was: female sex, age older than 21 years, not pregnant, suspicious lesion of the breast (mammography), patient with node-negative breast cancer, recommendation for excisional after mammography.

Mammographic lesions were categorized according to the *Breast Imaging Reporting and Data System (BI-RADS)* developed by the *American College of Radiology* [25].

Biopsy procedure

Radiologists trained in mammography using a dedicated stereotactic breast biopsy system, an automatic biopsy gun, and a 14-gauge biopsy needle with a long throw (2.3 cm excursion) performed stereotaxic localization.

The core needle biopsy was performed by first cleansing the skin overlying the lesion with alcohol; this was followed by skin and subcutaneous infiltration with approximately 1–2 mL of 1% lidocaine.

Usually one to three biopsies were taken from different areas in each lesion utilizing the same biopsy instrument. The core needle biopsy specimens were removed from the trough in the stylet by rinsed in a container filled with sterile saline. Surgical clip was placed in patients when the entire lesion was removed by the needle core biopsy.

Tissue specimens

It was obtained a mean of 2.6 specimens (range, 1 to 8) per lesion. To document the presence of calcification the core specimens were radiographed. Then the core specimens were fixed in 10% formalin, paraffin embedded, sectioned, leveled ×3, and stained with Hematoxylin and Eosin. Additional levels were requested, if necessary, for histological documentation of calcification. The use of a polarizing lens assisted in the microscopic identification of microcalcification in some cases. Two pathologists retrospectively reviewed the histological slides. At the retrospective review, the pathologists knew each lesion was later excised but did not know the excisional diagnosis.

Histological review

The same senior pathologist (SAPAG) in almost all cases made the original diagnosis of invasive malignancy.

For these cases, Hematoxylin and Eosin–stained slides of core biopsy samples were retrieved from the pathology archives, and reviewed by a second pathologist (S.V.) to confirm the diagnosis of invasive malignancy. Diagnoses were confirmed in all cases.

Immunohistochemical evaluation and scoring

Antibody

For the detection of progesterone receptor, the mouse monoclonal antibody (Novocastra, UK) was used. All the dilutions were done in phosphate buffered saline (PBS).

The antibody, clone, dilution, pretreatment conditions, and source for immunohistochemical studies are listed in Table 1.

Table 1 – *Antibody used for immunohistochemistry*

Antibody	Clone	Source	Dilution	Staining	Pretreatment
Progesterone receptor	1A16	Novocastra	1:100	N	H

N – nuclear staining, H – heating, 0.01M citrate buffer (pH 6.0).

Immunohistochemical staining

Immunohistochemical staining was performed on 10% formalin-fixed deparaffinized sections using the streptavidin–biotin method.

Immunohistochemistry was performed using an automated immunostainer VENTANA (NexES) according to the manufacturer's instructions. This system uses capillary action to draw up reagents to cover the specimens on the specially prepared slides.

Briefly, 4 μm sections were cut from the paraffin embedded blocks using a microtome. The glass slides were previously coated with poly-L-lysine. The sections were then incubated at 37°C overnight.

Thereafter, the sections were deparaffinised in xylene (30 minutes, twice), sequentially dehydrated by incubating in 1:1 xylene–alcohol mixture, 100% alcohol, 90% alcohol, 70% alcohol, 50% alcohol, 30% alcohol and 1×PBS (10 minutes each).

The slides were subjected to heat-induced epitope retrieval by immersing them in 0.01 M boiling citrate buffer (pH 6.0) in a pressure cooker for 3 minutes. They were subsequently cooled with the lid on for an additional 10 minutes.

After removing the lid, the entire pressure cooker was filled with cold running tap water for 2 to 3 minutes or until the slides were cooled. At 36°C, the stainer sequentially added an inhibitor of endogenous peroxidase, the primary antibodies (32 minutes), a biotinylated secondary antibody, an avidin–biotin-complex with horseradish peroxidase (30 minutes), 3,3'-diaminobenzidine (3,3'-diaminobenzidine tetra-hydrochloride) (15 minutes).

The sections were counterstained with Mayer Hematoxylin, dehydrated, cleared in xylene, and mounted.

The normal breast tissues adjacent to the tumor areas served as an internal control.

Negative controls were obtained by staining protocols omitting the first antibody, or by using non-immune mouse sera in place of the first antibody.

Interpretation of staining results

In almost all cases, immunoreactivity was evaluated semiquantitatively by the same senior pathologist (SAPAG).

Immunoreactivity was re-evaluated semiquanti-tatively by one pathologist (S.V.), and the interobserver concordance was more than 90%. Both pathologists were blinded to the clinicopathologic data and patients' outcome.

The type and distribution of immunostaining for progesterone receptor was recorded and compared to normal ductal breast epithelium present on the same slide.

The number of positive cells in 500 tumor cells within 4–6 non-overlapped microscopic fields at 400× magnification was counted.

Only nuclear immunostaining was interpreted as a positive result. Cytoplasmic reaction, if any, was ignored.

Immunostained slides were evaluated by light microscopy and the immunohistochemistry signal was scored using the so-called „Allred Score" [26]. Briefly, a proportion score was assigned representing the estimated proportion of positive staining tumor cells (0 = none; 1 <1/100; 2 = 1/100 to <1/10; 3 = 1/10 to <1/3; 4 = 1/3–2/3; 5 => 2/3). Average estimated intensity of staining in positive cells was assigned an intensity score (0 = one; 1 = weak; 2 = intermediate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0–8.

Based on these results, tumors were defined as PR-positive if their total IHC score was greater than 2 (corresponding to as few as 1% to 10% weakly positive cells) and PR-negative if their score was 0 or 2.

Quantification of tumor vascularity

Microvessel counts and density scoring were performed manually as a single microvessel count by light microscopy in areas of invasive tumor, without any knowledge of the subjects' previous investigations or clinical outcome, using a procedure based on a modification of the method by Weidner N *et al.* [27].

The slides from each tumor were at first scanned at 40× magnification, using a light microscope Olympus BX60 to select areas with the densest vascularization (hot spots). Normal mammary tissue, large areas of inflammation, granulation tissue, and tumor necrosis were excluded.

Vascularity was defined by the number of microvessels (capillaries and small venules) per area counted in the fields of highest vascular density ("hot spots") at 400× magnification.

After the individuation of the hot spots within the tumor, three adjacent, non-overlapping fields from each section were selected using a high-power magnification (40× objective and 10× ocular, 0.152 mm^2 per field). The count performed was the field thought to contain the highest number of microvessels found at low magnification, and each subsequent count was the field thought to be the next highest. MVD was quantified as the sum vessel count of the three fields (3×0.152 mm^2) from each tumor.

Microvessel counts and density scoring were repeated "blind" four months later and no discrepant results were found.

All microvessel counts were standardized. The standardized microvessel score was expressed as counts per square millimeter and was obtained by dividing the actual count by the size of three-microscope field (0.456 mm^2).

Statistical analysis

Descriptive statistics compared the microvesel density and PR status. Results are reported as mean ± standard deviation, medians and ranges for the microvessel counts performed for each subsets. A P-value equal to or less than 5% was considered statistically significant.

Independent group *t*–tests were used to compare the two patient groups on both the continuous and the ordinal measures. χ^2 tests of independence or Fisher's exact test was used to compare the two groups in regard to the categorical data.

If the *t* value that is calculated is above the threshold chosen for statistical significance (usually the 0.05 level), the null hypothesis that the two groups do not differ is rejected in favor of an alternative hypothesis, which typically states that the groups do differ.

Results

A total of 158 women met the eligibility criteria for this report. The progesterone receptor profile of the breast primary tumors was recorded in Table 2.

Table 2 – *Distribution of cases according to PR status*

Progesterone receptor (PR)	Frequency
Negative	90 (56.96%)
Positive	68 (43.04%)

Among the 158 case patients, 53 (33.54%) were PR$^-$ (Figures 1 and 2), and 105 (66.46%) were PR$^+$ (Figures 3 and 4).

The MVD ranged from 19.73684 to 72.36842 microvessels per mm^2 (median 30.70175, mean ± SD: 35.29591 ± 11.52149) for all patients. Thus, the cutoff was defined to be less than 30.70175 microvessels per mm^2 at 400× magnification. In this study low-MVD was defined as less than 30.70175 microvessels per mm^2 and high-MVD at least 30.70175 microvessels per mm^2.

The median microvessel density was 35.08772 microvessels per mm^2 (range: 19.73684 – 72.36842 microvessels per mm^2, mean ± SD: 37.3538 ± 11.7313) in patients with PR$^-$ status, 30.70175 microvessels per mm^2 (range: 19.73684 – 63.59649 microvessels per mm^2, mean ± SD: 32.5722385 ± 10.725086) in patients with PR$^+$ status.

In total, there were 53 (33.54%) patients in the low-MVC group and 105 (66.46%) in the high-MVC group, 22 cases in the low-MVC group and 68 in the high-MVC group in patients with PR$^-$ status, 31 cases in the low-MVC group and 37 in the high-MVC group in patients with PR$^+$ status (Table 3 and Figure 5).

Table 3 – *Correlation of groups defined by PR status with MVD in 158 patients with breast carcinoma*

MVD	PR status		Total (%)	P value[*]
	negative	*positive*		
Low (%)	22 (41.51)	31 (58.49)	53 (33.54)	2.03076E-05
High (%)	68 (64.76)	37 (35.24)	105 (66.46)	
Total (%)	90 (56.96)	68 (43.04)	158	

Data are no. of patients. [*]The χ^2 was used to evaluate the correlation between PR status and MVD. $P < 0.05$ indicates statistical significance.

When the mean values of MVD of the various groups defined by PR status were compared, significant difference was noted ($P = 0.008557$, *t*-Test: Two-Sample Assuming Unequal Variances).

MVD did show a relationship with groups defined by PR status ($P = 2.03076E-05$, Chi-square test).

Discussions

The goal was to study the relationship between angiogenesis and PR status, which is in contrast to other studies that assessed angiogenesis as a prognostic factor.

The College of American Pathologists considers PR to be a Category I breast cancer prognostic factor, meaning that it is of proven prognostic importance and is useful in patient management [9].

The major developmental role of progesterone in the normal breast has been postulated to be the formation of lobular-alveolar structures during pregnancy [1]. This is supported by the observation that mammary glands in PR null mice develop ductal structures that are relatively similar to the wild type but fail to form an interductal lobular-alveolar structure upon exposure to estrogen and progesterone [28].

The influence of progesterone is likely to be proliferative in this process, mediated by progesterone regulation of cell cycle genes, growth factors, and growth factor receptors. Progesterone also exerts a differentiating effect on the breast through its role in lactation. The role of progesterone in differentiated function at other times has not been extensively explored.

Progesterone effects are mediated by its nuclear receptor. Receptor proteins that specifically bind progesterone, and are induced by estrogen, were initially characterized in the mammalian uterus and chick oviduct in the early 1970's [29–32].

In comparison with the uterus there is less known of the mechanisms through which progesterone exerts its effect in the breast, primarily because of the difficulty of the normal breast tissue obtaining and the relative paucity of models of progesterone action in the normal breast.

Breast cancer cells have been used extensively as models to examine the role of growth factors and growth factor receptors in mediating progesterone effects. However, the limitation of studying progesterone regulation of gene expression in malignant cells, often derived from metastatic lesions, is the difficulty in extrapolating results to the normal breast. An illustration of this is the difference in progesterone effects on the PRL receptor in breast cancer cells and normal mammary gland.
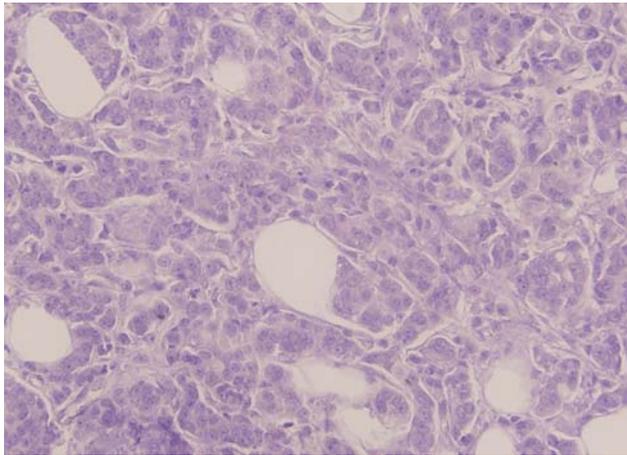
Figure 1 – 51-years-old women with invasive ductal carcinoma. Complete absence of PR immunoreactivity, 400×
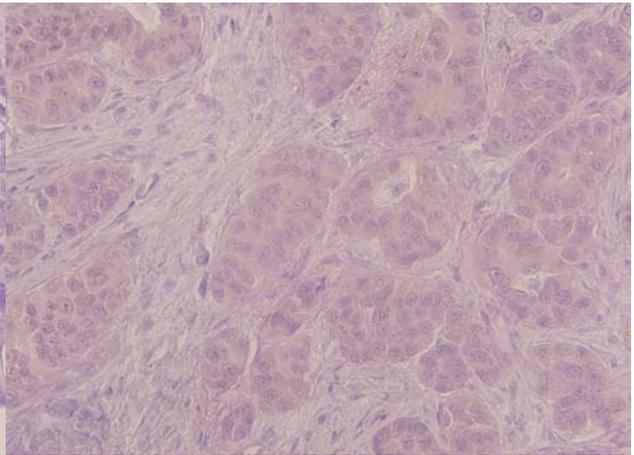
Figure 2 – 46-years-old women with invasive ductal carcinoma. PR-positive cells showed a dark brown or black nuclear signal, 400×. This tumor would get a total immunohistochemical score of 2 (proportion score [= 1] + intensity score [= 1])
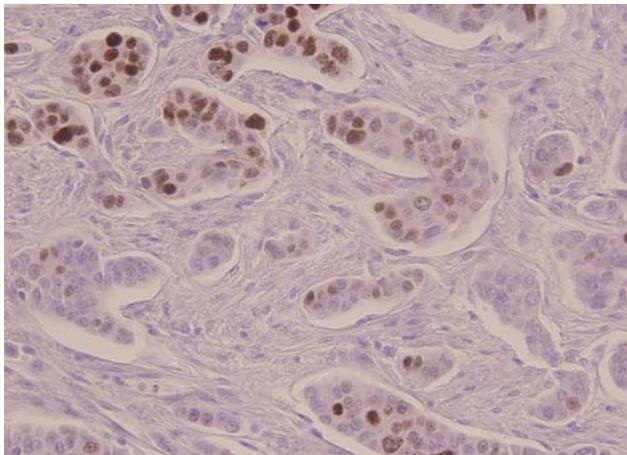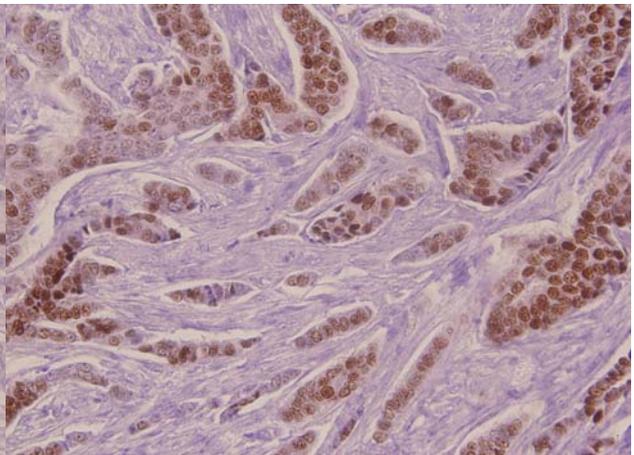
Figure 3 – 49-years-old women with invasive lobular carcinoma. PR-positive cells showed a dark brown or black nuclear signal, 400×. This tumor would get a total immunohistochemical score of 6 (proportion score [= 3] + intensity score [= 3])

Figure 4 – 43-years-old women with invasive lobular carcinoma. PR-positive cells showed a dark brown or black nuclear signal, 400×. This tumor would get a total immunohistochemical score of 8 (proportion score [= 5] + intensity score [= 3])
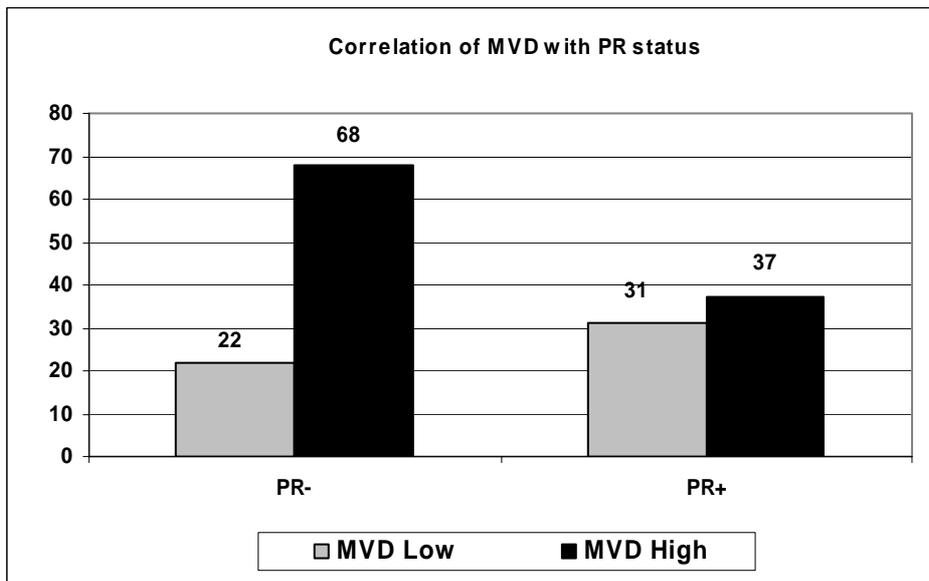

Figure 5 – Number of tumors with low and high microvessel density as a function of PR status

In addition to the developmental role of progesterone in formation of lobular-alveolar structures, there is an increasing body of *in vivo* evidence that supports a role for progesterone in the induction of cyclical proliferation in the breast. A number of studies have examined the effects of cyclical hormonal changes during the menstrual cycle on DNA synthesis in normal breast epithelium, and there is general agreement between studies that an increase in DNA synthesis is seen in the late luteal phase of the natural cycle [33–37].

The increase in DNA synthesis is consistent with the observation of a cyclical increase in the number of epithelial mitoses, which peaks toward the end of the luteal phase and is followed by an increase in apoptotic activity [38, 39].

These *in vivo* data are further supported by the observation that high circulating progesterone levels during pregnancy are responsible for inducing marked lobular-alveolar development of the breast in preparation for lactation. In contrast, a recent study examined proliferation in breast tissue from patients who had received percutaneous estrogen and progesterone administration to the breast before surgery. Epithelial mitoses and expression of proliferating cell nuclear antigen were lowest in progesterone-treated samples, compared with untreated controls and those receiving estrogen or estrogen plus progesterone [40].

However, these data should be interpreted with caution: while this is clearly an *in vivo* study, the duration of hormone administration, the percutaneous route of administration, and the levels of hormones applied were likely to result in tissue levels of hormones different from those found during the menstrual cycle, with attendant difficulty in extension of these effects on proliferative parameters to those observed in the breast during natural cycles.

In vitro studies of the involvement of progesterone in breast epithelial proliferation have produced inconsistent results [41]. Although estrogen consistently increases proliferation of normal breast epithelium *in vitro*, the progesterone effects either alone or combined with estrogen have been variable. Progesterone has been found to increase DNA synthesis in normal mammary epithelium in organ culture [42]. However, progesterone either decreases, or has no effect on, the proliferation of normal breast epithelium explanted into nude mice [43, 44].

The mechanism of PR action may also depend on an array of other proteins, such as the recently described nuclear receptor coactivators and corepressors. Nuclear receptors interact with coregulatory proteins, which may function as intermediates in transcription [45].

If they play a role in the transcriptional activity of PR, it is likely that they will be expressed in progesterone target tissues, although this has yet to be described. The role of coregulatory proteins in progesterone action needs further investigation to clarify whether progesterone regulates coregulatory protein expression and whether tissue levels of coregulatory proteins play a role in modulation of progesterone action.

Tumorigenesis is a multistep process that requires the acquisition of certain properties common to all tumors. These properties include uncontrolled cell division, suppression of senescence, inhibition of apoptosis and induction of angiogenesis [46].

The role of angiogenesis in the development and progression of human cancers has been widely studied [47].

New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated [48, 49]. This investigation was stimulated by the conflicting conclusions of some studies.

In this study, cases with high MVD were significantly more numerous.

Although significant progress has been made in understanding the physiological actions of progesterone in the mammalian reproductive system and the molecular structure and function of PR, there are still marked gaps in knowledge.

There is a great deal to be learned about the biology and hormone responsiveness of the normal breast, and the relative paucity of models has been a limitation in this regard. Importantly, there is a need for more information on the human breast, both normal and malignant, to provide baseline information that will be beneficial in model development, as well as in a better understanding of the physiology of hormone action in the breast.

The *College of American Pathologists* considers angiogenesis to be a Category III breast cancer prognostic factor, meaning that it is a factor not sufficiently studied to demonstrate their prognostic value [9].

Further studies to determine whether a specific number of microvessels within the primary tumors of patients with PR$^-$ or PR$^+$ status is predictive of occult metastasis is warranted, because this information could improve selection of patients for elective lymph node dissection and adjuvant therapy.

⌑ Conclusions

Neovascularization permits, but does not guarantee, progressive tumor spread. The quantitation of tumor angiogenesis and PR status in the primary tumor at the time of first diagnosis may be useful in predicting the prognosis of patients. Such information might prove valuable in deciding whether to administer adjuvant therapy to node-negative patients with breast carcinoma, a subject of considerable controversy and open the door to the development of other novel approaches to the treatment of breast cancer using antiangiogenic molecules.

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Corresponding author

Sorin Vameşu, Assistant, MD, PhD candidate, Department of Histology, Faculty of Medicine, "Ovidius" University, 58 Ion Vodă Street, 900573 Constanţa, Romania; Phone/Fax +40241–672 899, E-mail: sorinvamesu@yahoo.com