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# Novel DNA-methylation response marker vitronectin: Assessment in tissue specimens of normal and cancerous breast tissue by immunohistochemistry

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# 1. INTRODUCTION

## 1.1 Background: breast cancer epidemiology

All women, regardless of their racial or ethnic origin or heritage, are at risk of developing breast cancer. Variations in breast carcinoma incidence rates among different populations suggest that etiologic factors differ in their biologic expression and impact on disease outcome. In 2000, Hunter et al. noted that key factors affecting breast carcinoma development are the roles of genetics and the environment, the reproductive experience and the effects of endogenous and exogenous hormones (oral contraceptives) in women, the change in immune status and host vulnerability.

Cultural dynamics, geographic location, sociodemographic differences, and behavioral characteristics across population subgroups also modulate how biologic disease is expressed among different races and ethnic groups. This is evident from studies of migrants, which show quite clearly that incidence rises following migration from low- to high-incidence countries, particularly if this happens at young ages. Incidence rates are high in most of the developed countries except for Japan, with the highest age-standardized incidence in North America [Parkin et al., 2005]. The American cancer statistics of 2005 reported a continued increase in female breast cancer incidence rates since the mid1990s, although at a slower rate in the last couple of years. On the one hand, this trend is attributable to improved and earlier-applied detection methods, in particular mammography, making diagnosis more likely. On the other hand, increased prevalence of obesity and greater use of hormone replacement therapy after the menopause do also play a role [Jemal et al., 2005]. In contrast, the rates are low in the least affluent world areas, most of Africa and in most of Asia. Incidence is lowest in Central Africa [Global Cancer Statistics, 2002].

Stage, a measure of disease status, is used to assess prognosis, plan treatment, and evaluate outcome. Behavioral attributes unique to a particular multicultural population as well as societal issues such as access to care, dietary and socioeconomic conditions, all have an impact on the health measure called “stage at diagnosis “. The breast carcinoma is by far the most frequent malignant tumor in women in the western countries (about 25% of female malignancies, followed by

endometrial and cervical carcinoma), and, according to the American cancer statistics of 2005, the leading cause of death among women aged 20 to 59 years in the USA [Jemal et al., 2005].

According to Ferlay and Boyle [Annals of oncology 2005], the same applies to Europe, where breast cancer is followed by colorectal and lung cancer. These three represent the most commonly diagnosed forms of cancer, accounting for two-fifths of the total European cancer burden. Numerous studies have also reported a more advanced stage of breast carcinoma at diagnosis especially among women from African and African-American cultures, suggesting the possible influences of disparities in access to and receipt of quality health care [Hunter, American Cancer Society, 2000].

Approximately, every tenth woman develops breast cancer in her lifetime.

According to the report of the International Expert Consensus on breast cancer of 2005 by Goldhirsch et al., overall breast cancer mortality is decreasing in many countries, reflecting increased awareness and better treatment. However, the mortality for advanced metastatic breast cancer has remained essentially unchanged in the last couple of decades [Hölzel et al., Deutsche Aerzteblatt Nr. 40, 2005], even if quite recently some progress could be noted in the treatment of the subgroup of HER-2/new positive tumors with the humanized antibody Herceptin [Harbeck et al., 2003; Steger et al., New England Journal of Medicine, Oct. 2005]. Breast cancer incidence continues to rise worldwide even in countries where it is relatively low, such as India, Vietnam, Korea, Thailand, China and Gambia. Because of its high incidence and relatively good prognosis, breast cancer is the most prevalent cancer in the world today; there are an estimated 4.4 million women alive who have had breast cancer diagnosed within the last five years [Ferlay et al., 2004].

However, rapid advancements in knowledge of cancer biology and of genetic markers and tumor products are providing new mechanisms for identifying etiologic pathways that can be utilized for better screening, detection, treatment and monitoring of disease. Care for patients with breast cancer is essentially multidisciplinary, and there is an important general trend to more selective interventions to minimize acute and late toxicity without compromising efficacy. In particular, advancements in adjuvant therapy and the benefits of early detection

through screening leading to earlier stage diagnosis, especially in younger women, have contributed to a reduction in the overall mortality rate.

## 1.2 Risk factors of breast cancer

Breast cancer is a major public health burden world-wide and a variety of risk factors are involved in its etiology and development. The etiology of breast cancer has been investigated for decades and some cases can be explained by risk factors. Research conducted in different populations distinguishes between well-established and probable risk factors for breast cancer. The well-confirmed risk factors are a family history of breast cancer, genetic disposition (BRCA1/2-gene mutations), age, exposure to ionizing radiation, geographical location (USA and other western countries), history of benign breast disease, reproductive events (late age of menopause, over 54 years, early age of menarche, less than 12 years, nulliparity and advanced age at first pregnancy), high mammographic breast density, exogenous hormones (hormone replacement therapy, use of oral contraceptives), tall stature, lifestyle risk factors (alcohol, diet, obesity and lack of physical activity), high prolactin and insulin-like growth factors (IGF-1) levels.

Age, a risk factor for any type of cancer, combined with geographical location or country of origin, are strongly associated with breast cancer risk. The incidence of breast cancer is low before age 25 (less than 10 new cases per 100,000 women) and rises up to 100-fold by age 45 [Hulka et al., 2001]. It can be summarized that the cumulative risk of breast cancer increases with age [Feuer et al., 1993]. One possible reason would be the influence of hormones, which increases during the reproductive period.

!n 1998, Yang et al. noted that 5 to 10% of women have a mother or sister with breast cancer and about 10 to 20% of women do have a first-degree or a second-degree relative with a history of breast cancer, respectively. A family history of breast cancer remains a major well-established risk factor. These facts are confirmed by studies which estimate the relative risk of getting breast cancer with an affected first-degree relative at 2.1% (95% confidence interval (Ci) 2.0 – 2.2). The risk of developing breast cancer increases with the number of affected relatives and the closeness of their biologic relationship [Pharoah et al., 1997; Colditz et al., 1993].

The genetic disposition is also an etiologic factor which must be taken into account. Germ-line mutations in high-penetrance susceptibility genes like BRCA 1/2 and p53 are linked to a high risk of getting hereditary breast cancer. These mutations make up only 5–10% of breast cancers [Easton et al., 1993]. The majority of cancers are sporadic cancers which derive from an interaction between low-penetrance cancer susceptibility genes with endogenous and lifestyle risk factors [Johnson-Thompson et al., 2000]. Hereditary breast cancers are mainly diagnosed at an earlier age, being often multifocal in contrast to sporadic cancers which are bilateral and arise at advanced age [Rebbeck et al., 1999]. Depending on the stage of diagnosis, a history of benign breast disease could increase the risk of breast cancer. A previous primary breast cancer means a 3-fold to 4-fold increase in risk of developing a second breast cancer in the contralateral breast. While the risk of contralateral breast cancer persists for up to thirty years after the original diagnosis, the median interval between primary breast cancer and contralateral disease is approximately 4 years. Lobular carcinoma in situ (LCIS), which is often an incidental finding in breast biopsies, is associated with an increased risk of subsequent invasive cancer. Risks are higher for women diagnosed at a younger age and for those with a family history of breast cancer. Subsequent breast cancers are most often of ductal histology, and occur equally in either breast, suggesting that LCIS is a marker of risk rather than a precancerous lesion itself. The risk increases further up to ninefold if the woman also has a family history of breast cancer (first-degree relative).

The pattern, number and timing of reproductive events in a woman’s life contributes decidedly to the risk of breast cancer development. According to Colditz and co-workers, the hormonal exposure of women is influenced by different variables including age at menarche, age at first full-term pregnancy, age at menopause and higher parity. Early menarche at less than 12 years increases breast cancer risk, probably because of longer hormonal (estrogen and progesterone) exposure [Colditz et al., 1996]. Other studies indicate that high estradiol levels measured at patients with early menarche during their adolescence and lower sex-hormone-binding globulin (SHBG) after their adolescence lead to an increase of breast cancer risk [Bernstein et al., 2002]. Late menopause as well as early menarche suggests that these women are exposed to

more regular ovulatory cycles than other women during their lifetime, leading to a stronger estrogen and progesterone exposition. A protective effect was seen for an early first full-term pregnancy and also for a higher parity (3 or more). A study conducted in 2002 by the Collaborative Group on hormonal factors in breast cancer revealed that the longer women breast-feed the more they are protected against breast cancer. In contrast, nulliparity and late age at first full-term pregnancy lead to an increased risk of breast cancer. [McPherson et al., 2000; Colditz et al., 1996]

Ross and co-workers noted that the use of hormones after menopause correlated with an increased breast cancer risk, depending on exposure time and whether estrogen was used alone or in combination with progesterone. Long-term estrogen exposure and a family history of breast cancer are the two factors more consistently reported from previous studies. The use of oral contraceptives leads to a moderate increase in breast cancer risk among long-term users, but 10 or more years after stopping hormonal contraception, no difference to non-or short-term users was detectable anymore. Studies have demonstrated that independent of age at first use, dose, type of hormones within the contraceptive and duration of application, the intake of combined oral contraceptives results in a high risk of breast cancer. A report in 1996 by the Collaborative Group on hormonal factors in breast cancer revealed that current users of oral contraceptives showed a significant increase of breast cancer risk of 24%.

Fighting breast cancer, women have to take into account that lifestyle factors, which are modifiable risk factors, can be controlled by women themselves, thereby offering the chance to reduce breast cancer. These include alcohol consumption, diet, obesity after menopause, weight gain, and degree of physical activity. Alcohol increases risk of developing breast cancer. For every 10g-increment (approx. 0.75l–1l of drink) in daily consumption of alcohol the risk rises by 9% [Smith-Warner et al. 1998]. The carcinogen acetaldehyde, a metabolite of alcohol enhances procarcinogen activation and may cause breast cancer [Poschl et al., 2004]. Other findings indicate that alcohol enhances estrogen levels [Coutelle et al., 2004]. Specific diets rich in well-done meats or fat are in general correlated with a slight increase in developing breast cancer, while a high intake of fruits and vegetables decreases breast cancer risk [Zheng et al., 1998; Velie et al., 2000].

The survivors of the nuclear explosion in Chernobyl, Russia and the atomic bomb in Nagasaki and Hiroshima, Japan paid a heavy price in terms of the consequences on their health. They suffered a significantly increased cancer risk during their lifetime, which derived from exposure to high doses of ionizing radiation. Numerous retrospective studies provide strong evidence for the association between radiation exposure and breast cancer. For the same reasons, the patient's risk of breast cancer increases during therapeutic radiation treatments including fluoroscopy for tuberculosis and thymus-reduction.
Helzlsouer et al. observed that lymphocytes from affected family-members demonstrated reduced efficiency of repair of X-ray induced DNA breaks, suggesting that the breast cancers could have resulted from a genetic susceptibility to the mutagenic effect of radiation exposure [1995]. Other factors which are correlated with the risk of breast cancer include high prolactin levels and height. Studies conducted by Renehan et al. in 2004 demonstrate a relation between high IGF-levels, the anabolic effector of the growth hormone (GH) and breast cancer in pre-menopausal women.

## 1.3 Breast cancer classification

While it is evident that individual risk factors directly influence the prognosis for each patient, risk factors with their individual impact on breast cancer per se can not be considered to accurately estimate the overall risk of breast cancer formation. Furthermore, they cannot provide answers once the diagnosis *breast cancer* is established. In this case, the classical factors taken into consideration in order to determine, if or which kind of therapy is to be administered are tumor size, lymph node status, metastasis (TNM classification), morphology (histological type, grading) and steroid hormone receptor status. The strongest prognostic factor for breast cancer is the number of affected lymph nodes, which correlates directly with the risk of relapse or death. There is also a direct relation between the size of the primary tumor and the axillary nodal status [Harbeck et al., 2003]. This chapter gives an overview of the tumor features that presently play a role in the establishment of individual breast cancer therapy.

### 1.3.1 Tumor size and lymph node status

Breast cancer often spreads from the primary tumor to regional lymph nodes [Chambers et al., 2005]. Axillary lymph node status and tumor size are therefore two important parameters defining the extension and prognosis of a malignant tumor. Lymph node status provides clinically important information for treatment decisions. Spread via lymphatics is also important for the biology of breast cancer, as tumor cells in lymph nodes may provide a reservoir of cells leading to distant, lethal metastases. Improved understanding of the biology of lymphatic spread is thus important for improved breast cancer survival. Various studies by Fischer and co-workers, dating back several decades, have described the associations between large primary tumors and a positive lymph node status, and between a large tumor and shorter disease-free intervals and overall survival rate, respectively. It was supposed that tumor size rather indicates a certain stage in tumor progression than predicts the liability of a tumor to causing a relapse. Tumor aggressiveness is reflected much more accurately in the histological phenotype of tumor tissue. The determination of the axillary lymph node status is still one of the most important diagnostic means of identifying breast cancer patients with a higher risk of tumor recurrence. For this purpose, at least ten lymph nodes of levels I and II must be excised during surgery [Kreienberg et al., 2004]. The presence of lymph node metastases has long been regarded as one of the most important factors in evaluating the prognosis of breast cancer patients and was shown to correlate with disease-free and overall survival better than any other prognostic factor [McGuire, 1987; Nemoto et al., 1980].

In the past, node-positive patients were classified as “high-risk”. They usually received and profited from adjuvant systemic treatment. The recent St. Gallen consensus recommendations of January 2005 define three more specific risk categories – “low”, “intermediate” and “high” – based on endocrine responsiveness and menopausal status. The “intermediate-risk” group includes node-positive patients with up to three involved lymph nodes as well as node-negative patients with additional risk factors. In this category, the application of chemotherapy is in particular dependent on endocrine response [Goldhirsch et al., 2005]. With respect to node-negative patients, classification and therapeutic procedure remains controversial, as there is a significant percentage – approximately 30% – at risk of

suffering a relapse. Today, following the National Institutes of Health (NIH) [Eifel et al., 2001] and St. Gallen [Goldhirsch et al., 2005] consensus guidelines, up to 90% of node-negative patients receive adjuvant chemotherapeutic treatment. Evidently, there are a considerable number of unduly over-treated patients who could have been spared the unpleasant side-effects of chemotherapy. Additional prognostic factors are necessary to identify the group of patients with increased risk of relapse and thereby help to find the best possible treatment. For node-positive patients, predictive factors of the response to a particular adjuvant therapy may be useful to find alternative therapeutic approaches.

### 1.3.2 Histopathological classification

Pathomorphological parameters are important for breast cancer prognosis. Breast cancer is a histologically heterogeneous disease [Arpino et al., 2004]. The histological origin of the patient's primary tumor is therefore one of the most decisive components for her individual prognosis. Breast carcinomas derived from ductal or ductular epithelia are the most frequently diagnosed ones (85–95%). The remaining tumors originate from lobular epithelia, except the very rare and especially aggressive inflammatory carcinoma characterized by undifferentiated tumor cells and a lymphangiosis carcinomatosa of the skin, and the mammary sarcoma (less than 1%), respectively. The ductal breast carcinoma has a worse prognosis than other special types of breast cancer like the tubular, papillary and mucinous forms.

Breast carcinomas may be divided into invasive and non-invasive tumors.
The non-invasive forms – comprising the ductal carcinoma in situ (DCIS) and the carcinoma lobulare in situ (CLIS) – are considered to be early forms of invasive breast cancer. While in situ carcinomas can only be identified by apparative diagnostic measures, the majority of breast cancers are still discovered by the patients themselves, implying that the tumor stage is already advanced by the time of diagnosis. The American Cancer Society estimated that 41,000 new cases of ductal carcinoma in situ (also called DCIS or intraductal carcinoma) will be diagnosed in 2000, making DCIS the most common type of non-invasive breast cancer in women. DCIS accounts for nearly 25% of all breast cancer diagnoses.

An estimated 185,000 cases of invasive breast cancer are diagnosed each year, and approximately 20%–30% of breast cancers detected by mammography are carcinoma in situ. The invasive ductal carcinoma is the most frequent breast carcinoma. It also includes various special histological forms with own growth patterns, clinical features, and prognoses. For patients with invasive tumors without infested axillary lymph nodes, a good chance of cure does exist, although the applied adjuvant therapy schemes vary widely.

### 1.3.3 Tumor grading criteria – Elston modification of the Scarff, Bloom and Richardson system

The grade of any cancer is one of the most important pieces of information needed by physicians treating cancer. Indeed, the histological grading is important for survival without the recurrence of relapses. The Elston modification of the Scarff-Bloom-Richardson (SBR) system is the most commonly used and widely recommended type of cancer grading system. Generally, three characteristic features of a breast tumor – degree of glandular formation (percentage of carcinoma composed of tubular structures), nuclear pleomorphism and mitotic rate – are examined and evaluated by a pathologist and assigned to a total score ranging from three to nine points. The tumor grades 1, 2 and 3, distinguishing well-differentiated from poorly differentiated (or undifferentiated) tumors, are deduced from this score [Bloom et al., 1957; Scarff et al., 1968]. The “Elston grade” is calculated by evaluating the cancer for these three parameters in order to add up the designated points. For example: a tumor has 5% tubules (3 points), moderate nuclear abnormalities (2 points) and 18 mitotic figures in ten high-power microscopic fields (2 points), giving a total of 7 points. Elston grade I is defined as ranging fro 3–5 total points, grade II, 6–7 total points, and Elston grade III being defined as ranging from 8–9 points, respectively.

### 1.3.4 Steroid hormone receptors

The prognostic relevance of steroid hormone receptors in breast cancer adjuvant therapy has long been of research interest [Jensen 1971; Cooke 1979; Godolphin 1981; Clark 1983]. It is well-known that some breast tumors require steroid

hormones for their continuous growth. Colleoni, Viale and Zahrieh [Cancer Research, 2004] observed that steroid hormone receptors are indicative of endocrine responsiveness, though not all tumors expressing detectable hormone receptors will have a clinically useful response. For hormone receptor positive tumors, the anti-hormones applied include aromatase inhibitors, selective estrogen receptor modulators (SERM) like tamoxifen and estrogen receptor downregulators (ERD) – or ablative endocrine therapy. The rate of resistant tumors ranges from 30–40%. Resistance to endocrine therapy has been mainly attributed to steroid receptor polymorphism [Sluyser et al., 1992]. Tumors completely lacking such receptors were found to be sensitive to preoperative cytotoxic agents. Currently, steroid hormone receptors are the only biomarkers accepted and applied beyond clinical trials as predictors of response to endocrine therapy. This predictive function underscores their important role in breast cancer therapy.

## 1.4 Prognostic parameters of breast cancer

Prognostic parameters are important for the management of a risk-adapted and individual therapy. To date, several new prognostic factors have been discovered and are constantly being re-evaluated. A reliable marker quality is a pre-requisite for application in routine diagnostics. These factors have an influence on tumor cell growth and may predict the probability of relapse of the disease.

The need for new parameters is even more pressing with respect to nodal-negative breast cancer patients. Adjuvant radiotherapy is necessary for the one-third of patients developing a relapse, whereas the remaining, relapse-free two-thirds get cured by breast surgery. However, because a clear distinction cannot be made between these two groups of patients, prospectively, all nodal-negative patients are treated with an adjuvant therapy, radio-or chemo.

Schmitt and co-workers demonstrated that proteolytic enzymes and their receptors and inhibitors actively participate in tumor invasion and metastasis. Matrix metalloproteinases are tumor-associated and are involved in tumor progression and invasion. The serine protease uPA, its receptor uPAR, and inhibitor PAI-1 enable invasion of tumor cells and play an important role in the migration of tumor cells. High concentrations of uPA and PAI-1 in the primary tumor are accompanied by a higher risk of metastasis and a shorter total life span [Look et al., 2002].

Studies prove that nodal-negative patients with low levels of uPA and PAI-1 in the primary tumor have a good prognosis, so that adjuvant chemotherapy may not be necessary. In 2002, Harbeck and co-workers observed that breast cancer patients with high levels of uPA and PAI-1 show a better response to adjuvant therapy than those with low levels. The cell core-associated proliferation antigen Ki-67 is another parameter found in breast tumors that can be detected by immunostaining with the monoclonal antibody MIB-1 [Gerdes et al., 1984; Harbeck et al., 1998].

The oncogen c-erb-2 (HER-2/new), that was originally isolated from rat neuroblastomas, was shown to be present in human breast cancer. This new prognostic marker HER-2/new-onco-protein is overexpressed in 25% of invasive breast cancers. An overexpression based on an amplification of the HER-2/new-gene is correlated with a more aggressive evolution of the disease [Konecny et al., 2003]. A positive HER-2/new status correlates with response to a therapy with the humanized antibody Herceptin (Trastuzumab), which is directed against the HER-2/new-onco-protein. As previously stated, advanced cancer can be treated by Herceptin as adjuvant therapy, in combination with other therapeutic agents.

In contrast to steroid receptors, overexpression of the EGF receptor (EGFR) is an indicator of poor prognosis in breast cancer [Nicholson et al., 1991].

## 1.5 Tumor biology - invasion and metastasis

Breast cancer is a complex disease. In the transformation of normal cells to cancer cells, many processes are dysregulated that result in changes in the function and activity of genes. DNA damage in cells may be produced by environmental carcinogens, radiation or viruses, or by genetic alterations in genetic coding during cell division. Cells that have lost their regulatory controls continue to divide, giving rise to cancer [Hunter, American Cancer Society, 2000]. Breast cancer growth is controlled by numerous interacting factors, and breast tumors can consist of several cell populations.

Metastasis is the major cause of poor prognosis in breast cancer patients. Metastatic spread is indeed the leading cause of breast cancer deaths and spread to the lymph nodes is considered one of the earliest clinical indications that a breast tumor has a propensity for metastasis [Fidler et al., 2001; Van Trappen et al., 2002].

The process of metastasis depends on the ability of tumor cells to successfully complete a series of events that include breaking away from the primary tumor, invading the extracellular matrix (ECM), accessing the vasculature, surviving in the circulation, arresting in a new site and colonizing that site to form a metastatic tumor [Chambers et al., 2002; Pantel and Brakenhoff, 2004]. The process is illustrated in Fig. 1.

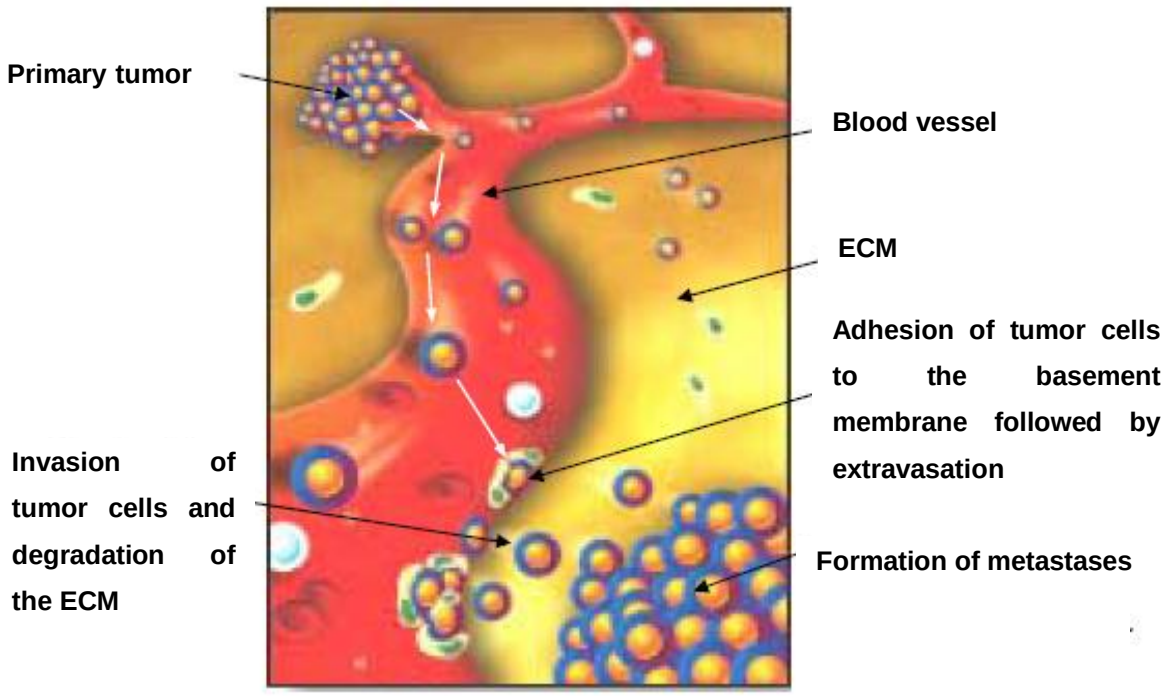


**Fig. 1** **Process of tumor invasion and metastasis**
(Graphic taken from www.dkfz.de/.../images/TumorCellInvasionLg.jp)

The ECM is a network of secreted proteins and carbohydrates that fills the intercellular spaces. It is essential for maintaining the structure and organization of tissues, but also restricts or promotes cell movement. It helps cells to bind together and consists of several major groups of molecules: collagens, fibrin, elastin, proteoglycans and glycoproteins [Teti et al., 1992].

Vitronectin (Vn) belongs to the group of adhesive glycoproteins present in blood and ECM, that play key roles in the attachment of cells to their surrounding matrix and may participate in the regulation of cell differentiation, proliferation and morphogenesis [Preissner et al., 1997; Tomasini & Mosher, 1990]. Cellular

invasion requires the proteolytic degradation of the ECM components [Fidler et al., 1997]. The serine proteases (*e.g.* of the uPA/plasmin system) are one of the major class of proteases involved in this degradation process.This chapter gives an overview of the important role that vitronectin may play in this regard.

### 1.5.1 The pluripotential role of vitronectin

Vitronectin (Vn) is an important molecule involved in coagulation, adhesion and cancer. By its localization in the ECM and its binding to plasminogen activator inhibitor 1 (PAI-1), Vn can potentially regulate the proteolytic degradation of this matrix [Schvartz et al., 1998], which is one of the first processes eventually leading to tumor cell invasion and metastasis. The ECM plays the dual role of substratum in which the cells move as well as a physical obstacle that the cells have to overcome. To degrade the physical obstacle which the ECM represents in the direction of cell migration, cells use proteolytic enzymes [Sidenius et al., 2003]. The binding of PAI-1 and uPAR to Vn suggests a functional interaction between Vn and the plasminogen activation system. The latter plays a causal role in growth, invasion and metastasis of malignant tumors. It is assumed that urokinase-type plasminogen activator (uPA) released in tumors catalyses the proteolytic conversion of the inactive zymogen plasminogen to the active protease plasmin, which in turn catalyses degradation of basal membranes and ECM, and thus facilitates cancer cell invasion into the surrounding tissue [Aaboe et al., 2003]. These observations raise the possibility that Vn may represent a unique link between cell adhesion and proteolytic enzyme cascades.

Although initially described as a plasma protein, recent studies provide evidence that Vn is present in various tissues under (patho-) physiological conditions, and that its biosynthesis may be differentially regulated in various disease states [Preissner et al., 1997]. The importance of gaining a clear understanding of the molecular aspects of Vn and its receptors in this context cannot be overemphasized.

## 1.6 Biochemical properties of vitronectin

Vn shows a high degree of conformational flexibility implicating the existence of different molecular forms of the molecule. In human blood, Vn occurs in the single-chain form with a $M_r$ of 75 kDa and in the two-chain form of $M_r$ 65+10 kDa due to an endogenous proteolytic cleavage site. The clipped form of two-chain Vn $_{65 + 10}$ is hold together by a disulfide bridge.

The majority of Vn in the circulation, about 80%, is present in the plasma form, in a "closed" conformation serving as a large pool of soluble latent Vn. A minor fraction, about 20% of circulating Vn, adopts the "opened", heparin-binding, reactive form. This form may either be associated with other Vn molecules, forming Vn multimers, or with other ligands such as the C5b-9 complex or the thrombin-antithrombin III complex. In the open form, the binding sites are accessible, thereby enhancing multimerization.

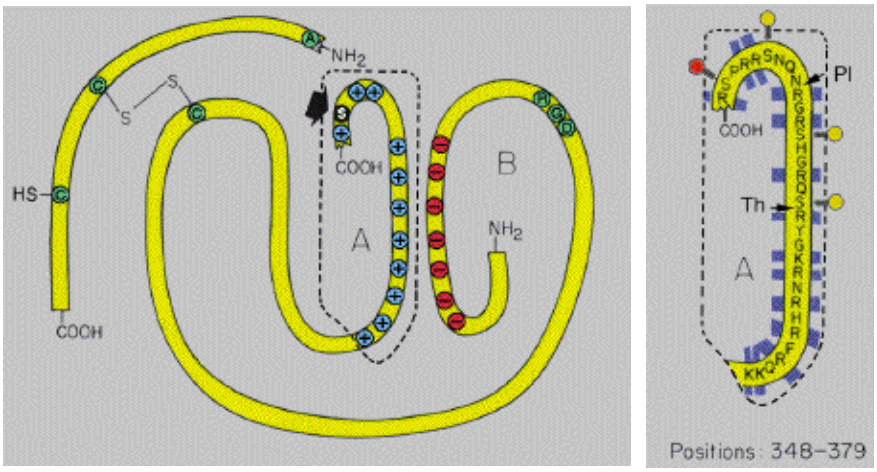


**Fig. 2** **Schematic representation of vitronectin [Shaltiel, 1996]**
**Left panel:** **Clipped form of vitronectin**
**Right panel:** **Sequence of the cluster of basic amino acids in Vn**

**Online reference:**

**http://bioinfo.weizmann.ac.il/_ls/shmuel_shaltiel/shmuel_shaltiel.html**

Vn is involved in the regulation of plasminogen activation by binding to PAI-1 and thereby stabilizing its "active" inhibitory form. Within the circulation, active PAI-1 is unstable unless it is bound to Vn. Vn slows down the inactivation of PAI-1, thereby prolonging the half-life of PAI-1, and it activates PAI-1 to inhibit fibrinolysis.

Furthermore, Vn contains an RGD-sequence, through which it binds to the integrin receptor $\alpha_v\beta_3$ and promotes cell migration, adhesion, motility and spreading.
In addition to its function in cell adhesion and complement regulation, Vn exerts several regulatory functions in the blood coagulation, complement and fibrinolytic systems. It binds glycosaminoglycans, collagen, plasminogen and the urokinase-receptor uPAR, and also stabilizes the inhibitory conformation of PAI-1 [Schvartz et al., 1998].
Vn was discovered as a 'serum spreading factor' which adheres to glass. It was also named 'epibolin' and 'S protein' and as such it was identified as an inhibitor of the membrane attack complex of complement [Schvartz et al., 1998]. It is a structural labile molecule, and its biological functions are dependent on and / or regulated by its conformational state(s) [Preissner & Seiffert, 1998]. Predominantly multimeric forms of Vn are recognized by several non-related cell surface receptors including integrins, the urokinase receptor and proteoglycans that mediate cell adhesion, migration and invasion relevant for tissue remodeling or bacterial tropism [Preissner & Seiffert, 1998].
In the plasma, Vn exists as a-*fold*ed monomer whereas in the ECM or after binding to complement factors, thrombin / antithrombin III-complexes, or active PAI-1, it exists as a disulfide linked multimer. Most of the Vn-ligands, including PAI-1, preferentially interact with the multimeric form of Vn [Schvartz et al., 1999].
A third form of Vn, the so-called two-chain Vn, is present in blood. Two-chain Vn results from the cleavage of full length Vn after R 379 by an unidentified protease and consists of two fragments connected via a single disulfide bond. Due to a polymorphism at position 381 (threonine versus methionine), various molecular forms and also amounts of two-chain Vn may exist *in vivo*, as threonine rather than methionine at this position will favor the cleavage. However, no obvious differences with respect to the multimeric state, heparin-binding activity and PAI-1 binding activity between full-length and two-chain Vn were observed [Gibson and Peterson, 2001]. Although deletion of the Vn gene is compatible with life [Zheng X et al., 1995], clarification of functions of tissue Vn are expected from challenging of Vn knock-out mice with different pathologies [Preissner et al., 1997].

## 1.7 Structure and function of vitronectin

The amino terminal segment of Vn (amino acids 1–44) is identical to somatomedin B. This domain harbours functional groups which are involved in the binding of plasminogen activator inhibitor-1 [Seiffert et al., 1997]. As seen in Fig. 3 (upper panel), following the somatomedin B domain (SMB), Vn has an Arg-Gly-Asp (RGD)-sequence (residues 45–47) through which it mediates the attachment and spreading of cells to the ECM, each via its specific integrin receptors.

**Localization of the binding domains of vitronectin**

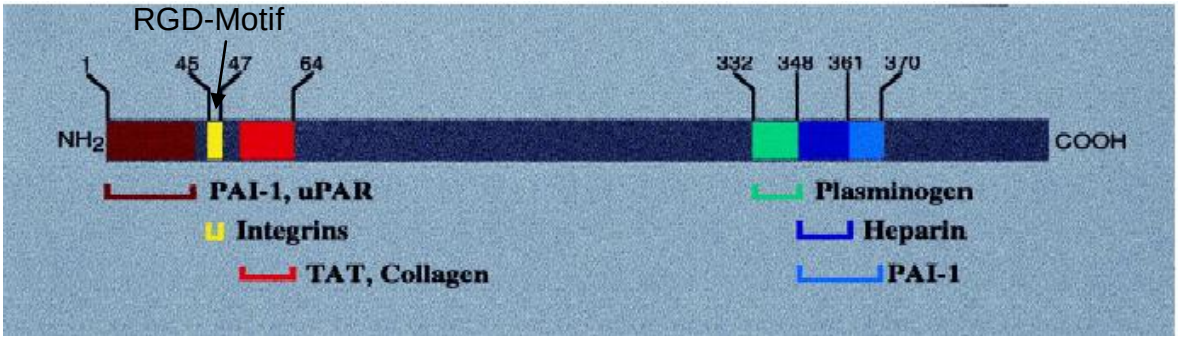


**Fig. 3 Structure of vitronectin, upper panel [Schvartz et al., 1991]**

Adjacent to the RGD sequence (residues 53–64), Vn has a stretch of acidic amino acids including two sulphated tyrosine residues (56 and 59). This segment is involved in the Vn binding of the thrombin-antithrombin III complex, and in neutralizing at least part of the polycationic domain (residues 348–379) at its carboxy-terminal end.

Ionic interaction between the above mentioned acidic and basic stretches is involved in stabilizing the three-dimensional structure of Vn, and in the formation of its multimers. Vn was reported to bind to collagen by means of functional groups from two regions: one adjacent to the RGD sequence, and the other adjacent to the heparin binding domain. The major part of the Vn molecule (residues 132–459) accommodates six hemopexin repeats. This region also anchors a disulfide bridge ($Cys^{274}$–$Cys^{453}$) linking the endogenously cleaved 10 kDa fragment of Vn (vitronectin-10) to the rest of the molecule (vitronectin-65). The carboxyl terminal edge of Vn (residues 332–348), accommodates its plasminogen binding site [Kost

et al., 1992]. Downstream of this domain, there is a cluster of basic amino acids (residues 348–376) which contains two consensus sequences for heparin binding, and is responsible for the glycosaminoglycan binding capacity of Vn (residues 348–361). This segment also harbors functional groups which are involved in the binding of plasminogen activation inhibitor-1 by Vn (residues 348–370) [Gechtman et al., 1997].

**Localization of the proteolytic cleavage and phosphorylation sites in vitronectin**

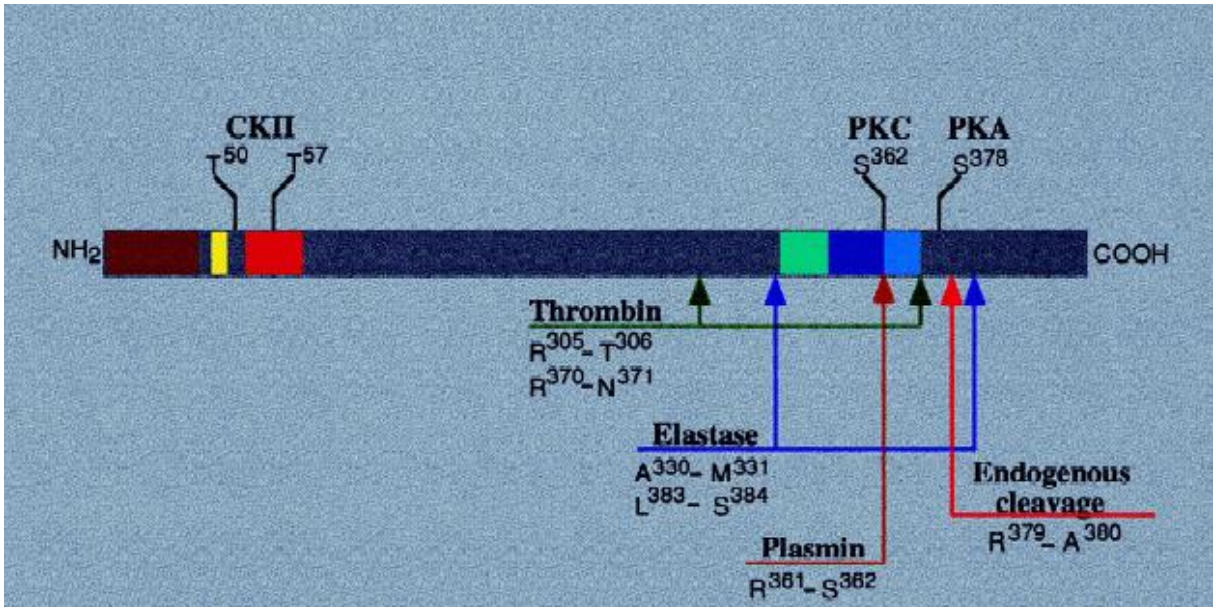


**Fig. 4 Structure of vitronectin, lower panel [Schvartz et al., 1991]**

Vn contains consensus sequences for phosphorylation by various protein kinases (Fig. 4 lower panel). cAMP-dependent protein kinase, released from thrombin stimulated platelets and also found in several blood cells as an ecto-enzyme, specifically phosphorylates Vn at $Ser^{378}$, modulating its conformation and function and abating its binding to PAI-1 [Shaltiel et al., 1993]. Vn can also be phosphorylated by protein kinase C (PKC), primarily at $Ser^{362}$. This phosphorylation attenuates the plasmin cleavage of Vn, and can thereby regulate plasminogen activation [Seger et al., 1998]. Phosphorylation of Vn by casein kinase II at $Thr^{50}$ and $Thr^{57}$ was demonstrated to promote cell adhesion and spreading [Seger et al., 1998].

Vn is predominantly found in plasma as a-*fold*ed monomer stabilized presumably by ionic interactions between its polyionic segment (53–64) and its polycationic

segment (348–376). In human blood, this adhesion protein is found in two molecular forms: a single-chain (75 kDa) and a clipped form of two chains: 65 and 10 kDa, which are held together by the disulfide bond (Cys $^{274}$—Cys $^{453}$) mentioned above. The presence of threonine rather than methionine at position 381 was proposed to be responsible for the susceptibility of Vn to cleavage at Arg$^{379}$–Ala$^{380}$ by an as yet unidentified protease. Under physiological conditions such as binding to either the thrombin-antithrombin III complex or the C5b-7 complex of the complement, unfolding of Vn presumably occurs, which exposes both the heparin binding and the amino terminal domain and leads to the formation of disulfide-linked Vn multimers [Seiffert et al., 1997].

Such Vn multimers are present in platelets releasate and in the ECM, and they were reported to exert preferential binding to several ligands, e.g. collagen, plasminogen activator inhibitor-1 or the urokinase receptor [Seiffert et al., 1997].

## 1.8 Biological functions of vitronectin

Vitronectin provides a unique regulatory link between cell adhesion and physiological proteolysis.

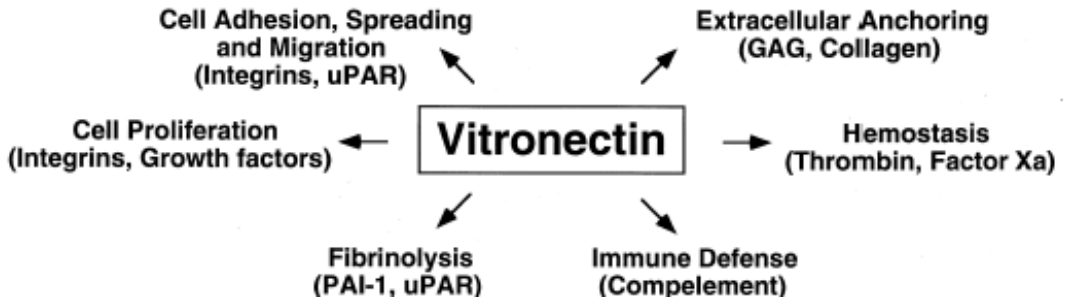


**Fig. 5 Major biological functions of vitronectin**

Vn is anchored to the extracellular matrix via its collagen binding domain, and its glycosaminoglycan (GAG) binding-domain. It promotes cell adhesion, spreading and migration by interaction with specific integrins as well as with the urokinase receptor (uPAR). The interaction of Vn with integrins, in the presence of growth factors (insulin, basic fibroblast growth factor), has been also implicated to play a role in cell proliferation.

Vn is involved in fibrinolysis, due to its ability to stabilize the active conformation of plasminogen activator inhibitor-1 (PAI-1), and to bind the urokinase receptor. It is also involved in the immune defense through its interaction with the terminal complex of complement, as well as in hemostasis through its binding to heparin and thereby neutralizing the antithrombin III inhibition of thrombin and factor Xa.
It is anchored to the extracellular matrix via its collagen binding or heparin binding domains, and it promotes cell adhesion, spreading and migration by interaction with the integrins αvβ3, αvβ5, αvβ1, αIIbβ3, αvβ6 and αvβ8 (Fig. 3). Upon binding of Vn, these integrins activate signaling pathways and regulate cytoskeletal reorganization, intracellular ion transport, lipid metabolism, and gene expression.
Vn has been shown to act as an inhibitor of the cytolytic reactions of the terminal complexes of the complement and of perforin. It is also involved in the formation and the dissolution of blood clots (coagulation and fibrinolysis). It protects thrombin from a rapid heparin-dependent inactivation by antithrombin III, and inhibits the fibrin-clot-induced activation of plasminogen by tissue-type plasminogen activator.
The binding of Vn to PAI-1 which is stabilizing its inhibitory activity is of great importance since plasminogen activation plays a role not only in key physiological processes such as fibrinolysis, cell migration and ovulation, but also in pathological processes such as tumor growth and metastasis.
In addition, Vn seems to act as a linking device between the ECM and urokinase-dependent events, directing the localization of urokinase receptors into focal adhesions so that localized urokinase mediated proteolysis can occur (Fig 6)

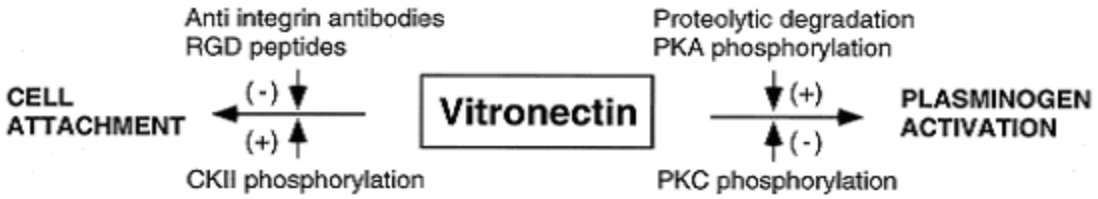


**Fig. 6 Schematic presentation of possible regulatory assignments of vitronectin**

The phosphorylation of vitronectin by cAMP-dependent protein kinase (PKA), or its proteolytic degradation by plasmin, thrombin or elastase leads to a reduced binding of plasminogen activator inhibitor-1 (PAI-1), and may thus lead to the

activation of plasminogen. PKC phosphorylation of Vn attenuates its cleavage by plasmin which probably culminates in attenuation of plasminogen activation.
The cell attachment activity of Vn can be enhanced by casein kinase II (CKII) phosphorylation or inhibited by either anti-integrin antibodies or by RGD-containing peptides.

## 1.9 Synthesis and degradation of vitronectin

Direct evidence of Vn biosynthesis, based on metabolic labeling followed by immunoprecipitation with antivitronectin antibodies, was provided for two human hepatoma cell lines: Hep-G2 and Hep-3B. In rodents, a high level of Vn mRNA is present in the liver, but also in many other organs, such as the brain, heart, skeletal muscle, lung, uterus, testis and thymus. In man, in normal organs Vn mRNA was detected exclusively in the male genital tract; however, it is expressed at high levels in tumors, indicating its playing a role in malignancy [Seiffert et al., 1997].
The susceptibility of Vn to protease degradation and the functional consequences thereby were recently studied in detail. Such degradation may take a part in the transition of Vn from an antifibrinolytic protein which binds PAI-1, stabilizes its inhibitory structure, and impedes the conversion of plasminogen to plasmin, into a profibrinolytic protein, which dissociates (‘unleashes') PAI-1, and converts it into a latent, noninhibitory protein (Fig. 4).

## 1.10 Distribution of vitronectin in health and disease

Vn is a multifunctional adhesive glycoprotein found in the circulation and different tissues.

### 1.10.1 Vitronectin in body fluids

Vn is present in normal plasma at concentrations of 200–400 µg/ml and thus makes up for 0.2–0.5% of total plasma protein. Unlike fibrinogen and fibronectin, the concentration of Vn in plasma does not significantly differ from that in serum [Preissner and Seiffert, 1997]. Reduced plasma levels of Vn have been reported in

patients with severe liver failure. In these patients, changes in plasma Vn levels closely parallel changes in biosynthetic markers of the liver parenchyma. These observations suggest that the liver is the major site of Vn biosynthesis *in vivo*.

Vn has also been found in urine, amniotic fluid and bronchoalveolar lavage fluid [Schaffer et al., 1984; Preissner et al., 1985b]. Although the Vn concentration in amniotic fluid is relatively low, its specific activity (defined as µg Vn/mg total protein) is identical to that in plasma [Schaffer et al., 1984]. The specific activity of Vn in urine is twice the one in plasma [Schaffer et al., 1984]. Although Vn is present in the lavage fluid from healthy subjects, the amount is approximately 10-fold higher in patients with interstitial lung disease, including idiopathic pulmonary fibrosis, sarcoidosis and hypersensitivity pneumonitis [Eklund et al., 1992; Teschler et al., 1993; Pohl et al., 1991]. This indicates that Vn may serve to regulate inflammatory reactions in the alveoli associated with lung disease. Cultured alveolar macrophages secrete a protein immunological identical to Vn [Pettersen et al., 1990], suggesting that the Vn in the lavage fluid may be synthesized locally by alveolar macrophages. On the other hand, the Vn concentration in the lavage fluid correlated with that of albumin [Eklund et al., 1992], indicating that this Vn is derived from leakage across the alveolocapillary membrane rather than being produced by alveolar cells. In general, there is no detectable age-dependent difference in the Vn-concentration in any of these body fluids. However, fetal cord blood contained only 60–70% of the Vn present in adult blood.

Increased plasma levels of Vn have been observed in patients undergoing orthopedic surgery and in rodents stimulated with acute phase mediators [Seiffert and Podor, unpublished observation], suggesting that Vn may be regulated like an acute phase protein. Platelets contain approximately 1µg Vn / $10^9$ platelets [Parker et al., 1989; Preissner et al., 1989], a value that could account for approximately 0.2% of Vn present in serum. Immunohistochemical studies reveal that Vn is present in platelet alpha-granules [Roger et al., 1992] and it can be released together with platelet factor 4 upon thrombin stimulation. It is unknown whether the Vn in platelets was actually synthesized by megakaryocytes or endocytosed from plasma and incorporated into alpha-granules. The majority of platelet Vn consists of disulfide-bonded multimers [Preissner et al., 1989].

## 1.11 Interactions of vitronectin with other binding proteins

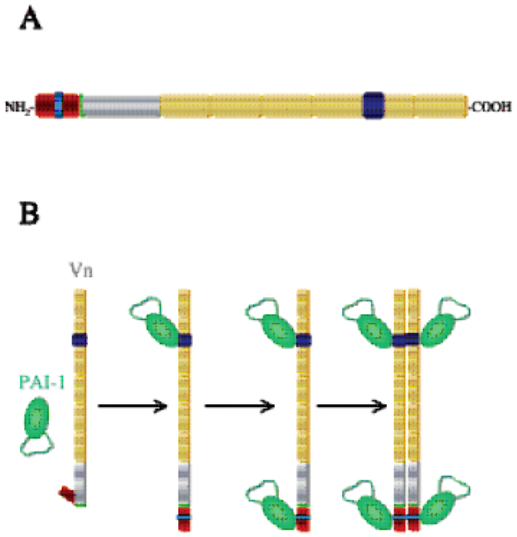


**Fig. 7 Interaction of Vn with PAI-1**

**(A)** Domain structure of Vn. The SMB domain (aa 1–44) is indicated in red, the RGD motif (aa 45–47) in green, the connecting region (aa 48–131) in silver, and the 6 hemoplexin repeats (aa 132–459) in ochre. The PAI-1 high affinity binding site (aa L24–S30) is depicted in cyan blue, the proposed low-affinity site within region K348–R370 in blue.
**(B)** Model describing the generation of PAI-1/Vn-complexes. PAI-1 initially interacts with monomeric Vn via the C-terminally located low-affinity binding site, which leads to a conformational change within the SMB domain and the unmasking of the high affinity PAI-1 binding site. The PAI-1/vitronectin complex (either the 1:1 or the 2:1 form) associates with a second complex via self-association of the vitronectin components. Higher order oligomers may be formed from the 4:2 complex or from associated vitronectin following conversion of PAI-1 to its latent form [Podor et al., 2000 ; Mior and Peterson, 2002].

Vn binds to PAI-1 with high affinity and stabilizes the inhibitor in its active conformation [Lawrence et al., 1997]. Vn can also associate with PAI-1 and assemble to form higher order complexes [Podor et al., 2000] that exhibit altered adhesive functions [Minor et al., 2002]. As one of the main physiological inhibitors of plasminogen activation, PAI-1 is likely to play a key role in the regulation of vascular homeostasis at sites of arterial injury. This function of PAI-1 is facilitated by Vn. [Konstantinides, Schäfer, Loskutoff, 2002]. The binding of PAI-1 to Vn also increases the specificity of the inhibitor, converting it into a thrombin inhibitor [Stoop et al., 2000] and a potent inhibitor of activated protein C (APC) [Rezaie et

al., 2001]. The complete active PAI-1 in blood circulates in complex with Vn. In summary, these observations suggest that Vn is a cofactor for PAI-1.

Only PAI-1 in its active conformation and neither latent nor RCL-cleaved nor PAI-1 in complex with its target proteases can bind to Vn [Lawrence et al., 1997]. Addition of uPA to a PAI-1/Vn complex leads to dissociation of this complex [Loskutoff et al., 1999]. Upon binding of Vn to active PAI-1, conformational changes in PAI-1 are induced, approximately doubling its half-life [Declerck et al., 1988] and providing it with inhibitory properties towards other serine proteases, namely thrombin and activated protein C [Ehrlich et al., 1990; Rezaie et al., 2001]. PAI-1 competes with cell surface receptors like uPAR and integrins for Vn-binding [Irigoyen et al., 1999]. Because of these important implications of the PAI-1/Vn interaction, this complex will be looked at more closely in this chapter.

**The PAI-1/Vn complex**

Most of PAI-1 in plasma is present in a high molecular weight complex with Vn [Declerck et al., 1988]. By ultracentrifugation, the PAI-1/Vn-complex was found to display a relative molecular mass of 324,000 ± 14,000 which could be either a complex of three PAI-1 and three Vn molecules (theoretical Mr: 345,000) or a complex of four PAI-1 and two Vn molecules (theoretical Mr: 316,000) [Podor et al., 2000]. The 4:2 complex is more likely, when considering the two possible PAI-1 binding sites on Vn (see 1.8.1). Additionally, in the ultracentrifugation experiments, free Vn was still detected after complex formation in the presence of equimolar amounts of PAI-1 and Vn. Based on these and other results, the following model for the formation of PAI-1/Vn-complexes was proposed [Podor et al., 2000].

PAI-1 initially interacts with monomeric Vn, resulting in a conformational change and to the unmasking of the high-affinity PAI-1 binding site. The PAI-1/Vn complex associates with a second complex.

The high-affinity PAI-1 binding site within the SMB domain of Vn is not exposed in monomeric Vn [Seiffert and Smith, 1997]. Therefore, PAI-1 initially interacts with monomeric Vn via the C-terminally located low-affinity binding site, leading to a conformational change within the SMB domain and unmasking of the high-affinity PAI-1 binding site [Seiffert, 1997a].

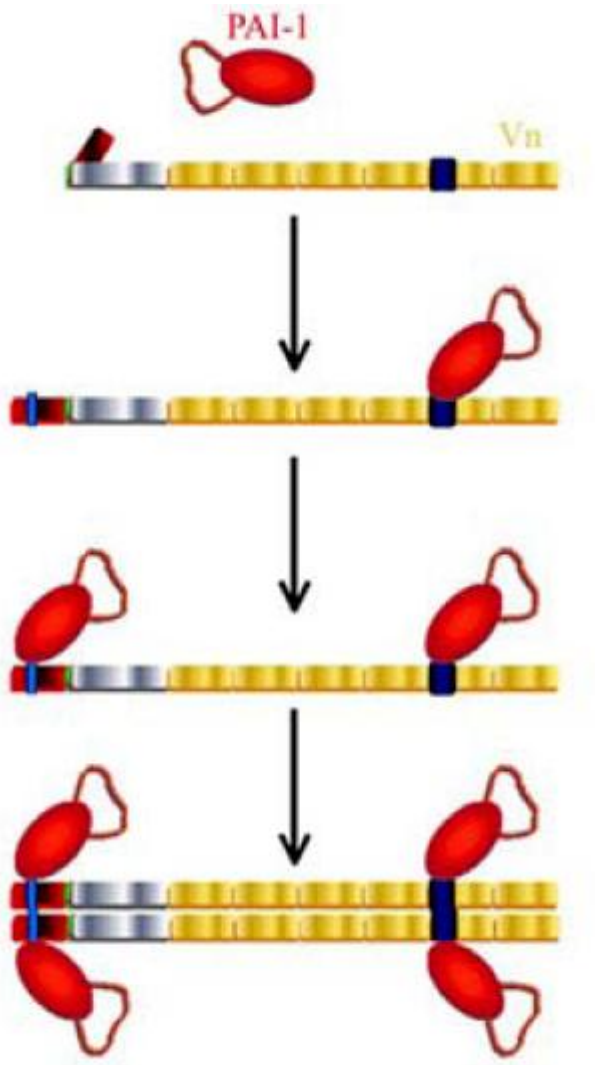


**Fig. 8 Model of the formation of PAI-1/Vn-complexes [Podor et al., 2000].**

Additionally, interaction of Vn with PAI-1 promotes oligo- /multimerization [Seiffert, 1997b] and thus enhanced affinity of these PAI-1/Vn complexes to the ECM and to smooth muscle cells [Minor and Peterson, 2002].

By binding to monomeric – but not multimeric – Vn, the substrate specificity of PAI-1 is altered, because this interaction enables PAI-1 to inhibit other serine proteases, e.g. thrombin, in addition to uPA and tPA. By using fluorescence-labeled PAI-1, it was demonstrated that binding of monomeric vitronectin to PAI-1 induces conformational changes in the vicinity of the P1–P1' bond rather than causes changes affecting on the whole RCL [Gibson et al., 1997]. The PAI-1/Vn-complex inhibits thrombin by formation of ternary PAI-1/Vn/thrombin-complexes as

well as binary PAI-1/thrombin-complexes, as determined by surface plasmon resonance and immunoprecipitation. In contrast, during inhibition of the tissue-type plasminogen activator (t-PA) by the PAI-1/Vn complex, only binary PAI-1/t-PA-complexes are formed [van Meijer et al., 1997]. The formation of the PAI-1/Vn/thrombin-complexes enables endocytosis of thrombin via low density lipoprotein receptor-related proteins (LRP) 1 and 2 [Stefansson et al., 1996].

## 1.12 OBJECTIVE

The prognosis for breast cancer patients varies strongly depending on several individual risk factors. The classical factors applied to decide if or which kind of adjuvant therapy is to be administered are lymph node status, tumor size and grade (TNM classification), patient age, menopausal status and steroid hormone status. The presence of estrogen and progesterone receptors is beneficial for prognosis in breast cancer [Clark et al., 1983]. For steroid hormone receptor positive patients, there exist diverse possibilities of endocrine treatment.
Martens et al. [Cancer Research, 2005], observed a correlation between endocrine Tamoxifen therapy response (reduced response) and DNA hypermethylation of the promotor region of vitronectin.
The question that arises is: Where do we find Vn in breast cancer tissue? So far, there has been no systematic approach to investigate this issue. The aim of this study therefore was to establish and optimize an immunohistochemical method of detection of Vn in tissue specimens of normal and cancerous breast by immunohistochemistry (IHC). This was done along the following lines:

- testing and comparing various Vn-specific antibodies
- testing of antibodies specific to the multimeric form of Vn using 13HI and 16AJ on a representative number of normal and cancerous breast tissue specimens
- testing of antibodies and a comparison of the results on a representative number of breast cancer specimens and identifying differences between tumor tissue and equivalent histologically normal tissue from the same breast

- comparing the results of IHC with ELISA to find out which antibodies are appropriate for both
- validation of data available on multiorgan arrays for normal and cancerous breast tissue.

Vn was also investigated as a potential biomarker for the therapy response of metastasized breast cancer patients to endocrine treatment with Tamoxifen. DNA methylation markers offer many exciting opportunities for clinical application of sensitive cancer detection. In brief, the greatest promise of DNA methylation markers lays in the power of diagnosis, in which panels of markers will provide a tool for risk assessment, early detection, molecular diagnostics of resected specimens, chemoprediction and monitoring for disease recurrence.

Consider the following scene from a clinic in future: As part of an annual routine examination of a patient, 10 ml of blood are drawn for a DNA methylation screen with a panel of the most common breast cancer-specific DNA methylation markers. The vitronectin marker comes up positive, and the sample is then selected for further analysis with more specific, diagnostic DNA methylation panels to determine the most likely tissue of origin. Based on this information, the physician uses sophisticated imaging technology and other non-invasive or minimally invasive techniques to confirm the diagnosis. An incipient mammary carcinoma is caught before it has spread [Laird et al., Nature Reviews Cancer, 2003]. This has to be considered the far aim to which this thesis may contribute a small but hopefully significant part.

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Patient collective

The tissue samples used in this study were obtained from selected female patients of the Department of Obstetrics and Gynecology of the *Klinikum rechts der Isar* of the Technische Universitaet Muenchen. Normal breast tissue samples were obtained from surgical reduction of breast hypertrophia while the tumor samples analyzed included a mixture of ductal and non-ductal (tubular, lobular, and medullary) invasive carcinomas as well as ductal carcinomas in situ. Twenty samples each of normal and cancerous breast tissue, classified according to histomorphological (tumor size, nodal status, metastasis), histological grading and tumor biological criteria (steroid hormone receptor status) were used. All patients involved in this research project have responded to an Informed Consent which was approved by the Ethical Committee of the Klinikum rechts der Isar, Technische Universitaet Muenchen, which accepted our project proposal.

### 2.1.2 Tissue processing and tissue fixation

Following surgical excision, the freshly acquired tissue is placed without delay in a buffered fixative solution made up of 37% formaldehyde and nine portions of 0.1 M phosphate buffer solution. This solution fixes proteins, preventing on the one hand autolysis of the tissue by inactivating lysosomal enzymes and on the other hand, the growth of bacteria and fungi which cause destructive changes. After 24 hours the protein components of the tissue are completely fixed and stabilized. The tissue is subsequently placed in an ascending order of graded ethanol (three times repeatedly in 70% ethanol for eight hours, another eight hours each in 80% ethanol and then 96% ethanol; then two times immersion in 100% ethanol and then washing in xylene for a period of four hours; followed by another three hours tissue wash in xylene for dehydration.

The tissue samples are then embedded in fluid paraffin for fifteen hours at 55 °C, a temperature slightly higher than the melting point of paraffin; then placed in a

paraffin bath for a further five hours at 55 °C to further increase the concentration of paraffin in the tissue sample (paraffin impregnation). Finally, the tissue is placed in a 50 °C-warm, fluid paraffin-plastic cassette. After 30 min of cooling, the paraffin blocks are ready to be cut into slices.

### 2.1.3 Preparation of paraffin sections

Special electrostatically active, silanized glass slides are employed for this purpose. The tissue embedded in the paraffin-block is sliced up by means of a microtome, into several layers of 2–3 μm, which are then picked up with a pair of tweezers or a brush, placed in a bath of distilled water and then carefully transferred onto the appropriate surface of the silanized glass slide. The slides are kept on a heated plate at 37 °C to straighten out all the folds. They are then placed in a heater at 37 °C for 24 h to dry; after which they can be stored at room temperature for several months.

### 2.1.4 Companies and Reagents

DAKO ChemMate$^{TM}$ Detection kit, Peroxidase /DAB, rabbit/mouse (Code Nr. K5001) were purchased from the company Dako Diagnostics AG in Zug, Switzerland and ChemMateTM antibody diluent (Code Nr. S 2022) for dilution of concentrated antibodies was purchased from DakoCytomation, Denmark.
Most of the chemicals used were obtained from Sigma, Munich, Germany, or Merck, Darmstadt, Germany. These for instance included citric acid monohydrate, sodium hydroxide, hydrogen peroxide, Tris-or phosphate-buffered saline (PBS).
Tris buffer solution was prepared as follows:
For a volume of 1000 mL, 60.5 g of Trizma-Base (Sigma T-1503) was dissolved in 700 mL of distilled water. A 2N HCl solution was added and the pH value adjusted to 7.6. Distilled water was again added, to 1000 mL and 90 g NaCl (Merck 1.06404) dissolved in the solution. This stock solution was kept at room temperature and diluted 1:10 with distilled water for use in immunohistochemistry.
Marker pens for IHC, Cat. No. S-2002 were purchased from the company Dako Diagnostics in Glostrup, Denmark.

## 2.2 Antibodies to vitronectin

For the immunohistochemical determination of Vn in the tissue sections, antibodies (AB) were used. AB to vitronectin were supplied by Prof. K.T. Preissner, Institute of Biochemistry, University Hospital Giessen.

- VN-7 monoclonal antibody from mouse
- VN-7 biotinylated monoclonal antibody from mouse
- VN-9 monoclonal antibody from mouse
- α-S-biotinylated polyclonal antibody from rabbit
- Rb 66/67 polyclonal antibody from rabbit

AB tested bind to monomeric and multimeric forms of Vn but none of the AB have been epitope-mapped for Vn-epitope specificity

- 13HI and 16AJ monoclonal antibody (bind multimeric forms of Vn)

AB play a central role in IHC. AB belong to the group of immunoglobulins. They are capable of specific binding to antigens and circulate either in free form or as immunocomplexes bound to blood. They form the specific, naturally occurring defense system of any organism. The following classes of immunoglobulins can be distinguished by immunoelectrophoresis: IgG, M, A, D and E. The commonly used AB for daily diagnostic purposes are the IgG antibodies. IgG has a half-life of three weeks in circulation and is more stable compared to the others. (IgM, the acute phase protein, has a short half-life of 4–6 days). The availability of antisera, immunoglobulin fractions and monoclonal AB for an increasing number of clinically relevant tissue antigens has lead to an expansion of the quantity and quality of immunohistochemical procedures. The AB were produced as follows:

### 2.2.1 Generation of mono- and polyclonal antibodies to vitronectin

#### 2.2.1.1 Polyclonal antibodies

Different animal species can serve as host for the production of polyclonal antibodies. The rabbit is the most commonly used source of polyclonal AB for vitronectin, with the New Zealand White being the most frequently used race [Harboe et al., 1983]. The first step is the process of immunization, in which the rabbit is injected subcutaneously with 0.1–0.5 mL of Vn immunogen, against which

antibodies of the IgG class would be produced by the B-cells of the rabbit and released into the blood stream. The production of AB starts within 20 min after the injection, although detectable amounts of AB can only be determined 5–10 days later. The procedure is repeated in definite intervals (boosts) to achieve a high AB yield. Booster injections (i.e. stimulating subsequent injections) of antigen are required once in a month to stimulate a steady production of AB.

Blood samples are taken in intervals of 15 days to monitor the AB production (titer) by immunochemical testing. When the AB titer is sufficiently high, blood is drawn from the ears of the immunized rabbit. The blood is then left to clot so that the immune serum gets separated from the solid blood components. The so derived anti-serum is frozen at –20°C as serum or lyophilized. There are companies specialized in this sort of process, which would challenge rabbits with provided antigen and guarantee a minimum titer. Thus, polyclonal antibodies or antiserum are easily commercially available.

Advantages:

- Polyclonal Vn antibodies recognize several Vn antigen determinants because the AB is produced from different B-cells of the rabbit. Mixing of anti-sera from different animals (pooling) is also possible. These polyclonal AB are therefore not as prone to epitope-changes (e.g. through fixation) as the monoclonal AB, but still give the required signals.
- Polyclonal Vn antibodies are not oversensitive to pH-changes and are often cheaper than monoclonal Vn AB.
-

Disadvantages:

- Polyclonal Vn AB are often not chromatographically purified, which in turn leads to unspecific background coloration by other rabbit serum proteins.
- Cross-reactivity of similar antigen structures may occur as a result of the differences in AB used which may give false positive test results.
- Because of the way and manner of manufacture, reproducibility cannot be guaranteed. If the animal producing a very specific antiserum is gone, it depends on chance whether the next cycle of production will give equally good results.

Conclusion:

- If there is no specific epitope known in an antigen, if only a general overview of immunoreactivity is desired as a first step, or a very strong signal is required, polyclonal antibodies are the means of choice.

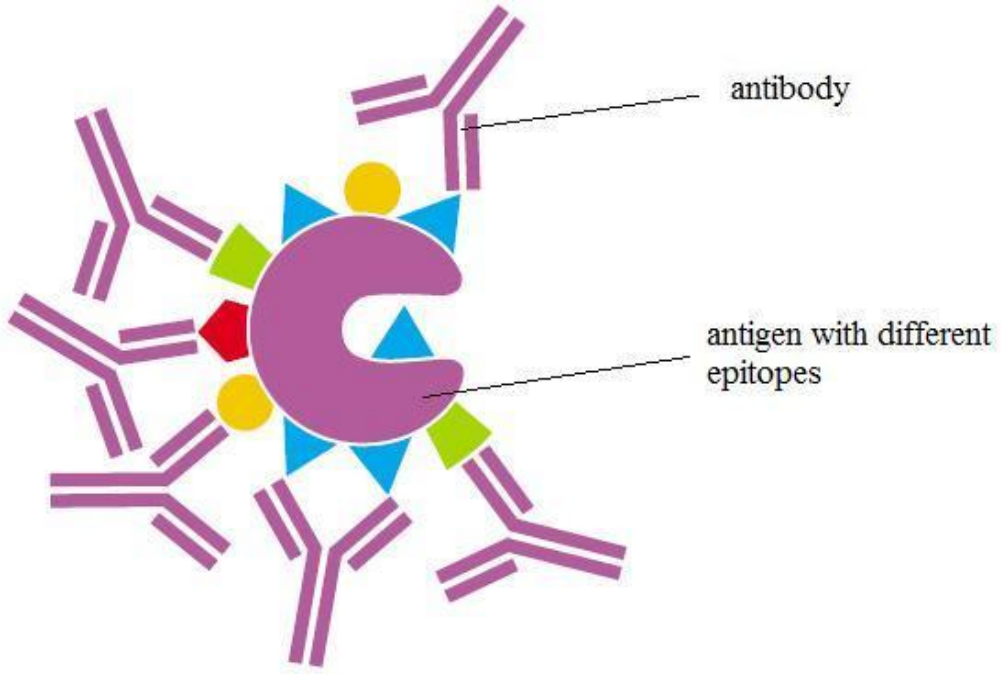


**Fig. 9** **Schematic representation of a polyclonal AB binding to different epitopes of an antigen [Boenisch, 2001]**

### Purification of polyclonal Vn antisera by chromatography

After production, the polyclonal Vn antisera are run through gel columns in the process of affinity chromatography. One of two methods can be used:

**I.** Gel chromatography: The immune serum containing the specific AB is run through a column of beads. During this process, all undesirable serum proteins of the rabbit will separate from the desired AB portion.

**II.** Affinity chromatography: This method differs from the first by the composition of the beads which contain the antigen specific for the antibody to purify. When the serum runs through the beads, the specific AB binds to the beads, leaving the undesired rest behind. A further step is required to release the desired AB fraction from the beads. The resulting product is better than that acquired by method I because method II is more specific.

Advantage:

- There is very little or no background coloration in immunohistochemistry because unspecific rabbit serum proteins were removed.

Disadvantage:

- The disadvantage over the unpurified immunoserum is with regard to the price. An additional step means additional costs.
- Affinity chromatography in itself reduces the amount of AB available. The AB may get damaged in the process of elution.

Conclusion:

- Purified polyclonal AB are more expensive but recommendable.

#### 2.2.1.2 Monoclonal antibodies

Monoclonal antibodies are the product of a single clone from plasma cells. They recognize only a specific epitope. They are mostly derived from mice and mostly consist of IgG antibodies.

**Production of monoclonal Vn antibodies**

As in the case of polyclonal AB, the first step is that of immunization. The mouse is injected with Vn immunogen in order to induce the production of AB. When the AB titer is adequate, the spleen and lymph nodes are removed. They contain considerable amounts of plasma cells which produce the specific antibodies, as well as several lymphocytes which produce AB that are not targeted against Vn. These lymphocytes have to be filtered out. A single-cell suspension is formed, whereby the spleen is separated into pieces of its components so that only the lymphocytes remain in suspension. Because plasma cells cannot survive long enough in cell culture, they first have to be “immortalized” to enhance antibody production. This purpose is achieved by utilizing myeloma cells, undifferentiated malignant cells which are able to survive in cell cultures for several decades. The utilized cells have an enzyme defect in their DNA-metabolism; they lack the hypoxanthine-guanine-phosphoribosyl transferase, which, under certain circumstances prevents the formation of AMP and GMP. Furthermore, they do not produce any AB.

The B-/T-lymphocytes are fused with these myeloma cells in the presence of polyethylenglycol (PEG), resulting in a hybridoma cell line as fusion product, further on referred to as hybrid.

During that process, several fusion combinations are possible, including the fusion of two identical cells. However, only the plasma cell / myeloma cell hybrid is suitable for Vn monoclonal AB production as it is immortalized and permanently releases AB into the medium. A selective medium based on hypoxanthine, aminopterine and thymidine, (HAT medium) is applied to distinguish between the various pairs. While myeloma cells and myeloma / myleoma hybrids cannot synthesize nucleotides in this medium and hence cannot multiply anymore, the lymphocytes and lymphocyte hybrids can survive on this diet, but not survive for long. The surviving hybrid cells are isolated and tested for the specificity of their AB, until eventually, cell lines are selected which produce identical AB with a defined specificity targeted at one epitope of the injected Vn antigen. These AB are referred to as monoclonal Vn antibodies because they stem from a cell line derived from a single ancestor hybrid cell, a so called “clone”. Commercial quantities may be obtained in a culture medium, from which the monoclonal AB can be isolated.

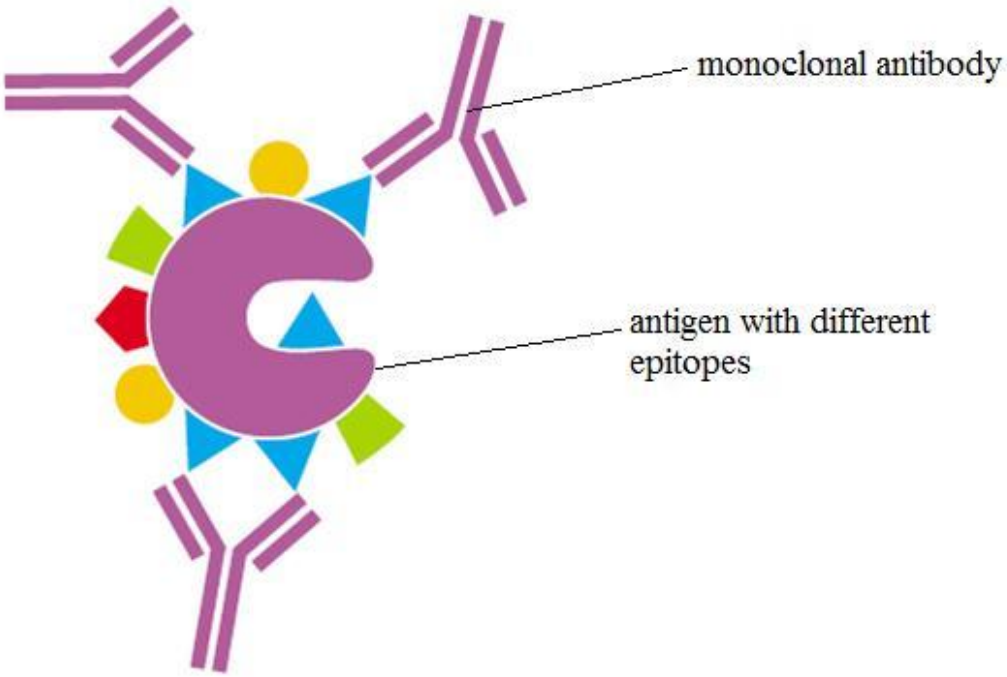


**Fig. 10** **A specific clone of the monoclonal AB reacts with a specific epitope of the antigen only [Boenisch, 2001]**

Advantages:

- Cross-reactions are possible but rare. The means of manufacture provides for a clean positive result without any disturbing background coloration.
- Reproducibility is guaranteed.

Disadvantages:

- Monoclonal AB are more sensitive with respect to epitope changes, e.g. through denaturation or association. “Over-fixed tissue” may lead to a false negative result by cross-reactivity, also slight changes in pH can lead to changes in reactivity or specificity.
- Monoclonal AB are usually more expensive than polyclonal AB.

Conclusion:

- If high specificity is required as well as reproducibility, e.g. in routine diagnostics, monoclonal AB are the reagents of choice.

The monoclonal antibodies to Vn used in this work were stored at -20°C and are brought to room temperature before use.

#### 2.2.1.3 Other Antibodies tested

- Mouse monoclonal antibodies to human CD31 (DakoCytomation, Denmark)
- Monoclonal antibodies to integrin $\alpha_v\beta_3$ (Abcam Ltd., United Kingdom, Chemicon$^R$, Germany)

The mouse monoclonal AB to human CD31, obtained from DakoCytomation, Denmark, Clone JC/70A, Isotype $IgG_{1,\ kappa}$, 345 mg/L, was tested at the recommended dilution of 1:25.

Human CD31 is an adhesion molecule expressed on platelets, endothelial cells, leukocytes and their bone marrow precursors. CD31 plays a role in homophilic adhesion and heterophilic transendothelial migration.

AB to CD31 reacts with domain 1 of CD31 and blocks homophilic interaction and transendothelial migration. The monoclonal AB is directed against the CD31-antigen, which is also expressed on human platelets. In immunohistology, the monoclonal AB reacts with endothelial cells, histiocytes (weak) and glomeruli.

For use within one month of purchase, AB were stored at 4°C, for long term storage antibodies were aliquoted into small volumes and stored at -20°C.

**Chemicon$^{R}$ International:**

- Mouse monoclonal antibody to human CD 51/61, Lot number: 544143PN Clone name: 23C6, Host/Isotype: Ms IgGI The combination of CD51 with CD61 produces the RGD-dependent Vn receptor. This complex ($\alpha_v\beta_3$) is widely distributed on monocytes and macrophages, endothelial, platelets, most cells and tumour cells. This antibody recognizes specifically the $\alpha_v\beta_3$ complex. So far, it has been applied in flow cytometry and immunoprecipitation but not yet in immunohistochemistry.

**Abcam Ltd., U. K:**

- Mouse monoclonal [272-17E6] to Integrin alpha V (ab16821) 100 µg (Protein G purified), Lot number: 142919 Clone number 272-17E6, Isotype: IgG1 Relevance: Integrin alpha V chain interacts with the integrin beta 3 subunit/CD61 to form the αvβ3 heterodimer/vitronectin receptor. It is expressed on endothelial cells, some activated leukocytes, macrophages, neutrophils, and platelets. Integrin alpha V also forms heterodimers with the integrin beta 1, beta 5, beta 6, and beta 8 subunits. αvβ3 is an activation-dependent receptor for platelet attachment and spreading on vitronectin and other matrix components. Recommended concentration for IHC: 2.5 µg/mL. Aliquots were stored at -20°C.

- Mouse monoclonal [BV4] to Integrin beta 3 (ab 7167) 50 µg (0.1 mg/mL), Lot number 162149 Clone number BV4, Isotyp: IgG1, Purity: IgG fraction Relevance: Human Integrin beta 3 (CD61) antigen is expressed on platelets, megakaryocytes, macrophages and osteoclasts. The integrin beta 3-chain of the vitronectin receptor and GPIIb/IIIa complex. The integrin beta 3-chain is a 90–110 kD glycoprotein polypeptide which is expressed on platelets, megakaryocytes, macrophages, osteoclasts and synovial lining cells. Aliquots were stored at –20°C.

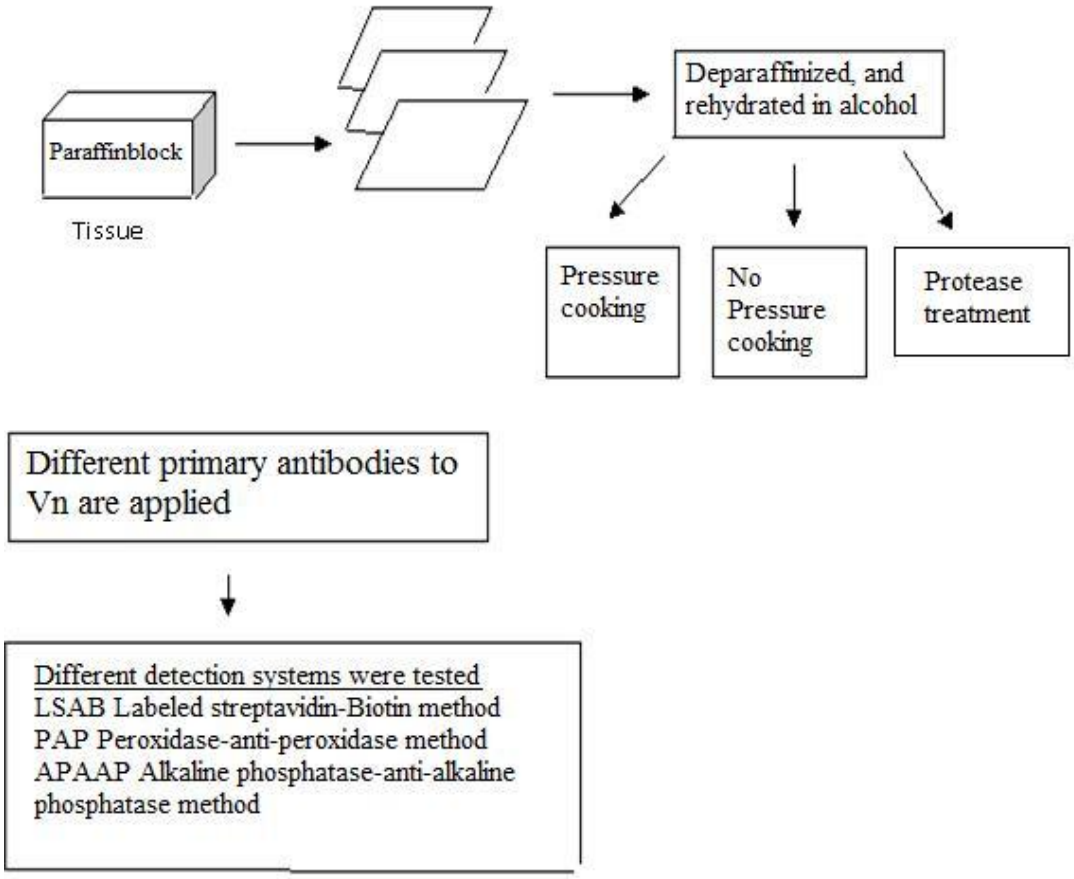


**Fig. 11 Schematic representation of applied IHC methods**

## 2.3 Immunohistochemistry (IHC) in breast cancer research

IHC has become established as an integral part of diagnosis and research in biology and medicine, due to the systematic development and manufacture of a wide range of highly specific antibodies to various antigens. Especially in the morphology-oriented subjects like pathology and anatomy, the field of application of IHC is extensive. Both specificity and sensitivity of IHC techniques have improved considerably, courtesy of significant improvement with respect to the methods of tissue pretreatment (e.g. antigen retrieval), which today allows for a broader routine application of formalin-fixed tissues.

Apart from the further development of applied IHC techniques (PAP, APAAP, LSAB etc., of which an overview is given below), the various techniques of intensive staining have brought about diversity in the applied methods, and, subsequently, new sources of error. All aspects of the methods of normal and cancerous breast tissue pre-treatment, and highly sensitive reinforcement

reactions as well as the selection of various antibodies in the detection of the extracellular matrix protein vitronectin are dealt with in detail in this work.

## 2.4 Technique of IHC

Formalin fixation in connection with proteolytic processes requires an optimal protocol which must be strictly adhered to. Antigen retrieval may be essential to restore the immunoreactivity of the formalin-fixed and paraffin-embedded tissue sections. Pretreatment with heating using citrate buffer was first described by Cattoretti et al.1992.

### 2.4.1 Protocol

1) **Preparation of tissue sections**
   Sections from various tissues are mounted on slides for immunohistochemical analysis.
2) **Pretreatment**
   Sections are deparaffinized, rehydrated in graded ethanols, and finally either pressure-cooked in citrate buffer (sodium citrate buffer, pH 6.0) for 4 min. in order to allow antigen retrieval; not pressure-cooked, or treated with protease, respectively.
3) **Blocking**
   Endogenous peroxidase activity is blocked by treating with 3% hydrogen peroxide for 20 min. at room temperature.
4) **Washing**
   Following an intervening wash in Tris-buffered saline (TBS: 0.005 M Tris/HCl; 0.015 M NaCl, pH 7.6), slides are either covered with 4% powdered milk (100 mg powdered milk + 2.5 mL Antibody Diluent) combined with 1% normal goat serum for 30 min. to block non-specific adsorption.
5) **Antibody application**
   Slides are covered with monoclonal antibody to Vn, diluted according to protocol and incubated in humidified chambers for 1 hour at room temperature.
6) **Detection**
   Kit used depends on protocol; PAP, APAAP or LSAB (see below). Antibody-linked peroxidase activity is revealed with diaminobenzidine, DAB. Nuclei of cells are subsequently counterstained with hematoxyline, dehydrated by using graded ethanols and mounted for microscopic evaluation.

### 2.4.2 Detection systems

The IHC-methods of staining allow a visualization of the Vn-antigen with the sequential use of antigen-specific primary AB, secondary AB directed to the primary AB, an enzyme complex (tertiary reagent) and a substrate-chromogen (color reaction). The enzymatic activation of the chromogen provokes a visible reaction product of the antigen.

#### 2.4.2.1 Peroxidase-anti-peroxidase method (PAP)

PAP is an indirect method of detection. An unconjugated primary AB (e.g. from a mouse) binds to a link AB (e.g. from a rabbit) targeted against the primary AB. A PAP-complex (from a mouse binding to rabbit IgG targeted against mouse IgG) then binds to the link AB. The PAP-complex consists of the enzyme peroxidase and an AB targeted against it (enzyme-anti-enzyme-complex). When a polyclonal primary AB e.g. from a rabbit is used, a corresponding PAP-complex from a rabbit is applied.

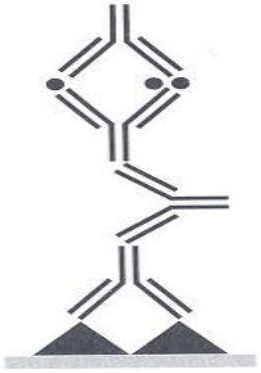


**Fig. 12 Schematic representation of the PAP-method [Noll et al., 2000]**

Advantage:

- A stronger dilution of the primary AB is possible compared with the direct staining method. The background staining is reduced, which in turn gives better results.

For detection, the 3,3'-diaminobenzidine (DAB) substrate solution is employed in this method. By reaction with free peroxidase in the PAP complex, DAB produces a brown end-product, which is insoluble in ethanol and other organic solvents.

Thus, staining for Vn was carried out according to the protocol shown above. After applying the DAB substrate, the sections were rinsed in normal tap water and placed in Meier's hematoxyline stain for a period of 90 sec. The stained sections were washed in normal tap water for 5 min, rinsed in aqua dist. and subjected to ethanol dehydration by incubation in graded ethanols for 5 min each and finally in xylene for a further 5 min. The sections were then glass-covered and sealed up using Pertex, an organic solution.

### 2.4.2.2 Alkaline phosphatase-antialkaline phosphatase-method (APAAP)

This is also one of the indirect complex methods. It is preferable to the PAP-method because it avoids the disturbing influence of endogenous peroxidase. The primary AB binds to the antigen contained in the tissue. Depending upon the animal source of the primary AB, a corresponding intermediary AB is employed so that the APAAP-complex can follow. Thus, employing a mouse monoclonal AB, the primary AB binds to the APAAP-complex via the intermediary AB. On the other hand, employing a primary AB from a rabbit would require an additional step using a mouse-anti-rabbit AB in order to allow for the binding of the APAAP-complex. The APAAP-complex comprises a monoclonal mouse-IgG, AB targeted against alkaline phosphatase. Like in the PAP-method, this is also an enzyme-anti-enzyme-complex. The APAAP-complex can intensify the reaction by a continuous repetition of the steps involving the intermediary AB and the APAAP-complex.

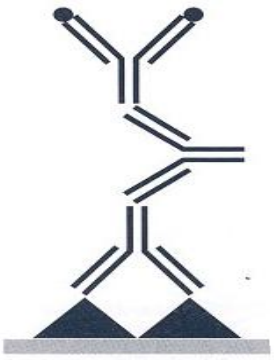


**Fig. 13 Schematic representation of the APAAP-method [Noll et al., 2000]**

Advantage:

- Compared to the direct method, a higher dilution of the primary AB is achievable. The possibility of repeating the steps involved ensures a stronger coloration.

Disadvantage:

- Unspecific coloration may arise due to insufficient suppression of the endogenous alkaline phosphatase by levamisol. Repetition of the steps involved can provoke unspecific background coloration.

To detect Vn by this method, a 10mg *fast red* tablet (substrate) was dissolved in aqua dist., the solution was then applied to the sections and incubated for 10 min at room temperature (for 10 sections:1 mL aqua dist. + 1 buffer tablet + 1 colored tablet). The sections were rinsed in normal tap water and placed in hematoxyline stain for 90 sec. The stained sections were again washed in normal tap water for 5 min, rinsed in aqua dist and subjected to ethanol dehydration by incubation in graded ethanol for 5 min each and finally in xylene for a further 5 min. The sections were then glass-covered and sealed up using Pertex, an organic solution.

#### 2.4.2.3 Labeled streptavidin-biotin method (LSAB)

This method is based on the affinity of avidin to biotin. Avidin is a tetrameric chicken glycoprotein present in chicken egg. It has four binding positions for biotin and has a molecular weight of 68 kDa. Streptavidin is isolated from the bacteria strain *Streptomyces avidinii*. This LSAB method requires a biotinylated intermediary AB. Biotin is a water-soluble vitamin which is conjugated to an AB. Either peroxidase or alkaline phosphatase is bound to avidin, which then interacts with the biotynilated antibody. All four possible binding positions of avidin for biotin remain free for binding.

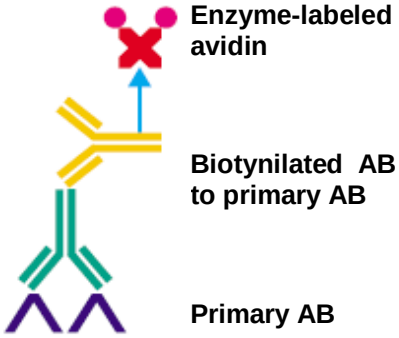


**Fig. 14 Enzyme-labeled Streptavidin reacts with the biotinylated link antibody [Boenisch et al., 2003]**

Advantages:

- Because the avidin-biotin complex is comparatively small in size, steric hindrance is minimized, leading to better staining results. The LSAB system is at present the most specific and most convenient one known.

After testing various antibodies to Vn on numerous tissue specimens using the various methods of staining enumerated above, it was concluded, in consultation with Dr. J. Nährig of the Institute of Pathology of the Technical University of Munich, that the best staining results were obtained when the sections were treated under the following circumstances:

- without pressure cooking
- using the monoclonal antibody to Vn, VN-7
- using the LSAB-method of detection

### 2.4.3 Adapted IHC protocol

**1) Preparation of sections**

Sections from normal and cancerous breast tissues are mounted on slides for immunohistochemical analysis. Liver sections are used as controls.

**2) Pretreatment**

Sections are deparaffinized and rehydrated in a descending order of graded ethanols (two times in a xylene bath for 10 min. each, two times in a 100% ethanol bath for 5 min. each and then in 96% and 70% ethanol baths for 5 min. each)

**3) Blocking**

Endogenous peroxidase activity is blocked by treatment with 3% hydrogen peroxide (180 mL aqua dist. + 20 mL $H_2O_2$ 30%) for 20 min. at room temperature.

**4) Washing**

Sections are rinsed in a Tris-buffered saline solution (TBS: 0.005 M Tris, pH 7.6) for 5 min.

**5) Antibody application**

Sections are covered with monoclonal antibody VN-7, diluted according to protocol (1:10,000) with a final concentration of 80 ng/mL and incubated in humidified chambers for 1 hour at room temp. No primary AB VN-7 is applied to the negative control liver section.

**6) LSAB detection**

This is followed by incubation of the slide with biotinylated rabbit anti-mouse IgG (Dako Detection kit) for 30 min. followed by streptavidin-biotin-peroxidase (HRP) (Dako Detection kit) for 30 min. with intervening washes with TBS.

**7) DAB substrate**

Peroxidase activity is revealed with the chromogen diaminobenzidine, DAB.

Then, nuclei of cells are counterstained with hematoxyline for a period of 90 seconds.

**8) Dehydration**

The stained sections are finally subjected to ethanol dehydration by immersion in an ascending order of graded ethanols (in 70% ethanol, 96% ethanol, two

times in 100% ethanol each for 5 min., then two times in xylene for 10 min. each) and finally mounted for microscopic evaluation.

### 2.4.4 Calculation for antibody dilution

Not only the antibody titer and dilution, but also the time of incubation and temperature are important determinants of the quality of the immunohistochemical staining. These factors can be manipulated either independently or in a complementary manner to bring about the desired staining results.

To achieve a dilution of 1:10,000 of the monoclonal AB VN-7, the following calculations were made.

1st dilution: 1:100 => 1 µl AB + 99 µl AD

(AB=Antibody, AD=Antibody Diluents)

Staining solution

For 15 Sections à 150 µl => 15 x 150 = 2250 µl. Using a total volume of 2500 µl,

1:10,000 => 25 µl AB (from 1st dilution) + 2475 µl AD.

Generally, when making any changes, the overriding goal should be the achievement of optimal specific staining accompanied by minimal interference from background staining.

### 2.4.5 Controls

Reagent and tissue controls are necessary for validating the results of immunohistochemical staining. Without appropriate controls, the interpretation of staining would be arbitrary and the results inconclusive. More specifically, controls give an indication whether or not staining protocols are strictly adhered to, whether there are differences in the processes involved or between different persons and whether the applied reagents functioned orderly.

Positive and negative controls were used to verify the method of Vn detection as well as a guarantee of quality control. In each of the tests performed therefore, a positive and negative control was included.

Positive tissue controls must be processed identically to the tissue specimen in question. However, they have to contain the target protein. In some cases it will be advantageous to have this control tissue stain only marginally positive, so as to

monitor not only for the presence of the antigen, but also for any possible loss of sensitivity. In this work, normal liver tissue was employed as a standard, since the liver is a known source of Vn biosynthesis.

Tissue specimens serving as negative tissue controls must also be processed (fixed, embedded, stained) identically to the unknown, but not contain the relevant tissue marker. An example would be normal liver serving as control for hepatitis B surface antigen-positive liver. In order to reveal unspecific reactions of the detection system (resulting in false positive signals), another option would be to apply the standard protocol to the negative control specimen while omitting the exposure to the primary AB. Any signals still detected in this case will be unspecific, false positive ones. In this work, liver sections were used for this purpose, without application of the primary Vn-directed antibody.

# 3. RESULTS

## 3.1 Testing of mouse monoclonal antibodies to human CD31

This antibody clone number JC-70A from DakoCytomation, Denmark reacts with CD31, also known as PECAM1 (Platelet Endothelial Cell Adhesion Molecule-1). It works well on formalin- and paraformaldehyde-fixed, paraffin-embedded tissue sections. CD31 is a 130 kDa integral membrane protein, a member of the immunoglobulin superfamily that mediates cell-to-cell adhesion. CD31 is a vascular endothelium -associated antigen and is used as a marker for benign and malignant human vascular disorders. CD31 is expressed constitutively on the surface of adult and embryonic endothelial cells. CD31 is involved in the transendothelial emigration of neutrophils, and neutrophil PECAM1 appears to be down-regulated after extravasation into inflamed tissues. CD31-mediated endothelial cell-cell interactions are involved in angiogenesis. CD31 has been used to measure tumor angiogenesis, which is a predictor of tumor recurrence. Other studies have indicated that CD31 and also CD34 can be used as markers of myeloid progenitor cells that may recognize different subsets of myeloid leukaemia infiltrates (granular sarcomas).

The mouse monoclonal antibody to human CD31 was tested at a recommended dilution of 1:25 with a stock concentration of 320 ng/mL along the same principles as mentioned above. Formalin-fixed, paraffin-embedded sections of liver and kidney tissue that display the known characteristic pattern were tested first before breast tissue samples were immunostained. In the kidney there was no difference in the distribution of Vn immunoreactivity between proximal and distal tubule epithelial cells (Fig.15A). A slightly positive reaction was detected in smooth muscle cells of capillaries and blood vessels (Fig.15E).

An immunohistochemical analysis of CD31 expression was performed using twenty samples each of normal and cancerous breast tissue, employing the mouse monoclonal antibody to human CD31 (Fig15. A -G). Lymphocytes within the normal and cancerous breast tissue did not react, whereas endothelial cells in the blood vessel of both normal and cancerous breast tissue sections were stained, indicating the presence of CD31 in the endothelial cell layer. Fig. 15C-E depicts staining of vessels in normal human breast tissue, Fig. 15F and G

illustrates staining in vessels within a breast cancer tissue section. The immunostainings demonstrated below using the monoclonal antibody to human CD31 were performed with a uniform antibody concentration, diluted at 1:25 with standard antibody diluent solution employing the LSAB method of detection**.** This primary antibody, however, was not applied to the negative control sections.

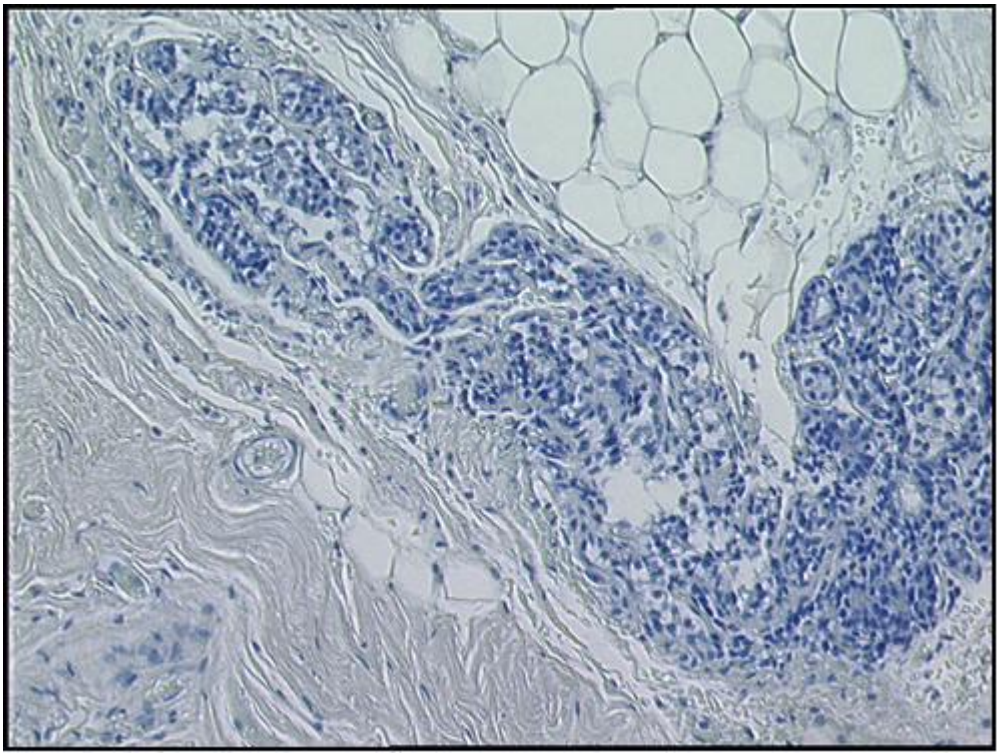

**Fig. 15 A Normal breast, negative control (no primary antibody)**
**magnification (objective)x10 LSAB staining method**
**Blue stain: hematoxylin to stain nuclei**

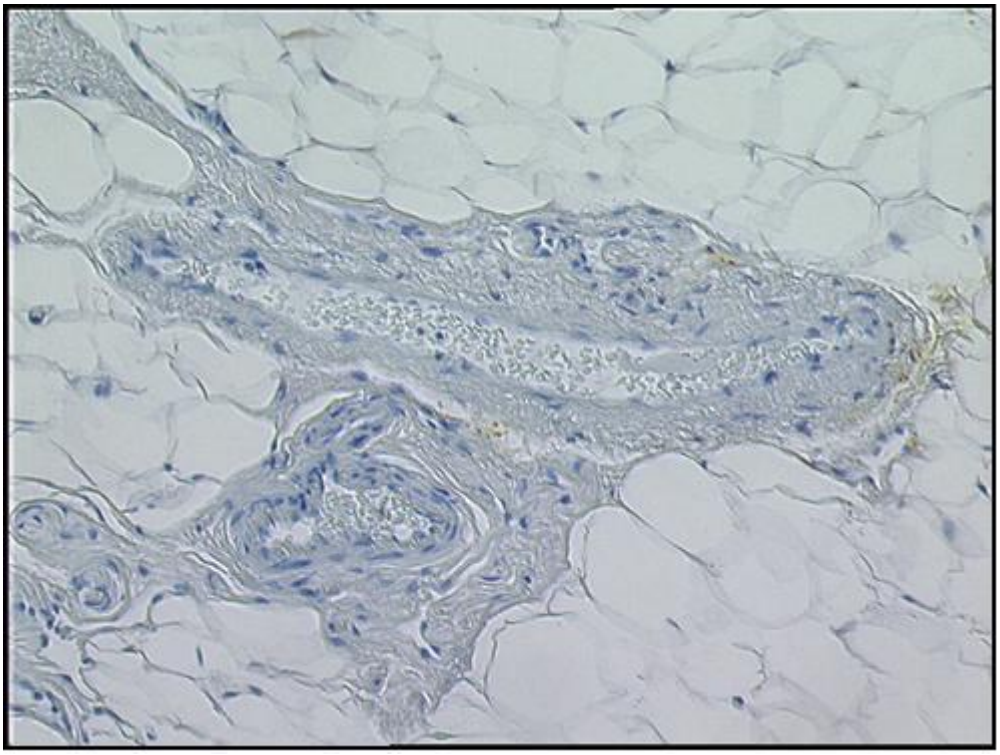

**Fig.15B Normal breast, negative control (no primary antibody)**
**magnification (objective)x10 LSAB staining method**
**Blue stain: hematoxylin to stain nuclei**

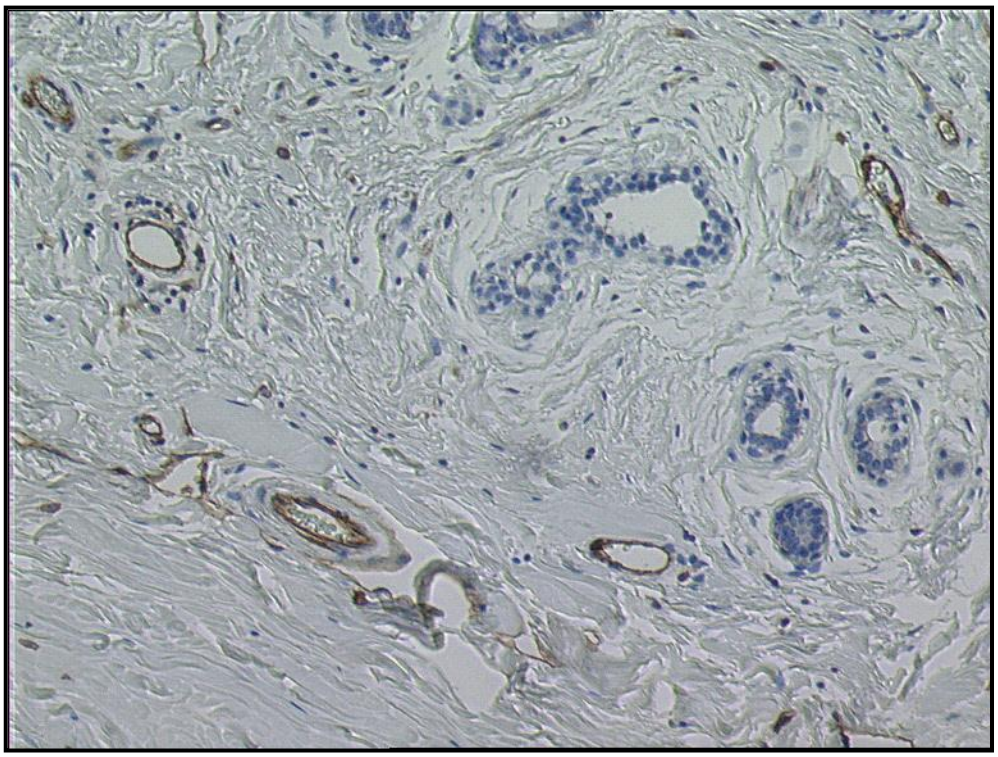

**Fig 15C** Normal breast, (primary antibody to human CD31), magnification (objective) x10 LSAB staining method Blue stain: hematoxylin to stain nuclei

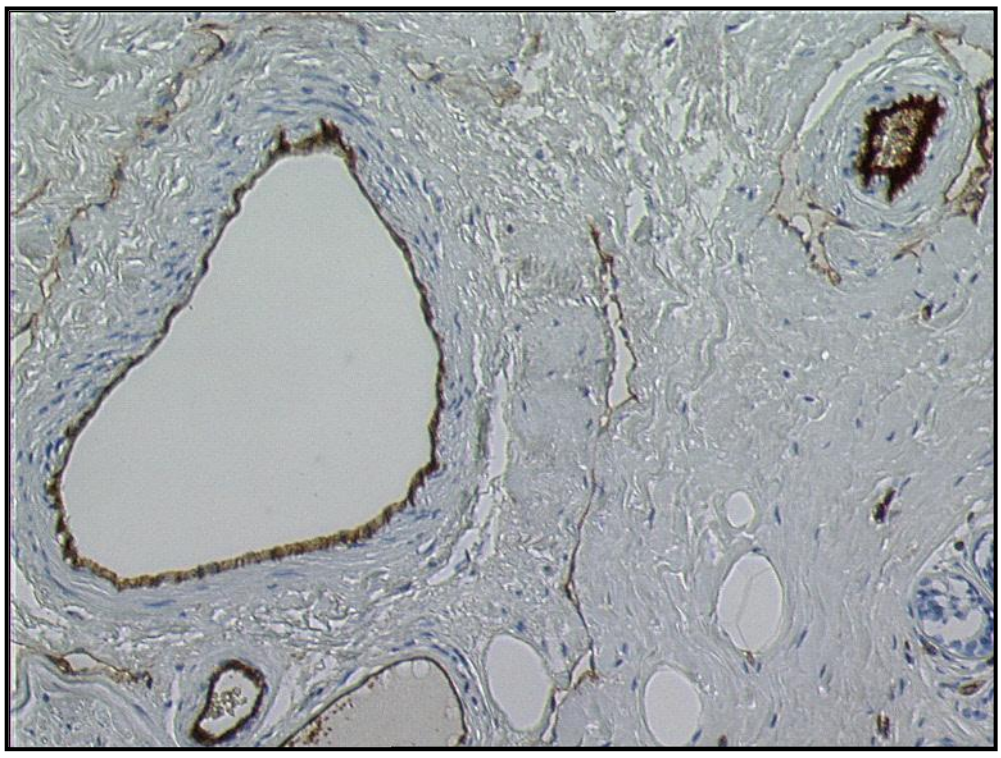

**Fig 15D** Normal breast, (primary antibody to CD31), magnification (objective) x 20 LSAB staining method Blue stain: hematoxylin to stain nuclei

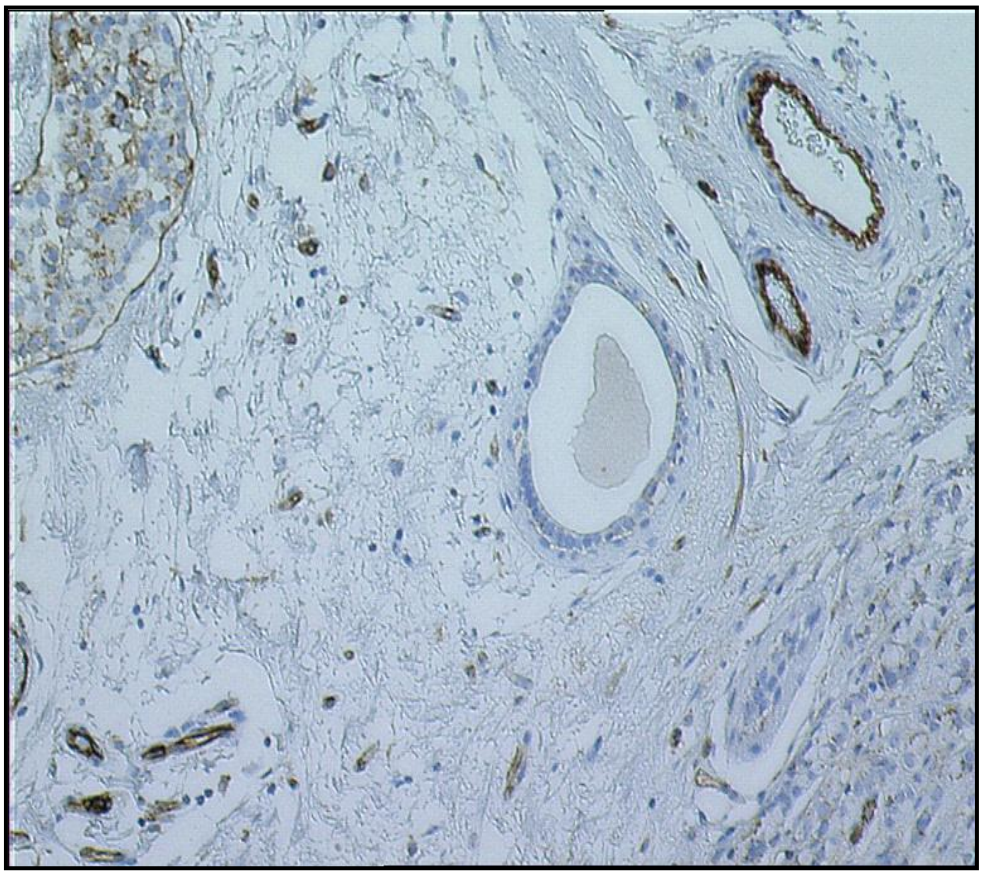

**Fig. 15E** **Breast cancer (primary antibody to CD31) magnification x10 LSAB staining method Blue stain: hematoxylin to stain nuclei**

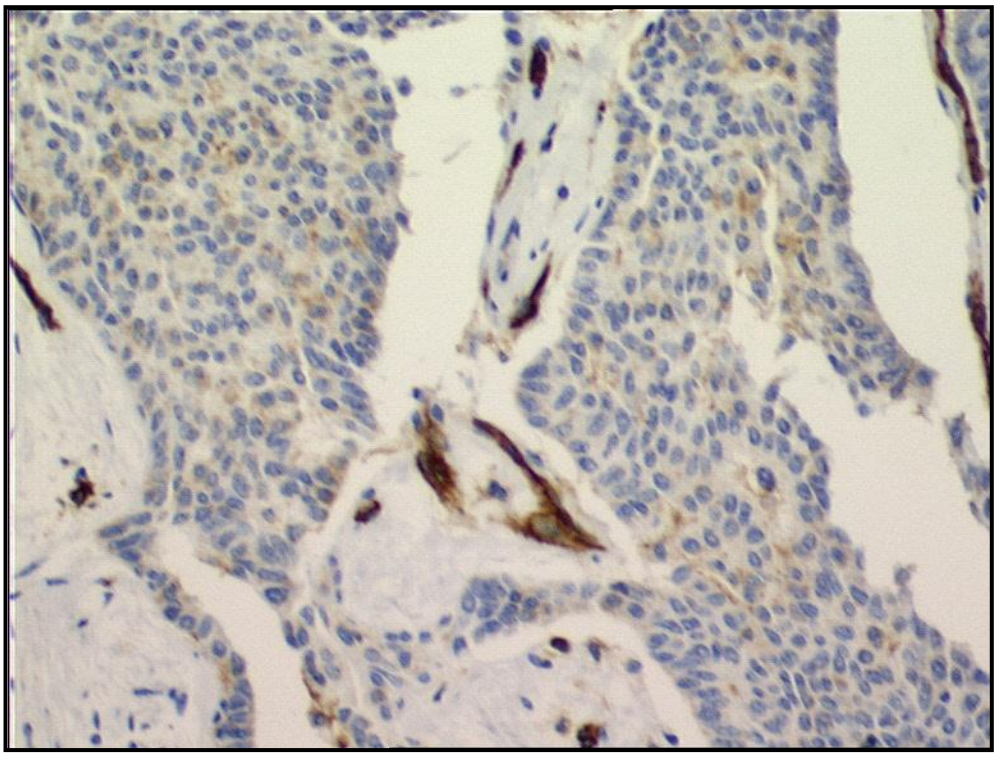

**Fig. 15F** **Breast cancer,(primary antibody to human CD31), magnification(objective) 10x LSAB staining method**

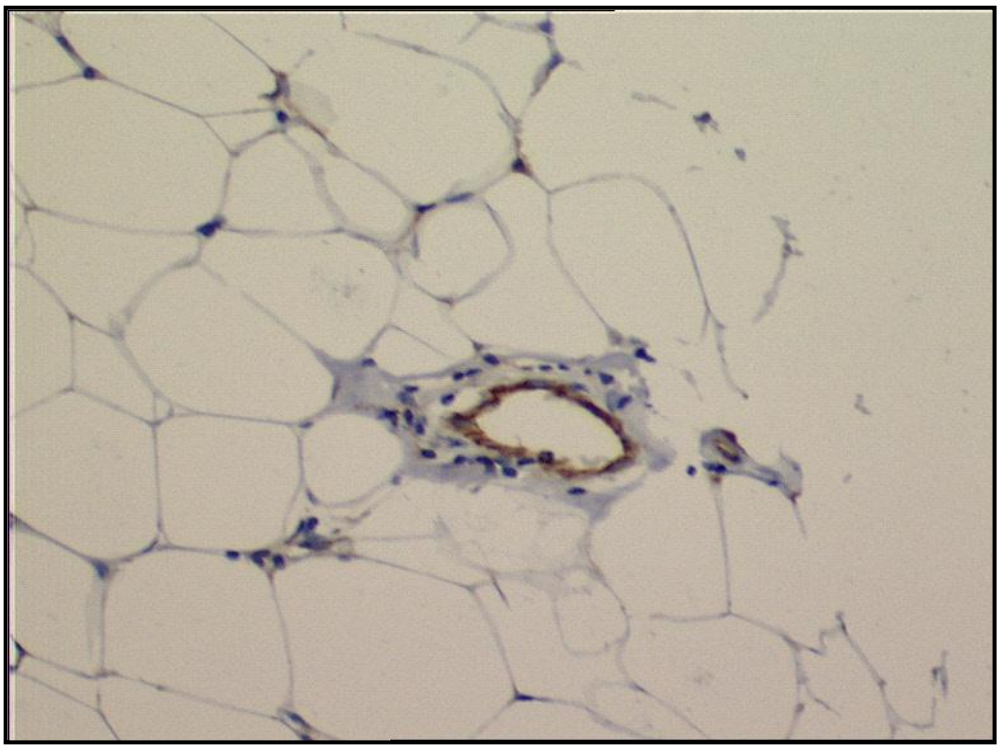

**Fig 15G Blood vessel in breast tissue, (primary antibody to human CD31), magnification (objective) 10x LSAB staining method**

## 3.2 Testing of antibodies to integrin αVβ3

Antibodies to human integrin $\alpha_V\beta_3$ clone number LM609 from Chemicon Int., Germany and Abcam Ltd., United Kingdom were tested on placenta and liver tissue sections to ascertain whether there is a colocalization of that integrin and Vn. Unfortunately, this antibody did not work well on formalin-fixed, paraffin-embedded tissue sections. Even after modifying and varying the methods of tissue section pretreatment, for example with and without pressure cooking, the staining remained entirely negative. We therefore could not apply the respective antibodies for the immunohistochemical analysis of breast tissue samples. It may be necessary to improve the process of manufacture of such antibodies to suit their application in immunohistochemistry of formalin-fixed tissue sections.

## 3.3 Immunohistochemical analysis of vitronectin in human breast carcinomas, human control tissues and normal human breast tissue

To study the localization of Vn in invasive ductal breast carcinomas, ductal carcinomas in situ and normal breast tissue, formalin-fixed, paraffin-embedded sections were assessed by IHC employing different antibodies to Vn. The antibodies tested were VN-7 monoclonal antibody from mouse, VN-7 biotinylated-monoclonal antibody from mouse, VN-9 monoclonal antibody from mouse, α-S-biotinylated polyclonal antibody from rabbit, Rb 66/67-polyclonal antibody from rabbit, 13HI and 16AJ monoclonal antibodies (all supplied by K.T. Preissner, Giessen) as well as monoclonal mouse antibodies to human CD31 (purchased from Dako, Hamburg) and an antibody to integrin $\alpha_V\beta_3$ (purchased from Chemicon, Hamburg). As determined by pilot experiments, the LSAB method of detection provided the best staining results.

Prior to the immunohistochemical staining of breast tissue samples, the immunohistochemical detection of the expression pattern of Vn was performed on a preliminary basis using stored formalin-fixed, paraffin-embedded sections of kidney, placenta and liver tissue as well as multi-norm arrays which were known to

show a characteristic staining pattern, for testing different methods and antibodies. The immunostaining of sections of human liver with the mouse monoclonal antibody VN-7 directed against Vn with a stock concentration of 320 ng/mL diluted according to protocol (1:10,000) with antibody diluent produced optimal results. A heterogeneous distribution of Vn immunoreactivity was observed throughout the central and portal vein regions. (Fig. 16T). These results were consistent with previous findings and provided the basis for the application of the same procedure to the immunostaining of the breast tissue samples. Although the other vitronectin-directed antibodies tested showed very similar staining pattern, they were not utilized for the immunohistochemical analysis of the breast tissue samples because their staining was weaker and less specific (not shown).

Using a series of parallel sections of twenty carcinomas and normal breast tissue, respectively, identical staining patterns were obtained for the antibodies tested. Polyclonal Vn-directed antibodies Rb 66/67 using the PAP and APAAP-methods of detection produced indistinct staining results with disturbing background coloration (not shown). Protease treatment and pressure cooking with citrate buffer only gave undesirable results that were not useful for our purposes. Monoclonal antibody VN-7 and employing the LSAB method of detection gave the best results.

Vn was often abundantly accumulated in the extracellular matrix and connective tissue around milk ducts (Fig. 16M, N and O). As judged from the staining, large areas with cancer cell burden did not contain any vitronectin. (Fig 16K and L). Also, Vn immunoreactivity surrounding cancer cells was localized in the vicinity of capillaries and small vessels (Fig. 16R and S). Both Fig.s 16R and S depict different areas of the same cancerous breast tissue. Fig. 16S demonstrates an overview of the tissue sample taken at an objective 2.5x whereas Fig. 16R shows a more focal view at objective 20x. Furthermore, in microvessels, there was no Vn deposition in the endothelial cells. Fig. 16I and J illustrate the negative control sections of cancerous breast tissue using the antibody of irrelevant specificity. Both displayed no staining.

On the other hand, normal breast tissue displayed a heterogeneous distribution of Vn immunoreactivity. Vn immunoreactivity had a homogenous periductal

occurrence in normal breast tissue as can be clearly seen in Fig 16 (F, G and H) below, but the staining pattern was clearly different from that seen around the malignant epithelial cells in the carcinomas, the local accumulation of Vn in cancerous tissue being more distinct and intense as compared to normal tissue. Normal breast tissue showed occasional staining in some of the small vessels (Fig. 16E). The negative control section of normal breast tissue remained entirely blank and unstained as shown in Fig. 16C and D. Liver tissue was used as a positive control. As shown in Fig. 16 B, the hepatocytes were partially positively stained. On the other hand, negative control (antibody diluent of irrelevant specificity) using liver tissue always gave completely blank sections (Fig. 16A). Again, Fig. 16V shows the staining pattern for Vn in normal breast tissue at a dilution of 1:10,000, with a Vn concentration of 80 ng/ml. Compared to a similar section in Fig. 16W at the same antibody concentration, a more focal view distinguishes the various cell layers. The epithelial cells of the mammary ducts are largely unstained and the distribution of Vn in the surrounding connective tissue is heterogeneous. Fig 15V displays the cutis surrounding the mammary gland. A positive reaction was detected in the underlying smooth muscle cells.

**Immunostainings using the monoclonal antibody VN-7 to vitronectin and the LSAB method of detection**

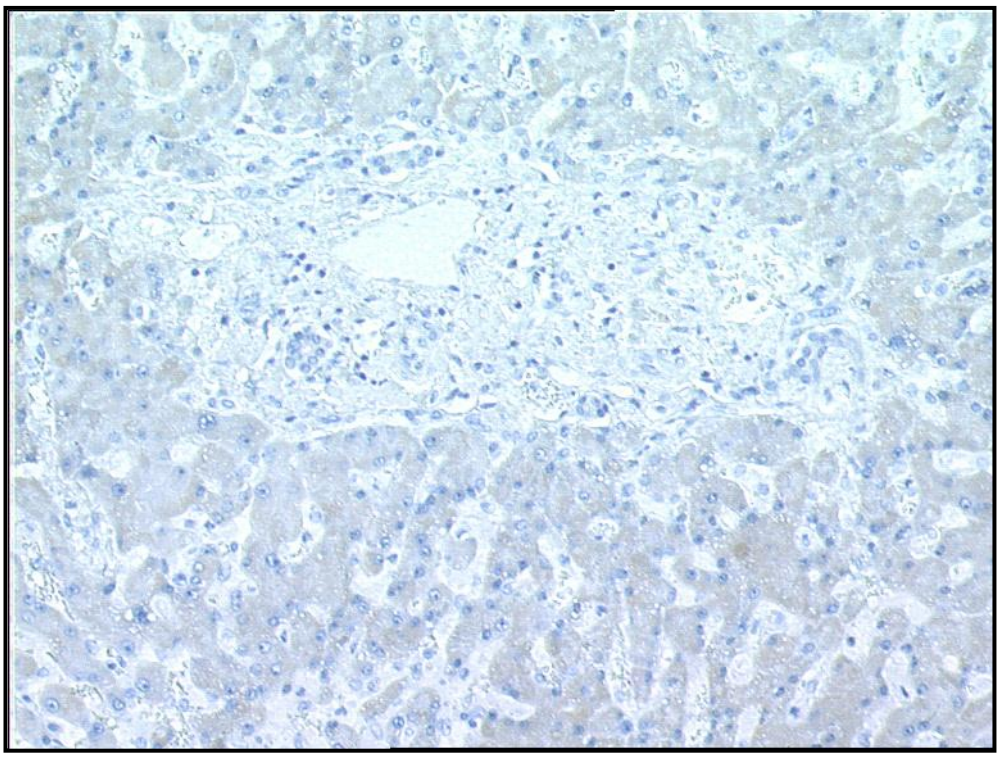

**Fig 16A** Liver , negative control (no primary antibody)
magnification (objective) x10 LSAB staining method
Blue stain: hematoxylin to stain nuclei

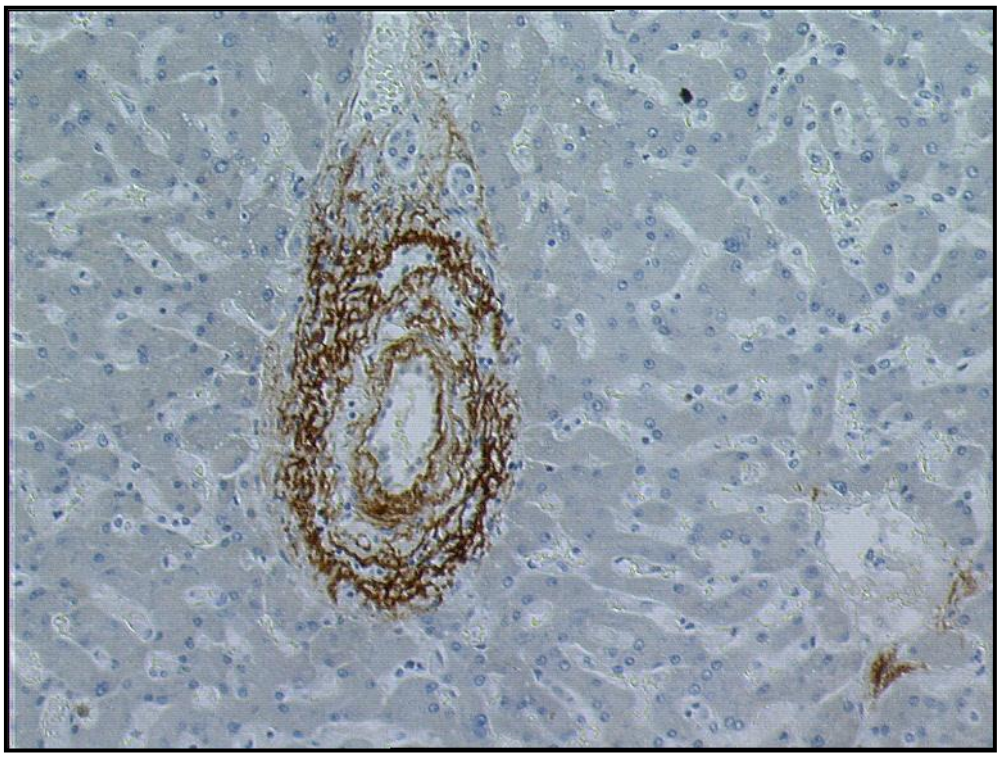

**Fig. 16B** Liver ( primary antibody VN-7, 80 ng/mL)
magnification (objective) x10 LSAB staining method
Blue stain: hematoxylin to stain nuclei

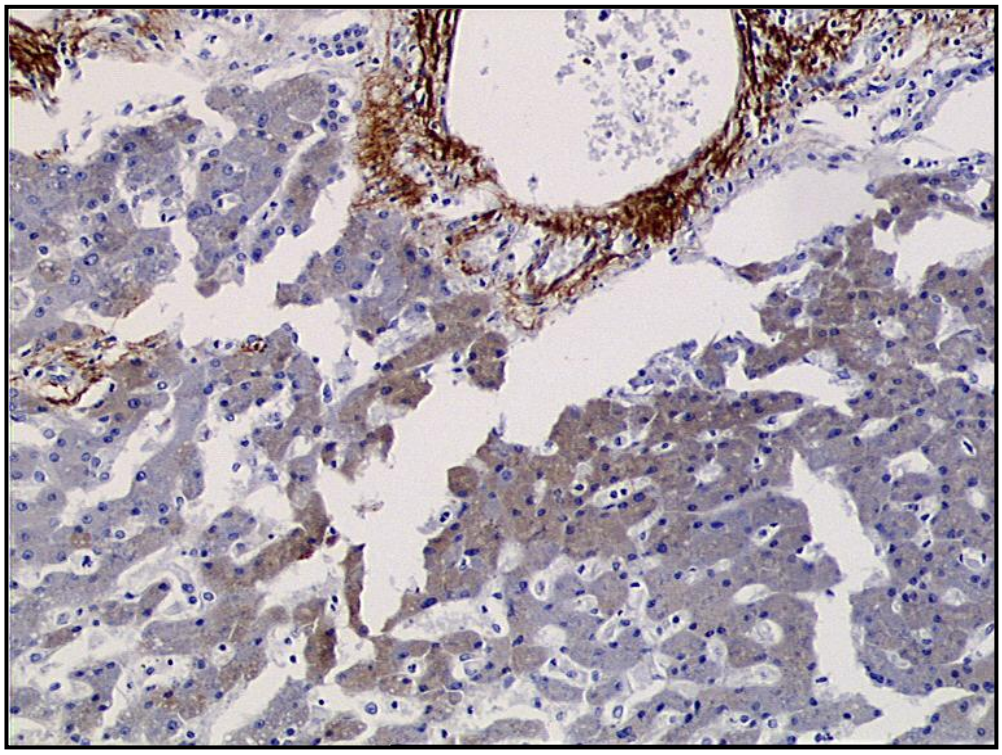

**Fig. 16C** **Liver (primary antibody VN-7) 112 ng/ml magnification (objective) x 10 LSAB staining method**

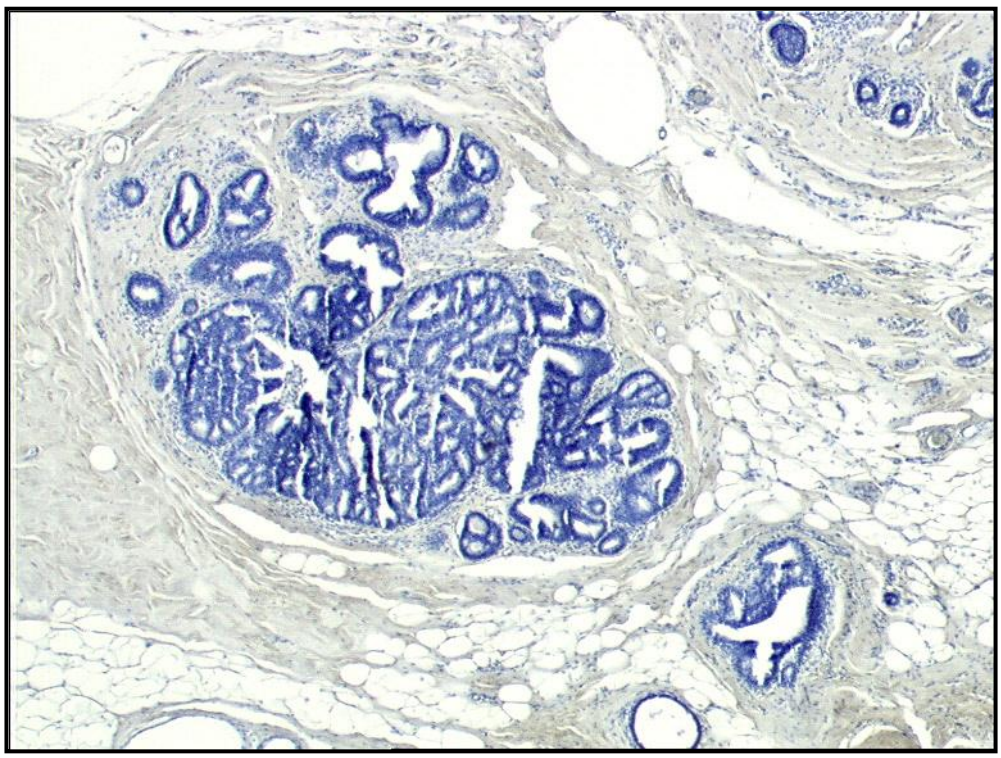

**Fig. 16D** **Normal breast, negative control (no primary antibody) magnification (objective) x2.5 LSAB staining method Blue stain: hematoxylin to stain nuclei**

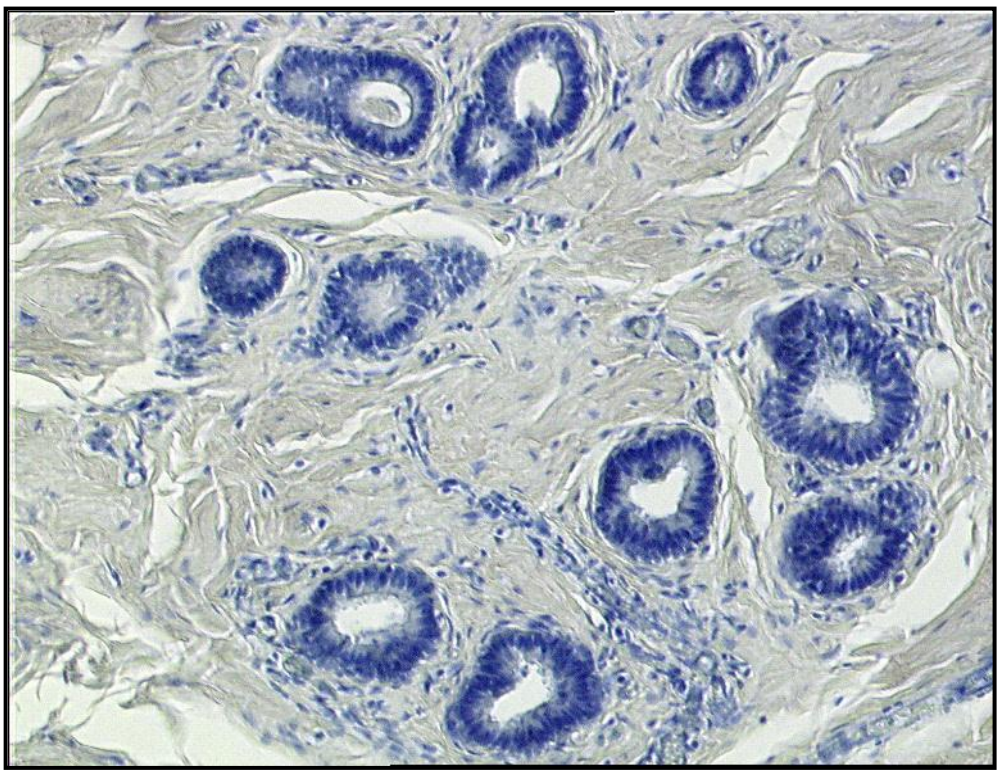

**Fig 16E** **Normal breast, negative control (no primary antibody) magnification (objective) x10 LSAB staining method Blue stain: hematoxylin to stain nuclei**

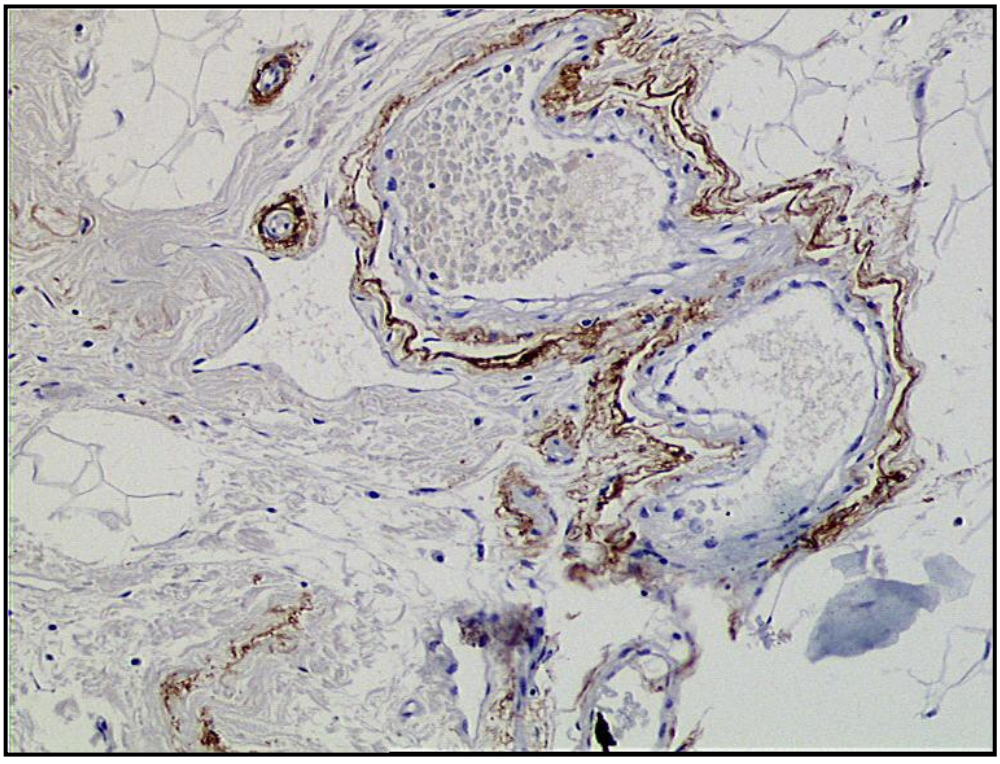

**Fig. 16F** **Normal breast (antibody VN7, 80 ng/mL) magnification (objective) x10 LSAB staining method**

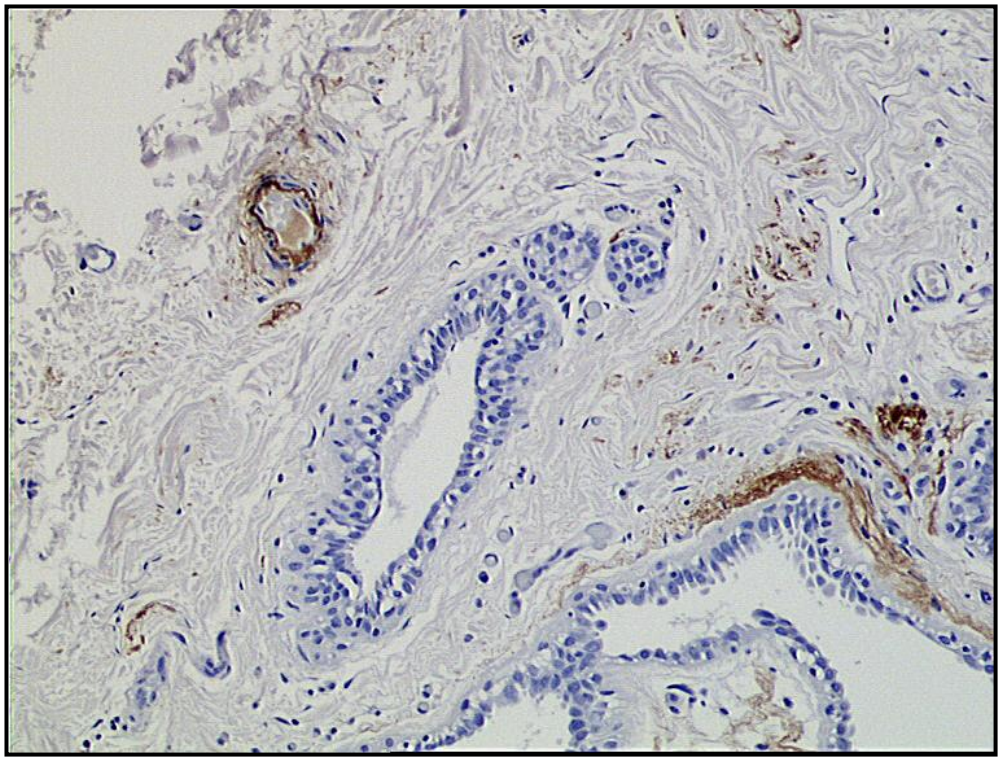

**Fig. 16G** **Normal breast (antibody VN7, 80 ng/mL) magnification (objective) x10 LSAB staining method**

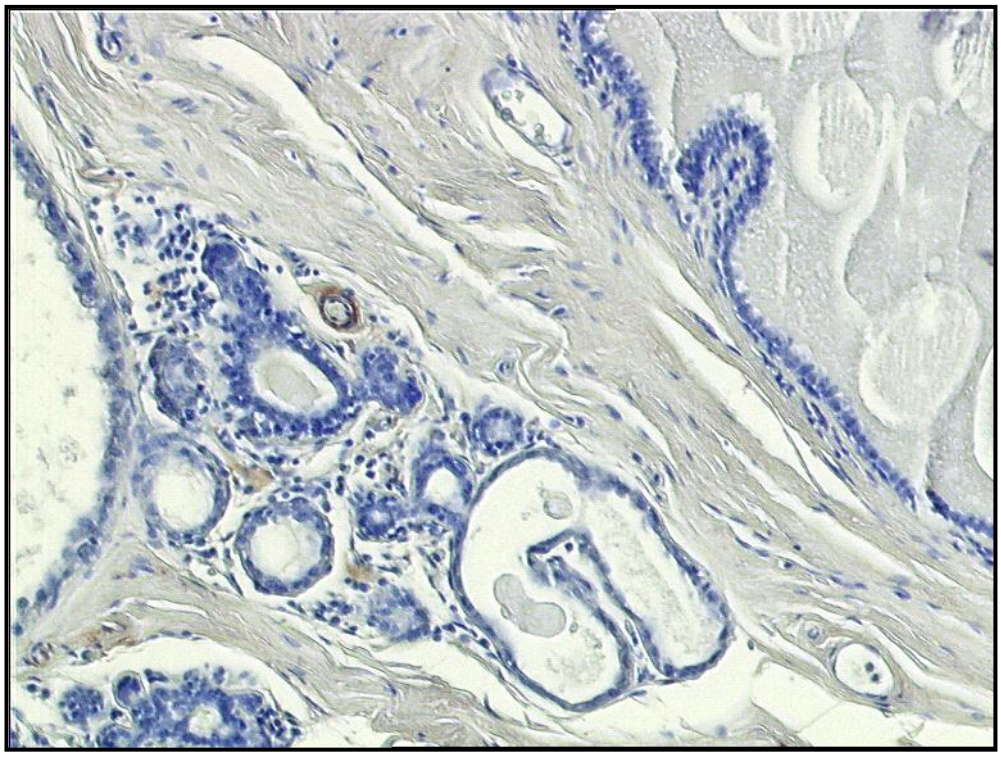

**Fig 16H** **Normal breast (antibody VN7 80 ng/mL) magnification (objective) x10 LSAB staining method**

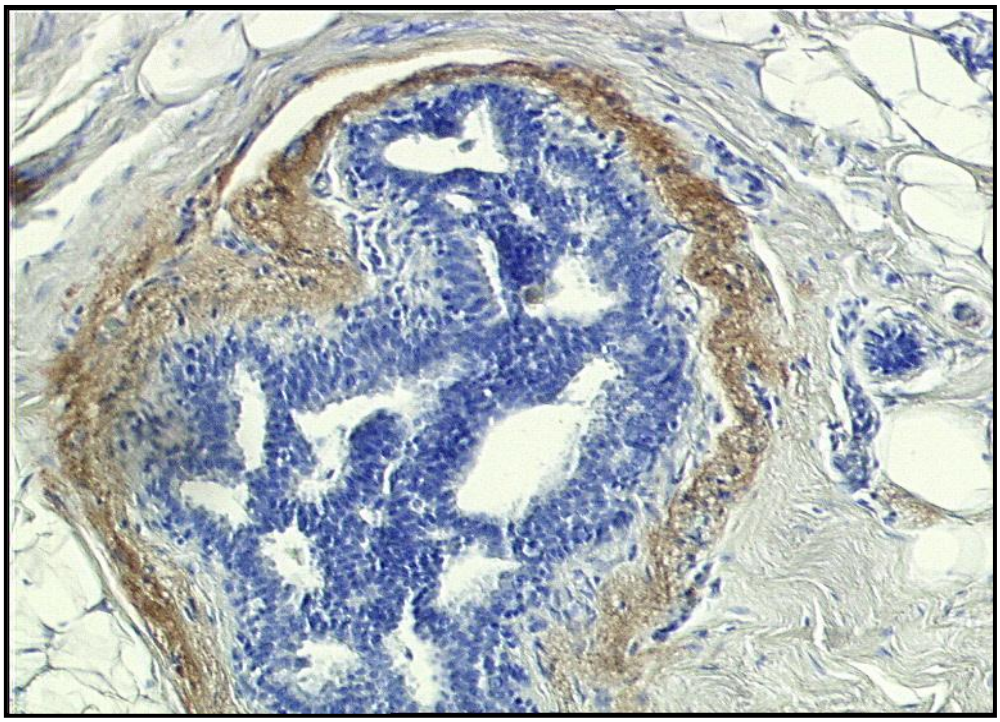

**Fig. 16I** **Normal Breast (antibody VN7, 80 ng/mL)**
**magnification (objective) x10 LSAB staining method**

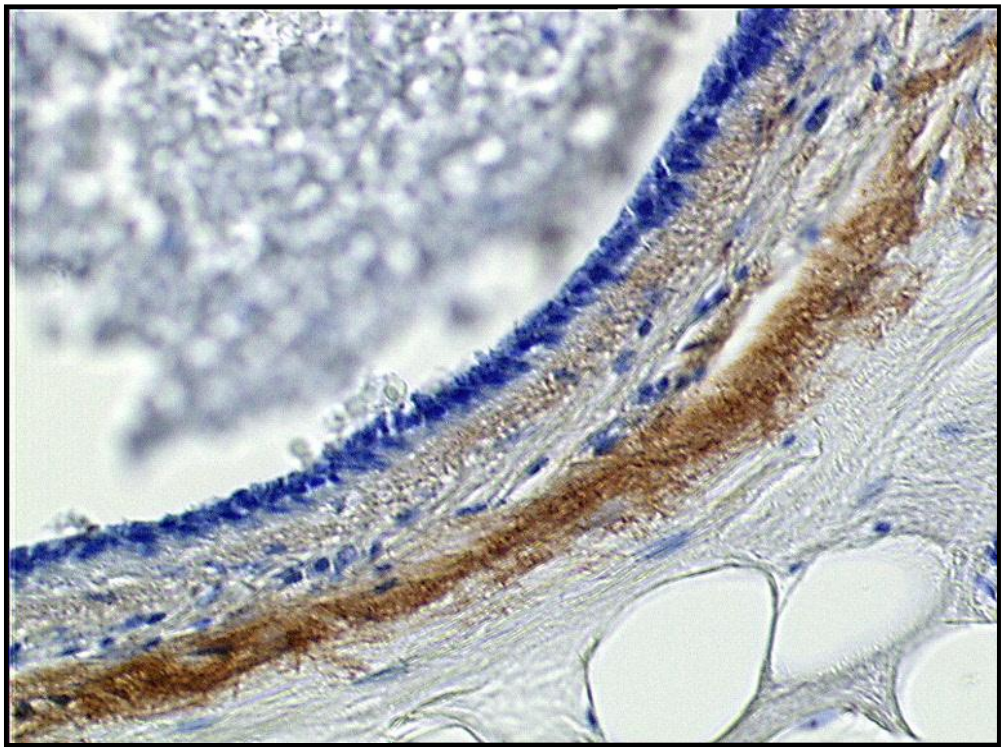

**Fig. 16J** **Normal breast (primary antibody VN7, 80 ng/mL)**
**Magnification (objective) 20x LSAB staining method**

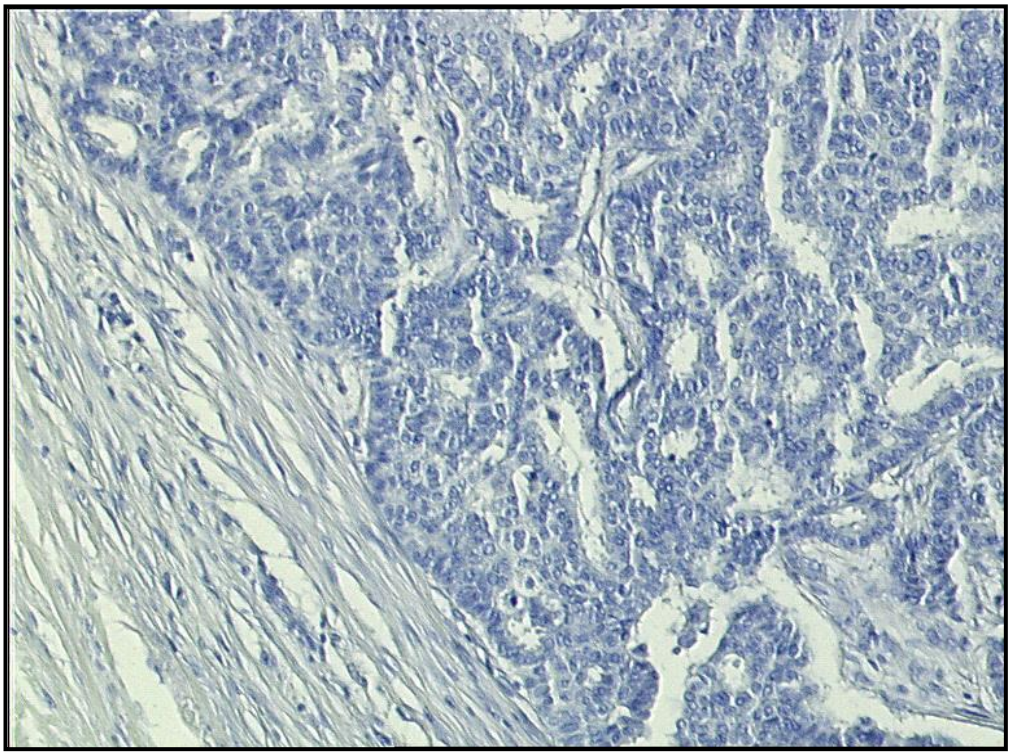

**Fig 16K** **Breast cancer, negative control (no primary antibody) magnification (objective) x10 LSAB staining method Blue stain: hematoxylin to stain nuclei**

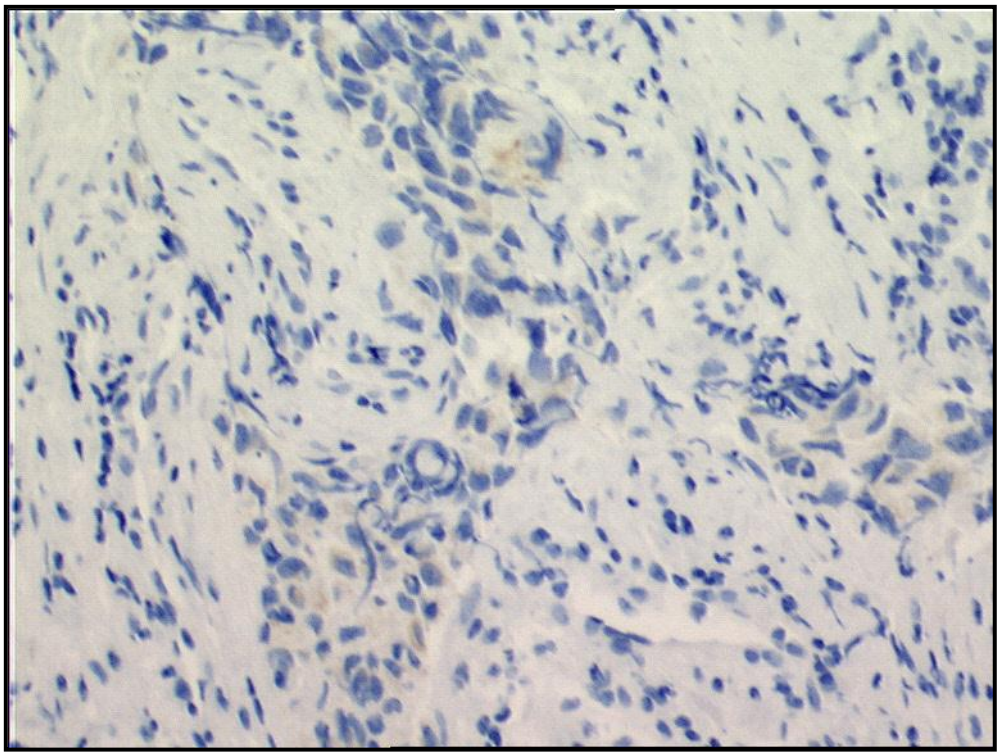

**Fig 16L** **Breast cancer, negative control (no primary antibody) magnification (objective) x10 LSAB staining method**

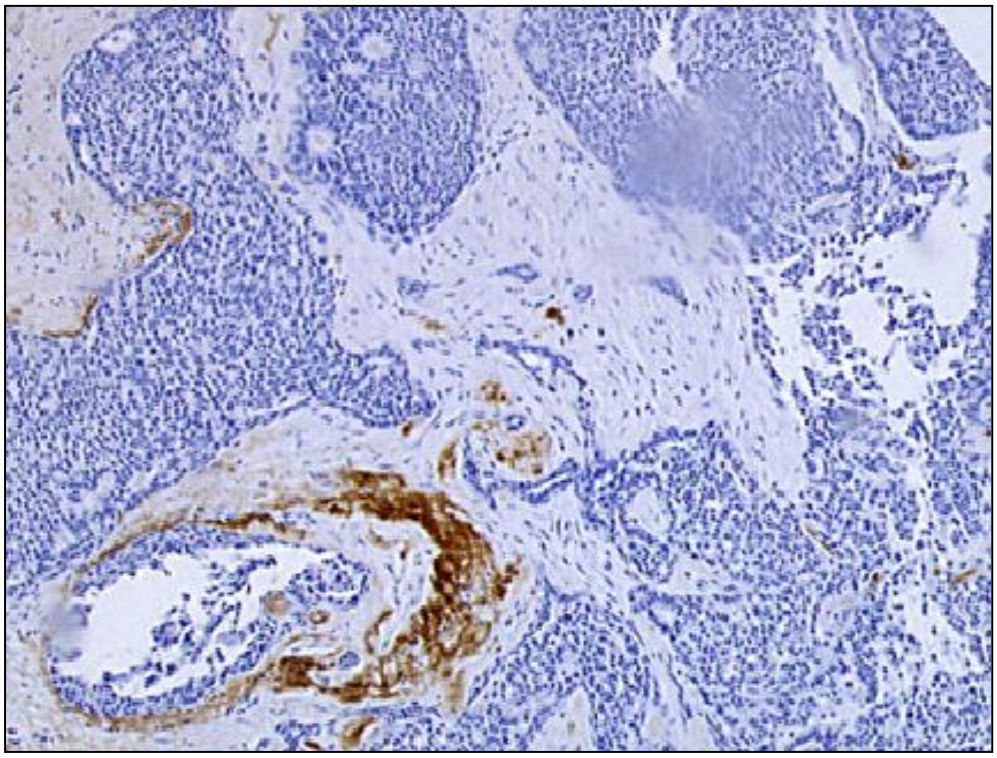

**Fig. 16M** **Breast cancer (antibody VN7 320 ng/mL) magnification (objective) 2.5x LSAB staining method**

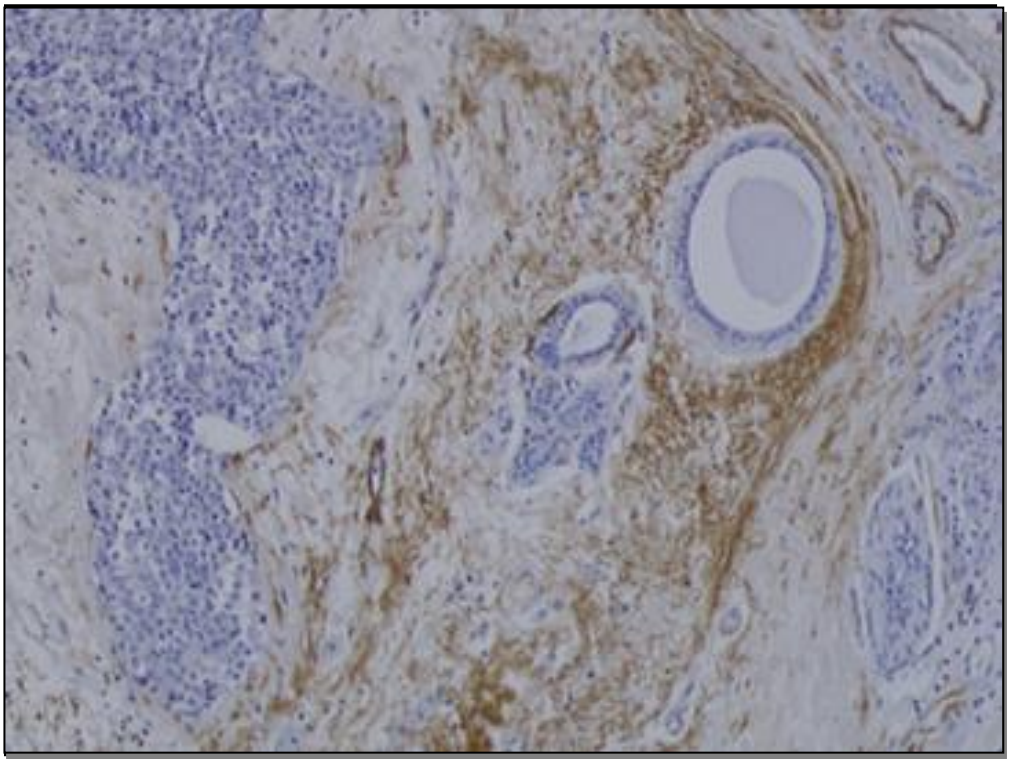

**Fig.16N** **Breast cancer, (primary antibody VN7, 80 ng/mL) magnification (objective) x 2.5 LSAB staining method**

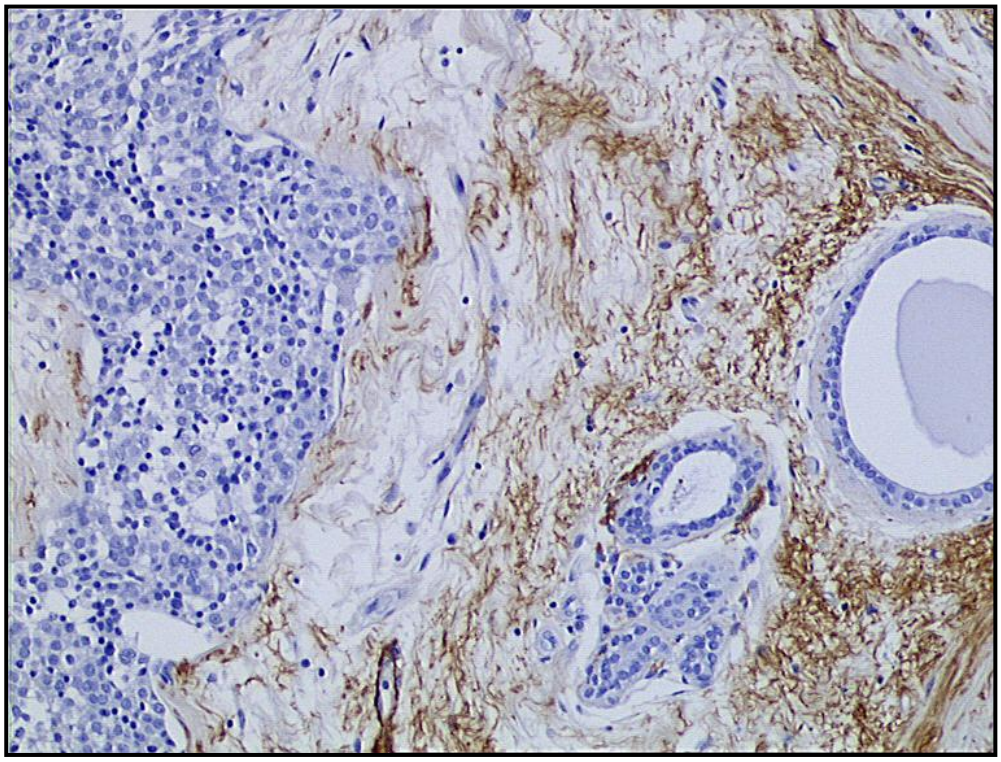

**Fig. 16O** **Breast cancer (primary antibody VN7, 160 ng/mL) magnification (objective) 20x LSAB staining method**

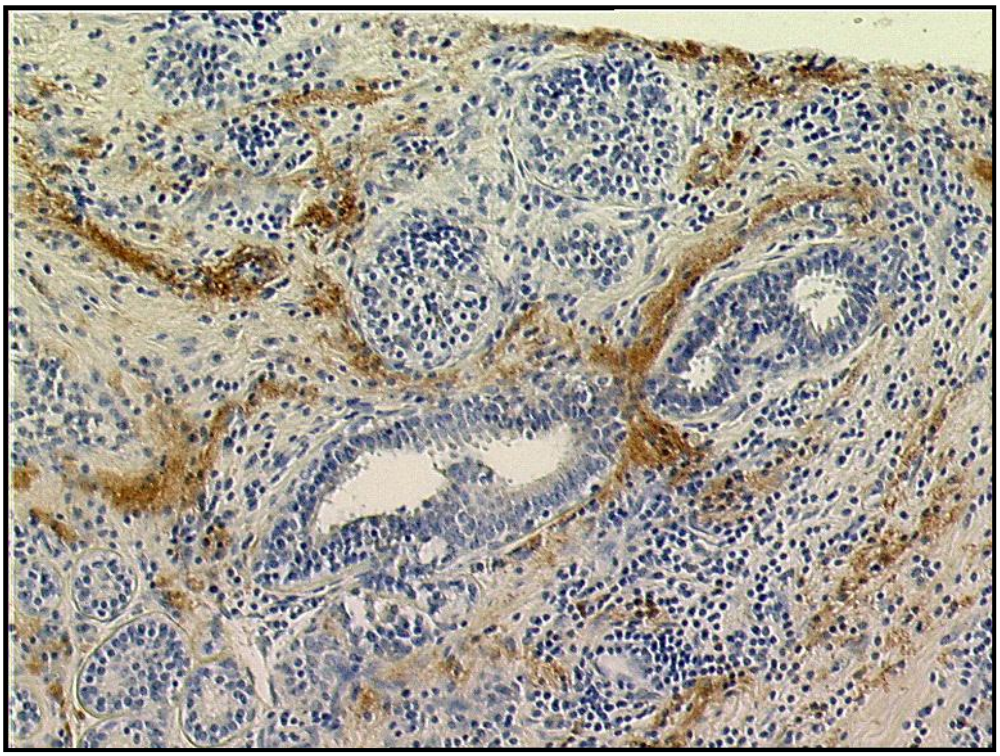

**Fig. 16P** **Breast cancer (primary antibody VN-7, 80 ng/mL) magnification (objective) 20x LSAB staining method**

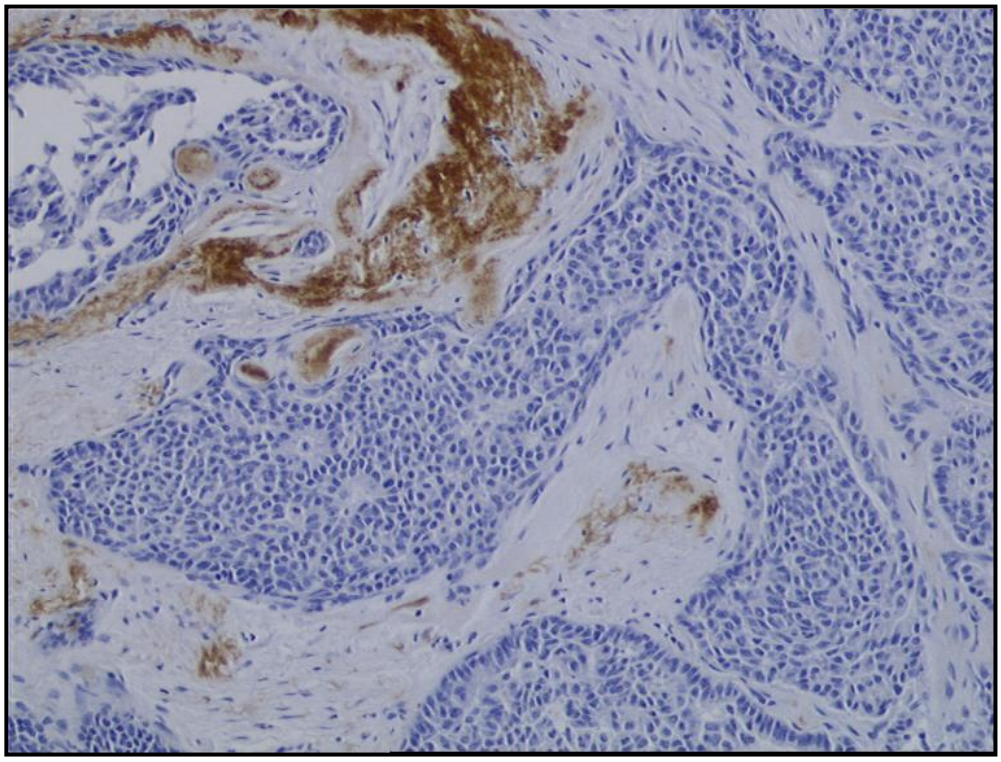

**Fig. 16Q** **Breast cancer (primary antibody VN-7, 80 ng/mL) magnification (objective) 10x LSAB staining method**

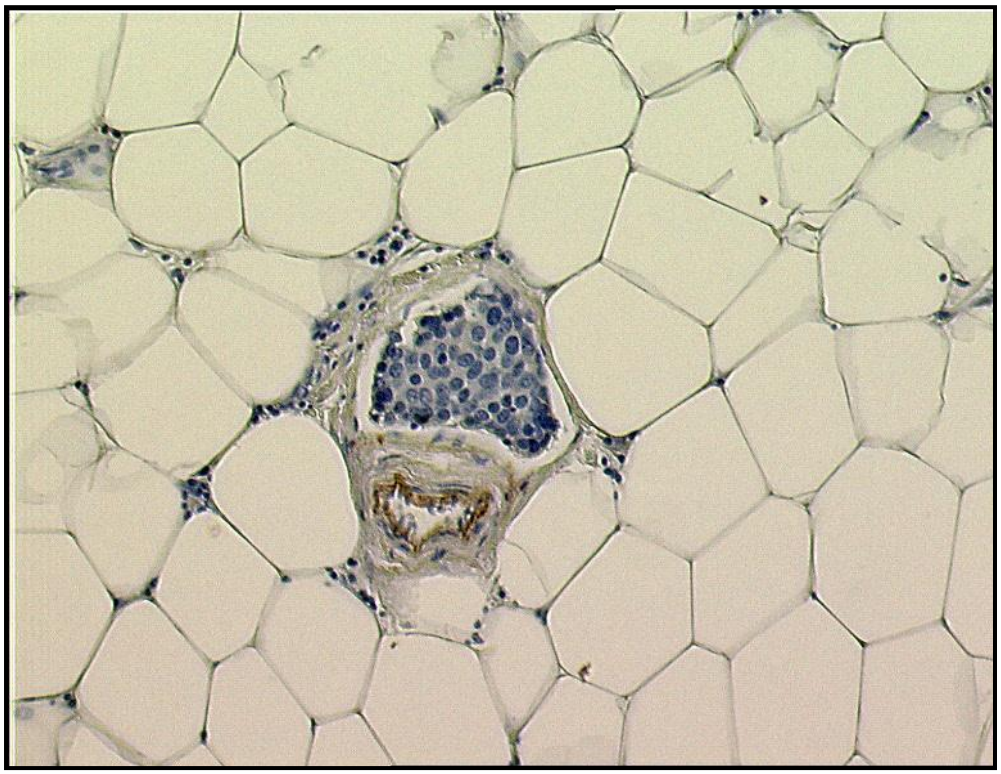

**Fig. 16R** **Blood vessel next to tumor nest in fat tissue (primary antibody VN-7, 80ng/mL) magnification (objective) x10 LSAB staining method**

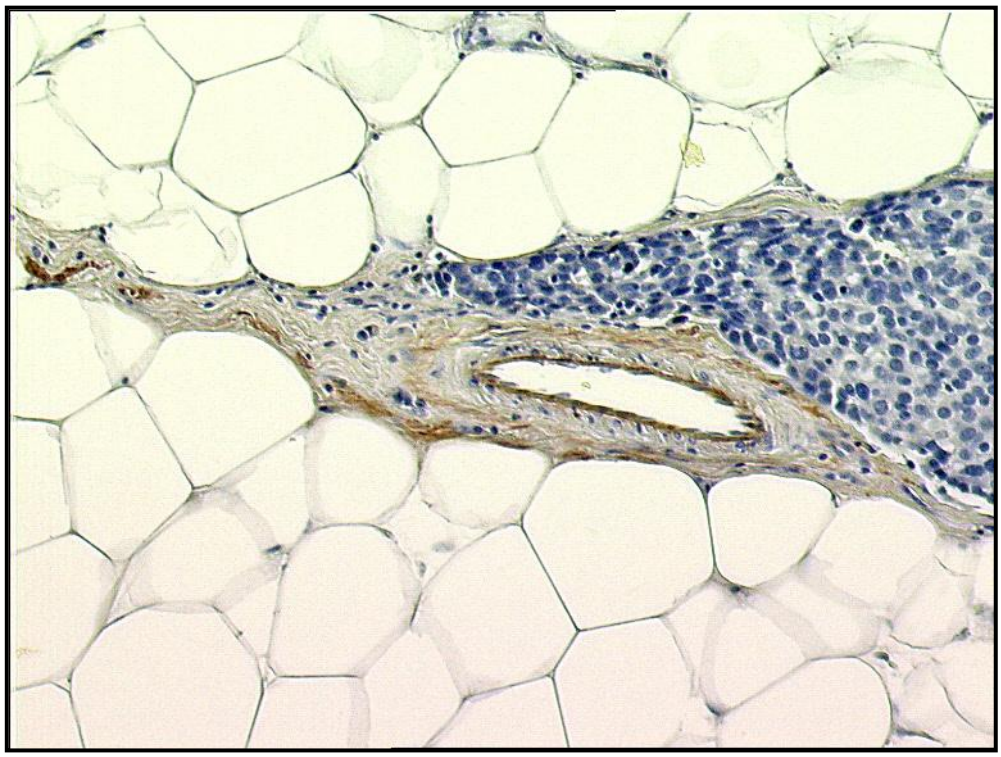

**Fig. 16S** **Blood vessel next to breast cancer nest in fat tissue (antibody VN-7, 80 ng/mL) magnification (objective) x10 LSAB staining method**

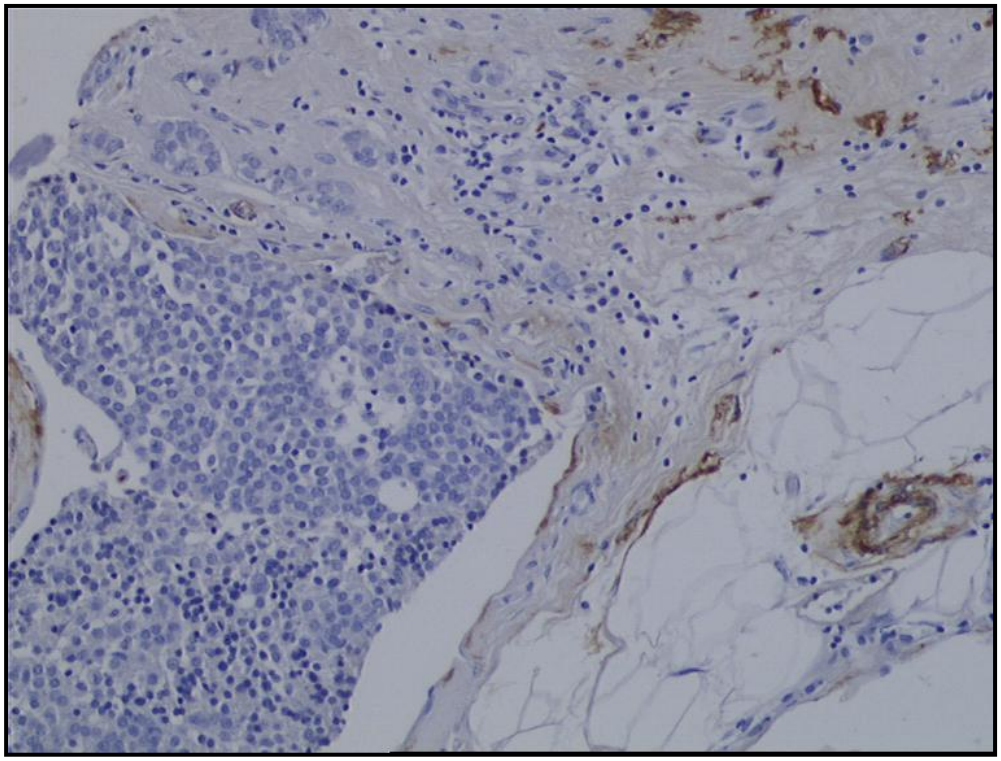

**Fig: 16T** **Breast cancer (primary antibody VN-7, 80 ng/mL) magnification (objective) 20x LSAB staining method**

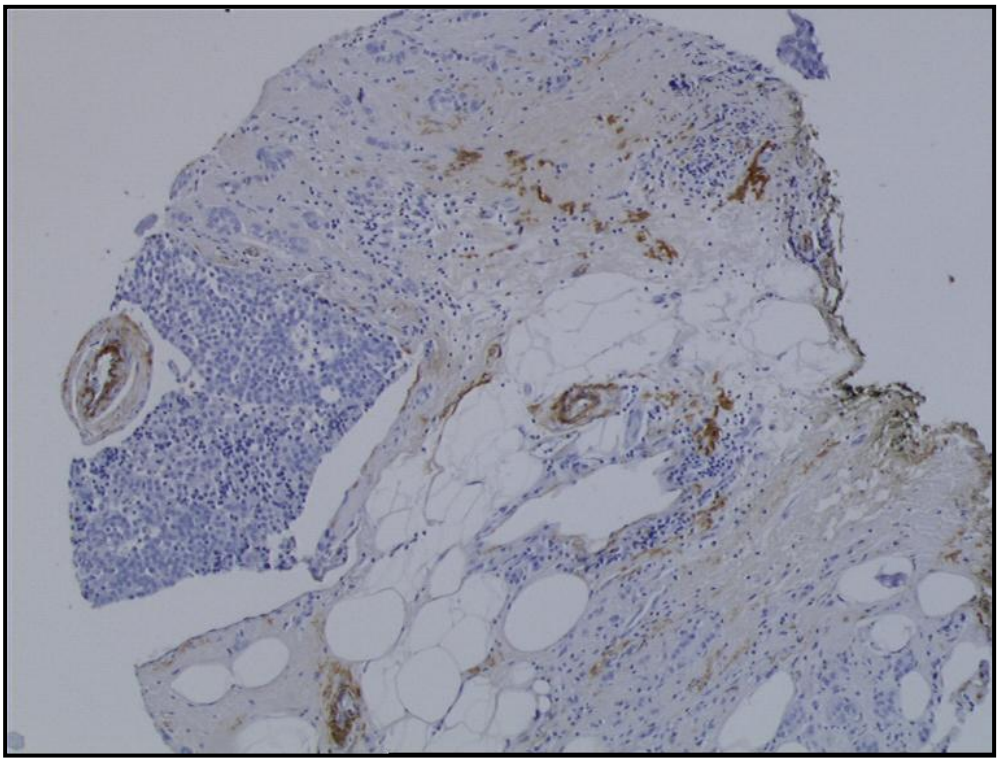

**Fig. 16U** **Breast cancer (primary antibody VN-7, 80 ng/mL) magnification (objective) 2,5x LSAB staining method**

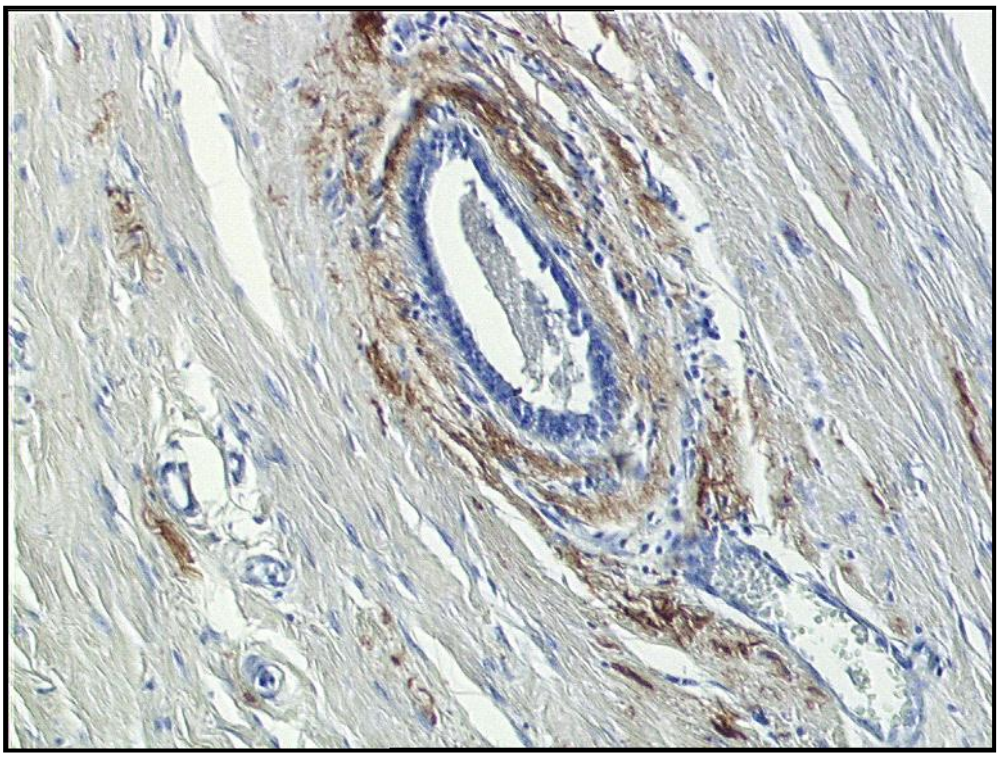

**Fig. 16V** **Breast cancer (primary antibody VN7, 80 ng/mL) magnification (objective) 10x LSAB staining method**

### 3.4 Immunohistochemistry for Vn in ductal carcinomas in situ (DCIS)

Using a series of parallel sections each of ten ductal carcinomas in situ (DCIS), identical staining patterns were obtained similar to those observed in the ductal invasive cancerous human breast tissue described in 3.2. Monoclonal antibodies VN-7, employing the LSAB method of detection, gave the best results of immunostaining. DCIS refers to the most common type of noninvasive breast cancer in women. With DCIS, the cancer cells are confined to milk ducts in the breast but have not spread yet into the surrounding breast tissue or to any other part of the body (such as the axillary lymph nodes).

According to Tsikitis and Chung (American Journal of Clinical Oncology, 2006 June; 29(3): 305-10), incidence of DCIS has increased significantly during the last decade, comprising almost 20% of all breast cancers diagnosed today. DCIS is composed of malignant breast duct epithelial cells that have clonally proliferated and accumulated within the mammary duct lumen. It comprises a group of heterogeneous tumors with varying biologic behaviour rendering its classification and management challenging. By definition, DCIS does not penetrate the basement membrane; it is a preinvasive malignancy and systemic disease is nonexistent. The basis of treatment is to prevent progression into an invasive cancer such as invasive ductal carcinoma. Most current DCIS classification schemes do not predict its potential to progress to invasive disease. There are two categories of DCIS: non-comedo and comedo. The term, comedo, describes the appearance of the cancer. When comedo type breast tumors are cut, the dead cells inside of them (necrosis) can be pressed out just like a comedo or blackhead on the skin. Comedo type DCIS is the most common form and also the most aggressive. Non-comedo DCIS has low mitotic rates and does not exhibit central necrosis. It is further classified into cribiform, solid, micropapillary, and papillary subtypes. Below are illustrations of DCIS immunostainings performed. Epithelial cells lining the numerous small ducts (Fig. 17F, G and H) are completely unstained, indicating the absence of Vn immunoreactivity in these areas of breast tissue. In Fig. 17B and C, a clearly heterogeneous distribution of Vn immunoreactivity can be seen in the surrounding ECM, while the carcinoma cells remain unstained. The same pattern of distribution has been described in 3.2 for cancerous breast tissue. Negative control sections of DCIS (Fig. 17A, D and I)

remained unstained as well. In Fig. 17 J, the small ducts are unstained. The carcinoma in situ cells are unstained and can be said to demonstrate no Vn immunoreactivity. Fig. 17K illustrates the same area of breast tissue at another optical magnification.

**Immunostainings of ductal carcinomas in situ using the monoclonal antibody VN-7 to vitronectin and the LSAB method of detection**

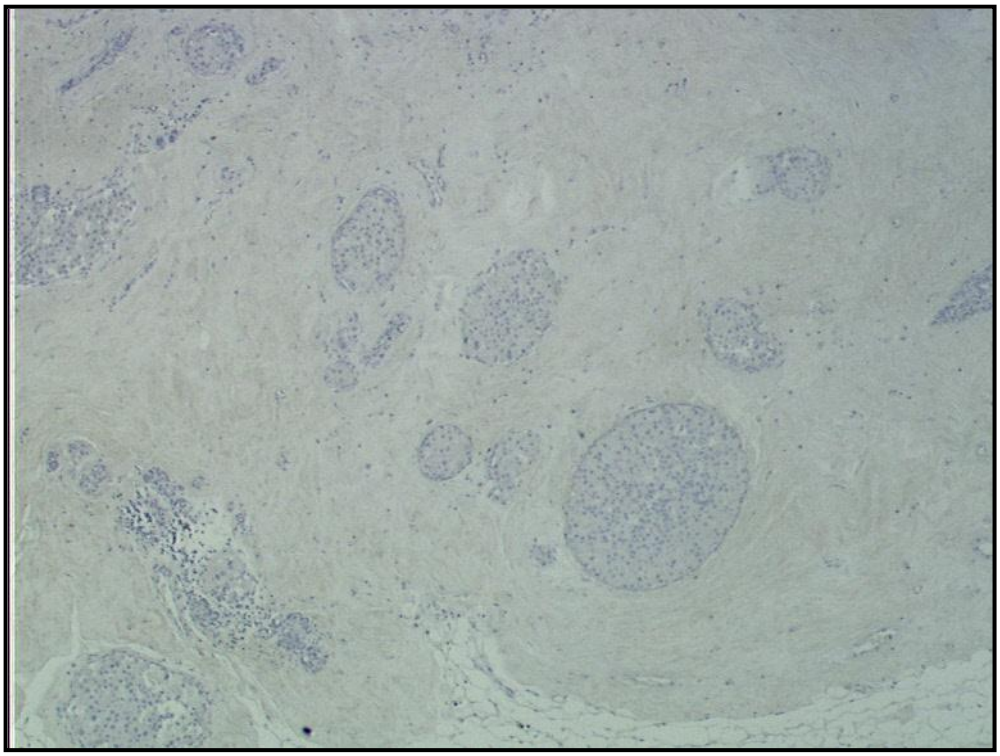

Fig. 17A DCIS negative control (no primary antibody)
magnification (objective) x2.5 LSAB staining method

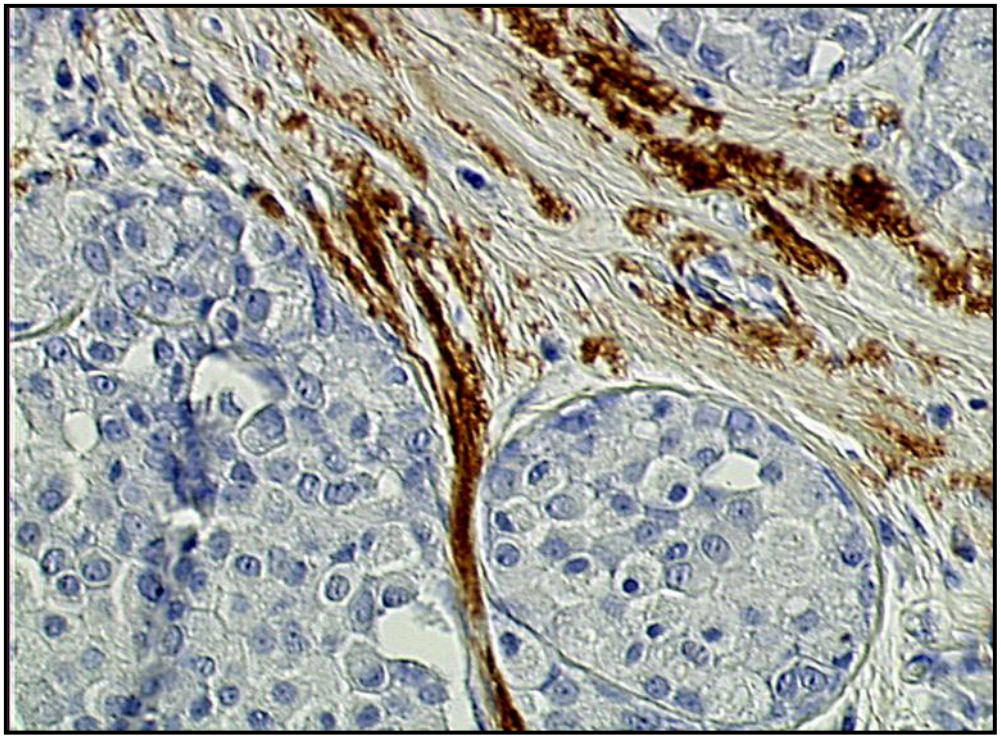

Fig. 17B DCIS (antibody VN-7, 130 ng/mL)
magnification x20 LSAB staining method

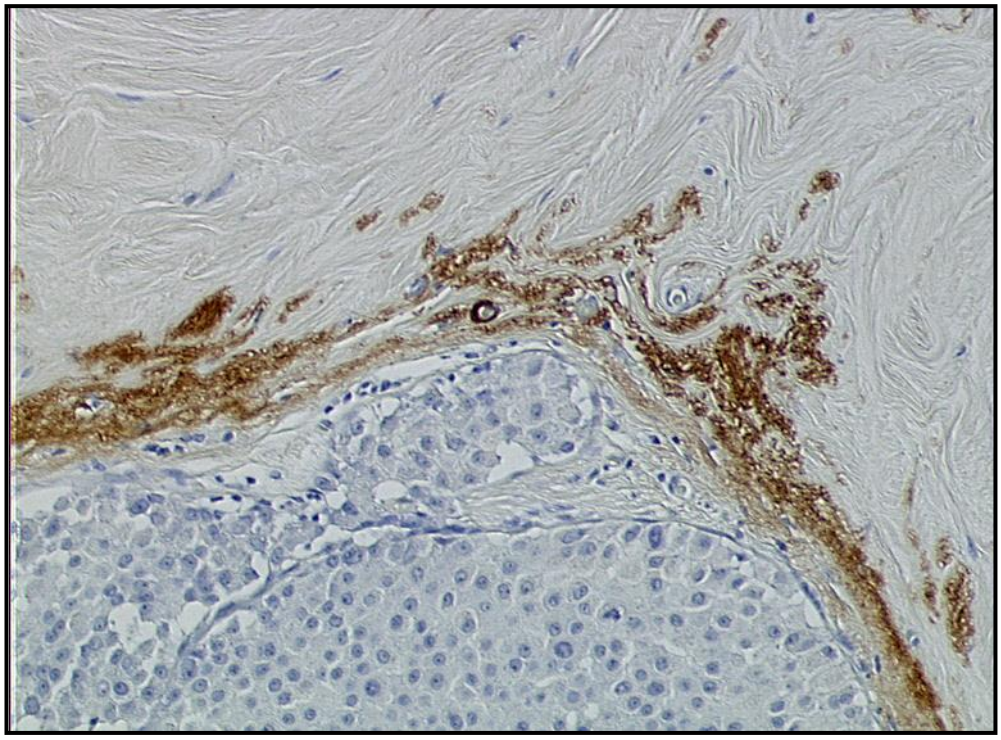

Fig. 17C DCIS antibody VN-7, 130 ng/mL)
magnification x20 LSAB staining method

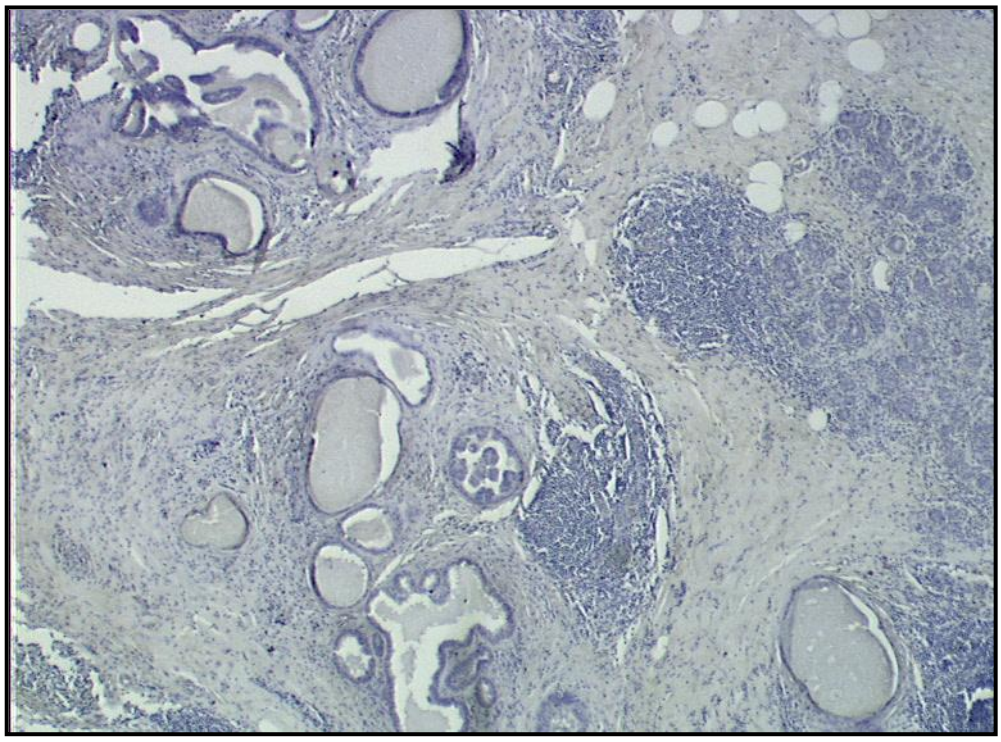

Fig. 17 D DCIS negative control (no primary antibody)
magnification (objective) x2.5 LSAB staining method

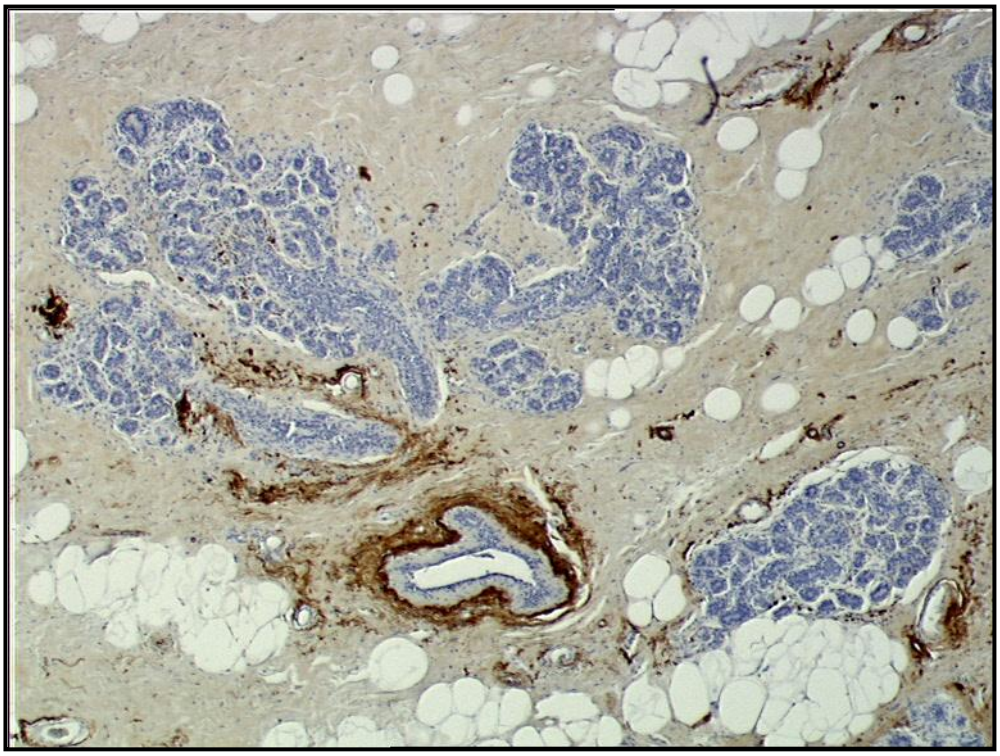

Fig. 17E DCIS (primary antibody VN7, 80 ng/mL)
magnification (objective) x2.5 LSAB staining method

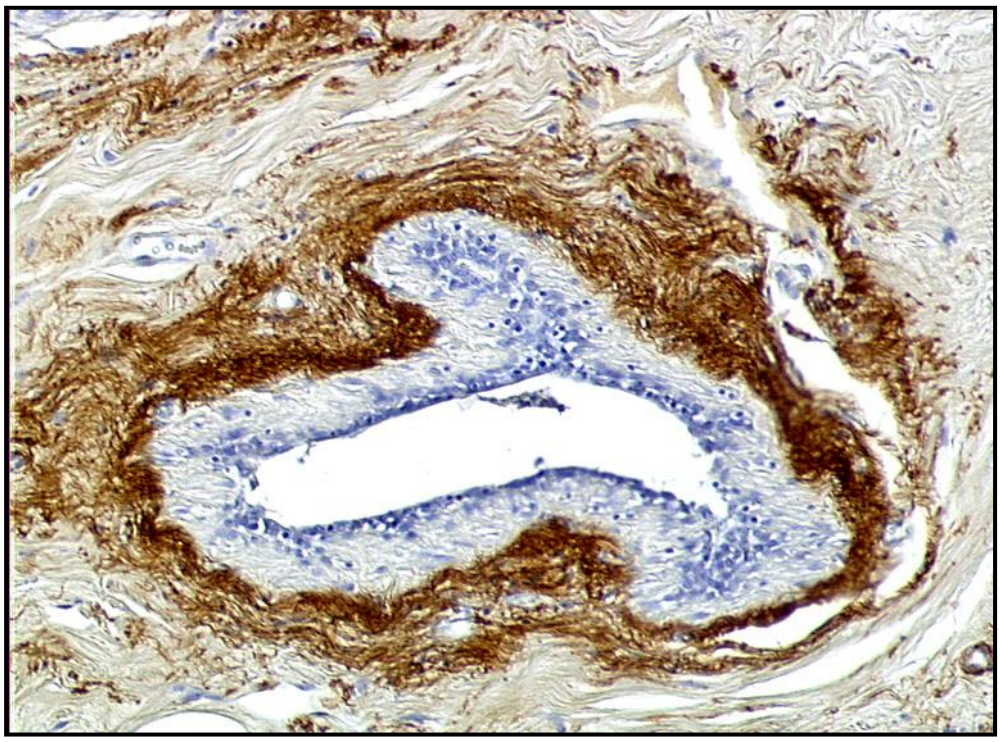

Fig. 17F DCIS (primary antibody VN7, 80 ng/mL)
magnification (objective) x10 LSAB staining method

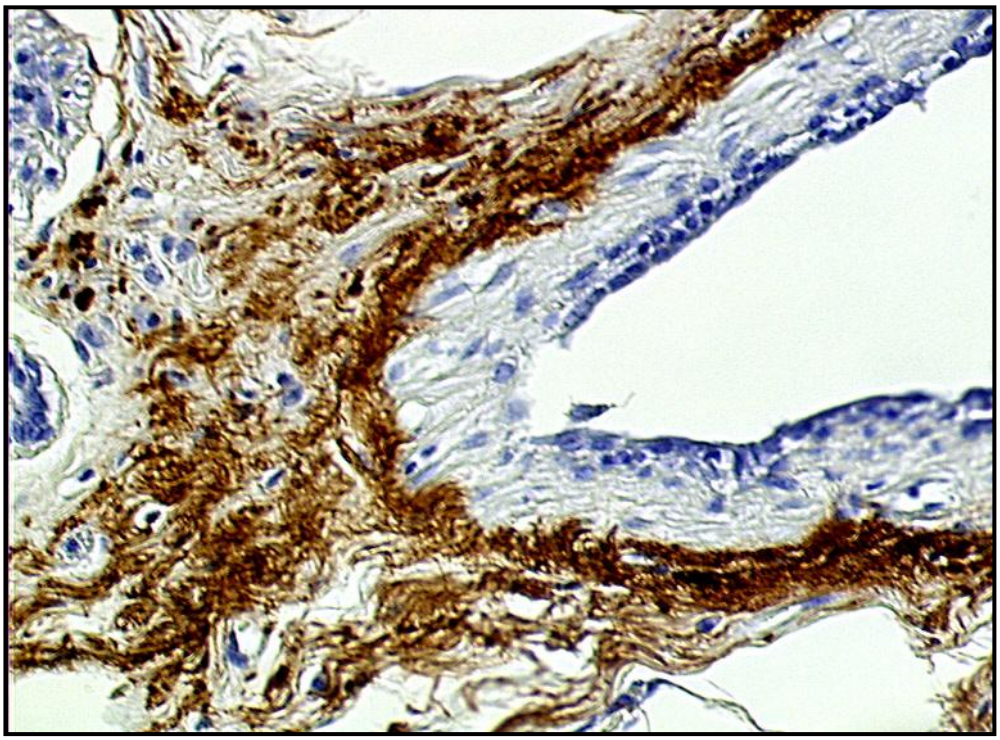

Fig. 17G DCIS antibody VN-7, 80 ng/mL)
magnification x20 LSAB staining method

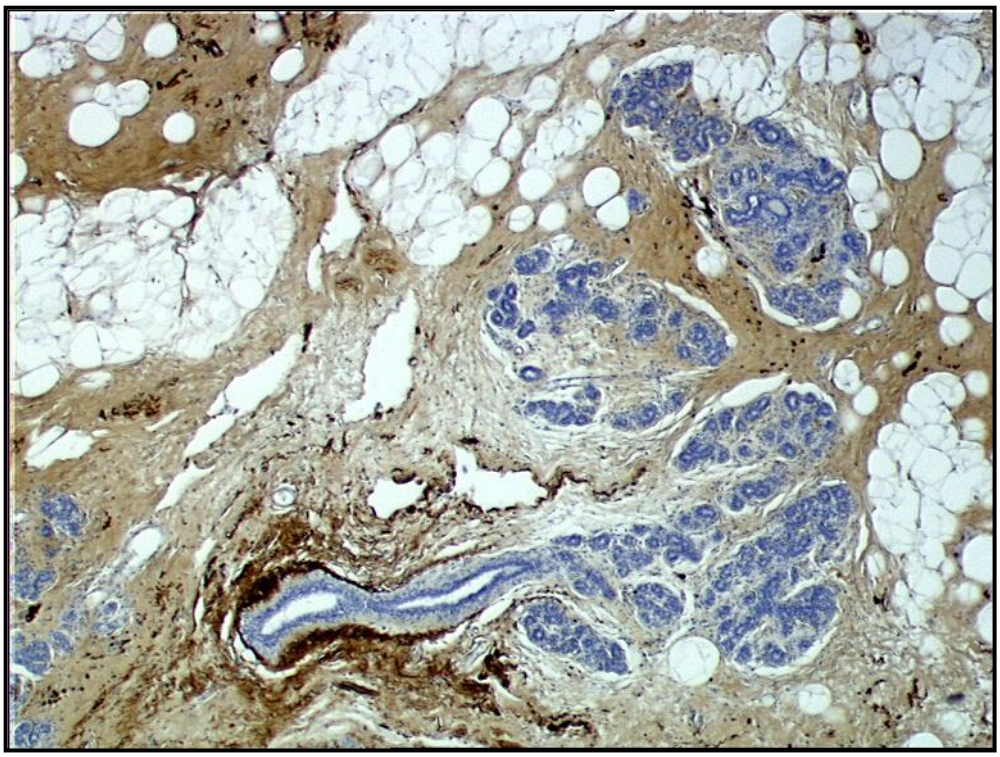

Fig. 17H DCIS antibody VN-7, 130 ng/mL)
magnification x2,5 LSAB staining method

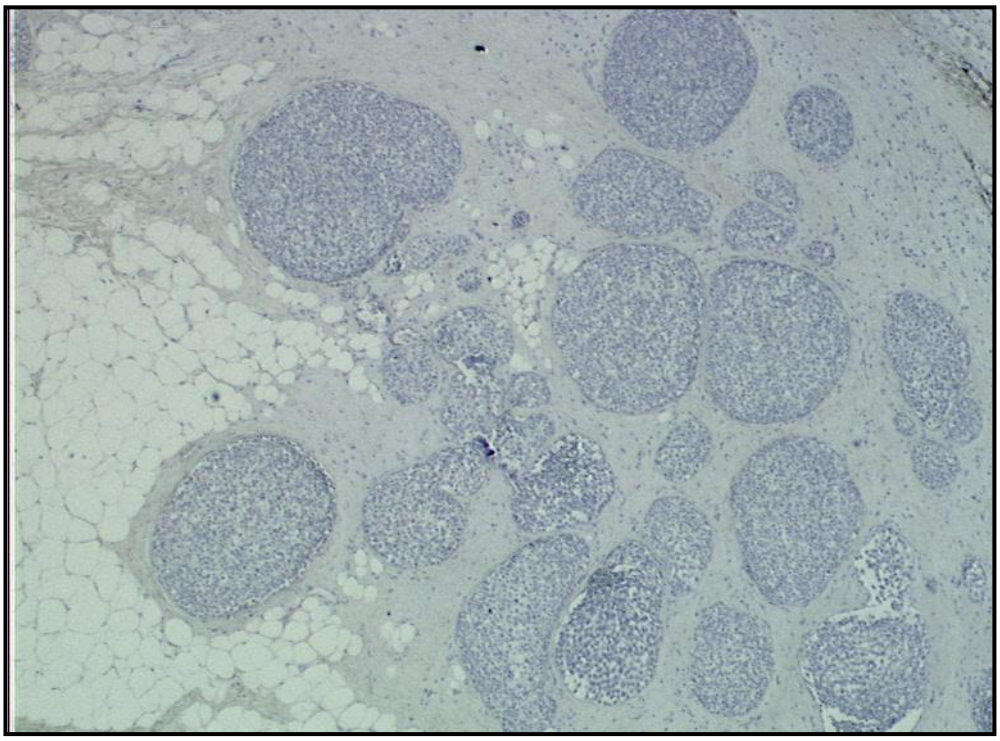

Fig. 17I DCIS negative control (no primary antibody)
magnification x2.5 LSAB staining method

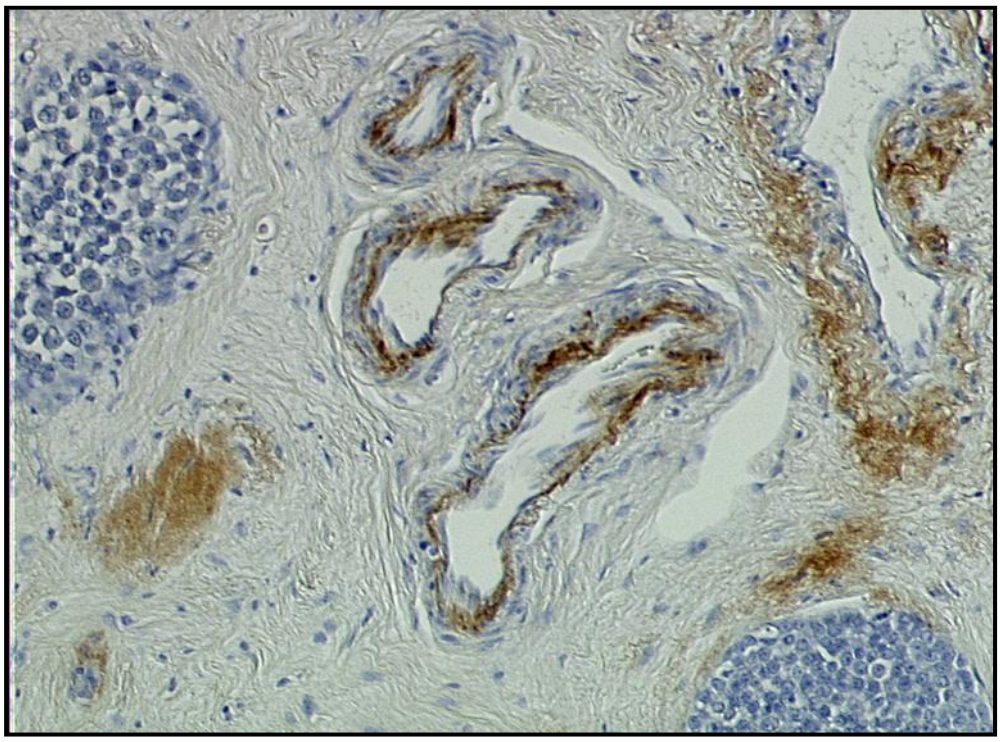

Fig. 17J DCIS (antibody VN-7, 130 ng/mL)
magnification x2.5 LSAB staining method

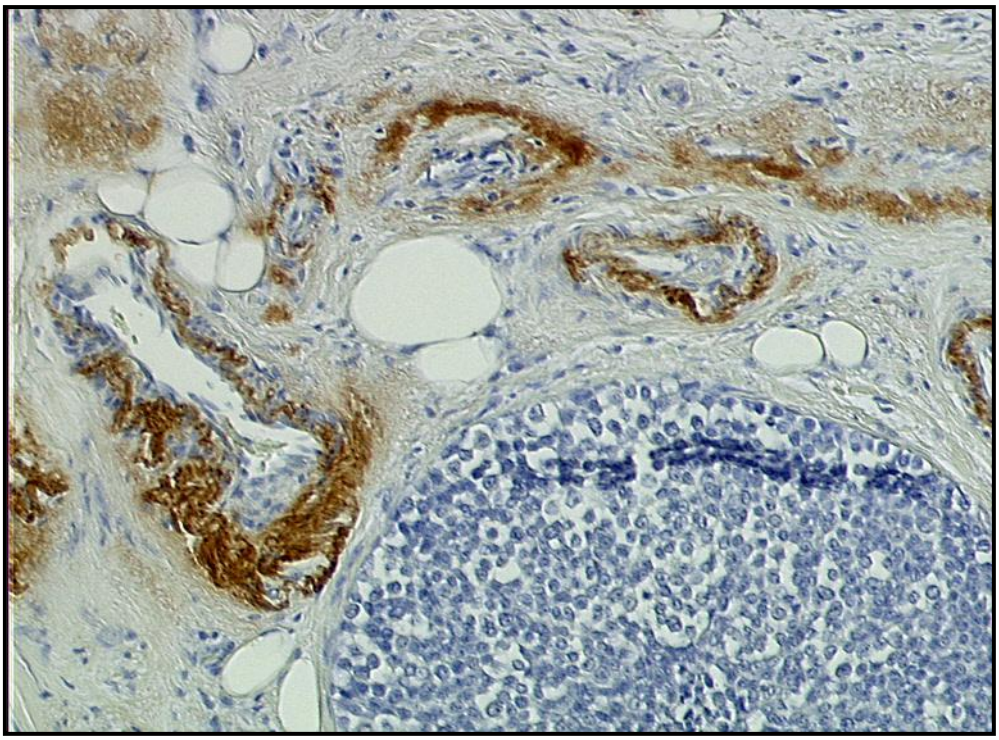

Fig. 17K DCIS antibody VN-7, 130 ng/mL)
magnification x10 LSAB staining method

# 4. DISCUSSION

In this work, an immunohistochemical analysis of the occurrence of Vn in human breast carcinomas, ductal carcinomas in situ and normal human breast tissue is described with the aim to search for proteins as novel biomarkers in human breast cancer for cancer prognosis and therapy response.

For the stainings, the monoclonal antibody VN-7 to human VN (K.T. Preissner) and the monoclonal antibody to human CD31 (Dako, Hamburg) were employed. The two antibodies stained well on formalin-fixed, paraffin-embedded sections even at very low antibody concentrations. The immunohistochemical detection was first done on a preliminary basis using archived tissue sections of kidney, placenta, lungs and skin, testing different methods of staining and antibodies. No data shown. In the course of these investigations, a number of challenges arose and revisions in strategies and approaches were necessary. After establishing the optimal methods of staining for Vn, these were applied to our samples of normal and malignant breast tissue. Liver sections were used as a control tissue. The negative control antibody of irrelevant specificity gave no staining in all cases.

The testing of different antibodies against the antigen of interest, Vn, using different methods of staining and the use of the antibody of irrelevant specificity are necessary to assure the specificity of the stainings and thus to obtain reliable and conclusive results.

In immunohistochemistry, background staining is a frequent problem of staining. Ionic interactions are one of the prime forces that control immunochemical interaction between antigens and their corresponding antibodies. They may, however, also be one of the factors contributing to low affinity and/or non-specific background signal. Most of the diffuse background staining is the result of a combination of ionic and hydrophobic interactions. When using polyclonal antibodies, the immunohistochemical analysis must be supported by absorption controls and immunoblotting analysis. [Andreasen et al., 1997; Larsson et al., 1981]. In 1997, Carriero et al. reported the presence of Vn in blood vessel walls, fibroblasts and in the peritumoral stroma. In 1992, Niculescu et al. reported the accumulation of Vn as diffuse deposits around tumor cells. However, to date, no

comprehensive study exists for the assessment of Vn in tissue specimens of normal and cancerous breast tissue by immunohistochemistry.

## 4.1 Vitronectin

### 4.1.1 Vitronectin in tissues

It has been reported that the Vn antigen is present in embryonic tissue, fetal membranes, smooth and skeletal muscle, kidney, supporting stroma of portal triads, and the capsular surface of viscera [Hayman et al., 1983]. However, the monoclonal antibody employed in these studies was later found to cross-react with a 30 kDa extracellular protein distinct from Vn [Tomasini-Johansson et al., 1993], thus bringing into question the interpretation and significance of these findings [Tomasini-Johansson et al., 1993]. Unlike fibronectin, Vn appears to be absent from the basement membrane of most tissues studied. However, Vn antigen was detected in diseased renal tubular basement membranes [Falk et al., 1987]. A strong correlation between the deposition of the membrane attack complex of complement and Vn was found in kidneys of patients with glomerulonephritis, arteriosclerosis and systemic lupus erythematosus [Falk et al., 1987; Bariety et al., 1989; French et al., 1992].

There was no evidence for Vn deposits in the absence of membrane attack complex [French et al., 1992]. These observations suggest that Vn may serve to regulate immune reactions in the diseased kidney. Although Vn immunoreactivity seems to be absent in embryonic and fetal spleen, Vn antigen has been detected in the red and white pulp of adult spleen [Liakka et al., 1992]. Light microscopic studies of the liver revealed Vn deposition in the area of focal necrosis and in the portal tracts in patients with acute and chronic viral hepatitis, and in areas of fibrous deposition in the liver of patients with cirrhosis [Inuzuka et al., 1992]. Vn antigen has also been localized to elastic fibers of the endometrial stroma [D'Cruz et al., 1992].

Vn deposition has also been reported in areas of fibrosis and necrosis for a number of different tissues including kidney [Dahlback et al., 1988] and fibrillar deposits in the connective tissue matrix around all types of breast cancer

[Niculescu et al., 1992]. Vn was also detected in apparently normal vessels in organs including the uterus [D'Cruz et al., 1992] and kidney [French et al., 1992], suggesting that Vn accumulation in the vessel wall is not a specific marker for vascular disease. Still, a more detailed analysis of its tissue distribution and mechanism of deposition, and the identification of the molecules to which Vn binds *in vivo* will be necessary to further increase our knowledge of its function(s) under both normal and pathophysiological conditions.

### 4.1.2 Vitronectin in biological systems

Biological systems depend on the adherence of cells to the extracellular matrix, and this phenomenon is central for the maintenance of tissue integrity, cellular movement or extracellular recognition processes. Owing to its multidomain structure with various ligand binding sites and its high degree of conformational flexibility, Vn appears to be an adhesive component, versatile for multiple interactions at pericellular sites.

### 4.1.3 Vitronectin and cellular adhesion

Initial cell-substratum contacts with a transient character may lead to stabilized interactions between cell surface components and constituents of the extracellular matrix [Woods and Couchman, 1988] and result in different morphological types of contact sites such as focal adhesions, close contacts or extracellular matrix contacts [Chen and Singer, 1982]. Focal adhesions of adherent cells constitute tight interactions in which extracellular matrix components and the cytoskeleton are linked by transmembranous junctions involving integrin and non-integrin type receptors.

The presence of Vn in focal adhesions [Neyfakh et al., 1983; Baetscher et al., 1986] was demonstrated in cultured cells by immunofluorescence techniques and coincided with characteristic clustered distribution of integrins. The majority of cell attachment activity in cell culture media containing bovine or human serum is mediated by Vn and, to a lesser extent, by fibronectin, and is at least in part due to a 40fold higher adsorption of Vn from plasma or serum to culture dishes compared

to fibronectin [Bale et al., 1989]. Moreover, certain anchorage-dependent cell lines attach to and grow on plastic dishes only when Vn is present in the cell culture medium [Hayman et al., 1985; Ruoslahti et al., 1987; Edwards et al., 1987; Underwood & Bennet, 1989] which appear to indicate that in addition to its direct attachment-promoting activity, Vn may indirectly affect cell growth in a yet unidentified manner. For a given cell, provision of endogenous Vn may have profound effects on its serum requirements for attachment, growth and phenotypic morphology in vitro, as has been documented for human yolk sac carcinoma cells [Cooper and Pera, 1988].

### 4.1.4 Role in disease processes

In view of the involvement of the Vn-binding integrin $\alpha_v\beta_3$ in angiogenesis, Vn may play an important role in wound healing and in tumor progression. Since antibodies to integrin and integrin antagonists (e.g. cyclic RGD peptides or disintegrins) have been shown to inhibit angiogenesis and to induce programmed cell death (apoptosis), such reagents are currently studied in animal models of cancer and of blinding eye diseases. These studies are aimed at devising new therapeutic strategies against human pathologies. Vn deposition has been detected in areas of fibrosis and necrosis in a variety of diseases, including membranous nephropathy, arteriosclerosis and degenerative central nervous system disorders.

Deposition of Vn in association with PAI-1 (i.e. formation of Vn-PAI-1 complexes) alters the balance of the fibrinolytic system to favor inhibition of fibrinolysis and development of necrosis. The urokinase receptor (CD87) has recently been identified as a cell surface receptor for Vn. It was also shown that active PAI-1 dissociates Vn from the urokinase receptor and thus detaches cells from their Vn substratum. Such activity of PAI-1 may explain why high levels of this inhibitor are considered a marker of poor prognosis for several types of cancers. Developing compounds targeted to the urokinase receptor-Vn binding may also be an option for therapeutic use.

Interestingly, a genetic deficiency of Vn has not been reported so far in humans or in other mammals. Null mice completely deficient in Vn were found to be normal

with respect to fertility, development and survival, suggesting that this protein is either dispensable, or that in its absence the functions of Vn are replaced by alternative constituents of the extracellular matrix. In view of the above, it is rather difficult to establish unequivocally the overall regulatory role of Vn in (patho)physiology, its involvement in pathogenesis and its importance as a target for pharmacological intervention.

### 4.1.5 Vn and integrin receptors

The ability of Vn and other adhesive proteins such as fibronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin or collagen to engage in cell attachment activities, resides in the versatile RGD-epitope [Pierschbacher & Ruoslahti, 1984; Ruoslahti & Pierschbacher, 1986], which is recognized by a growing number of integrins. A variety of cell types express these receptors, and they are composed of non-related α-and β-subunits, which share about 50% structural homology within the different classes and whose non-covalent association is promoted by divalent cations [Hynes 1987;Ruoslahti & Pierschbacher 1987].

The cloning and sequence analysis of cDNAs as well as the ligand-binding properties and the cell line distribution of β-subunits revealed that $\alpha_v$–$\beta_5$ and $\alpha_v$-$\beta_1$ heterodimers, which are present on a variety of adherent cells, may be responsible for the widespread and specific cell adhesion to Vn.

Both $\alpha_v$–$\beta_3$ and $\alpha_v$–$\beta_5$ recognize Vn in an RGD-dependent manner, but only the $\alpha_v$-$\beta_3$ integrin was localized to focal contacts on transformed cells, which indicates that the $\beta_5$–subunit may be unable to associate with cytoskeletal elements [Wayner et al., 1991]. Consequently, relative expression of $\alpha_v$-$\beta_5$ and / or $\alpha_v$-$\beta_3$ on a cell surface may have profound effects on the biological behavior of a particular cell in the presence of Vn. The specific binding of Vn to $\alpha_v$-$\beta_3$ receptor on endothelial cells, partly competed for by fibrinogen and von Willibrand factor [Preissner et al., 1988], resulted in the promotion of attachment and of spreading of these cells.

### 4.1.6 Non-integrin receptors and vitronectin complexes

In addition to their RGD-containing cell-attachment site, adhesive proteins, including Vn, fibronectin or laminin contain heparin-binding or other domain(s) that are crucial for the establishment of stable focal adhesions [Woods & Couchman 1988; Obara et al., 1988; Basson et al., 1990]. Experimental evidence suggest that the majority of non-integrin matrix receptors involve different types of cell surface-associated proteoglycans [Ruoslahti et al., 1988], which thereby facilitate cell attachment and spreading events, whereas soluble proteoglycans may have an opposing effect by regulating cell adhesion processes. It is critical for interactions with proteoglycans that the heparin-binding domain of Vn be exposed as it is in the ternary Vn-thrombin-antithrombin III complex [Tomasini & Mosher 1988], which is the ultimate reaction product of thrombin in plasma following blood clotting.
In this work, we tested an antibody to $\alpha_v\beta_3$ on different tissue sections by immunohistochemistry with the view to ascertaining a colocalization of Vn with the receptors mentioned above. The receptors are known to facilitate spreading and cell attachment to the adhesive glycoprotein Vn.

## 4.2 Evaluation of work

One of the objectives of this work depends on the observation made by Martens et al., [Cancer Research 2005] that the methylation of the Vn gene correlated with a reduced response to Tamoxifen-based endocrine therapy in metastasized breast cancer patients. The detection of Vn in tissue samples of normal and cancerous breast by immunohistochemistry would under these circumstances surely be of clinical value to the physician for deciding if and which kind of adjuvant endocrine therapy should be administered to the metastasized breast cancer patient.
Immunohistochemical analysis of normal breast and carcinoma tissue showed a particularly prominent accumulation of Vn in the extracellular matrix structures, also those surrounding ducts while normal epithelium or cancer cells were found to contain no Vn. In normal breast tissue, Vn was seen in relation to mammary ducts and occasionally in relation to small vessels. Thus, when comparing tissues with one another, there occurred the interesting observation that the differences in tissue architecture of normal and cancerous tissue are correlated with distinctly

different Vn distribution patterns. This could be explained as follows: Vn binds to other ECM constituents after it is derived by leakage from vessels. It is reported to bind to elastin [Dahlbäck et al., 1991], sulfated glycosaminoglycans [Tomasini et al., 1990], fibrin [Podor et al., 2000] and several collagen types [Ruoslahti et al., 1986].

As expected, the negative control liver section, without addition of the primary antibody to Vn, did not stain whereas the positive control liver section stained with primary monoclonal antibody VN-7 showed a partially positive staining of the hepatocytes. The endothelial cell layer of the blood vessels was positively stained with the monoclonal anti-human CD31 antibody as demonstrated above. In this work, also using other detection methods like PAP and APAAP in addition to LSAB, no staining could be detected in normal and cancerous breast tissue. The absence of Vn in breast epithelium and breast cancer cells may suggest that Vn is not synthesized locally by tumor cells but that the presence of Vn in the ECM is due to leakage from vessels, especially as Vn is present in the blood in large quantity. A variety of mechanisms for trans-endothelial transport of Vn have been discussed (for a review, see [Preissner and Seiffert, 1998]).

Following transfer into the tissue, Vn may accumulate in specific areas in patterns distinctly different in carcinomas and in normal tissue as has been demonstrated in this work. An extravasation mechanism is in agreement with the observation that the Vn concentrations of tissues were found to be around 10-fold lower than that of plasma [Harada et al., 1994, Carreiras et al., 1996]

In the introduction to this work, mention was made of the role of Vn in tumor cell invasion and metastasis. Being a ligand for uPAR and several integrins, Vn may play an important role in tumors by providing traction and direction to migrating tumor cells during invasion and/or migrating endothelial cells during angiogenesis. The observed immunohistochemical localisation of Vn is not supporting this but also not in contradiction to this hypothesis. A more elaborate substantiation is required, however, by future studies of the inter-relationships between Vn, integrins and the plasminogen activation system at the single-cell level.

## 4.3 SUMMARY

An assessment of the occurrence of the novel DNA-methylation response marker vitronectin in tissue specimens of normal and cancerous breast was done by immunohistochemistry. Different antibodies to the extracellular glycoprotein vitronectin were tested in a representative number of tissues using various methods of detection. Immunohistochemical analysis in normal breast tissue revealed a homogeneous periductal occurrence with local deposition much lower than in the carcinomas. In carcinomas, large areas of cancer cells and tumor nests were found to contain no vitronectin, whereas strong vitronectin accumulation in the extracellular matrix around cancer cell clusters and some blood vessels was observed.

The best staining results were achieved using the monoclonal antibody VN-7 and applying the labeled streptavidin biotin method of detection. A comparison of the pattern of distribution differs distinctly in normal tissue and carcinomas. There was no vitronectin immunoreactivity observed in tumor nests. It can be said that vitronectin is not synthesized locally in breast tissue by tumor cells. A correlation between DNA-hypermethylation of vitronectin and reduced response to Tamoxifen-based endocrine therapy in metastasized breast cancer patients observed by Martens et al. [Cancer Research 2005; 65(10) May 15 2005], contributes to the need for a further clarification of the role of vitronectin in tumor biology and in the development of new therapeutic strategies for human pathologies.

# 5. APPENDIX

## 5.1 List of Figures

## 5.2 One letter amino acid code

| | | | |
|---|---|---|---|
| A | Alanine | M | Methionine |
| C | Cysteine | N | Asparagine |
| D | Aspartic acid | P | Proline |
| E | Glutamic acid | Q | Glutamine |
| F | Phenylalanine | R | Arginine |
| G | Glycine | S | Serine |
| H | Histidine | T | Threonine |
| I | Isoleucine | V | Valine |
| K | Lysine | W | Tryptophan |
| L | Leucine | Y | Tyrosine |

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