TECHNISCHE UNIVERSITÄT MÜNCHEN

Frauenklinik und Poliklinik des Klinikums rechts der Isar

(Direktorin: Univ.-Prof. Dr. M. B. Kiechle)

Assessment of clinical utility of breast cancer biomarkers uPA and PAI-1

Kerstin Regner

Vollständiger Abdruck der von der Fakultät für Medizin
der Technischen Universität München
zur Erlangung des akademischen Grades eines Doktors der Medizin
genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. E. J. Rummeny
Prüfer der Dissertation:	1. Univ.-Prof. Dr. M. Schmitt
	2. Univ.-Prof. Dr. M. B. Kiechle

Die Dissertation wurde am 14.08.2012 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 30.01.2013 angenommen.

Meiner Familie

Table of Contents

1. Introduction

1.1 The new concept of personalized therapy in breast cancer

According to the World Health Organization (WHO) 500,000 women die from breast cancer each year. It is the most common cause of death in women between the age of 35 and 55 years in industrialized countries. In Germany, about 58,000 women contract breast cancer per year which accounts for about 28 % of female malignancies. Roughly every tenth woman develops breast cancer during her lifetime. Early menarche, late menopause, nullipara, obesity, genetic disposition (e.g. BRCA1/2-gen) and postmenopausal hormone-replacement therapy are factors known to increase the risk of disease.

Figure 1 shows trends in incidence and mortality rate for breast cancer in women during the period 1980-2004 in Germany. Since the middle of the 1990's, a decline in mortality rate is observed due to enhanced awareness, improved adjuvant treatment therapy options, and early detection programs leading to diagnosis in earlier cancer stages. However, incidence rate of breast cancer is still increasing since 1970. This can supposedly be seen as a result of enhanced collection of data. In addition, incidence rate is influenced by early detection programs such as mammography screening which has been introduced nationwide in Germany 2007 (*Manual Mammakarzinome 2009, Tumorzentrum München; Levi et al. 2007; www.rki.de*).

Today, breast cancer has become a curable disease for the vast majority of early-stage patients due to effective loco-regional as well as systemic treatment options. The basis for breast cancer classification are pathologic-anatomic features including tumor size, lymph node status, metastasis (pTNM-classification, see **9.1**, UICC-stadium, see **9.2**), grading (see **9.3**), histopathological subtype (see **9.4**), R-classification with incomplete or complete resection, peritumoral lymph and vessel invasion, steroid hormone receptor status (estrogen and progesterone receptor), and HER2 status.

Chapter **9.4** in the appendix gives an overview of the different types of breast tumor histopathology. In about 80 % the mammary carcinoma derives from the ductal epithelia, whereas only the remaining approximately 20 % have their origin from the lobular epithelia. Very rare are distinct subtypes such as tubular carcinoma, invasive cribriform carcinoma, medullary carcinoma, invasive papillary carcinoma, mucinous carcinoma, or neuroendocrine tumors of the breast. There also exist precursor lesions such as the ductal carcinoma *in situ* (DCIS) and the lobular carcinoma *in situ* (LCIS).

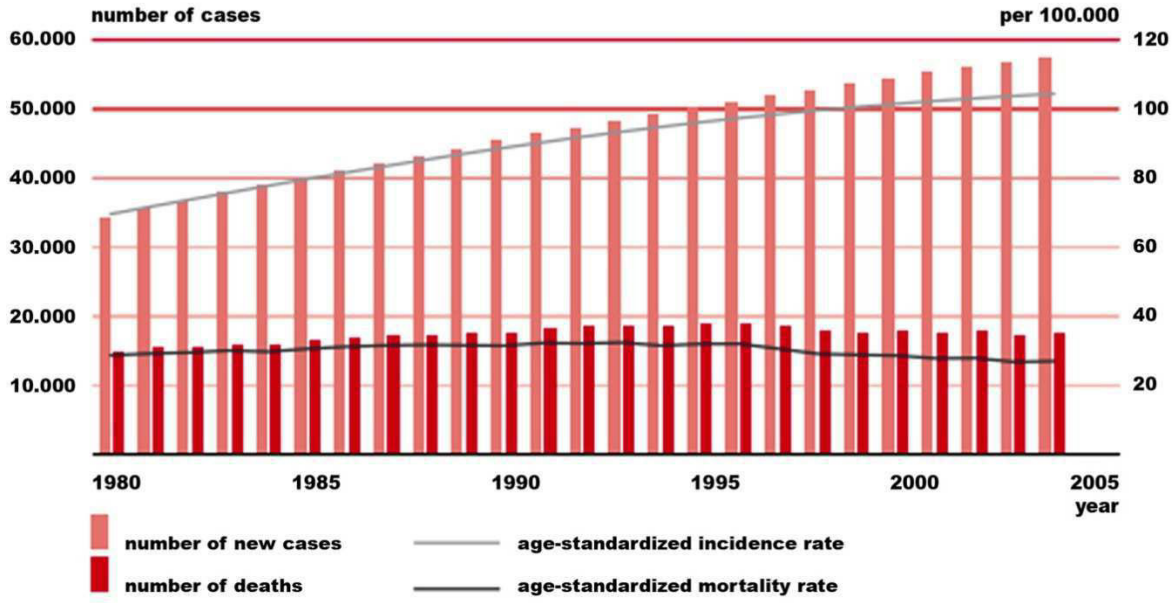


Figure 1 Number of new diagnosed breast cancer cases and number of deaths per year as well as age-standardized (per 100,000 population) incidence and mortality rates for breast cancer in Germany, 1980-2004 (Taken from *www.rki.de*).

Criteria for the selection of breast cancer therapy are the extent of disease and biological features. After primary surgery (with either breast-conserving therapy or modified radical mastectomy), systemic adjuvant therapy is chosen. According to the St. Gallen consensus recommendations, a general guidance underlining main principles for tailoring therapeutic choice, patients are divided into different risk categories: “low”, “intermediate”, and “high” (*Goldhirsch et al. 2007*). According to these groups, respective adjuvant treatment is allocated to the patients (see **9.5**). If indicated, chemotherapy is given to eradicate microscopic deposits of cancer cells which may have escaped from the primary tumor. In case of positive steroid receptor status, the application of anti-hormones e.g. tamoxifen (selective estrogen receptor modulator) or anastrazole (aromatase inhibitor)

selected according to menopausal status can be beneficial. In case of HER2 positivity, Trastuzumab (trade name Herceptin®), a monoclonal antibody that interferes with the HER2 receptor, can be given simultaneous and after chemotherapy *(Goldhirsch et al. 2007, Fornier et al. 2005).*

However, clinical benefits of adjuvant systemic treatment are relatively small for patients with lymph node-negative disease because primary loco-regional treatment cures 60-70 % of these patients. Following the St. Gallen consensus guidelines, more than 90 % of patients with node-negative disease would obtain adjuvant systemic therapy, even though only 30 % of patients with node-negative disease will eventually relapse *(Harbeck et al. 2002b, Bernard-Marty et al. 2004).* Hence, 70 % of patients receiving adjuvant treatment would have survived the disease but were subjected to unnecessary and toxic side-effects of the therapy. In order to avoid over- as well as under-treatment, it is important to select the appropriate treatment strategy on the basis of accurate risk assessment for each individual patient. The aim is to distinguish between patients with low and high risk of disease recurrence and to identify those breast cancer patients who will benefit from a distinct chemotherapy regimen after primary surgery.

The use of traditional prognostic factors, such as tumor size, tumor grade, steroid hormone receptor status, age or menopausal status is not sufficient to identify the subgroup of patients who are likely to develop recurrent disease *(Look et al. 2002)*. Taking into consideration tumor biology, different molecular subtypes and clinical outcomes, breast cancer is still a very heterogeneous disease. That's why the period of "one size fits all" therapy concept should be left behind *(Harbeck et al. 2010*). Hence, the major future challenge in oncology is the individualized, personalized, tailored care of cancer patients. This means to choose the optimal therapy for every individual patient preferentially based on cancer biomarker-based diagnostic tests.

To realize this concept, detailed information about a tumor's gene and protein-expression profile has to be observed first. Second, meaningful parameters are needed to characterize individual cancer patients and subgroups with different biological behaviors have to be defined in order to determine their course of disease and to predict response to a given therapy *(Schmitt et al. 2010)*.

During the last years, a range of biomarkers has been studied at the gene and protein level. The most desirable tumor markers to predict prognosis are

molecules functionally involved in the regulation of tumor invasion and metastasis because metastatic disease is the main cause of cancer patient morbidity and mortality *(Hayes 1993, Stephens et al. 1998)*. While many different markers have been reported, so far only the steroid hormone receptors and the oncogene HER2 are in widespread clinical use, as mentioned before.

The American Society of Clinical Oncology (ASCO) guidelines, first published in 1996, are evidence-based clinical practice guidelines intended to assist physicians in clinical decision-making. The update of the ASCO guidelines for the use of tumor markers in breast cancer published in 2007 by *Harris et al.* focuses especially on recommendations for use of the tumor marker tests in prevention, screening, treatment, and surveillance of breast cancer. For these recommendations 13 tumor markers were considered regarding clinical outcome, like for example overall survival (OS), disease-free survival (DFS), quality of life, lesser toxicity as well as cost-effectiveness.

Next to CA 15-3, CEA, steroid hormone receptors and HER2, uPA (urokinase-type plasminogen activator) and PAI-1 (plasminogen activator inhibitor type-1) are new tumor markers which were recommended for use in practice as they showed evidence of clinical utility.

There is a battery of studies which shows that high concentrations of uPA and PAI-1 in primary cancer tissue are associated with tumor spread and metastasis and therefore with short disease-free survival and early death *(e.g. Duffy et al. 1988a,b,c, Jänicke et al. 1989, 1990, 1991, 1993, 1994, 2001, Foekens et al. 1992, 1994, 1995, 2000, Grøndahl-Hansen et al. 1993, 1995, Duggan et al. 1995, Schmitt et al. 1997, Harbeck et al. 2002, Look et al. 2002, Zemzoum et al. 2003*). uPA and PAI-1 are suggested for determination of prognosis in newly diagnosed, node-negative breast cancer patients independent of size, grade, and steroid hormone receptor status and therefore incorporated into the ASCO guidelines.

Patients with low levels of both factors are associated with a low risk of disease recurrence. These patients do not benefit from adjuvant chemotherapy and may be spared of its toxic side effects, increasing quality of life and reducing healthcare costs. Patients with high levels of uPA and PAI-1 are related with poor prognosis comparable with that of patients with tumor cell positive axillary lymph nodes. These patients do benefit from adjuvant chemotherapy *(Harbeck et al. 2004a, Harris et al. 2007*).

1.2 Characteristics of the plasminogen activator system

The malignant potential of solid tumors depends on their proliferation rate as well as on their potential for invasion and metastasis. During tumor invasion and metastasis, tumor cells cross host cellular and extracellular matrix (ECM) barriers by attachment to and interaction with components of the basement membrane and the extracellular matrix. As the structure of the penetrated tissues consists mainly of proteins e.g. fibronectin, fibrin, and proteoglycans, the primary substances used by tumor cells for invasion and metastasis are proteases, such as matrix metalloproteases, cysteine proteases, and serine proteases. The serine protease uPA and its inhibitor PAI-1 play an important role with multiple interactions and members participating in fibrinolysis and extracellular matrix degradation, including cancer invasion, angiogenesis and metastasis in many different tumors *(Duffy 1996)*. So the plasminogen activator system directly influences tumor behavior, being related to an aggressive tumor phenotype and patients' poor progression-free survival in breast cancer.

The plasminogen activator system mainly consists of the serine proteinase plasmin, uPA (urokinase-type plasminogen activator), the uPA-receptor (uPA-R or CD87), and four major inhibitors: α2-antiplasmin and α2-macroglobulin targeting plasmin, and plasminogen activator inhibitors type-1 and type-2 (PAI-1 and -2). The most important members of this system are the serine protease uPA, its inhibitor, PAI-1 and its receptor uPA-R. Imbalance of these factors leads to efficient focal proteolysis, adhesion, migration, and consequently subsequent tumor cell invasion and metastasis *(Jankun et al. 1993, Carriero et al. 1994, Bianchi et al. 1995, Bajou et al. 1998, Andreasen et al. 1997, 2000, Annecke et al. 2008).* In tissues the primary role of uPA, when bound to its cellular receptor uPA-R is to convert plasminogen into plasmin. There, plasmin leads either directly or indirectly, through other proteases, to degradation of the tumor stroma surrounding the tumor cell nests. uPA is involved in numerous cell-signaling cascades and therefore affects cell adherence, cell migration, chemotaxis, cell growth, cell apoptosis, and turnover of the ECM. This leads to invasion of cancer cells into the surrounding tissue, access of cancer cells to blood and lymphatic vessels, and consequently formation of colonies at distant locations *(Andreasen et al. 1997, Duffy 2002)*. uPA-R

itself interacts with various other proteins such as vitronectin and certain types of integrins, modifying cell adhesion and migration (see **Figure 2**). Compared to normal tissues, uPA, uPA-R, and PAI-1 levels are increased in a variety of malignant solid tumors, including primary breast cancer *(Andreasen et al. 1997, Harbeck et al. 2004b, Bevan et al. 2008)*.

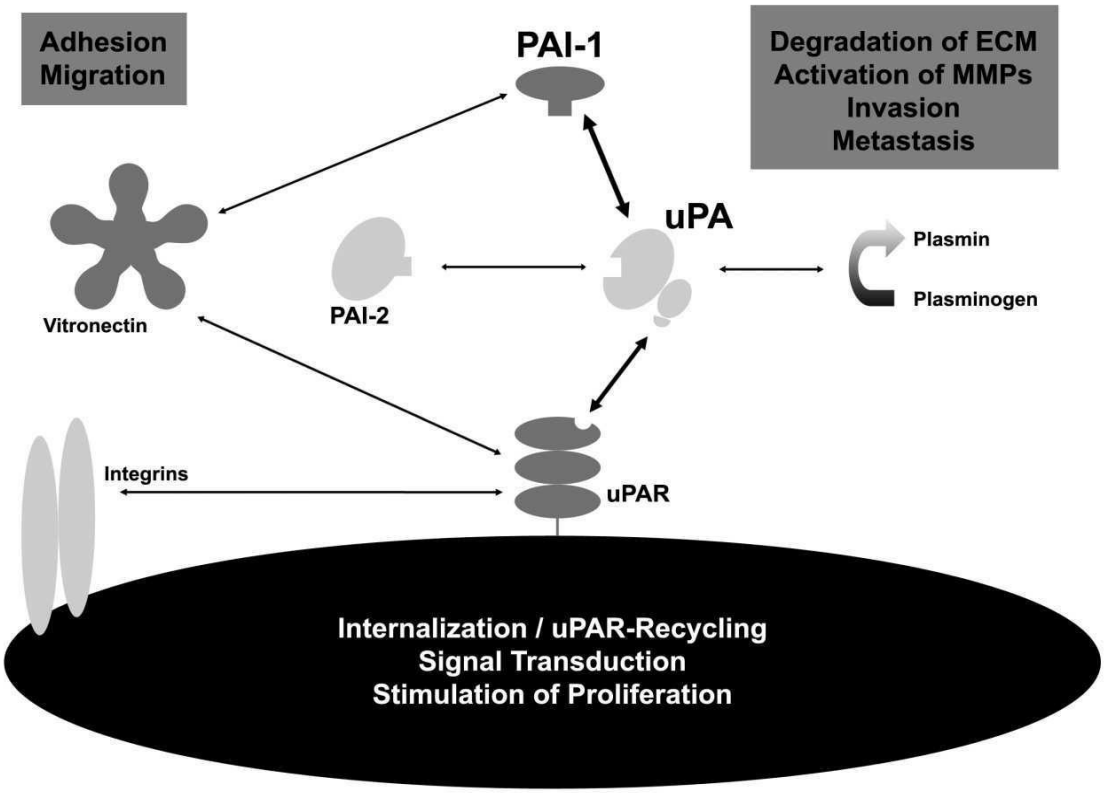


Figure 2 The multifunctional role of uPA and PAI-1 in tumor growth, invasion and metastasis (Taken and modified from *Harbeck et al. 2004b*).

The prefix “urokinase” refers to the original observation and isolation of uPA from urine, where uPA is high concentrated (200-300 ng/ml) compared to the plasma mounts (3.5 ng/ml) *(Schmitt et al. 2008)*. uPA content released into the blood stream of healthy individuals is about 1 ng/ml (~20 pM), but it can increase considerably in case of inflammatory, infectious or malignant process. The uPA gene (PLAU) is located on chromosome 10q24, spanning a chromosomal region of around 6.34 kb. PLAU comprises 11 exons and 10 introns, coding for a proform of 431 amino acids. Gene expression is induced for example by UV light, radiation and by oncogenes such as p53. Some cytokines, growth factors (e.g. TNF-α, VEGF, EGF), and lipopolysaccharides trigger the synthesis of uPA. The uPA content consists mainly of the 52,000 kDa proenzyme (pro-uPA), a 411 amino acid-long, enzymatically only weakly active single-chain zymogen, generated after

removing the signal peptide (amino acids 1-20) from the proform. This pro-uPA is produced and secreted by many different cell types, such as fibroblasts, specific types of leukocytes, muscle cells, endothelial and epithelial cells, and of course cancer cells. Conversion of secreted one-chain zymogen form pro-uPA to its enzymatically active two-chain high molecular weight form (HMW-uPA) is catalyzed by plasmin but also by other proteins, e.g. plasma kallikrein, cathepsin B or even by bacteria like *Pseudomonas aeruginosa* *(Beaufort et al. 2010)*. The other way round, when bound to the uPA-R, HMW-uPA catalyses the conversion of the inactive zymogen plasminogen to the active broad-spectrum serine protease plasmin. In addition to plasminogen, there are many other substrates such as fibrinogen, fibronectin, α6-integrin, and uPA itself *(Andreasen et al. 1997, Stephens et al. 1998, Mengele et al. 2010)*.

PAI-1 is a single-chain glycoprotein including 379 amino acids and belongs to family of the serpins (serine protease inhibitors). Serpins trap the proteinases in inactive forms in stable complexes of 1:1 stoichiometry. The PAI-1 gene (SERPINE1) is located on chromosome 7, spanning a chromosomal region of 11.85 kb. SERPINE1 comprises nine exons and eight introns, coding for a proform of 402 amino acids. PAI-1 is secreted by many different cell types such as endothelial cells, stromal cells, monocytes, smooth muscle cells, trophoblasts, adipocytes, hepatocytes, myofibroblasts, and cancer cells. The PAI-1 expression is influenced by different factors such as insulin, estrogen, hyperglycemic conditions, and certain cytokines (e.g. TNF-α, angiotensin II, VEGF), which may explain the variable plasma levels, ranging from 5 to 85 ng/ml. PAI-1 can increase considerably in case of inflammatory, hypoxia, tissue injury or malignant process. An interesting example reported by *Keates et al.* in 2008 is, that *Helicobacter pylori* infection stimulates PAI-1 production by gastric epithelial cells. The main function of PAI-1 is the inhibition of proteases such as uPA. Other major targets are extracellular matrix components such as vitronectin. When uPA is bound to uPA-R, PAI-1 can bind to uPA which leads to internalization of the trimeric uPA-uPA-R-PAI-1 complex via particular members of the low-density lipoprotein receptor family such as the very low-density lipoprotein receptor (VLDL-R) *(Andreasen et al. 1997, Mengele et al. 2010)*.

1.3 Clinical relevance of uPA and PAI-1 in breast cancer

O'Grady et al. were the first to report in 1985 that uPA activity was significantly higher in malignant tumors compared to benign ones. The fact that primary breast cancer patients with high enzymatic activity of uPA have a significantly shorter disease-free survival published in 1988 by *Duffy et al.* has led to the conclusion that uPA may serve as a new prognostic marker for breast cancer patients *(Duffy et al. 1988a,b,c)*. Soon after these initial observations, *Jänicke et al.* showed in 1989 using an immunoenzymometric test (ELISA) that next to enzymatic activity, also the antigen level of uPA in tumor tissue is of prognostic relevance. One year later the same group reported that in addition to uPA, its inhibitor PAI-1 is also of significant prognostic value in breast cancer patients *(Jänicke et al. 1989, 1990; 1991)*.

In the following, numerous independent studies evidenced that high uPA/PAI-1 antigen levels are contributing to tumor cell spread and metastasis in breast cancer patients and therefore are associated with poor prognosis. For risk-adapted individualized therapy decisions the combination of both factors (both factors low versus either or both factors high) is superior to either factor taken alone *(Jänicke et al. 1993; Harbeck et al. 2002b)*.

The prognostic and predictive value of uPA and PAI-1 was also shown for several cancer types apart from breast cancer such as cancer of the urinary bladder, cervix uteri, endometrium, ovary, stomach, esophagus, colorectum, lung and kidney *(Kuhn et al. 1994, Duggan et al. 1995, Foekens et al. 1995, Duffy 2002, Schmitt et al. 1997, 2008, 2010)*.

Concerning breast cancer, the prognostic and predictive value of uPA and PAI-1 was shown in several validated retrospective and prospective studies and the fact that there is no contradictory evidence on the impact of uPA and PAI-1 in breast cancer is unique for any tumor biomarker *(Harbeck et al. 2002c)*. To date, regarding their clinical utility in primary breast cancer, uPA and PAI-1 are the only novel biological factors that have reached the highest level of evidence (LOE-1) based on the Tumor Marker Utility Grading System (TMUGS), a consistent and objective process for evaluating tumor markers, published in 1996 by *Hayes et al.* (see **9.6**). According to these criteria, LOE-1 can be achieved either by a prospective high-powered study of a tumor-associated marker or by a suitable meta-analysis of prospective or retrospective datasets *(Look et al. 2002)*. Both of

them have been carried out for uPA and PAI-1. The clinical utility of uPA and PAI-1 was proved by the Chemo-N0-trial, an international multicenter prospective randomized therapy trial of 689 node-negative breast cancer patients. Between 1993 and 1999 patients with high uPA/PAI-1 levels in tumor tissue measured by ELISA were randomized to adjuvant CMF-chemotherapy or observation only, whereas patients with low uPA/PAI-1 levels in tumor tissue were observed. Patients with high uPA and PAI-1 levels showed to be of high risk for disease recurrence and death and therefore to benefit of chemotherapy whereas low risk patients have a 5-year overall survival rate of 95 % without any adjuvant systemic treatment *(Jänicke et al. 2001)*.

These findings were validated by a pooled analysis encompassing 8377 patients from 18 different study centers from nine European countries with a median follow-up of 6.5 years *(Look et al. 2002 Harbeck et al. 2008, Schmitt et al. 2008)*. In this study uPA and PAI-1 were measured in primary tumor tissue using commercially available ELISA (American Diagnostica Inc., Greenwich, CT). Individual patient data was validated by members of the European Organization for Research and Treatment of Cancer-Receptor and Biomarker Group (EORTC-RBG). As published in different reports from various authors, uPA and PAI-1 were confirmed as strongest independent prognostic factors for disease-free and overall survival in the analysis of all patients, next to lymph node status *(Harbeck et al. 2004a)*.

Considering the increasing percentage of node-negative patients because of early detection programs and consequently the rising important issue of overtreatment in potentially cured women, adequate risk group assessment is getting more and more important. uPA and PAI-1 have their greatest clinical relevance in node-negative breast cancer and allow such a risk-adapted therapy. As mentioned at the beginning even though only 30 % of node-negative patients will eventually relapse, 90 % of node-negative patients would be candidates for adjuvant systemic therapy, strictly following the St. Gallen criteria. The patient group with low risk identified by uPA/PAI-1 is considerably larger than that characterized by the St. Gallen consensus guidelines *(Goldhirsch et al. 2003)* and much closer to the actual 70 % of node-negative patients cured by locoregional treatment (surgery and radiotherapy, if indicated) alone *(Harbeck et al. 2002c)*. As a result of all these studies, uPA/PAI-1 were included in the German AGO guidelines

in 2002, in the National Academy of Clinical Biochemistry Laboratory Medicine Practice guidelines in 2008, and in the American 2007 Breast Cancer Treatment Guidelines of ASCO for the use of tumor markers in breast cancer management mentioned at the beginning *(Annecke et al. 2008, Sturgeon et al. 2008)*.

Apart from the prognostic impact, uPA and PAI-1 also have predictive value foretelling response to chemotherapy or endocrine therapy in breast cancer. In 2002, *Harbeck et al.* demonstrated in a collective of 3,424 patients with primary breast cancer that patients with high uPA/PAI-1 attained a significantly higher benefit from early adjuvant chemotherapy with regard to DFS than those with low uPA/PAI-1 levels *(Harbeck et al. 2002a)*.

The different clinical trials with uPA and PAI-1 as well as important steps during the ELISA test development are summarized in **Figure 3**. As a follow-up trial to the Chemo-N0 trial mentioned above, the prospective phase III Node-Negative Breast-Cancer (NNBC-3) trial, a multicenter intergroup trial conducted in cooperation with the AGO, the EORTC group, and the German Breast Group recruited 4,149 patients from 2002 to 2009. The two main aims of the NNBC-3 trial are to compare clinical outcome and risk assessment based on uPA/PAI-1 versus established, clinical and histomorphological factors and to determine the benefit of a sequential anthracycline-docetaxel regimen in high risk node-negative breast cancer patients compared to the current standard of anthracycline-based chemotherapy. To date, a first interim analysis is still pending *(Annecke et al. 2008, Schmitt et al. 2011)*.

Other promising trials are based on gene expression profiling, a technique which uses microarray technology to measure the simultaneous expression of up to thousands of genes. Currently, a 70-gene signature including uPA and PAI-1 is undergoing prospective validation as part of the Microarray In Node-Negative Disease May Avoid Chemotherapy (MINDACT) trial. This trial was set up to find out if lymph node-negative patients with low risk of disease recurrence based on the gene signature, but with high risk of disease recurrence based on clinicopathological factors can be spared adjuvant chemotherapy without influencing distant metastasis-free survival. Another multigene signature, the Oncotype DX™ recurrence score, is not using microarray technology but reverse transcription-polymerase chain reaction (RT-PCR) to measure the expression of 21 genes. This assay quantifies the likelihood of disease recurrence in women

with newly diagnosed, early stage breast cancer and is currently being tested within large prospective trials, e.g. the Trial Assigning Individualized Options for Treatment (TAILORx) *(van de Vijver et al. 2002, van't Veer et al. 2002, Paik et al. 2004, Fan et al. 2006, Sparano 2006, Sturgeon et al. 2008, Marchionni et al. 2008)*.

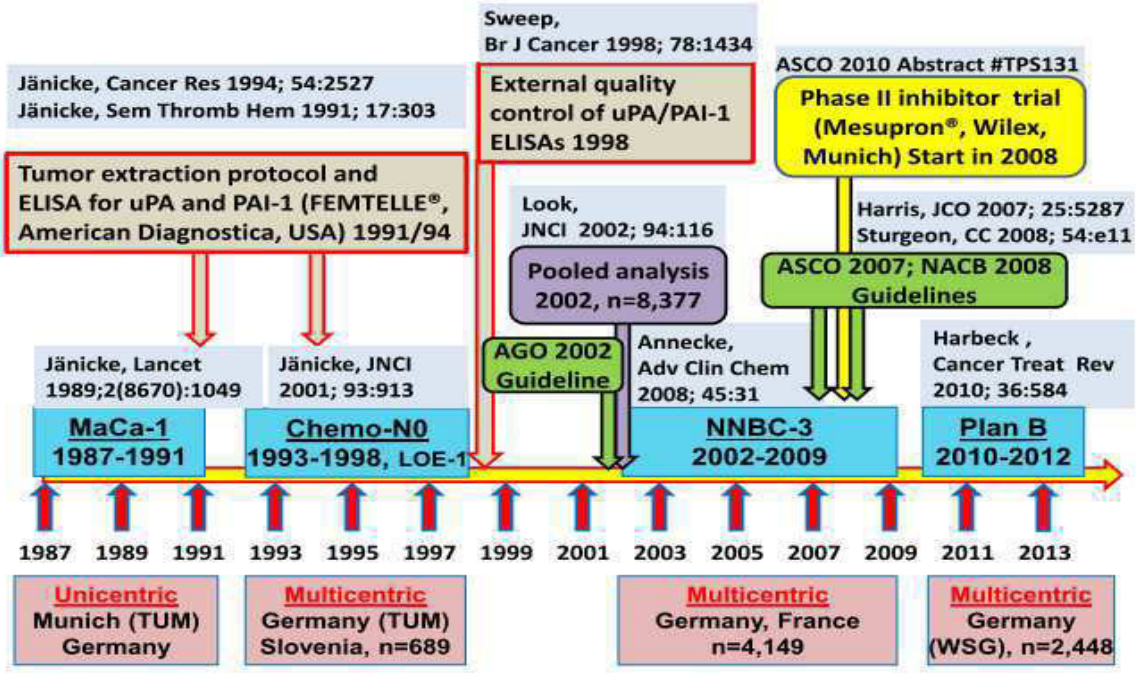


Figure 3 Summary of important clinical trials associated with uPA and PAI-1 and important steps during the ELISA development starting in the year 1987 (Taken from *Schmitt et al. 2011*).

Another large-scale multicenter trial, the West-German Study Group (WSG) Plan B trial (n=2,448) is the first trial comparing the prognostic and predictive impact of both, the uPA/PAI-1 status and the Oncotype DX™ recurrence score. The design of the study is to select two groups of breast cancer patients for adjuvant chemotherapy: first, high-risk node-negative breast cancer patients and second, node-positive breast cancer patients presenting with 1-3 affected lymph-nodes, using the Oncotype DX® test and optionally the uPA/PAI-1 Femtelle® test for selection. Secondary objectives of this trail are to compare overall survival and toxicity between the two chemotherapy arms (sequential adjuvant epirubicin, cyclophosphamide (EC)/ docetaxel with docetaxel/ cyclophosphamide chemotherapy) in patients with HER2-negative early breast cancer *(Harbeck et al. 2010, Degenhardt et al. 2010, Schmitt et al. 2011)*.

1.4 Techniques for determination of uPA and PAI-1 in breast cancer

So far, most clinically relevant, validated data concerning uPA and PAI-1 in breast cancer have been obtained by standardized, quality-assured enzyme-linked immunosorbent assays (ELISAs). For clinical use of risk-adapted, individual therapy decisions, particularly in patients with lymph node-negative disease, such ELISAs are commercially available (Femtelle®, American Diagnostica Inc., Greenwich, CT). This ELISA has been standardized and quality assessed by the EORTC Receptor and Biomarker Group (EORTC-RBG) *(Schmitt et al. 2008)*. It should therefore be considered as the gold standard. To perform an ELISA, a representative piece of fresh breast tumor (>200-300 mg) must be stored in liquid nitrogen immediately after surgery *(Sturgeon et al. 2008)*. Patients are categorized as low risk when the ELISA levels are under the cut-offs (3 ng uPA/mg protein and 14 ng PAI-1/mg protein).

It is worth mentioning that measurement of uPA and PAI-1 in blood is not recommended for assessing prognosis in breast cancer patients, because no significant correlation has been reported between plasma and tumor levels of uPA and PAI-1, indicating that determination of these factors in plasma does not reflect their concentration in tumor tissue *(Grebenchtchikov et al. 2005, Schmitt et al. 2008).*

Even though uPA and PAI-1 have reached the highest level of evidence (LOE-1) as biomarkers and the clinical validity is not being disputed, they are not yet part of international guidelines. One reason for this is certainly the need of fresh-frozen tumor tissue for the determination by ELISA and adequate capacity for the storage of tumor samples which is not available as standard of care in the hospitals of many industrialized countries of the world. Especially smaller breast care centers are not equipped for such analyses, and transport and storage of fresh-frozen tissue specimens are complex and often too expensive *(Annecke et al. 2008).* That's why it has been searched for alternative methods of analysis using worldwide available formalin-fixed paraffin-embedded (FFPE) tissue.

One new method published by *Becker et al.* in 2007 is the extraction of uPA and PAI-1 from routinely prepared FFPE sections followed by the quantification of protein content of uPA and PAI-1 in these extracts by ELISA or western blot *(Becker et al. 2007, Malinowsky et al. 2010).*

Another option is the assessment of uPA and PAI-1 at the transcriptional level measuring mRNA by a quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay *(Biermann et al. 2008)*. There are so far a few studies which show only a borderline correlation between uPA and PAI-1 mRNA expression and patient prognosis *(Spyratos et al. 2002, Leissner et al. 2006, Sternlicht et al. 2006, Urban et al. 2006, Castello et al. 2007).*

Another method published by *Lamy et al.* 2007 using the NASBA (nucleic acid sequence-based amplification) showed high correlation with the respective ELISA data. A different approach is based on the epigenetic level: cytosine methylation within the context of CpG dinucleotides in the genome is a molecular mechanism that affects epigenetic changes in the chromatin structure and leads to transcriptional silencing of genes in many human cancers. For example, *Pakneshan et al.* examined the methylation status of the uPA promoter and the levels of uPA expression by bisulfate sequencing analysis and reverse transcription PCR. They found a strong correlation of the uPA methylation status with the mRNA expression: demethylation of the uPA promoter is associated with high mRNA levels which is associated with poor prognosis in breast cancer patients *(Pakneshan et al. 2004)*.

So far, these novel techniques still need to be methodologically standardized, quality assured and clinically validated in prospective multicenter clinical trials before they can be recommended for routine clinical decision-making. Until now, no clinically relevant data has been published yet applying other determination techniques than ELISA.

According to the ASCO guidelines, routinely available and cost-effective techniques such as immunohistochemistry (IHC) are favored, even if different technologies are getting more and more popular, e.g. molecular assays at the DNA, RNA, and protein level, as mentioned above. In routine work, FFPE tissue sections have to be prepared of every excised breast cancer tissue for evaluation of the tumor's histopathology. And as IHC is routinely performed on every breast tumor tissue to identify the steroid hormone receptor status and the HER2 status, it is obvious to investigate the assessment of uPA and PAI-1 by IHC.

2. Aim of the study

Although international consensus panels are highly acknowledging the idea of therapy individualization by new prognostic factors such as uPA and PAI-1, the routine use of these cancer biomarkers is often hampered by the fact that determination using ELISA has to be performed on extracts obtained from fresh-frozen tumor tissue specimens. That's why alternative, routinely available and cost-effective methods, like immunohistochemistry, using formalin-fixed paraffin-embedded tissue are urgently needed.

The aim of the study therefore was to establish protocols (standard operating procedures) for quantitative assessment of uPA and PAI-1 in formalin-fixed paraffin-embedded primary breast cancer tumor tissue by immunohistochemistry and to explore the prognostic impact and clinical utility of this method as an alternative to the clinically relevant ELISA. To achieve this, the following steps were taken:

- Standard operating procedures (SOPs) were developed for manual staining procedure for uPA and PAI-1 and then transferred to two different automatic staining instruments: the Dako Autostainer and the VENTANA Benchmark® XT.
- Tissue microarrays (TMAs) and core biopsies with breast cancer tissues were stained by immunohistochemistry for uPA and PAI-1 following the SOPs.
- Immunohistochemically stained tissue was scored by pathologists and cutoffs were defined to distinguish between "weak" and "high" staining intensity.
- Scoring results were compared to uPA/PAI-1 protein expression measured by ELISA.
- Statistical associations between scoring results and clinical factors, overall and disease-free survival were explored to investigate the clinical utility of this method.

3. Materials and methods

3.1 Patients and tissue collection

For the development of different standard operating procedures (SOPs) for staining for uPA and PAI-1, normal kidney tissue and primary breast cancer tissue are used provided by the Department of Obstetrics and Gynecology and the biobank of the Klinikum rechts der Isar, Technical University of Munich, Germany.

The main part of this study is the examination of three TMAs which are prepared by members of the Institute of Pathology of the Technical University of Munich in 2003 and 2004. For these TMAs tissue sections of FFPE tumors are applied from 210 patients with breast cancer, who have undergone surgical intervention from 1987 to 2000. “TMA 1” includes a collective of 62 patients, who received CMF-chemotherapy as adjuvant therapy. The other two TMAs include 105 patients (“TMA 2”) and 43 patients (“TMA 3”), receiving adjuvant endocrine therapy with tamoxifen. Follow-up is performed for all 210 patients. uPA and PAI-1 ELISA data are available for 208 of the 210 primary breast cancer tissue specimens measured by commercially available ELISA kits (uPA: Imubind #894; PAI-1: Imubind #821, American Diagnostica Inc., Greenwich, CT).

Another small part of this study is the examination of ten cases of immunohistochemically stained core biopsies and their respective primary tumor tissue taken from breast cancer patients in 2003 and 2004. uPA and PAI-1 ELISA data are available for all ten core biopsies as well as for their respective primary tumor tissues.

3.2 Preparation of full-face sections and core biopsies

As in immunohistochemistry antibody binding capability is diminished by prolonged or inappropriate fixation, prompt and adequate fixation is crucial to guarantee the conservation of cell morphology and tissue architecture and to obtain reproducible

staining. Breast cancer tissue obtained from surgery is routinely fixed and paraffin-embedded in the Institute of Pathology of the Technical University of Munich using the following protocol: first, the respective tissue is examined and sectioned by a pathologist and then fixed in 3.7 % buffered formalin for eight hours. After that, the tissue is rehydrated by passing through the ascending row of alcohols using an automatic tissue processor: 70 % ethanol for one hour, 96 % ethanol once for 45 minutes and once for one hour, isopropanol once for 45 minutes and twice for one hour, and finally xylene twice for one hour. After four baths in liquid paraffin at a temperature of 60 °C (15 min, 15 min, 30 min, 60 min), the samples are embedded in paraffin, cooled and ready to get cut. The 3 µm thick adjacent serial sections are cut by the use of a microtome and floated on a 40 °C water bath. Afterwards the tissue is transferred onto glass slides and dried overnight at room temperature. Next day the slides are ready for the respective staining procedures. At first, routine histopathological examination is performed with hematoxylin-eosin staining (HE stain) for every tissue specimen in the routine lab of the Institute of Pathology. For breast cancer, pathologists evaluate every slide by applying the conventional histopathological classification of the WHO (see appendix **9.4**). Concerning breast cancer, the tissue specimens are subsequently immunohistochemically stained for HER2 as well as for steroid hormone receptors (ER, PR).

Not only full-face sections but also core needle biopsies can be used for staining procedures. A core needle biopsy is an effective tool in evaluating and diagnosing suspect lumps or masses, as a relatively large sample can be removed through a small single incision in the skin. In general, the breast area is first locally anesthetized and then the core needle biopsy is performed under image-guidance using ultrasound, stereotactic mammography, or magnetic resonance imaging. The biopsy tissue specimens are processed in the Institute of Pathology of the Technical University of Munich following exactly same procedure as the tissue preparation of full-face sections mentioned above. Examples of core biopsy sections are shown in **Figures 14-17**.

3.3 Construction of tissue microarrays

Tissue microarray technology facilitates a massive acceleration of studies correlating histopathological findings with clinico-pathological information.

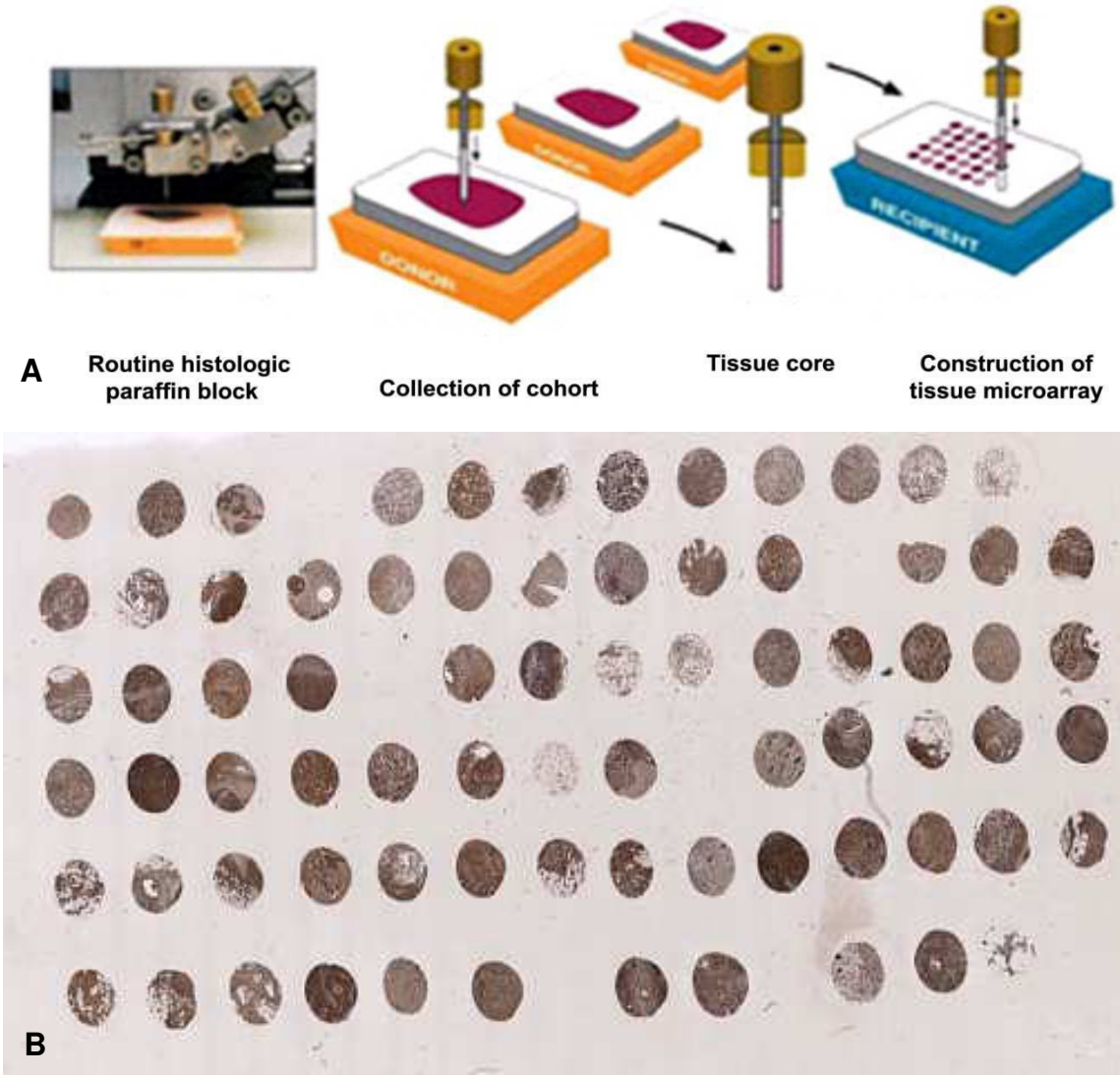


Figure 4 A Construction of a tissue microarray (TMA). With the use of a tissue microarray instrument (see on the left picture) single 1 mm tissue cores with 1 mm diameter are punched from the defined area of the respective donor blocks and inserted into 1 mm core holes of an empty paraffin block until the TMA is completed. (Taken and modified from *http://www.yalepath.org/YPTS/TMA.htm*) **B** Example of a slide of a tissue microarray, immunohistochemically stained using the automated staining instrument Ventana Benchmark® XT.

In order to prepare tissue microarrays, a pathologist has to select a representative area of the tumor in the respective HE stain of each tissue block. With the use of a manual tissue microarray instrument, single tissue cores (diameter 1 mm) are punched from the defined area of the donor block and inserted into a 1 mm core hole of an empty recipient paraffin block. According to a drawn tissue microarray

map, punches from other donor blocks are arranged by the same procedure until the TMA is completed (**Figure 4**). Then 3 µm sections are prepared analogically to normal paraffin blocks.

With this method cylindrical tissue samples from up to 100-1000 different tissues can be analyzed on one microscope glass slide. TMAs are highly representative of their donor tissues, despite the small size of the individual tissue specimens. Moreover, TMAs provide a high degree of standardization as all tissues are analyzed simultaneously with the same reagents and under same condition *(Bubendorf et al. 2001, Simon et al. 2005, Conway et al. 2008)*.

3.4 Immunohistochemical staining procedure

Immunohistochemistry is based on the affinity of antibodies to the according antigens. These antibodies are linked with a detection system whereby already very low amount of antigen can be presented. **Table 1** gives an alphabetically arranged overview of all used reagents, solutions and equipment.

Table 1 Alphabetical overview of the used reagents, solutions and equipment.

Material	Company
Antibody Diluent, Dako REAL™	Code S2022, Dako, Glostrup, Denmark
Antibody Diluent, Ventana	Cat. No. 251-018, Ventana, Tucson, AZ
Automated coverslipping machine	Medite glass coverslipper promounter RCM 2000, Medite GmbH, Burgdorf, Germany
Bluing reagent	Cat. No. 760-2037, Ventana, Tucson, AZ
CC1 (Cell Conditioning 1)	Cat. No. 950-124, Ventana, Tucson, AZ
Coverslips	Code K12450, Engelbrecht Medizin- und Labortechnik GmbH, Munich, Germany
DAB Chromogen	Code K5001, Dako, Glostrup, Denmark
Dako Cytomation EnVision™+ Dual Link System-HRP	Code K4061, Dako, Glostrup, Denmark
Dual Endogenous Enzyme Block	Code S2003, Dako, Glostrup, Denmark

Dako REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse	Code K5001, Dako, Glostrup, Denmark
Dako Autostainer Link 48	Code AS480, Dako, Glostrup, Denmark
Deionized water	In-house provided by the Institute of Pathology, Technical University, Munich
Ethanol 96 %/ 70 %	In-house provided by the Institute of Pathology, Technical University, Munich
EZ Prep™ Solution	Cat. No. 950-102, Ventana, Tucson, AZ
Formalin	In-house provided by the Institute of Pathology, Technical University, Munich
Goat Serum (Normal)	Code X0907, Dako, Glostrup, Denmark
Harris' Hematoxylin	HHS32, SIGMA-ALDRICH, Steinheim, Germany
Hematoxylin (in-house provided)	Recipe by the Institute of Pathology, Technical University Munich: 1.875 ml Harris' Hematoxylin, 1.875 ml Mayer's Hematoxylin, 3.500 ml aqua dest., 3.250 ml 100 % acetic acid (glacial), Merck, 1.00063.1000, Darmstadt, Germany
Hematoxylin	Cat. No. 760-2021, Ventana, Tucson, AZ
Dako REAL™ Hematoxylin	Code S2022, Glostrup, Denmark
Humid chamber	M920 Stain Tray™, Slide Staining System, Simport, Beloeil, Canada
Hydrogen peroxide 3 %	Aqua dest. + hydrogen peroxide (30 %), 1.07210.0250, Merck, Darmstadt, Germany
Imubind PAI-1 ELISA Kit	#821, American Diagnostica Inc., Greenwich, CT
Imubind uPA ELISA Kit	#894, American Diagnostica Inc., Greenwich, CT
Isopropanol	in-house provided by the Institute of Pathology, Technical University, Munich
iView® DAB Detection Kit	Cat. No. 760-091, Ventana, Tucson, AZ
Liquid DAB+ Substrate Chromogen System	Code K3468, Dako, Glostrup, Denmark
Mayer's Hematoxylin (Lillie's Modification)	"Saures Hämalaun nach Mayer", Code 9249, Merck, Darmstadt, Germany

Microtome	Microm HM 35 E, Microm GmbH, Walldorf, Germany
Light microscope	Zeiss Axioskop, Zeiss GmbH, Jena, Germany
Microscope slides	Code 4951PLUS, Superfrost Plus microscope slides, Thermo Scientific, Braunschweig, Germany
Mounting medium	Pertex®, Code 41-4012-00, Medite GmbH, Burgdorf, Germany No. 1025-250, Assistent, Sondheim, Germany
Nanozoomer	Nanozoomer 2.0 HAT slide scanner, Hamamatsu Photonics, Herrsching am Ammersee, Germany
Paraffin	in-house provided by the Institute of Pathology, Technical University, Munich
Pipettes	Pipetman Neo, Gilson, Middleton, WI
Pipette tips	Sarstedt, Nümbrecht, Germany
Reagent Vials, for Dako Autostainer	Code S3425, Dako, Glostrup, Germany
Reagent Rack, for Dako Autostainer	Code S3424, Dako, Glostrup, Germany
Scanning software	Nanozoomer Virtual Microscopy, Hamamatsu Photonics, Hamamatsu, Japan
Sodium chloride	Code 1.06404, Merck, Darmstadt, Germany
SPSS software	IBM SPSS Statistics 19, IBM, Armonk, NY
Staining jars	Code 46-3551-00, MEDITE GmbH, Burgdorf, Germany
Tissue microarray instrument	AlphaMetrix GmbH, Rödermark, Germany
Tris-buffered saline (TBS)	TBS-stock: 60,5g Trisma Base, 1L aqua dest., 2 N hydrogen chloride, 90g sodium chloride, adjust ad a pH 7.6, 10x diluted for application
Trizma Base	Code T-1503, Sigma, St. Louis, MO
ultraView® DAB detection Kit	Cat. No. 760-500, Ventana, Tucson, AZ

Ventana Benchmark® XT	Cat. No. N750-BMKTX-FS, Ventana, Tucson, AZ
Vortex shaker	MS1 Minishaker, Roth, Karlsruhe, Germany
Wash Buffer, 10x	Code S3006, Dako, Glostrup, Denmark
Water bath	Paraffin Streckbad, TFB 35, Medite GmbH, Burgdorf, Germany
Xylene	in-house provided by the Institute of Pathology, Technical University, Munich

3.4.1 Tissue pretreatment

The term “pretreatment” indicates the steps of the staining procedure before the primary antibody is applicated. First, in order to perform immunohistochemistry on paraffin-embedded tissue slides, they have to pass the descending row of graded alcohols for deparaffinization and rehydration to avoid incomplete removal of paraffin which would result in increased unspecific or reduced staining.

In the following an antigen demasking can be performed to reconstitute the lost immunoreactivity of the FFPE tissue. The antigen retrieval is a technique that reexposes epitopes in paraffin sections and makes them detectable by IHC. Formaldehyde is known to induce conformational changes in the antigen molecules by forming intermolecular cross-linkages and therefore diminish specific staining. “Heat Induced Epitope Retrieval” (HIER) includes different methods that can significantly improve the demonstration of many antigens. The most commonly used heating devices are microwave oven, pressure cooker and steamer. Other methods which are called “Proteolytic Induced Epitope Retrieval” (PIER) bring back immunoreactivity to tissue antigens. This includes treatment with proteinase k, trypsin, pepsin and many other proteases.

In addition unwanted protein interactions have to be reduced. In order to avoid unspecific background staining, the activity of the endogenous peroxidase of the tissue sections has to be blocked effectively. To achieve this, incubation with 3 % hydrogen peroxide is applied. Moreover, delayed or inadequate fixation may lead to unspecific background staining due to passive uptake of serum protein and

diffusion of the antigen. The most common cause of unspecific background staining is non-immunological binding of the specific immune serum by hydrophobic and electrostatic forces to certain sites within tissue sections. This can be reduced by blocking sites with normal serum. Besides, some tissues like especially kidney and liver have endogenous biotin. The use of a biotin-avidin detection system would lead to unwanted avidin binding to endogenous biotin. This can be avoided by the pretreatment with unconjugated avidin, which is then satured with the endogenous biotin *(DAKO handbook, 4th edition, www.Dako.com)*.

3.4.2 Antibody incubation

In immunohistochemical pretests with a panel of monoclonal antibodies accomplished by members of the Clinical Research Unit of the Department of Obstetrics and Gynecology, four monoclonal antibodies (mAbs) have been giving best results are chosen to establish protocols to assess uPA and PAI-1 by immunohistochemistry as listed in **Table 2**. The monoclonal antibodies #3689, #3785, #3786 are delivered lyophilized, whereas #ADG25 was sent as aqueous liquid or frozen liquid by American Diagnostica (Greenwich, CT). To avoid repeated freezing and thawing the lysate is dissolved in distilled water, aliquoted into convenient volumes (depending on the dilutions respectively) and stored in the freezer. Fresh dilutions of the antibodies are made prior to use and unused portions of antibody preparations and opened aliquots are discarded.

Table 2 Primary antibodies used for the staining procedures (Taken from *American Diagnostica, www.american-diagnostica.de*).

Antibody	Epitope specifity	Species	Concentration of antibody stock solution
#3689 (LOT 70830)	Serine proteinase domains/ B-chain of uPA	Monoclonal mouse IgG1	1 mg/ml
#3785 (LOT 3100)	Not determined	Monoclonal mouse IgG1	1 mg/ml
#3786 (LOT 71004)	Not determined	Monoclonal mouse IgG1	0.5 mg/ml
#ADG25 (LOT 27801)	Not determined	Monoclonal mouse IgG2a	1.7 mg/ml

Anti-uPA mAb #3689 shows a clear and specific staining pattern against uPA. To detect most of the free and complexed uPA in breast tumor sections, this monoclonal antibody is directed against different domains of uPA. Thus, #3689 reacts with free and receptor bound, single and two chain (HMW) urokinase and the B-chain (33 kD) fragment *(American Diagnostica, www.american-diagnostica.de).* The other three antibodies directed against PAI-1 show all excellent specific staining pattern, so that the protocols are performed with all three antibodies. Epitope specificity is not available for any of the three mAbs.

3.4.3 Visualization of the antigen-antibody-reaction

Immunohistochemistry is a technique used in laboratories for diagnostic purposes. The basic concept of IHC is the localization of antigens in tissue sections using specific primary antibodies. The antibody-antigen binding must be visualized with a colored reaction visible by light microscopy or through the use of fluorescence microscopy. There are direct methods in which a specific primary antibody carries the signal generating molecule providing direct visualization of the binding. Indirect methods need additional steps to localize the specific antibody and generate a signal. The most common indirect method is the application of a secondary antibody directed against the species of primary antibody and an enzyme with a corresponding substrate chromogen system which then leads to a colored precipitate at the site of the specific antibody binding (*www.ventanamed.com*). In this study two different detection systems are used: labeled streptavidin-biotin technology (3.4.3.1) and chain-polymer-conjugated technology (3.4.3.2).

3.4.3.1 Labeled streptavidin-biotin technology – LSAB method

The labeled streptavidin-biotin method (LSAB) is one of the avidin-biotin methods, which are based on the high affinity of the protein streptavidin to the vitamin biotin.

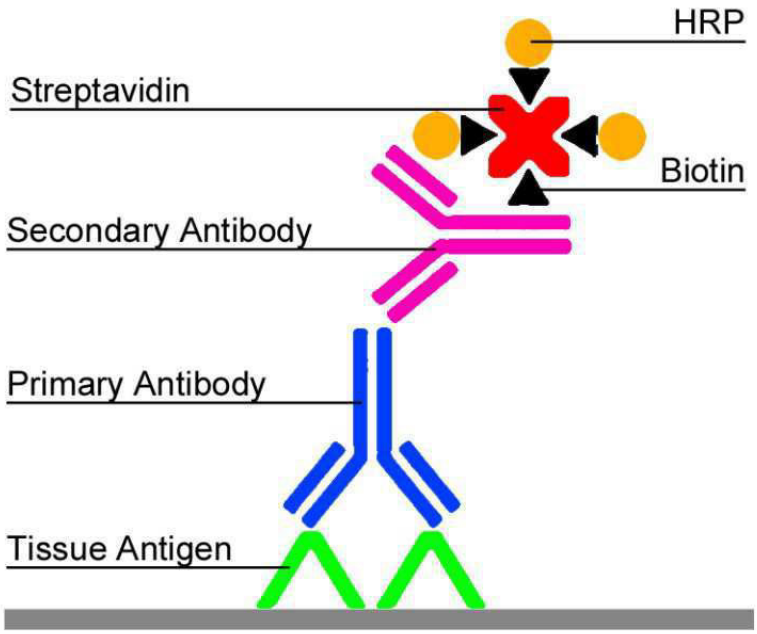


Figure 5 Labeled streptavidin-biotin method. A biotinylated secondary antibody is attached to the primary antibody. Signal is generated via the HRP-streptavidin-biotin-enzyme-complex converting DAB to a brown colored complex (Taken from *Dako Handbook 4th edition, www.dako.com*).

A species specific biotinylated antibody binds to the primary antibody, on which afterwards a horseradish peroxidase (HRP)-streptavidin enzyme complex is conjugated. Streptavidin (a protein from *Streptomyces avidinii*) has four binding sites for biotin. This enzyme complex converts in the following step the chromogen diaminobenzidin (DAB) to a brown colored complex. Thus, the secondary antibody links the primary antibody with the streptavidin-peroxidase conjugate (see **Figure 5**). Compared to direct peroxidase-conjugate methods a considerable increase of sensitivity is achieved because of the large enzyme-to-antibody-ratio as a single primary antibody subsequently is associated with multiple peroxidase molecules.

But there are certain limitations in this method. Especially the presence of endogenous biotin in tissues can lead to significant background staining. Because of that, polymer-based immunohistochemical methods which are not based on biotin are getting more and more popular. One of those is the EnVision™ system which is explained in the following *(DAKO handbook 4th edition, www.dako.com)*.

3.4.3.2 Chain-polymer-conjugated technology – EnVision™ method

Figure 6 shows the components of the EnVision™ system, a two-step method in which the application of the primary antibody is followed by a polymeric conjugate. This consists of up to 15 secondary antibodies (goat anti-mouse or goat anti-rabbit) directly bound to a dextran backbone containing up to 100 HRP molecules. These convert in the following step the chromogen diaminobenzidin (DAB) to a brown colored complex. The benefits are reduction of the number of steps compared to other IHC techniques, increased sensitivity and minimization of unspecific background staining *(DAKO handbook 4th edition, www.dako.com, Kämmerer et al. 2001).*

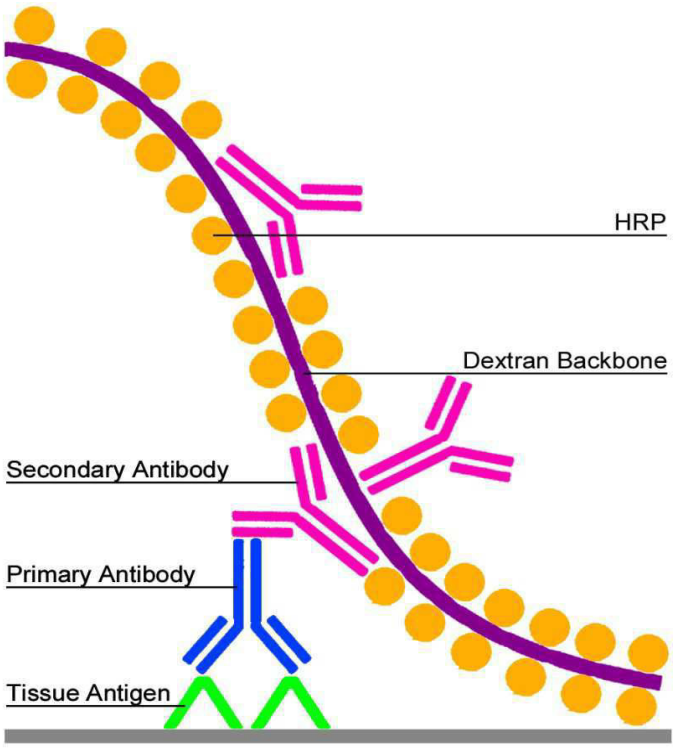


Figure 6 Chain-polymer-conjugated technology. A dextran polymer chain labeled with secondary antibodies and multiple HRP molecules is attached to the primary antibody. Signal is generated via the HRP molecules converting DAB to a brown colored complex (Taken from *Dako Handbook 4th edition, www.dako.com*).

3.4.4 Counterstaining

The DAB staining reaction leads to a water insoluble colored end-product. For counterstaining a water-based hematoxylin is used which is suitable for visualization of nuclei in tissue sections. Depending on the potency of the hematoxylin, slides are immersed in a bath of hematoxylin for 5-50 seconds until a blue coloration of cell nuclei results. Then, slides are “blued”, which means that they are placed in a tap water bath (or any neutral or alkaline liquid) and rinsed softly with running tap water whereby an insoluble blue aluminum hematein complex is formed. Hematoxylin solutions contain hematein and a metal mordant. The mordant provides the stain color. The main aluminum hematoxylin solutions

employed are Harris's Hematoxylin and Mayer's Hematoxylin *(www.dako.com)*. The hematoxylin used in the Institute of Pathology of the Technical University, Munich, in routine clinical use is following a house intern recipe described in **Table 1**.

3.5 Different staining procedures

Figure 7 gives an overview about the workflow. First, SOPs are worked out for the manual staining procedure for the LSAB/DAB method as well as for the EnVision™ method. Then, the protocol is transferred to the automatic staining machines: first to the Dako Autostainer (LSAB/DAB method, EnVision™ method), and then to the Ventana Benchmark® XT (iView®, ultraView®).

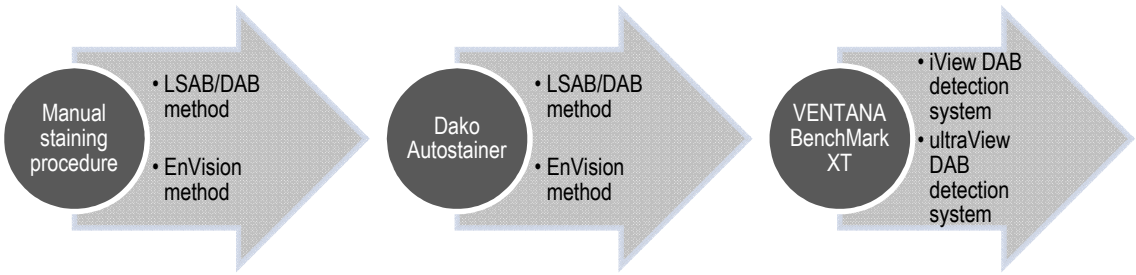


Figure 7 Overview about the workflow. First, manual staining procedure is performed using two different methods, the LSAB/DAB and the EnVision™ method. These protocols are transferred to the Dako Autostainer, an automatic staining machine. Afterwards protocols are modified for the Ventana Benchmark® XT using the iView® and the ultraView® detection system.

3.5.1 Manual staining procedure

Manual staining is optimized prior the protocol is transferred to automatic staining machines. According to standard staining protocols used by members of the Research Unit of the Department of Obstetrics and Gynecology of the Klinikum rechts der Isar, Technical University of Munich, the first step is to optimize this protocol for each antibody. All subsequent steps are carried out using commercially available kits. The whole staining procedure is accomplished at room temperature. In order to prevent drying up the tissue, the incubation of the respective reagent is performed in a humid chamber. Unless otherwise noted 130

µl of the different reagents are applied per section during the different staining steps.

First, the deparaffinization and rehydration is performed by passing the FFPE tissue sections through xylene twice for ten minutes and then through the descending row of graded alcohols with twice isopropanol, once 96 % ethanol and once 70 % ethanol, each for five minutes. After that, slides are transferred into a staining jar filled with tris-buffered saline (TBS) with an intervening buffer change.

Using the LSAB-method, the following steps are taken: first, the endogenous peroxidase activity is quenched by incubating all slides with 3 % hydrogen peroxide solution for ten minutes in a staining dish. Afterwards slides are rinsed in tap water shortly and then transferred into a staining jar filled with TBS for five minutes with an intervening buffer change. Thereafter 10 % goat serum in TBS is applied to each slide using a pipette and incubated for 30 minutes at room temperature without any following washing step. The slides are just drained off and in the next step the primary antibody diluted in antibody diluent (Dako) is applied by using a pipette and incubated for a certain time according to the particular antibody, e.g. two hours, whereas pure antibody diluent is applied to the negative control tissue. According to the LSAB kit manual, sections are washed with TBS with an intervening buffer change and then four drops of bottle A (goat anti-mouse antibody, biotinylated) of the LSAB kit (code K5001) are put on each section for 30 minutes at room temperature. Subsequent to a five minutes washing step with TBS, four drops of bottle B (streptavidin-complex, labeled with HRP) are applied to each section. After another five minutes washing step, DAB substrate is prepared by mixing bottle D (HRP substrate buffer) and C (DAB chromogen) of the LSAB kit (1500 µl (D) + 30 µl (C) for 10 slides) and applied for ten minutes at room temperature. After the final washing step, sections are counterstained in hematoxylin for 50 seconds, rinsed under flowing tap water for five minutes and transferred into distilled water. Then sections are dehydrated in the ascending row of graded alcohols (70 % ethanol, 96 % ethanol, 2x isopropanol, 2x xylene, each for 5 minutes) and finally mounted with permanent mounting medium either manual or by using an automated coverslipping machine.

Using the EnVision™/DAB-method, following steps are taken: first, blocking of endogenous peroxidase is performed by preincubating the slides in Dual Endogenous Enzyme Block (S2003, Dako) for ten minutes. After a washing step in

TBS for five minutes with an intervening buffer change, the primary antibody is diluted in antibody diluent (Dako) and applied to the sections according to its particular dilution and incubation time. Subsequent to a following washing step in TBS, the labeled polymer-HRP-antibody (Dako Cytomation EnVision™ + Dual Link Peroxidase, K4061) is put on the slides and incubated for 30 minutes according to the kit manual. After another five minutes washing step, DAB substrate is prepared by mixing bottle D and C of the LSAB kit (1500 µl + 30 µl for 10 slides) and applied for ten minutes. After a final washing step, nuclei are counterstained with hematoxylin for 50 sec, rinsed under flowing tap water for five minutes and transferred into distilled water. Then slides are passed through the ascending row of graded alcohols (70 % ethanol, 96 % ethanol, 2x isopropanol, 2x xylene, each for 5 minutes) and finally mounted with permanent mounting medium either manual or by using an automated coverslipping machine.

3.5.2 Automatic staining procedure – Dako Autostainer

As the SOPs should serve for future routine clinical use, it is important to transfer the manual staining protocol to automated staining instruments. The Dako Autostainer instrument (**Figure 8**) consists of a slide processor, a desktop personal computer with software, and a printer. It is an “open” system, which implies that various parameters of the protocol may be modified. It can process a maximum of 48 microscope slides in a single staining run. For each slide an independently designed protocol can be used. First, a protocol template with defined elements has to be designed and saved for future use. There standard protocol elements can be chosen such as *Endogenous Enzyme Block, Protein Block, Primary Antibody, Secondary Reagent, Tertiary Reagent, Labeled Polymer, Substrate Batch, Substrate, Auxiliary, Buffer* and *Water.* Thereby specific reagents with respective incubation times are assigned to each slide. When reagent programming is complete, the *Slide Layout Map* appears on

Figure 8 Dako Autostainer (Taken from *Dako Autostainer Handbook 2006, www.dako.com*).

the screen. Here, the dispense volume and the drop zone can be arranged for each slide. Next, the Dako automated stainer instrument calculates the fill volume of reagents required including wash buffer and deionized water, the time required for the staining run, and the placement for the reagent vials. Then the reagents are transferred from the kit bottles into graduated autostainer reagent vials and placed into the *Autostainer Reagent Rack* according to the computer-generated *Reagent Layout Map*. After that the slides are loaded on the slide racks of the autostainer according to the computer-generated *Slide Layout Map* and sprinkled with wash buffer (Dako) to prevent the tissue drying up. The "*start run"* button has to be pressed in order to start the staining run *(Dako Autostainer Handbook 2006, www.dako.com)*. After the run has completed, slides are transferred into a staining jar filled with deionized water with an intervening water change. Then according to the manual staining procedure, the slides are manually counterstained, dehydrated in the ascending row of graded alcohols and finally mounted with permanent mounting medium either manual or by using an automated coverslipping machine.

3.5.3 Automatic staining procedure – Ventana Benchmark® XT

Figure 9 Ventana Benchmark® XT (*www.ventanamed.com*).

The Ventana Benchmark® XT is an automated staining instrument (**Figure 9**), which is routinely used for the immunohistochemical staining at the Department of Pathology of the Hospital Klinikum rechts der Isar of the Technical University in Munich. It consists of a staining module with air vortex mixers, liquid coverslip, heater pad, and the E-Bar barcode slide label system. Compared to the "open" system of the Dako Autostainer Instrument, the Ventana Benchmark® XT is a "closed" system which means that the ability to modify staining protocols is limited and that only the reagents of the instrument's distributor can be used, except the primary antibody. In a single staining run, the Ventana Benchmark® XT can process a maximum of 30 microscope slides. See **Table 3** for comparison of the two instruments.

Table 3 Comparison Dako Autostainer versus Ventana Benchmark® XT (Taken from *Myers et al. 2004*).

	Dako Autostainer	**Ventana Benchmark® XT**
Company	Dako	Ventana Medical Systems
Slide placement format	Matrix/Array	Carousel/Rotary
Operating modes	Open	Barcode
Intended staining capabilities	ICH	IHC, ISH, FISH, FITC
Reagent application method	Rinsed probe	Disposable dispenser
Slide capacity	48	30
Reagent capacity	64	35
Continuous Processing	No	Yes
Slide Heating	No	Yes
Variable dispense volume	Yes	No

Staining is performed with the help of a laboratory technician at the routine lab of the Institute of Pathology of the Technical University of Munich. The procedures for staining on the Ventana Benchmark® XT are as follows. First of all, all the staining information of the respective staining procedure has to be entered into the software system according to the operator's manual. Then barcode labels with the individual staining information are printed and applied to each respective slide. According to manual staining procedures, slides pass through the descending row of alcohols started with xylene to gradient alcohols. Then dispensers are filled with the individual diluted primary antibodies and are put together with the other prefilled barcode-labeled dispensers of the particular detection kit and the hematoxylin onto the reagent tray and then placed on the carousel of the automated slide stainer. Two different detection systems are used: the ultraView® and the iView®. As the kits are optimized for Ventana automated slide stainers neither mixing nor diluting of kit reagents is required. Then the deparaffinized, labeled slides are loaded into the instrument. The respective parameters are chosen in the Benchmark® XT software. To remove unbound material, the slides are washed with buffer at the end of each incubation step. In addition a liquid cover slip is applied during every incubation step to minimize evaporation of the aqueous reagents from the slides. After completion of the run, slides are removed from the automated slide stainer by hand and washed in alcohol or a mild dishwashing detergent to remove the cover slip solution. The next steps are analog to the manual procedure: dehydration by the ascending row of alcohols and xylene and afterwards coverslipping with permanent mounting media.

The Ventana ultraView® method is quite similar to the EnVision™ System. It is a multimer-technology based detection system intended for the specific and sensitive detection of mouse and rabbit primary antibodies by utilizing a cocktail of enzyme labeled secondary antibodies. The primary antibody-HRP labeled antibody complex is visualized using DAB resulting in a brown target signal. This detection system is optimized for use on the Benchmark® automated slide stainers and mainly used in Europe and also at the Department of Pathology of the University Hospital Klinikum rechts der Isar for routine clinical use.

The Ventana iView® DAB detection kit is similar to the LSAB/DAB method and quite common in the USA. It is an indirect biotin streptavidin system for detecting mouse and rabbit primary antibodies. The technique uses biotinylated secondary antibodies that locate the bound primary antibody, followed by the binding of Streptavidin-HRP conjugate. Again, the primary antibody-HRP labeled antibody complex is visualized using DAB resulting in a brown target signal. All reagents are provided pre-diluted and ready-to-use for convenient operation on Benchmark® automated slide stainers *(www.ventanamed.com)*.

3.6 Scanning of stained slides by use of the Nanozoomer 2.0 HAT slide scanner

All slides are scanned using the Nanozoomer 2.0 HAT slide scanner, Hamamatsu in Herrsching am Ammersee, Germany. All slides are scanned with 20x or 40x zoom according to the operator's manual and with the help of the staff of Hamamatsu.

3.7 Evaluation of immunostaining

Histological evaluation is performed in collaboration with the Institute of Pathology of the Technical University of Munich. Immunohistochemical staining intensity is categorized from 0.5 to 3. The absence of chromogenic reaction in tumor tissue is allocated in class negative (0). The scoring is done blinded to information about the used antibodies as well as to clinical data. Four different areas are considered

in every core of the tissue microarray: nuclei of the tumor cells, cytoplasm of the tumor cells, cells of the tumor stroma and non-cellular part of the tumor stroma. In order to perform statistical analysis two groups (strong vs. weak staining) are performed using the median as cut-off.

Positive and negative controls run simultaneously with every staining procedure to make sure any loss in sensitivity or titer. A positive tissue control is important in every staining procedure performed to ascertain a proper performance of all applied reagents. Kidney or breast tissues which are fixed in identical manner to the test sections are used as control tissues. If the positive tissue control failed to demonstrate positive staining, results with the test tissue specimens are considered invalid. A negative reagent control is required in every staining run to identify any non-specific staining. Therefore, slides are stained with negative control reagent or just with antibody diluent in place of the primary antibody for the same incubation period. Non-specific staining can be recognized as a diffuse, brown staining on the negative control slides *(www.dako.com, www.ventanamed.com)*.

3.8 Statistical analyses

Statistical analyses are performed using the SPSS software (IBM SPSS Statistics Version 19, Armonk, NY). A p-value ≤ 0.05 is defined as statistically significant. Correlations are Spearman rank correlations and are regarded as strong when R ≥ 0.5. Outcome variables are disease-free survival (DFS) and overall survival (OS). DFS is defined as the period between date of primary surgery and date of disease recurrence. OS is defined as the period between date of primary surgery and date of death. The impact of different histopathological and clinical factors as well as immunohistochemical scoring results on OS and DFS are assessed by univariate and multivariate Cox regression models. The resulting hazard ratio and p-value reflect their respective statistical significance. Significant findings are shown in Kaplan-Meier curves. Significance is assessed by log-rank.

4. Results

4.1 Optimization of immunohistochemical staining protocols for uPA and PAI-1

For optimization of the different staining procedures, numerous parameters such as antibody concentration, incubation time of the primary antibody, incubation temperature, antigen retrieval, and blocking reagents are evaluated.

Well-established incubation times and temperatures are overnight incubation at 4 °C, incubation at room temperature for 2 hours, or incubation for 30-60 minutes at 30-37 °C. Bearing in mind these protocols are meant for future clinical routine use, it is very important to use, as far as possible, practical incubation times and easy reproducible procedures. The testing procedure starts at room temperature in order to avoid additional equipment e.g. an oven and unnecessary time wasting procedures such as overnight incubation. After several manual staining runs, it has turned out that results are similar either incubating the primary antibody at room temperature for two hours or for one hour in the oven at 37 °C. In addition, same results are achieved with the double antibody dilution (e.g. 1:500 instead of 1:250) for two hours at 37 °C in the oven.

According to the study of *Ferrier et al. 1999*, systematically evaluating the influence of different antigen retrieval regimens on immunoreactivity, neither HIER nor PIER does improve the staining results using anti-uPA mAb #3689 or anti-PAI-1 mAbs #3785, #3786, #ADG25. Instead, it leads to fuzzy background staining.

The most difficult part of the optimization is certainly the determination of the best antibody dilution. First, a testing row of different dilutions starting with 1:50, 1:100, 1:250, 1:500 and 1:1000 is performed for each antibody. Then, slides are studied with the microscope by members of the Clinical Research Unit of the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technical University of Munich. Depending on the staining intensity and specificity, the dilutions with best results are chosen and a more detailed staining dilution around this dilution is established. For this testing easily available kidney tissue

specimens are used first. When best dilution is found, valuable breast cancer tissue specimens are applied for the staining. The staining procedure with the optimized antibody dilution is repeated several times and results are compared in order to guarantee reproducibility. **Table 4** gives on overview of the antibody dilutions of the different staining systems and methods.

Table 4 Overview of the recommended antibody dilutions using different staining kits and instruments.

	Manual staining LSAB/DAB method	Manual staining EnVision™ method	Dako Autostainer LSAB/DAB method	Dako Autostainer EnVision™ method	Ventana Benchmark® XT iView® detection Kit	Ventana Benchmark® XT ultraView® detection Kit
Temperature	RT	RT	RT	RT	37 °C	37 °C
Incubation time (hrs)	2	2	2	2	1	1
Anti-uPA #3689 (LOT 70830)	1:500	1:600	1:500	1:700	1:300	1:300
Anti-PAI-1 #3785 (LOT 3100)	1:150	1:200	1:200	1:250	1:250	1:250
Anti-PAI-1 #3786 (LOT 71004)	1:35	1:60	1:60	1:60	1:15	1:15
Anti-PAI-1 #ADG25 (LOT 27801)	1:125	1:125	1:125	1:125	1:30	1:30

The manual staining procedure is performed using the DAB detection Kit from Dako. In addition, the same testing procedure is performed for the EnVision™ system from Dako. For these different kits standard operating procedures (SOPs) are established (see **4.1.1**). Examples of the manual staining results are shown in **Figures 10-13, A and B**. The next step is to transfer the staining procedure onto automatic staining instruments (see **Figure 7**).

As it is possible to use the same reagents in the Dako Autostainer, the good and reproducible manual staining protocols can easily be transferred to the instrument. In order to avoid wasting valuable breast cancer tissue specimens, again kidney tissue specimens are used first. Staining quality and pattern between the manual staining results and automatic staining results are almost identical.

After a short period of optimization primary antibody dilutions, the protocol has been established (see **4.1.2**). Staining results are presented in **Figures 10-13, C and D**.

The next step is to transfer the protocol to the Ventana Benchmark® XT (iView® and ultraView® detection Kit), the routinely used staining instrument of the Institute of Pathology of the Technical University of Munich and of many Pathology Departments worldwide. As the working temperature of this instrument is fixed at 37 °C and compared to manual staining different reagents are required, several runs and variations have to be performed in order to establish reproducible protocols for each antibody. An interesting point to mention is the fact that using the Dako Antibody Diluent leads to considerably better staining results than using the Ventana Diluent. Examples of staining results for each antibody are presented in **Figures 10-13, E and F.**

All different methods employed produce similar staining results. In general, automatic staining is stronger than manual for tumor cells and tumor stroma, but in the same context.

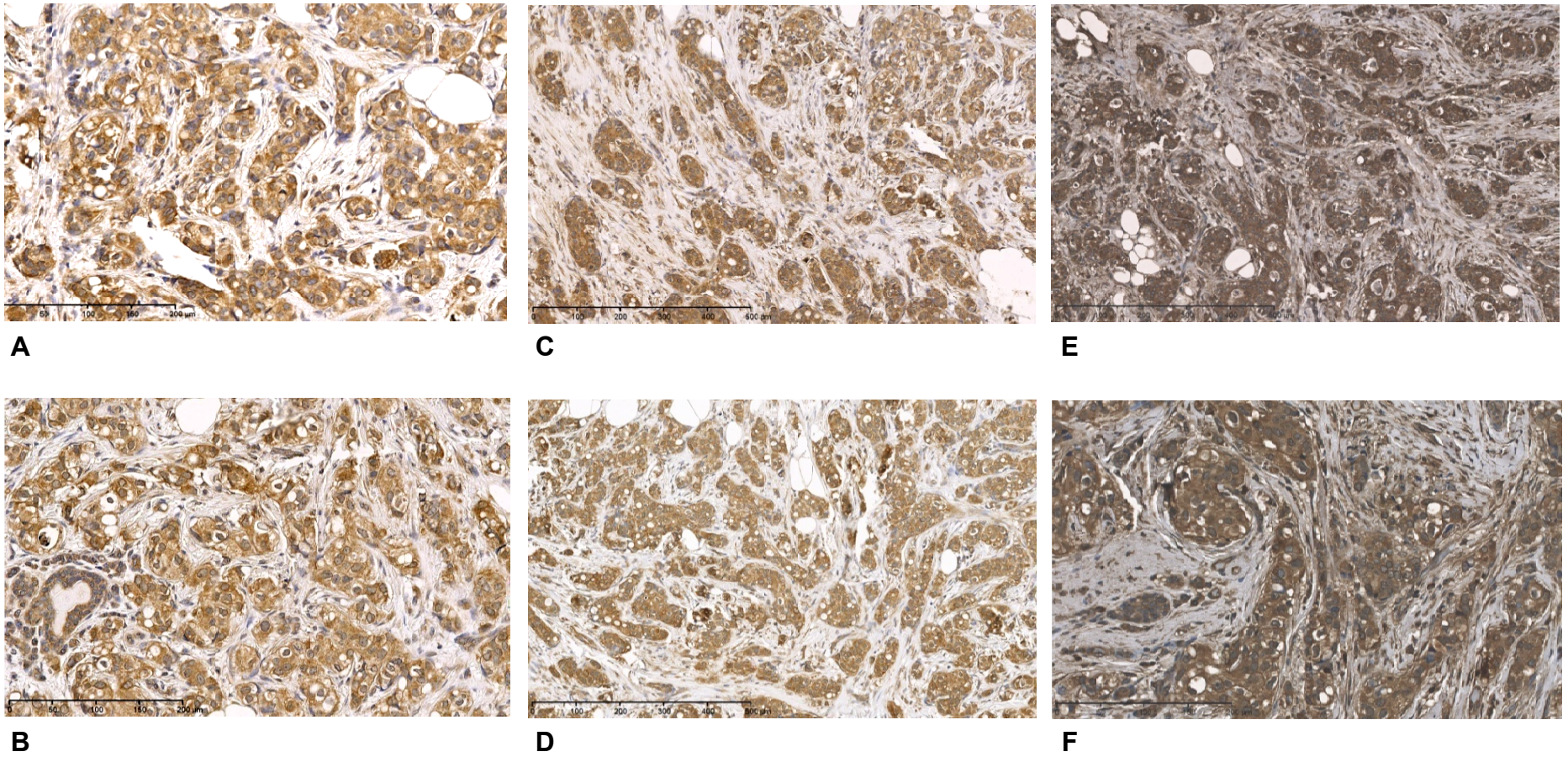


Figure 10 Examples of immunohistochemical staining of FFPE breast cancer tissue specimens using anti-uPA mAb #3689 by different staining procedures and antibody dilutions. **(A)** manual staining, LSAB method, antibody dilution 1:500, **(B)** manual staining procedure, EnVision™ method, antibody dilution 1:600, **(C)** automatic staining procedure using Dako Autostainer, LSAB method, antibody dilution 1:500, **(D)** automatic staining procedure using Dako Autostainer, EnVision™ method, antibody dilution 1:700, **(E)** automatic staining procedure using Ventana Benchmark® XT, iView® method, antibody dilution 1:300, **(F)** automatic staining procedure using Ventana Benchmark® XT, ultraView® method, antibody dilution 1:300. All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

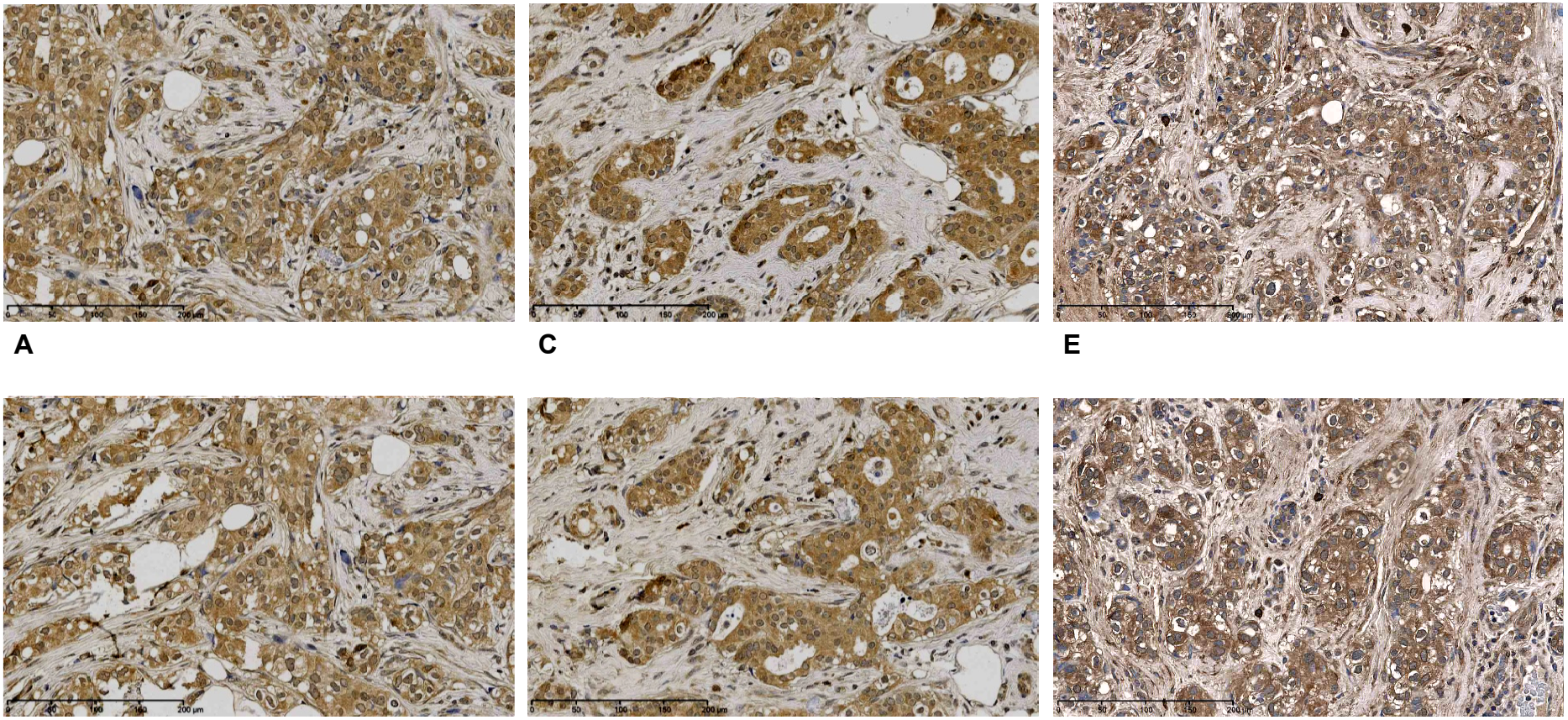


Figure 11 Examples of immunohistochemical staining of FFPE breast cancer tissue specimens using anti-PAI-1 mAb #3785 by different staining procedures and dilutions. **(A)** manual staining, LSAB method, antibody dilution 1:150, **(B)** manual staining procedure, EnVision method, antibody dilution 1:200, **(C)** automatic staining procedure using Dako Autostainer, LSAB method, antibody dilution 1:200, **(D)** automatic staining procedure using Dako Autostainer, EnVision method, antibody dilution 1:250, **(E)** automatic staining procedure using Ventana Benchmark® XT, iView® method, antibody dilution 1:250, **(F)** automatic staining procedure using Ventana Benchmark® XT, ultraView® method, antibody dilution 1:250. All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

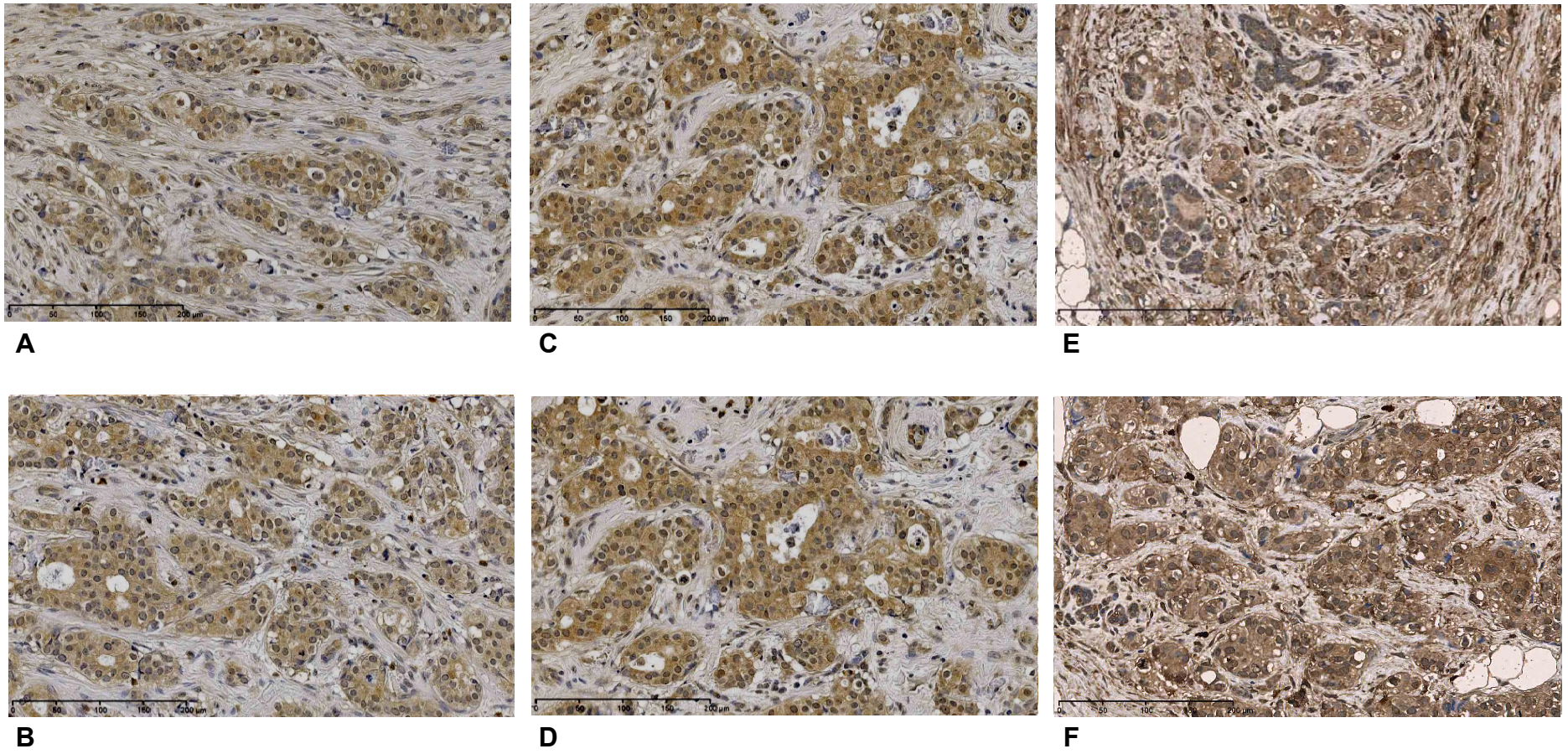


Figure 12 Examples of immunohistochemical staining of FFPE breast cancer tissue specimens using anti-PAI-1 mAb #3786 by different staining procedures and dilutions. **(A)** manual staining, LSAB method, antibody dilution 1:35, **(B)** manual staining procedure, EnVision method, antibody dilution 1:60, **(C)** automatic staining procedure using Dako Autostainer, LSAB method, antibody dilution 1:60, **(D)** automatic staining procedure using Dako Autostainer, EnVision method, antibody dilution 1:60, **(E)** automatic staining procedure using Ventana Benchmark® XT, iView® method, antibody dilution 1:15, **(F)** automatic staining procedure using Ventana Benchmark® XT, ultraView® method, antibody dilution 1:15. All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

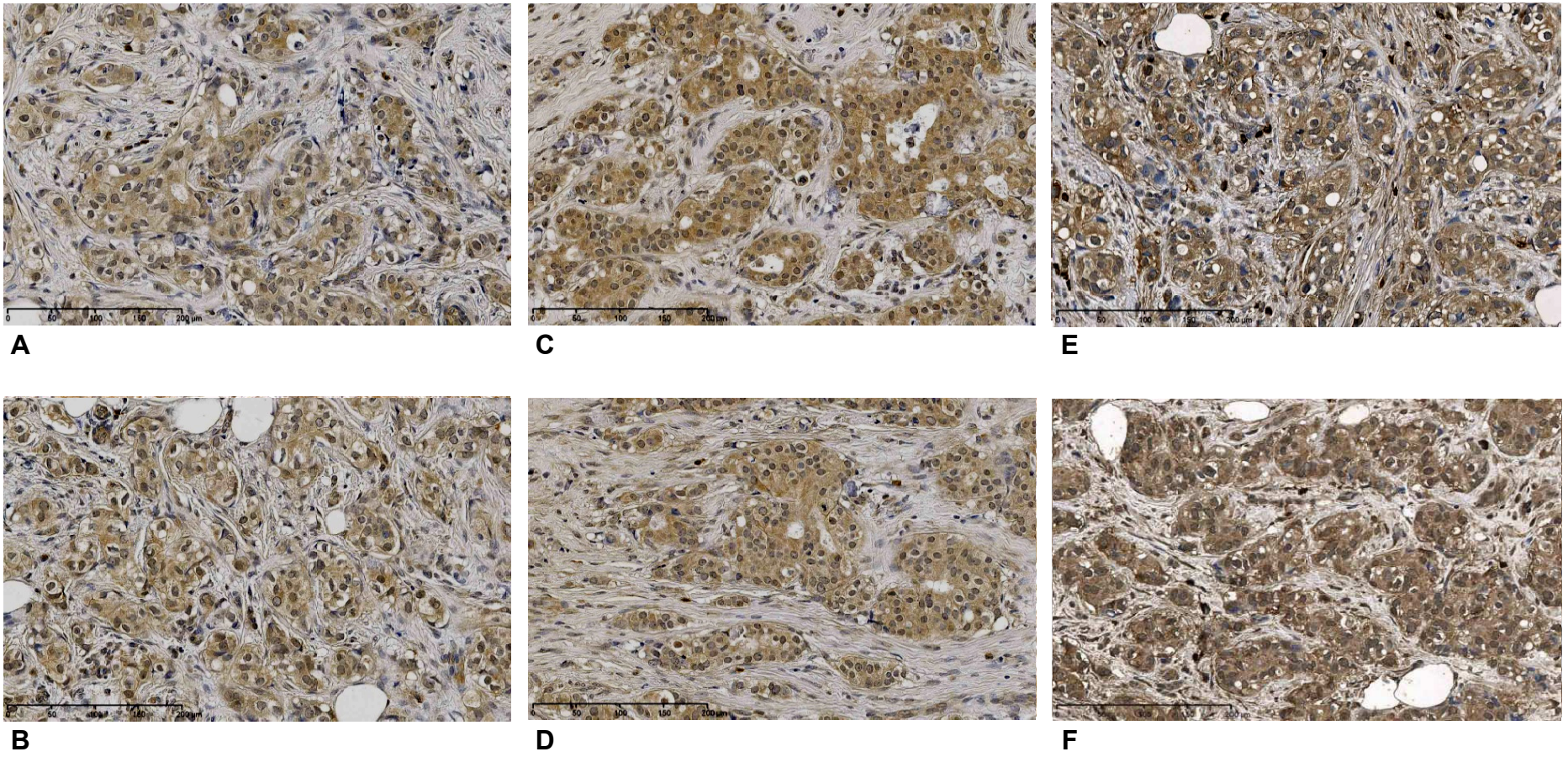


Figure 13 Examples of immunohistochemical staining of FFPE breast cancer tissue specimens using anti-PAI-1 mAb #ADG25 by different staining procedures and dilutions. **(A)** manual staining, LSAB method, antibody dilution 1:125, **(B)** manual staining procedure, EnVision method, antibody dilution 1:125, **(C)** automatic staining procedure using Dako Autostainer, LSAB method, antibody dilution 1:125, **(D)** automatic staining procedure using Dako Autostainer, EnVision method, antibody dilution 1:125, **(E)** automatic staining procedure using Ventana Benchmark® XT, iView® method, antibody dilution 1:30, **(F)** automatic staining procedure using Ventana Benchmark® XT, ultraView® method, antibody dilution 1:30. All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

4.1.1 SOP to assess uPA and PAI-1 by immunohistochemistry – manual staining procedure

The respective optimized protocols for the manual staining procedure are described in **Table 5** (LSAB/DAB method) and **Table 6** (EnVision™/DAB method).

Table 5 SOP to assess uPA and PAI-1 by immunohistochemistry: Manual staining procedure for LSAB/DAB method on slides from FFPE tissue specimens.

1. Deparaffinization and rehydration in descending row of graded alcohols (2 x 10 min xylene, 2 x 5 min 100 % isopropanol, 1 x 5 min 96 % ethanol, 1 x 5min 70 % ethanol)
2. 5 min washing in TBS, with an intervening buffer change
3. Blocking of endogenous peroxidase activity with 3 % hydrogen peroxide for 20 min at RT
4. Short rinse in tap water
5. 5 min washing in TBS, with an intervening buffer change
6. Blocking with 10 % goat serum in TBS for 30 min at RT, 130 µl/slide
7. No washing step! Just tap off the serum from the slides
8. Application of the primary antibody diluted in Dako Antibody diluent (S2022) for 2 hrs at RT, 130 µl/slide #3689 (LOT 70830) 1:500 #3785 (LOT 3100) 1:150 #3786 (LOT 71004) 1:35 #ADG25 (LOT 27801) 1:125
9. 5 min washing in TBS, with an intervening buffer change
10. Application of bottle A of DAB detection Kit (code K5001, Dako) for 30 min at RT, 130 µl/slide
11. 5 min washing in TBS, with an intervening buffer change
12. Application of bottle B of DAB detection Kit (code K5001, Dako) for 30 min at RT, 130 µl/slide
13. 5 min washing in TBS, with an intervening buffer change
14. Application of DAB substrate (mixture of bottle C+D of DAB detection Kit (code K5001, Dako)) for 10 min at RT, 130 µl/slide
15. 5 min washing in TBS, with an intervening buffer change
16. Counterstaining with Mayers' Hematoxylin for 50 sec/ with in-house provided hematoxylin for 7 min
17. Blue-dying: washing under flowing normal tap water for 5 min
18. Transfer into aqua dest. for 2 min
19. Dehydration in ascending row of graded alcohols (1 x 70 % ethanol, 1 x 96 % ethanol, 2 x 100 % isopropanol, 2 x xylene, each for 1 min)
20. Coverslipping of the slides with permanent mounting media either manual or by using the automated coverslipping machine

Table 6 SOP to assess uPA and PAI-1 by immunohistochemistry: manual staining procedure for EnVision™/DAB method on slides from FFPE tissue specimens.

1. Deparaffinization and rehydration in descending row of graded alcohols (2 x 10 min xylene, 2 x 5 min 100 % isopropanol, 1 x 5 min 96 % ethanol, 1 x 5 min 70 % ethanol)
1. 5 min washing in TBS, with an intervening buffer change
2. Blocking with Dual Endogenous Enzyme Block (S2003) for 10 min at RT, 130 µl/slide
3. 5 min washing in TBS, with an intervening buffer change
4. Application of the primary antibody diluted in Dako Antibody diluent (S2022) for 2 hrs at RT, 130 µl/slide: #3689 (LOT 70830) 1:600 #3785 (LOT 3100) 1:200 #3786 (LOT 71004) 1:60 #ADG25 (LOT 27801) 1:125
5. 5 min washing in TBS, with an intervening buffer change
6. Application of the labeled polymer HRP antibody (code K4061) for 30 min at RT, 130 µl/slide
7. 5 min washing in TBS, with an intervening buffer change
8. Application of DAB substrate (mixture of bottle C+D of DAB detection Kit (code K5001)) for 10 min at RT, 130 µl/slide
9. 5 min washing in TBS, with an intervening buffer change
10. Counterstaining with Mayers' Hematoxylin for 50 sec/ with in-house provided hematoxylin for 7 min
11. Blue-dying: washing under flowing normal tap water for 5 min
12. Transfer into aqua dest. for 2 min
13. Dehydration in ascending row of graded alcohols (1 x 70 % ethanol, 1 x 96 % ethanol, 2 x 100 % isopropanol, 2 x xylene, each for 1 min)
14. Coverslipping of the slides with permanent mounting media either manual or by using the automated coverslipping machine

4.1.2 SOP to assess uPA and PAI-1 by immunohistochemistry – automatic staining procedure for Dako Autostainer Instrument

1. Deparaffinization and rehydration of tissue sections in descending graded row of alcohols: 2 x 10 min xylene 2 x 5min 100 % isopropanol, 1 x 5 min 96 % ethanol, 1 x 5 min 70 % ethanol, followed by 5 min washing in TBS, with an intervening buffer change.
2. Programming of the Autostainer Instrument (Refer to the template below (**Table 7**) and to Operators Manual for the respective Dako Autostainer Instrument)
3. Loading of the template into the "Program Staining Run Screen"
4. Loading of reagents according to the "Slides Layout Map Screen"
5. Loading of slides according to the "Reagents Layout Map Screen"

6. Checking water and buffer pumps
7. Starting the program
8. After end of program: washing slides under flowing tap water for 5 min
9. Dehydration in ascending row of graded alcohols: 1 x 70 % ethanol, 1 x 96 % ethanol, 2 x 100 % isopropanol, 2 x xylene, each for 1 min
10. Coverslipping of the slides with permanent mounting media either manual or by using the automated coverslipping machine.

Table 7 Template of the staining program of the Dako Autostainer using LSAB/DAB method (left column) and EnVision™/DAB-method (right column).

LSAB/DAB- method	**EnVision™/DAB-method**
1. Rinse buffer	1. Rinse buffer
2. Peroxide Block [10 min]	2. Endogenous Enzyme Block[10 min]
3. Rinse Water	3. Rinse buffer
4. Rinse buffer	4. Primary antibody [120 min]
5. NGS [30 min]	5. Rinse buffer
6. Blow	6. Secondary Reagent [30 min]
7. Primary antibody [120 min]	7. Rinse Buffer
8. Rinse buffer	8. Substrate Batch [10 min]
9. Secondary reagent [30 min]	9. Rinse buffer
10. Rinse buffer	10. Auxiliary [Hematoxylin, 7 min]
11. Tertiary reagent [30 min]	11. Rinse water
12. Rinse buffer	
13. Substrate batch [10 min]	
14. Rinse buffer	
15. Auxiliary [Hematoxylin, 7 min]	
16. Rinse water	

4.1.3 SOP to assess uPA and PAI-1 by immunohistochemistry – automatic staining procedure for Ventana Benchmark® XT

1. Deparaffinization and rehydration of tissue sections in descending graded row of alcohols: 2 x 10 min xylene; then 2 x 100 % isopropanol, 1 x 96 % ethanol, 1 x 70 % ethanol, each step for 5 min followed by 5 min washing in TBS, with an intervening buffer change.
2. Preparation of required reagents: dilute Reaction Buffer, EZ Prep Solution, and SSC Wash Solution according to the manufacturer's instruction *(www.ventanamed.com)*.

3. Dilution of primary antibodies in DAKO REAL™ antibody diluent (S2022) as the follows and fill Prep Kit Dispenser with the diluted primary antibodies respectively (#3689 1:300; #3785 1:250; #3786 1:15; #ADG25 1:30).
4. Application of slide code label to the slides.
5. Loading of the detection kit and the primary antibody dispenser as well as the required accessory reagents onto the reagent tray and place them on the automated Ventana Benchmark® XT slide stainer.
6. Checking of bulk fluids and waste.
7. Starting the staining run of the Ventana Benchmark® XT (follow the manufacturer's instructions).
8. Selection of the parameters from the Benchmark® software, as described in **Table 8**.
9. Loading of the slides onto the instrument.
10. After completion of the run, removal of the slides from the instrument, and washing in a mild dishwashing detergent to remove the cover slip solution. Last washing step with filtered deionized H_2O.
11. Dehydration the slides with the ascending row of alcohols: 70 % ethanol, 96 % ethanol, 2 x 100 % isopropanol, 2 x xylene, each for 1 min.
12. Coverslipping of the slides with permanent mounting media either manual or by using the automated coverslipping machine.

Table 8 Selected parameters from the Benchmark® software.

Paraffin	[Selected]
Deparaffinization	[Selected]
Cell Conditioning	[Selected]
Conditioner Nr.1	[Selected]
Mild CC1	[Selected]
Define antibody incubation temperature 37 °C	[Selected]
Antibody PREP KIT	[Selected], 60 min
Counter Staining HEMATOXYLIN	[Selected], 7 min
After Counter Staining BLUING REAGENT	[Selected], 4 min

4.2 Core biopsies and respective primary tumor tissues

4.2.1 Scoring results of immunohistochemical staining of uPA and PAI-1

10 cases of core biopsies together with their respective primary tumor tissue are immunohistochemically stained with the four antibodies described above (#3689, #3785, #3786, #ADG25) by the Dako Autostainer instrument. The mean time period between core biopsy and breast cancer surgery is 7.5 (1-15) days. Slides are scored by a pathologist and staining pattern and intensity of the core biopsies are compared to that of the respective primary tumor. Four different staining areas are considered: nuclei of the tumor cells, cytoplasm of the tumor cells, the cellular and non-cellular part of the tumor stroma. Normal breast cancer tissue is not available on any of the slides.

In general, the staining results of the different antibodies are quite similar: the nuclei of the tumor cells present only weak staining in some cases, whereas the cytoplasm of the tumor cells shows weak to moderate in most of the ten cases. The cellular part of the tumor stroma offers no staining positivity at all, while the non-cellular part of the tumor stroma is weakly stained in most of the cases. Concerning detailed staining results of the core biopsies using the anti-uPA antibody #3689, the nuclei show weak staining in two cases whereas the remaining eight present no staining. The nuclei of the tumor cells in the primary tumor tissue are stained weakly in three cases. The cytoplasm of the tumor cells offers weak to moderate staining in nine of the ten cases. The non-cellular part of the tumor stroma of the core biopsy specimens shows weak staining only in four cases while the non-cellular part of the tumor stroma of the primary tumor is weakly stained in nine cases. With regard to the three anti-PAI-1 mAbs (#3785, 3786, #ADG25), the staining results are quite similar: the nuclei show positive staining in about half of the cases. The cytoplasm of the tumor cells presents weak to moderate staining in almost all cases. The non-cellular part of the tumor stroma demonstrates weak staining in about eight cases of the ten cases and the cellular part of the tumor stroma is not stained in any of the ten cases. In **Figures 14-17** examples of staining results for the core biopsy compared to the respective primary tumor are shown.

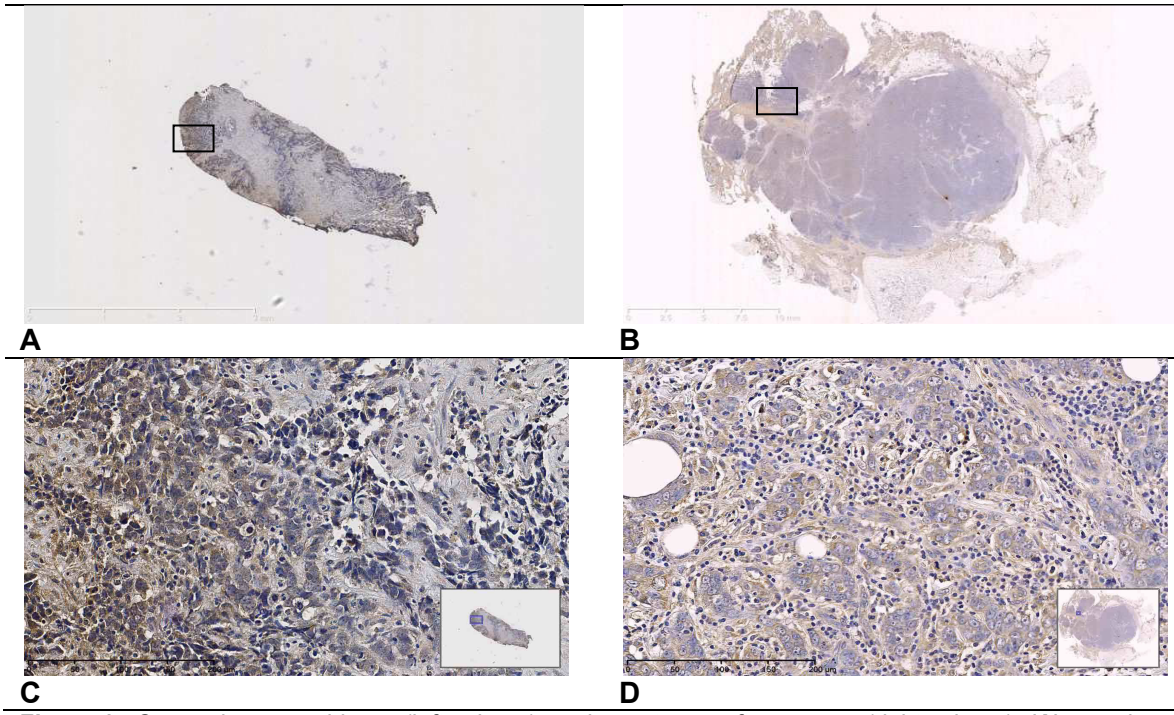


Figure 14 Comparison core biopsy (left column) – primary tumor after surgery (right column). **(A)** overview of the core biopsy, 5x zoom; **(C)** detailed view of the core biopsy, 20x zoom; IHC score: TC nucleus 0/ TC cytoplasm 1/ TS cellular 0/TS non-cellular 0. ELISA 1.8 ng uPA/ mg protein. **(B)** overview of a piece of the primary breast tumor, 5x zoom; **(D)** detailed view of the primary tumor, 20x zoom; IHC score: TC nucleus 0/ TC cytoplasm 1/ TS cellular 0/TS non-cellular 0.5. ELISA 2.6 ng uPA/ mg protein. All four slides were stained with anti-uPA mAb #3689 by automatic staining procedure using the Dako Autostainer and scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

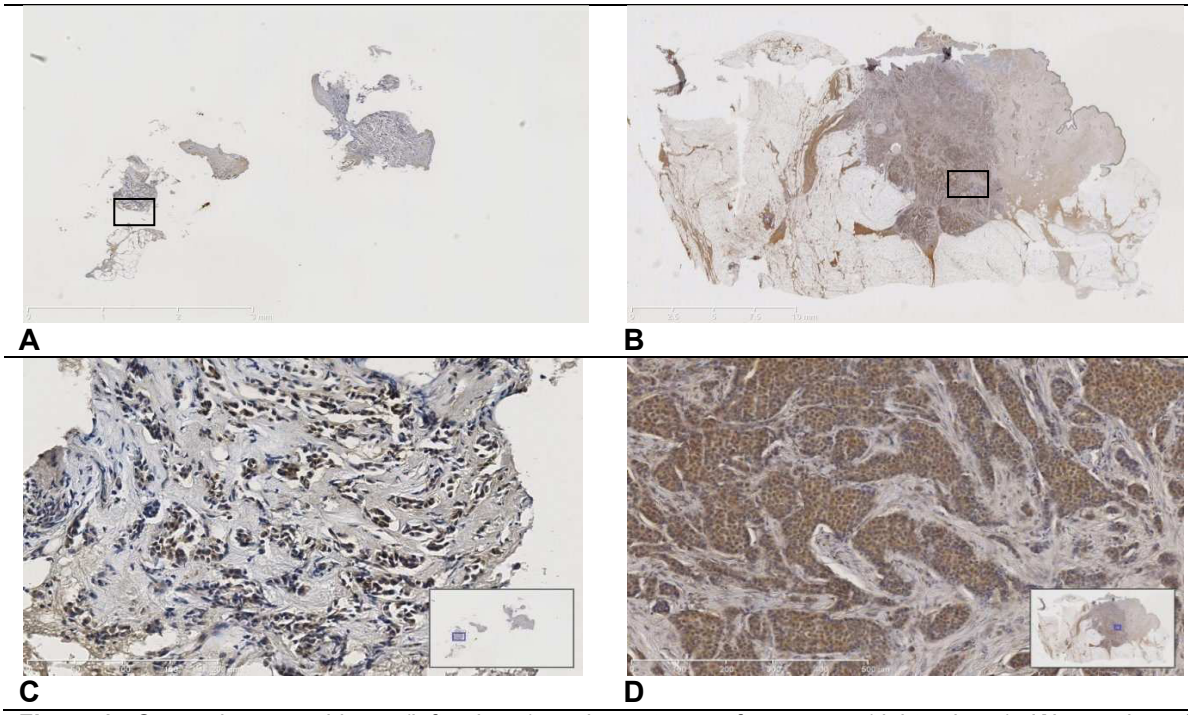


Figure 15 Comparison core biopsy (left column) – primary tumor after surgery (right column). **(A)** overview of the core biopsy, 5x zoom; **(C)** detailed view of the core biopsy with 20x zoom; IHC score: TC nucleus 0/ TC cytoplasm 1/ TS cellular 0/TS non-cellular 0. ELISA 12.37 ng PAI-1/mg protein. **(B)** overview of a piece of the primary breast tumor, 5x zoom; **(D)** detailed view of the primary tumor with 20x zoom; IHC score: TC nucleus 1/ TC cytoplasm 2/ TS cellular 0/TS non-cellular 1. ELISA 63.17 ng PAI-1/mg protein. All four slides were stained with anti-PAI-1 mAb #3785 by automatic staining procedure using the Dako Autostainer and scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

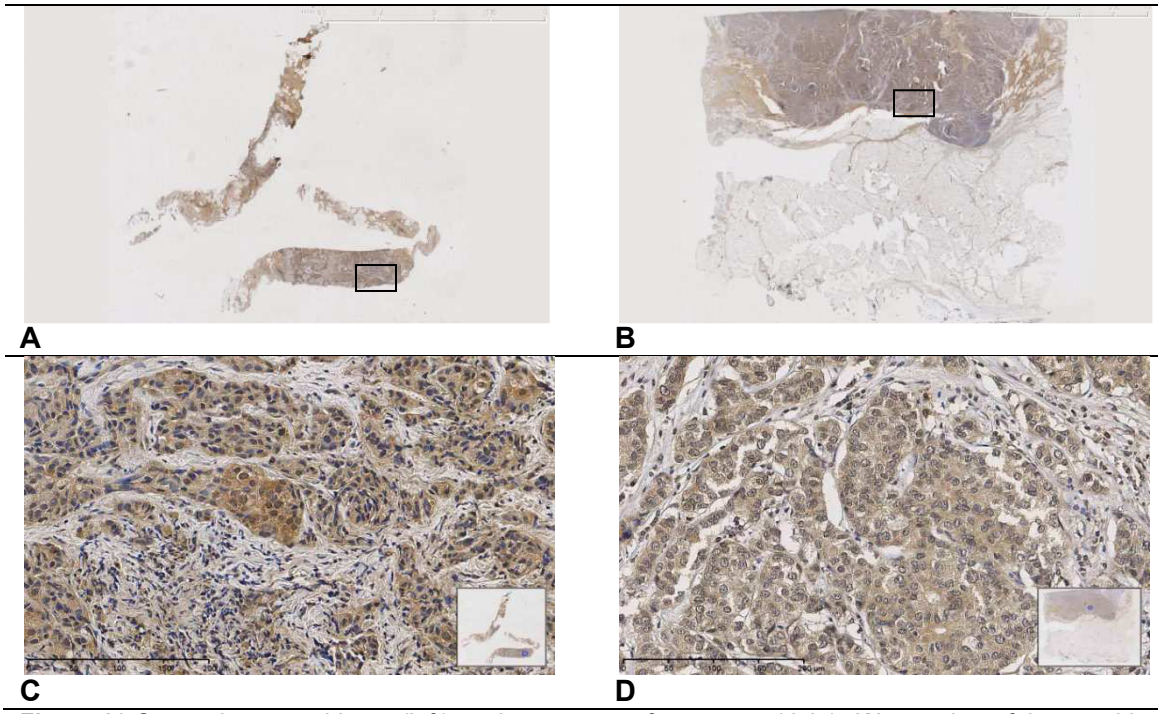


Figure 16 Comparison core biopsy (left) – primary tumor after surgery (right). **(A)** overview of the core biopsy, 5x zoom; **(C)** detailed view of the core biopsy with 20x zoom; IHC score:TC nucleus 1/ TC cytoplasm 2.5/ TS cellular 0/TS non-cellular 1. ELISA 6.2 ng PAI-1/mg protein. **(B)** overview of a piece of the primary breast tumor, 5x zoom; **(D)** detailed view of the primary tumor with 20x zoom. IHC score: TC nucleus 0/ TC cytoplasm 1.5/ TS cellular 0/TS non-cellular 1. ELISA 7.6 ng PAI-1/mg protein. All four slides were stained with anti-PAI-1 mAb #3786 by automatic staining procedure using the Dako Autostainer and scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

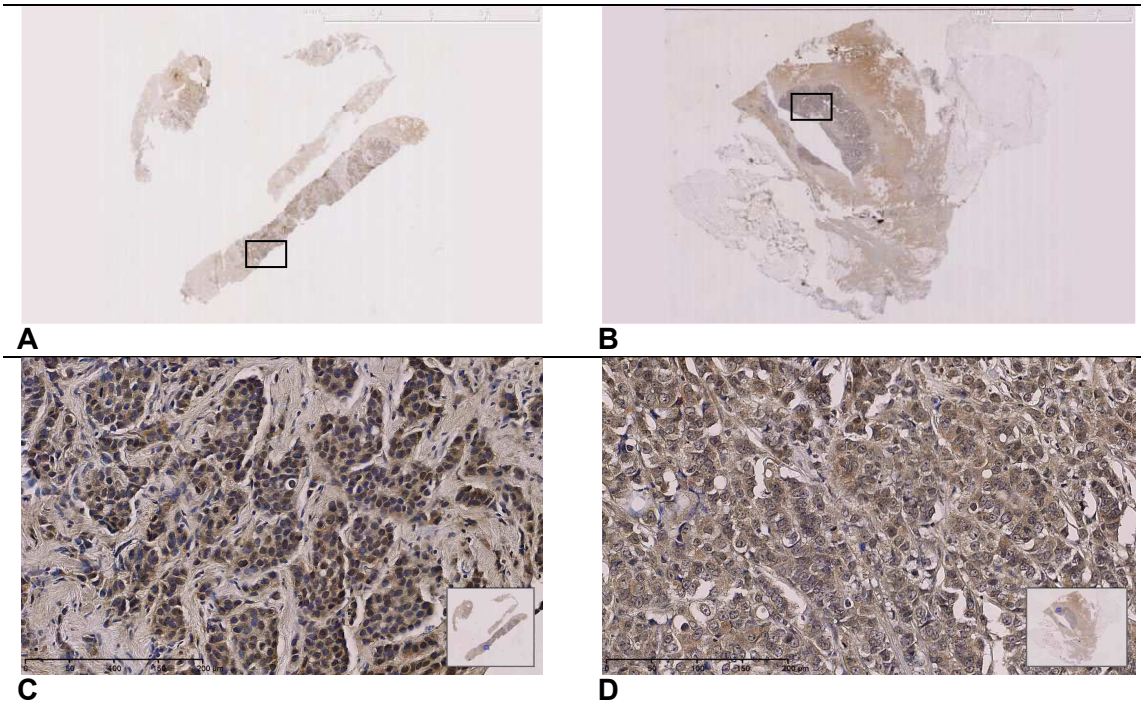


Figure 17 Comparison core biopsy (left column) – primary tumor after surgery (right column). **(A)** overview of the core biopsy, 5x zoom; **(C)** detailed view of the core biopsy with 20x zoom; IHC score: TC nucleus 1/ TC cytoplasm 2/ TS cellular 0/TS non-cellular 1. ELISA 47.4 ng PAI-1/ mg protein. **(B)** overview of a piece of the primary breast tumor, 5x zoom; **(D)** detailed view of the primary tumor with 20x zoom; IHC score: TC nucleus 0/ TC cytoplasm 1.5/ TS cellular 0/TS non-cellular 1. ELISA 32.4 ng PAI-1/ mg protein. All four slides were stained with anti-PAI-1 mAb #ADG25 by automatic staining procedure using the Dako Autostainer and scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

4.2.2 ELISA data – comparison to staining results of uPA and PAI-1

uPA and PAI-1 content of all core biopsy and primary tumor specimens is measured by ELISA using the Imubind detection Kit (uPA: Imubind #894; PAI-1: Imubind #821, American Diagnostica, Greenwich, CT). ELISA data are available at the tumor bank of the Department of Obstetrics and Gynecology of the Klinikum rechts der Isar, Technical University of Munich, for all 10 core biopsy specimens as well as their respective primary tumors. uPA values in core biopsy tumor specimens range from 0.68 to 10.87 ng/mg protein with a mean value of 3.78 ng/mg and a median value of 2.76 ng/mg protein. For PAI-1, minimum value is 2.03 ng/mg protein and maximum value is 47.70 ng/mg protein. Mean value is 15.83 ng/mg protein and median value is 11.59 ng/mg protein. Concerning primary tumor specimens uPA values range from 0.60 to 6.21 ng/mg protein with a mean value of 3.12 ng/mg and a median value of 2.94 ng/mg protein. PAI-1 levels vary from 4.21 to 63.17 ng/mg protein with a mean value of 27.36 ng/mg protein and a median value of 25.51 ng/mg protein. In **Table 9** these ELISA data are compared to immunohistochemical staining results. Spearman's Rho and the p-value are calculated using the SPSS software (IBM SPSS Statistics 19). Considering all four different tumor areas, no significant correlation between immunohistochemical staining results and ELISA data can be found for any of the four antibodies.

Table 9 Correlation between uPA and PAI-1 content measured by ELISA and uPA/PAI-1 expression in four different areas measured by IHC in core biopsies and respective primary tumors of 10 breast cancer patients (R= Spearman's Rho = Spearman's rank correlation coefficient; n.p.= not possible, p-value and Spearman's Rho can not be calculated because all cases were scored "0").

		ELISA							
		uPA				**PAI-1**			
		Core biopsy		**Primary tumor**		**Core biopsy**		**Primary tumor**	
IHC		p-value	R	p-value	R	p-value	R	p-value	R
TC cytopl.	#3689	0.422	-0.287	0.085	-0.570	-	-	-	-
	#3785	-	-	-	-	0.557	-0.212	0.746	-0.118
	#3786	-	-	-	-	0.270	-0.386	0.755	0.114
	#ADG25	-	-	-	-	0.671	-0.154	0.706	-0.137
TC nucleus	#3689	0.334	-0.342	0.451	-0.270	-	-	-	-
	#3785	-	-	-	-	0.426	-0.284	0.924	0.035
	#3786	-	-	-	-	0.554	-0.213	0.695	0.142
	#ADG25	-	-	-	-	0.275	0.383	0.695	-0.142
TS cellular	#3689	n.p.	n.p.	n.p.	n.p.	-	-	-	-
	#3785	-	-	-	-	n.p.	n.p.	n.p.	n.p.
	#3786	-	-	-	-	n.p.	n.p.	0.873	0.058

	#ADG25	-	-	-	-	n.p.	n.p.	n.p.	n.p.
TS non-	#3689	0.008	-0.777	0.981	-0.009	-	-	-	-
cellular	#3785	-	-	-	-	0.147	-0.494	1.000	0.000
	#3786	-	-	-	-	0.082	-0.576	n.p.	n.p.
	#ADG25	-	-	-	-	0.873	-0.058	0.631	-0.174

4.3 Tissue microarrays

4.3.1 Scoring results of immunohistochemical staining of uPA and PAI-1

210 cases of breast tumor tissue are immunohistochemically stained with the four monoclonal antibodies (#3689, #3785, #3786, #ADG25) by automatic staining using the Ventana Benchmark® XT. Slides are scored by a pathologist. Again, four different staining areas are considered: the nucleus of the tumor cells, the cytoplasm of the tumor cells, the cellular part of the tumor stroma, and the non-cellular part of the tumor stroma. Neither normal breast epithelium (NBE) nor normal breast stroma (NBS) is available on any of the slides. The detailed scoring results are listed in **Table 10**. Representative staining results are shown in **Figures 18-21** for each antibody.

All four antibodies show positive staining reaction (>"0") in all four considered areas: the cytoplasm of the tumor cells reveals the highest staining positivity with staining in 82.5- 97.6 %. The nuclei of the tumor cells show positive expression in 38.1-67.2 %.The staining of the cells of the stroma vary depending on the antibody from 6.2-39.5 % positive staining, whereas the non-cellular part of the stroma is stained in 56.7-72.4 %.With regard to the anti-uPA mAb #3689, the cytoplasm shows positive staining in almost all cases (97.6 %), with a strong staining intensity of "2" in 46.2 % and a very strong one ("2.5", "3.0") in 25.2 %. A staining of the nucleus of the tumor cells is observed in 67.2 % with a staining intensity "1" in 98.6 %. Considering the tumor stroma, the cells show positive staining reaction in 49 cases, while the non-cellular part is stained in 70.5 % with moderate to strong staining in 17 cases (8.1 %) (see **Table 10 A**, **Figure 18**).

Table 10 Scoring results of 210 immunohistochemically stained breast cancer cases. Four different areas are considered: nuclei of the tumor cells (TC nucleus), cytoplasm of the tumor cells (TC cytoplasm), cells of the tumor stroma (TS cellular), and the non-cellular part of the tumor stroma (TS non-cellular). n= number of patients (%, percentage) **(A)** anti-uPA mAb #3689, **(B)** anti-PAI-1 mAb #3785, **(C)** anti-PAI-1 mAb #3786, **(D)** anti-PAI-1 mAb #ADG25, n=210.

A anti-uPA mAb #3689

	IHC Scoring result, n (%)						
Area	0	0.5	1	1.5	2	2.5	3
TC nucleus	69 (32.8)	0 (0.0)	139 (66.2)	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)
TC cytoplasm	5 (2.4)	2 (1.0)	41 (19.5)	12 (5.7)	97 (46.2)	22 (10.5)	31 (14.8)
TS cellular	161 (76.7)	0 (0.0)	48 (22.9)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
TS non-cellular	62 (29.5)	2 (1.0)	129 (61.4)	4 (1.9)	11 (5.2)	2 (1.0)	0 (0.0)

B anti-PAI-1 mAb #3785

	IHC Scoring result, n (%)						
Area	0	0.5	1	1.5	2	2.5	3
TC nucleus	130 (61.9)	0 (0.0)	78 (37.1)	0 (0.0)	2 (1.0)	0 (0.0)	0 (0.0)
TC cytoplasm	36 (17.1)	4 (1.9)	59 (28.1)	15 (7.1)	72 (34.3)	16 (7.6)	8 (3.8)
TS cellular	197 (93.8)	0 (0.0)	13 (6.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TS non-cellular	79 (37.6)	4 (1.9)	114 (54.3)	4 (1.9)	8 (3.8)	0 (0.0)	1 (0.5)

C anti-PAI-1 mAb #3786

	IHC Scoring result, n (%)						
Area	0	0.5	1	1.5	2	2.5	3
TC nucleus	106 (50.5)	0 (0.0)	99 (47.1)	0 (0.0)	5 (2.4)	0 (0.0)	0 (0.0)
TC cytoplasm	36 (17.1)	1 (0.5)	56 (26.7)	16 (7.6)	81 (38.6)	9 (4.3)	11 (5.2)
TS cellular	173 (82.4)	0 (0.0)	37 (17.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TS non-cellular	91 (43.3)	2 (1.0)	103 (49.0)	5 (2.4)	8 (3.8)	0 (0.0)	1 (0.5)

D anti-PAI-1 mAb #ADG25

	IHC Scoring result, n (%)						
Area	0	0.5	1	1.5	2	2.5	3
TC nucleus	70 (33.3)	0 (0.0)	116 (55.2)	0 (0.0)	24 (11.4)	0 (0.0)	0 (0.0)
TC cytoplasm	9 (4.3)	2 (1.0)	48 (22.9)	15 (7.1)	85 (40.5)	21 (10.0)	30 (14.3)
TS cellular	127 (60.5)	0 (0.0)	68 (32.4)	0 (0.0)	15 (7.1)	0 (0.0)	0 (0.0)
TS non-cellular	58 (27.6)	2 (1.0)	121 (57.6)	0 (0.0)	28 (13.3)	1 (0.5)	0 (0.0)

Concerning the three anti-PAI-1-antibodies, following differences in the staining intensities are found: the mAb #3785 offers the weakest staining in general. The cytoplasm demonstrates positive staining in 82.9 %, but only the nuclei show positive staining in 38.1 %. In the non-cellular part of the tumor stroma positive staining reaction is observed in 62.4 % with a staining of “0.5” or “1” in most of the cases (90.0 %). The cells of the stroma bare a positive staining in 6.2 % (13 cases) (see **Table 10 B**, **Figure 19**). The mAb #3786 reveals a similar staining pattern as the mAb #3785, but with higher positive staining of the cells of the tumor stroma (17.6 %, 37 cases). The non-cellular part is stained in 56.7 %. The cytoplasm is stained in 82.5 % and the nucleus of the tumor cells show in 49.5 % positive staining reaction (see **Table 10 C**, **Figure 20**). Compared to #3785 and #3786, the mAb #ADG25 demonstrates more often positive staining reaction: the cytoplasm is stained in 95 % with a percentage of 74.6 % with “2” staining. In addition, the nuclei of the tumor cells manifest staining in 66.6 %. 39.5 % of the cases present positive staining of the cellular part of the tumor stroma, and 72.4 % of the non-cellular part of the tumor stroma (see **Table 10 D**, **Figure 21**).

In **Figures 18-21** representative examples of staining results are shown for each antibody in four different combinations of staining intensity of epithelial tumor cells with staining intensity of cells of tumor stroma: **A** presents tumors with weak staining of both cells types. **B** shows strong staining in tumor cells and weak staining of cells of the tumor stroma. **C** illustrates tumors with weak staining of tumor cells and strong staining of cells of the tumor stroma, while **D** shows tumors with strong staining in both areas.

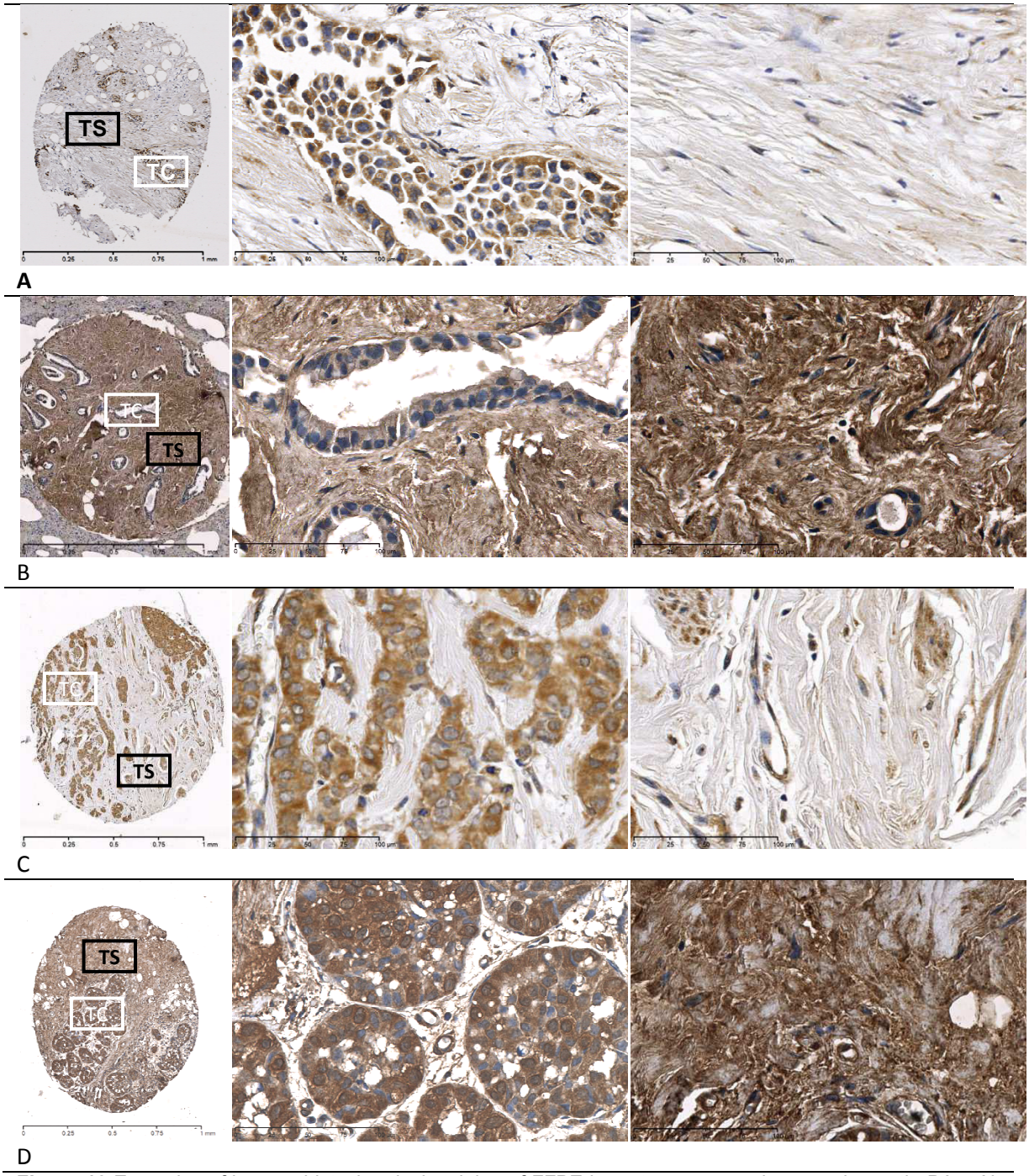


Figure 18 Examples of immunohistochemical staining of FFPE breast cancer specimens using anti-uPA mAb #3689 by automatic staining procedures. **(A)** Left: overview of the TMA: UICC Stadium II, G2, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 1 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0 (WEAK). **(B)** Left: overview of the TMA: UICC Stadium I, G3, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 1 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 2 (STRONG). **(C)** Left: overview of the TMA: UICC Stadium III, G2, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 1/ TC cytoplasm 2 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0 (WEAK). **(D)** Left: overview of the TMA: UICC Stadium II, G3, lobular invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 1/ TC cytoplasm 3 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 2 (STRONG). All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

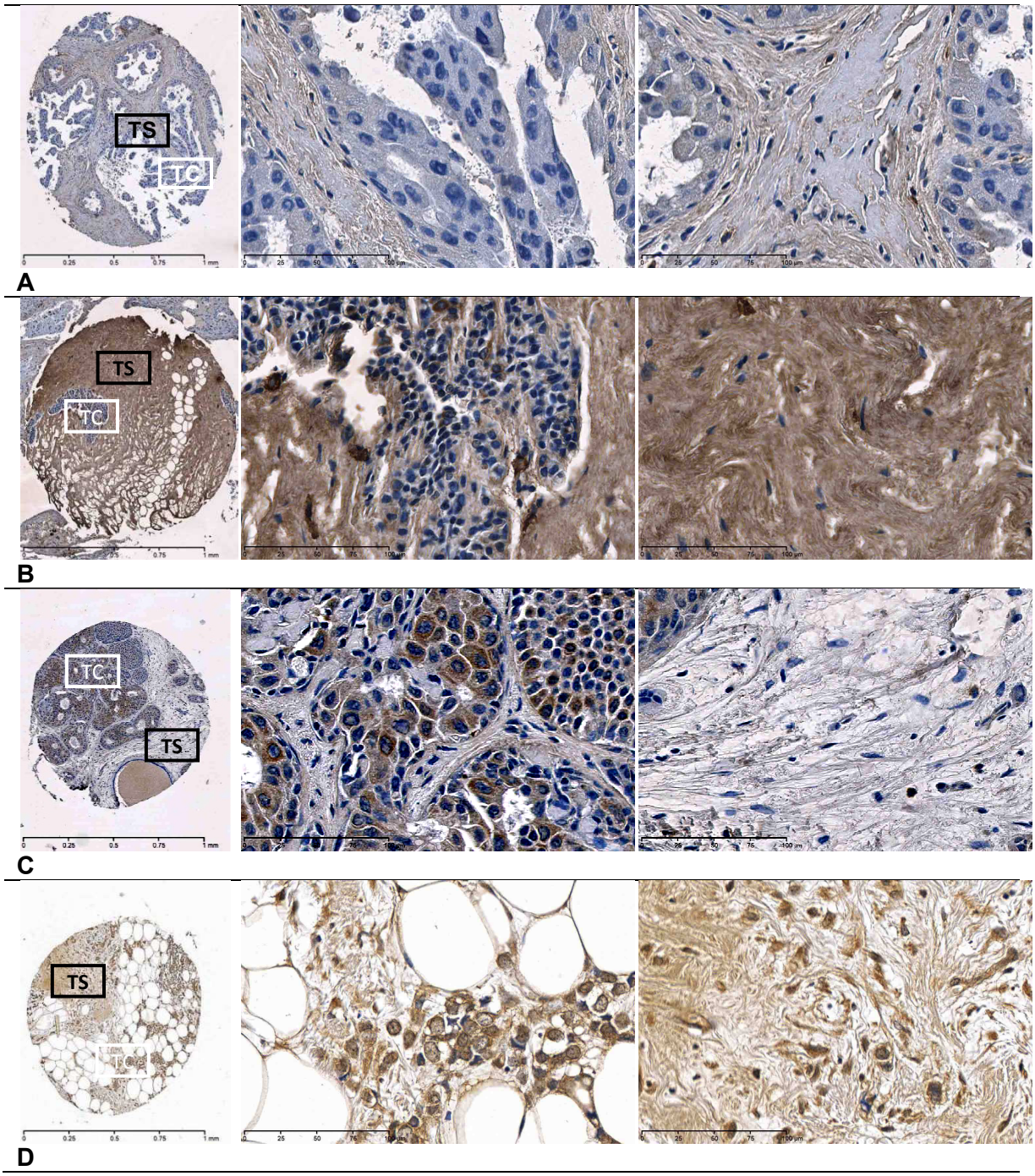


Figure 19 Examples of immunohistochemical staining of FFPE breast cancer specimens using anti-PAI-1 mAb #3785 by automatic staining procedures. **(A)** Left: overview of the TMA: UICC Stadium II, G3, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 0 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0.5 (WEAK). **(B)** Left: overview of the TMA: UICC Stadium I, G2, lobular invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/TC cytoplasm 1 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 2 (STRONG). **(C)** Left: overview of the TMA: UICC Stadium III, G3, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 2 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0 (WEAK). **(D)** Left: overview of the TMA: UICC Stadium III, G3, lobular invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 1/ TC cytoplasm 2 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 2/ TS non-cellular 1 (STRONG). All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

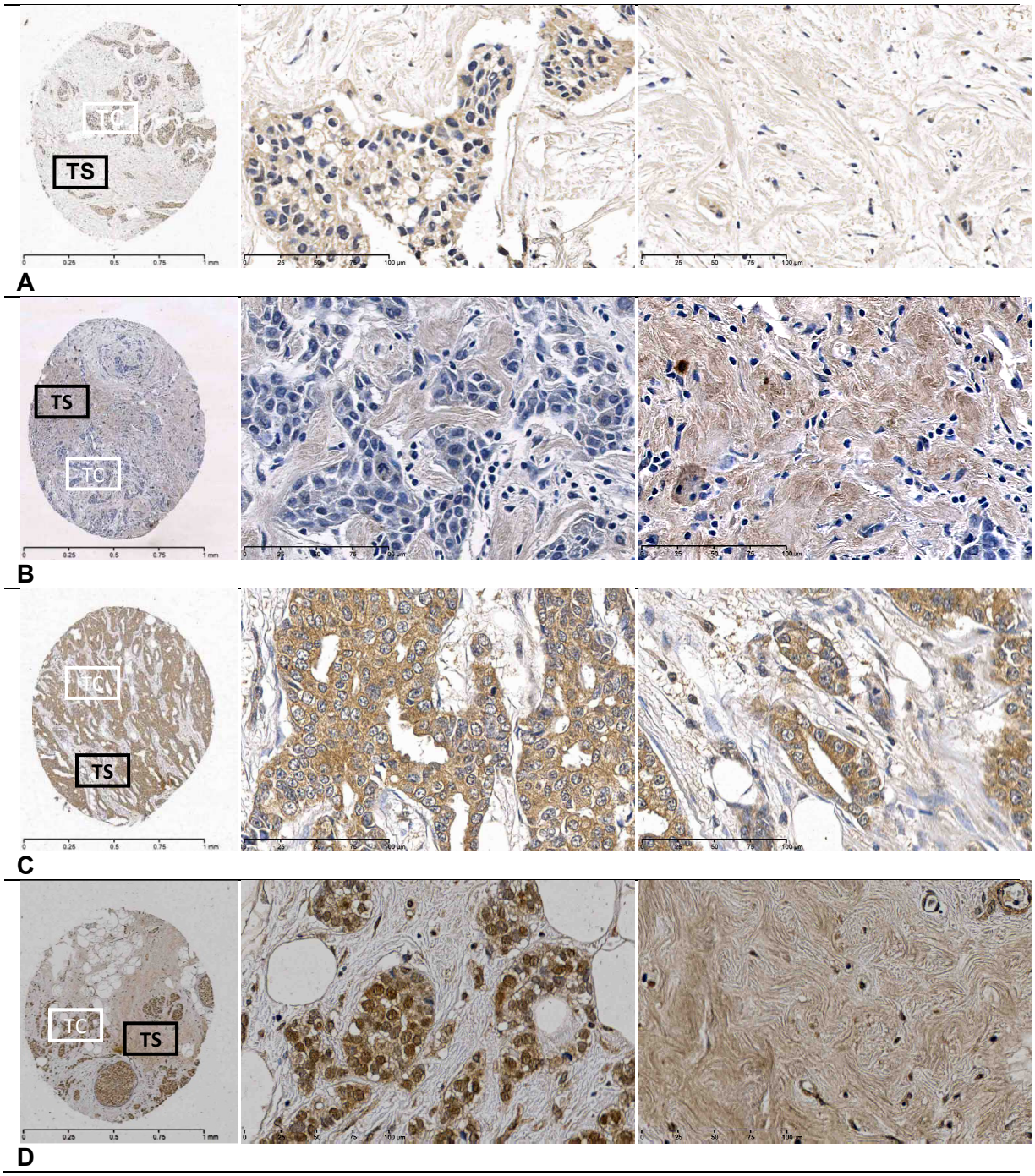


Figure 20 Examples of immunohistochemical staining of FFPE breast cancer specimens using anti-PAI-1 mAb #3786 by automatic staining procedures. **(A)** Left: overview of the TMA: UICC Stadium II, G2, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 1 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0 (WEAK). **(B)** Left: overview of the TMA: UICC Stadium II, G2, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 1 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 2 (STRONG). **(C)** Left: overview of the TMA: UICC Stadium II, G3, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 1/ TC cytoplasm 2 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/TS non-cellular 0 (WEAK). **(D)** Left: overview of the TMA: UICC Stadium II, G2, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 1/ TC cytoplasm 2 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 1 (STRONG). All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

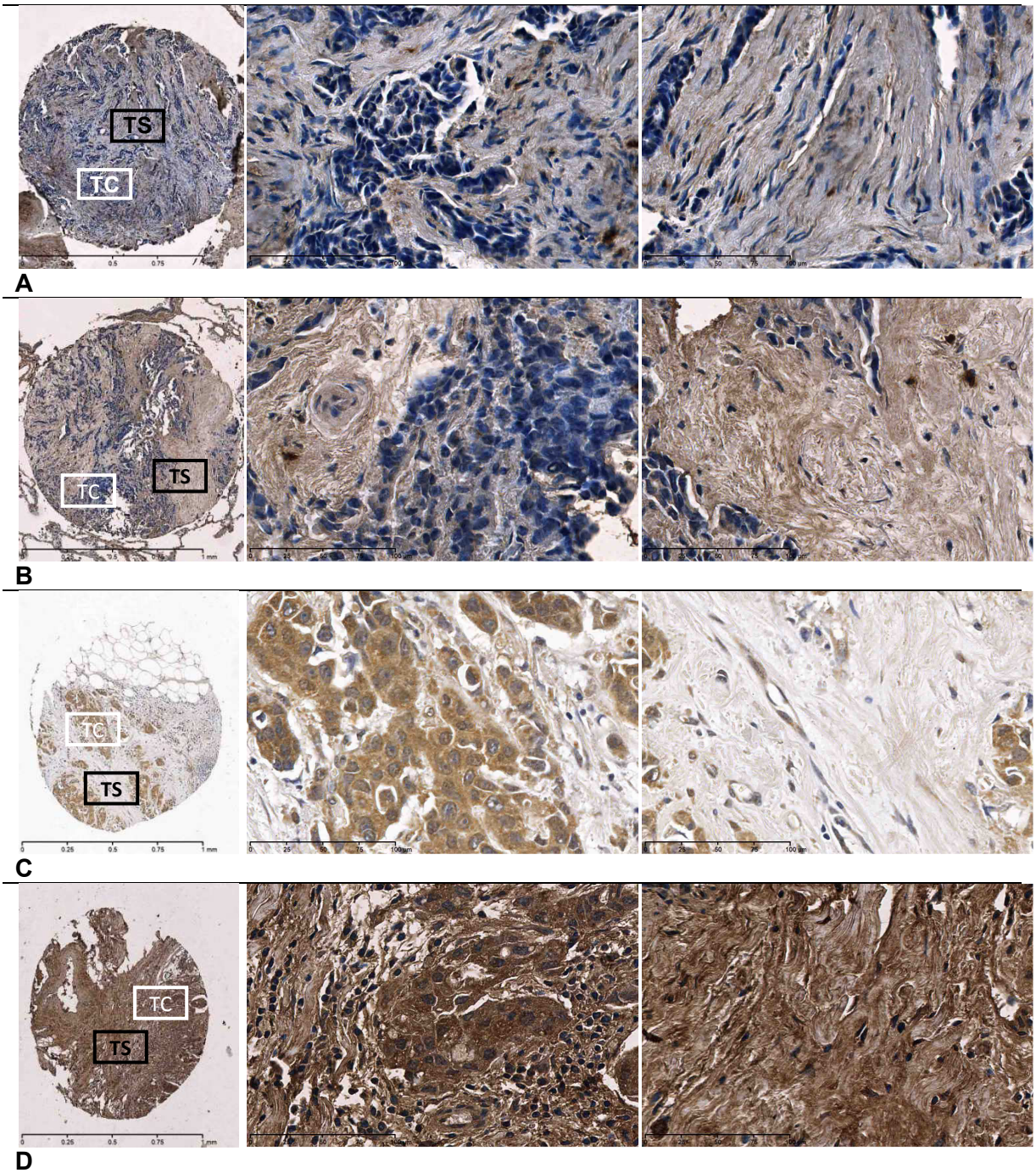


Figure 21 Examples of immunohistochemical staining of FFPE breast cancer specimens using anti-PAI-1 mAb #ADG25 by automatic staining procedures. **(A)** Left: overview of the TMA: UICC Stadium I, G1, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 0 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0.5 (WEAK). **(B)** Left: overview of the TMA: UICC Stadium II, G3, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 0/ TC cytoplasm 0 (WEAK); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 1 (STRONG). **(C)** Left: overview of the TMA: UICC Stadium III, G2, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 1/ TC cytoplasm 3 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 0/ TS non-cellular 0 (WEAK). **(D)** Left: overview of the TMA: UICC Stadium II, G3, ductal invasive carcinoma, 5x zoom; Middle: detailed view of the tumor cells with 40x zoom: TC nucleus 2/ TC cytoplasm 2.5 (STRONG); Right: detailed view of the tumor stroma with 40x zoom: TS cellular 1/ TS non-cellular 2 (STRONG). All slides are scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.

4.3.2 ELISA data – comparison to staining results of uPA and PAI-1

uPA and PAI-1 levels in extracts of the tumors are determined by staff of the lab by ELISA using the Imubind detection Kit (uPA: Imubind #894; PAI-1: Imubind #821, American Diagnostica Inc., Greenwich, CT). The ELISA data are available at the tumor bank of the Department of Obstetrics and Gynecology, Klinikum rechts der Isar of the Technical University of Munich, for 208 of the 210 patients. uPA values in tumors vary from 0.04 to 15.63 ng/mg protein with a mean value of 2.55 ng/mg and a median value of 1.73 ng/mg protein. For PAI-1, minimum value is 0.19 ng/mg protein and maximum value is 71.26 ng/mg protein. Mean value is 12.61 ng/mg protein and median value is 9.14 ng/mg protein. Spearman's Rho and the p-value are calculated using SPSS (IBM SPSS Statistics 19). Considering all four different tumor areas, no statistically significant correlation ($p<0.05$) between immunohistochemical staining results and ELISA data can be found for any of the four antibodies (**Table 11**).

Table 11 Correlation between uPA and PAI-1 content measured by ELISA and uPA/PAI-1 expression in four different areas measured by immunohistochemistry in 208 breast cancer patients (R= Spearman's Rho= Spearman's rank correlation coefficient). *uPA/PAI-1 ELISA data was not available for 2 patients

		ELISA			
		uPA		PAI-1	
IHC		p-value	R	p-value	R
TC cytoplasm	#3689	0.653	0.031	-	-
	#3785	-	-	0.917	0.007
	#3786	-	-	0.865	0.012
	#ADG25	-	-	0.765	0.012
TC nucleus	#3689	0.246	-0.081	-	-
	#3785	-	-	0.170	-0.095
	#3786	-	-	0.695	-0.027
	#ADG25	-	-	0.522	-0.045
TS cellular	#3689	0.630	-0.034	-	-
	#3785	-	-	0.134	-0.104
	#3786	-	-	0.256	-0.079
	#ADG25	-	-	0.651	-0.032
TS non-cellular	#3689	0.458	-0.052	-	-
	#3785	-	-	0.841	-0.014
	#3786	-	-	0.768	0.021
	#ADG25	-	-	0.531	0.044

Furthermore it is investigated whether there is an association between high ELISA values and strong staining measured by immunohistochemistry. Therefore two groups are formed: high vs. low uPA/PAI-1. ELISA values considering the well-

established cut-offs for these proteases (3 ng/mg protein for uPA and 14 ng/mg protein for PAI-1). In addition, scoring results of the immunohistochemical stained slides are divided in two groups using the median as mentioned above. P-value is calculated using Pearson's chi square test. Even though there is a tendency for high uPA ELISA levels being correlated with more intense staining intensity, no significant correlation can be found for any of the four antibodies.

4.3.3 Patient cohort

Follow-up is performed with the help of staff of the Department of Obstetrics and Gynecology of the Klinikum rechts der Isar, Technical University of Munich. The period between date of primary surgery and death is defined as overall survival (OS). The period between date of primary surgery and date of disease recurrence is described as disease-free survival (DFS).

Concerning the 210 breast cancer cases of the TMAs investigated (see **Table 12**), the mean observation period is almost 10 years (109.3 months) with a range from 7 to 271 months (from year 1987 until 2010). 81 patients (38.5 %) relapsed during follow-up period (thereof 16 local recurrences, 65 distant recurrences). 101 (48.1 %) patients deceased, of these 73 (72.3 %) because of breast cancer. Median disease-free survival is 91.5 months, median progression-free survival is 21.0 months. Median patient age is 62.2 years. Age of the patients ranges from 28.0 to 88.6 years (median age 62.2 years). Concerning menopausal status, four women are perimenopausal, 42 women premenopausal, and the remaining 164 women postmenopausal at time of diagnosis. 165 (78.6 %) out of 210 tumors are categorized as invasive ductal carcinomas, 28 (13.3 %) as invasive lobular carcinomas and 17 (8.1 %) as other histological types. Size of the tumors ranges from 0.5 to 11.0 cm with a mean size of 3.1 cm and a median size of 2.5 cm. 49 tumors are smaller than 2 cm, 32 larger than 5 cm and the remaining 129 tumors are between the two categories. Regarding to tumor stage (see **9.1**) 54 (25.7 %) patients are categorized in tumor stage pT1, 101 (48.1 %) pT2, 23 (11.0 %) pT3 and 32 (15.2 %) pT4. 55 (26.2 %) patients are node-negative, whereas 155 (73.8 %) patients are categorized as lymph node-positive. None of the patients has distant metastasis at time of diagnosis. With reference to the

UICC tumor stadium (see **9.2**) 23 (11.0 %) patients belong to the stadium I, 122 (58.1 %) to stadium II, 65 (30.9 %) to stadium III and none of them to stadium IV. Concerning the steroid hormone receptor status 196 (93.3 %) are steroid hormone receptor positive and 14 (6.7 %) are steroid hormone receptor negative. Concerning the HER2 status, 104 (49.5 %) are categorized “negative”, 21 (10.0 %) “positive”, and for 85 (40.5 %) cases HER2 status is not available. The grading according Bloom-Richardson (see **9.3**) is G1 (n=7, 3.3 %), G2 (n=105, 50.0 %), and G3 (n=98, 46.7 %). None of the patients has received any neoadjuvant therapy. Primary treatment consists of modified radical mastectomy or breast-conserving surgery including axillary lymph node dissection. The 210 patients are composed of 3 different TMAs. These differ in the adjuvant therapy given to the patients: the 62 patients of “TMA 1” have received 6 cycles of chemotherapy (CMF), whereas the 105 patients of the “TMA 2” and the 43 patients of the “TMA 3” have obtained tamoxifen, an antagonist of the estrogen receptor, for at least 60 months. It is worth mentioning that in the 80’s and 90’s the traditional CMF was in common use for adjuvant chemotherapy regimen in breast cancer patients. Today anthracyclines such as epirubicin or doxorubicin, and taxanes, e.g. docetaxel, are considered to be beneficial in high-risk node-negative breast cancer patients. The three TMAs vary also concerning the age of the patients (mean age in the “TMA 1” was 47.9 years compared to the patients of the “TMA 2” (67.6 years) and the “TMA 3” (65.6 years). Furthermore, tumor size and lymph node status vary, by means that the “TMA 2” has more advanced stage disease to the “TMA 1” and even more to the “TMA 3”. These differences in tumor stage and adjuvant treatment translate into different survival rates: the CMF-treated “TMA 1” shows the longest median overall survival (OS) and the longest disease-free survival (DFS). The worst survival rate has “TMA 2”. “TMA 3” ranges in between. Regarding relapse rates, almost half of “TMA 2”, nearly 40 % of patients of “TMA 1” and less than 20 % of “TMA 3” suffered of recurrent disease. According to tumor stage at time of diagnosis nearly 43 % of the patients in “TMA 2” died because of breast cancer, whereas approximately 30 % of “TMA 1” and 20 % of patients of “TMA 3” deceased. All studied clinical and pathological features of the tumors are summarized in **Table 12**.

Table 12 Distribution of histomorphological and clinical factors of 210 tumors of the three TMAs.

Parameter category		TMA 1 (n= 62)	TMA 2 (n= 105)	TMA 3 (n= 43)	n=210
Age	**Mean**	47.9	67.6	65.6	61.5
	Range	28.0-74.4	37.7– 88.6	49.3- 86.3	28.0-88.6
	Median	48.4	68.7	64.0	62.2
Menopausal status	**Premenopausal**	39	3	0	42
	Postmenopausal	20	101	43	164
	Perimenopausal	3	1	0	4
Histological type	**Ductal invasive**	51	84	30	165
	Lobular invasive	8	13	7	28
	Other	3	8	6	17
Tumor size (cm)	**Mean**	2.9	3.6	2.3	3.1
	Range	1.0-7.0	1.1-11.0	0.5-7.0	0.5-11.0
	Median	2.5	3.0	2.1	2.5
	< 2	15	16	18	49
	≥ 2 and < 5	42	63	24	129
	≥ 5	5	26	1	32
Tumor stage	**pT1**	17	18	19	54
	pT2	35	47	19	101
	pT3	8	14	1	23
	pT4	2	26	4	32
Lymph node status	**pN-**	12	0	43	55
	pN+	50	105	0	155
Tumor stadium UICC	**I**	4	0	19	23
	II	45	57	20	122
	III	13	48	4	65
	IV	0	0	0	0
Steroid hormone receptor status	**Positive**	48	105	43	196
	Negative	14	0	0	14
	Not available	0	0	0	0
HER2 status	**Negative**	33	67	4	104
	Positive	7	12	2	21
	Not available	22	26	37	85
Nuclear grading (Bloom-Richardson)	**G1**	0	4	3	7
	G2	25	52	28	105
	G3	37	49	12	98
Adjuvant therapy	**Chemotherapy**	62	0	0	62
	Endocrine	3	105	43	151
Overall survival (months)	**Mean**	137.4	86.8	123.2	109.3
	Range	11-271	7-263	24-219	7-271
	Median	151.5	80.0	123.0	105.5
Disease-free survival (months)	**Mean**	122.2	74.7	116.6	97.5
	Range	7-271	0-263	2-219	0-271
	Median	146.5	63.0	123.0	91.5
Disease Recurrence	**No recurrence**	38	55	36	129
	Local	6	9	1	16
	Distant	18	41	6	65
	Not defined	0	0	0	0
Deaths	**Breast cancer**	19	45	9	73
	Others	4	17	7	28
Survivors		39	43	27	109

4.3.4 Association between uPA/PAI-1 staining intensity and clinical and histomorphological factors

Staining intensity of the four different areas of the tumor (cytoplasm of the tumor cells, nucleus of the tumor cells, cells of the tumor stroma, non-cellular part of the stroma) of the 210 breast cancer tissues is categorized as strong or overexpressed versus weak using the median as cut-off. uPA and PAI-1 expression is associated with clinical and pathological factors using Pearson's chi-square test. p is defined significant when ≤0.05.

Regarding the cytoplasm of the tumor cells, following results are demonstrated: high staining intensity (>1.0) of the anti-uPA mAb #3689 is statistically significant associated with positive lymph node status (p=0.012). High staining intensity of the anti-PAI-1 mAb #3785 is associated with age (≥50 years) (p<0.001), menopausal status (postmenopausal) (<0.001), positive lymph node status (p=0.039), high UICC stadium (p=0.030) and positive steroid hormone receptor status (p<0.001). Concerning anti-PAI-1 mAb #3786 high staining intensity is related to age (≥50 years) (p<0.001), menopausal status (postmenopausal) (<0.001), high pT (p=0.006), positive steroid hormone receptor status (p<0.001), and shorter overall survival (p=0.037). High staining intensity of anti-PAI-1 mAb #ADG25 is only associated with menopausal status (premenopausal) (p=0.026) (see **9.8 A**).

Considering cells of the tumor stroma, uPA staining is significantly associated with longer overall survival (p=0.034) and disease-free survival (0.014). Strong staining of anti-PAI-1 mAb #3785 is associated with negative lymph node status (p<0.001), lower UICC stadium (p=0.005), longer overall survival (p=0.045), and disease-free survival (p=0.045). Positive staining of anti-PAI-1 mAb #3786 is associated with age (≥50 years) (p=0.001), menopausal status (postmenopausal) (p=0.001), negative lymph node status (p<0.001), low UICC stadium (p=0.005), absence of disease recurrence (p=0.002) and deaths (p=0.014). Regarding staining intensity of anti-PAI-1 mAb #ADG25 in cells of the tumor stroma, statistically significant association is found for age (<50 years) (p<0.001), menopausal status (premenopausal) (p<0.001), negative lymph node status (p=0.013), low UICC stadium (p<0.001), longer overall survival (p<0.001) and disease-free survival (p=0.001) (see **9.8 B**).

Considering the nuclei of the tumor cells, statistical relevant p-value can be shown for positive lymph node status (p=0.006) using anti-uPA mAb #3689. Concerning anti-PAI-1 mAb #3785 staining intensity is associated with age (≥50 years) (p<0.001), menopausal status (postmenopausal) (<0.001) and positive steroid hormone receptor status (p=0.002). In addition, mAb #3786 staining results are related with age (≥50 years) (p<0.001), menopausal status (postmenopausal) (p<0.001), and steroid hormone receptor status (p<0.001). Anti-PAI-1 mAb #ADG25 is only associated with menopausal status (premenopausal) (p=0.028) (see **9.8 C**).

With respect to the non-cellular part of the tumor stroma, statistically significant association of staining intensity using the anti-uPA mAb #3689 is shown for negative lymph node status (p<0.001), low UICC stadium (p=0.009) and longer disease-free survival (p=0.035). For mAb #3785 following clinical factors are associated with high staining intensity: age (≥50 years) (p=0.014), menopausal status (postmenopausal) (p=0.009). For mAb #3786 following clinical factors are associated with high staining intensity in the non-cellular part of the tumor stroma: age (≥50 years) (p=0.005), menopausal status (postmenopausal) (p=0.004), tumor size (<2 cm) (p=0.011), negative lymph node status (p=0.001), low UICC stadium (p=0.005). High staining of mAb #ADG25 is associated with negative lymph node status (p<0.001) and low UICC stadium (p=0.024) (see **9.8 D**).

All results of the analyses with the respective p-values were shown in **9.8 A-D** in the appendix. To sum it up, it is noteworthy that high PAI-1 staining intensity of tumor cells is associated with advanced tumor parameters (e.g. positive lymph node involvement, high UICC stadium) and consequently poor prognosis in general whereas high staining intensity of tumor stroma is linked in most cases with better prognosis (negative lymph node status, longer overall and disease-free survival). Especially anti-PAI-1 mAb #3786 shows statistically significant results for these findings.

4.3.5 Univariate and multivariate Cox regression analysis of clinical and histomorphological factors and uPA/PAI-1 proteases' influence on overall and disease-free survival

To assess the clinical impact of uPA and PAI-1 expression on patients' prognosis, the univariate and multivariate Cox model are used (see **Tables 13 and 14**). Included in the analysis are clinical factors such as age, tumor size, tumor stage, lymph node involvement, UICC stadium, grading, histological subtype, menopausal status, steroid hormone receptor status, HER2 status, adjuvant therapy, disease recurrence, uPA/PAI-1 ELISA levels, and immunohistochemical staining results of the four different areas using the four antibodies.

In the univariate setting, the model shows that age in addition to tumor size, pT-stage, lymph node involvement, UICC stadium, menopausal status, adjuvant therapy and disease recurrence are of statistical significance to predict the probability of overall survival (see **Table 13**). Using anti-PAI-1 mAb #3786, immunohistochemical staining in the nuclei of tumor cells (p=0.007, HR 1.674) as well as in cells of the tumor stroma (p=0.004, HR= 0.597) is of statistical significance. Staining in the cytoplasm of the tumor cells just fails statistical significance (p=0.086, HR=1.546). Moreover PAI-1 expression in the cells of the tumor stroma stained by anti-PAI-1 mAb #ADG25 is of statistical significance to predict the probability of overall survival (p=0.004, HR=0.597). Age, tumor size, UICC stadium, disease recurrence and PAI-1-ELISA results evolve as statistically independent prognostic factors in the multivariate analyses.

In univariate Cox analysis, following parameters are statistically associated with disease-free survival in the univariate setting: tumor size, pT, pN, UICC stadium, uPA ELISA, PAI-1 expression in the nucleus of the tumor cells and cells of the tumor stroma stained by anti-PAI-1 mAb #3786 as well as cells of the tumor stroma stained by anti-PAI-1 mAb #ADG25. Whereas only tumor size, lymph node involvement, grading, histology and uPA ELISA results evolve as statistically independent prognostic factors (see **Table 14**).

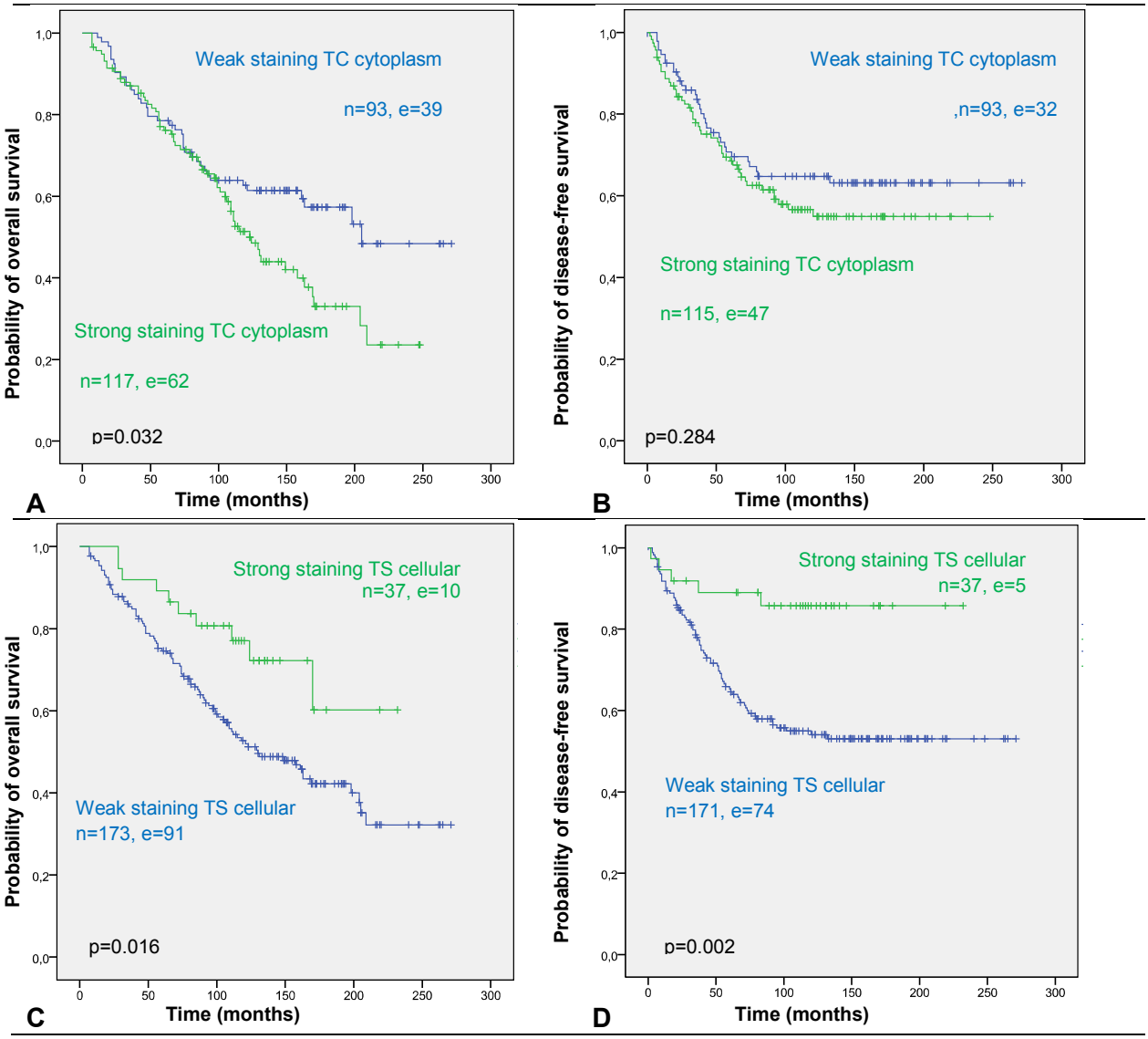
Table 13 Univariate and multivariate Cox proportional hazard regression analyses of different pathological and clinical factors for OS in 210 patients. Statistical significant results are printed in bold.

	Univariate			**Multivariate**		
Factor	p-value	HR	95 % CI	p-value	HR	95 % CI
Age	**< 0.001**	**1.030**	**1.013- 1.046**	**<0.001**	**1.041**	**1.022- 1.060**
Tumor size	**< 0.001**	**1.338**	**1.219- 1.468**	**0.009**	**1.189**	**1.044-1.353**
pT	**< 0.001**	**1.573**	**1.308- 1.891**	-	-	-
pN	**0.008**	**1.965**	**1.189- 3.247**	-	-	-
Stadium UICC	**< 0.001**	**2.433**	**1.739- 3.404**	**0.024**	**1.630**	**1.066- 2.492**
Grading	0.363	1.180	0.826- 1.685	-	-	-
Histology	0.199	1.411	0.834- 2.386	-	-	-
Menopausal status	**0.004**	**2.366**	**1.316- 4.252**	-	-	-
Hormon receptors	0.995	1.002	0.464- 2.166	-	-	-
HER2 status	0.753	0.896	0.453- 1.771	-	-	-
Adjuvant therapy	**0.005**	**1.964**	**1.228- 3.143**	-	-	-
RTx	0.958	0.989	0.667- 1.467	-	-	-
Disease recurrence	**< 0.001**	**2.437**	**1.919- 3.095**	**<0.001**	**3.181**	**2.387- 4.240**
uPA ELISA high/low	0.062	1.479	0.980- 2.234	-	-	-
PAI-1 ELISA high/low	0.122	1.408	0.912- 2.174	**0.045**	**1.565**	**1.010- 2.426**
IHC#3689 TC cytoplasm	0.630	0.937	0.719- 1.221	-	-	-
IHC#3785 TC cytoplasm	0.200	1.164	0.923- 1.468	-	-	-
IHC #3786 TC cytoplasm	0.086	1.546	1.290-1.802	-	-	-
IHC #ADG25 TC cytoplasm	0.375	0.892	0.692- 1.149	-	-	-
IHC #3689 TC nucleus	0.795	1.056	0.701- 1.589	-	-	-
IHC #3785 TC nucleus	0.322	1.208	0.831- 1.755	-	-	-
IHC #3786 TC nucleus	**0.007**	**1.674**	**1.149- 2.440**	-	-	-
IHC #ADG25 TC nucleus	0.371	0.867	0.635- 1.185	-	-	-
IHC #3689 TS cellular	0.132	0.691	0.427- 1.118	-	-	-
IHC #3785 TX cellular	0.244	0.552	0.203- 1.501	-	-	-
IHC#3786 TS cellular	**0.019**	**0.457**	**0.237- 0.878**	-	-	-
IHC#ADG25 TS cellular	**0.004**	**0.597**	**0.419- 0.852**	-	-	-
IHC #3689 TS non-cellular	**0.038**	**0.699**	**0.498- 0.981**	-	-	-
IHC #3785 TS non-cellular	0.734	1.058	0.766- 1.460	-	-	-
IHC#3786 TS non-cellular	0.529	0.903	0.657- 1.241	-	-	-
IHC #ADG25 TS non-cellular	0.137	0.797	0.590- 1.075	-	-	-

Table 14 Univariate and multivariate Cox proportional hazard regression analyses of different pathological and clinical factors for DFS in 210 patients. Statistical significant results are printed in bold.

	Univariate			**Multivariate**		
Factor	p-value	HR	95 % CI	p-value	HR	95 % CI
Age	0.196	0.989	0.972- 1.006	-	-	-
Tumor size	**<0.001**	**1.307**	**1.179-1.449**	**0.002**	**1.341**	**1.113- 1.617**
pT	**<0.001**	**1.513**	**1.229-1.861**	-	-	-
pN	**<0.001**	**2.632**	**1.800- 3.849**	**0.007**	**2.594**	**1.293- 5.202**
Stadium UICC	**<0.001**	**2.300**	**1.576- 3.357**	-	-	-
Grading	0.097	1.421	0.939- 2.149	**0.027**	**1.921**	**1.076- 3.429**
Histology	0.059	1.729	0.980- 3.052	**0.005**	**3.029**	**1.389- 6.607**
Menopausal status	0.585	1.155	0.689- 1.936	-	-	-
Steroid hormone receptors	0.594	0.797	0.347- 1.834	-	-	-
HER2 status	0.566	1.224	0.615- 2.437	-	-	-
Adjuvant therapy	0.757	1.079	0.667-1.745	-	-	-
RTx	0.571	1.139	0.727- 1.784	-		-
uPA ELISA high/low	**0.019**	**1.718**	**1.091- 2.704**	**0.011**	**1.939**	**1.166- 3.225**
PAI-1 ELISA high/low	o.266	1.318	0.810- 2.145	-	-	-
IHC#3689 TC cytoplasm	0.216	1.233	0.889- 1.684	-	-	-
IHC#3785 TC cytoplasm	0.216	1.177	0.909- 1.525	-	-	-
IHC #3786 TC cytoplasm	0.385	1.123	0.864- 1.460	-	-	-
IHC #ADG25 TC cytoplasm	0.678	1.061	0.797- 1.413	-	-	-
IHC #3689 TC nucleus	0.261	1.310	0.818- 2.097	-	-	-
IHC #3785 TC nucleus	0.977	1.007	0.648- 1.564	-	-	-
IHC #3786 TC nucleus	**0.093**	**1.441**	**0.940- 2.208**	-	-	-
IHC #ADG25 TC nucleus	0.615	1.093	0.773- 1.545	-	-	-
IHC #3689 TS cellular	0.272	0.740	0.433- 1.266	-	-	-
IHC #3785 TX cellular	0.299	0.542	0.171- 1.721	-	-	-
IHC#3786 TS cellular	**0.004**	**0.262**	**0.106- 0.648**	-	-	-
IHC#ADG25 TS cellular	**0.290**	**0.818**	**0.563- 1.187**	-	-	-
IHC #3689 TS non-cellular	0.088	0.712	0.482- 1.052	-	-	-
IHC #3785 TS non-cellular	0.644	0.916	0.630- 1.330	-	-	-
IHC#3786 TS non-cellular	0.829	0.961	0.671- 1.377	-	-	-
IHC #ADG25 TS non-cellular	0.297	0.833	0.591- 1.175	-	-	-

Concerning immunohistochemical staining results of the four different antibodies, all three anti-PAI-1 antibodies show same tendency, but only anti-PAI-1 mAb #3786 show statistically significant impact on DFS and OS. This result is demonstrated in **Figure 22**. With respect to the four different scoring areas, staining results of cells of the tumor stroma (p=0.016 for OS, p=0.002 for DFS) and of the cytoplasm of the tumor cells (p=0.032 for OS) present statistical significance. Staining results of the nuclei of the tumor cells just fail to reach statistical significance. It turns out that PAI-1 overexpression in the cytoplasm of tumor cells is associated with poor overall and disease-free survival (**Figure 22 A, B**), whereas PAI-1 overexpression in the cells of the tumor stroma is significantly linked with better survival (**Figure 22 C, D**).



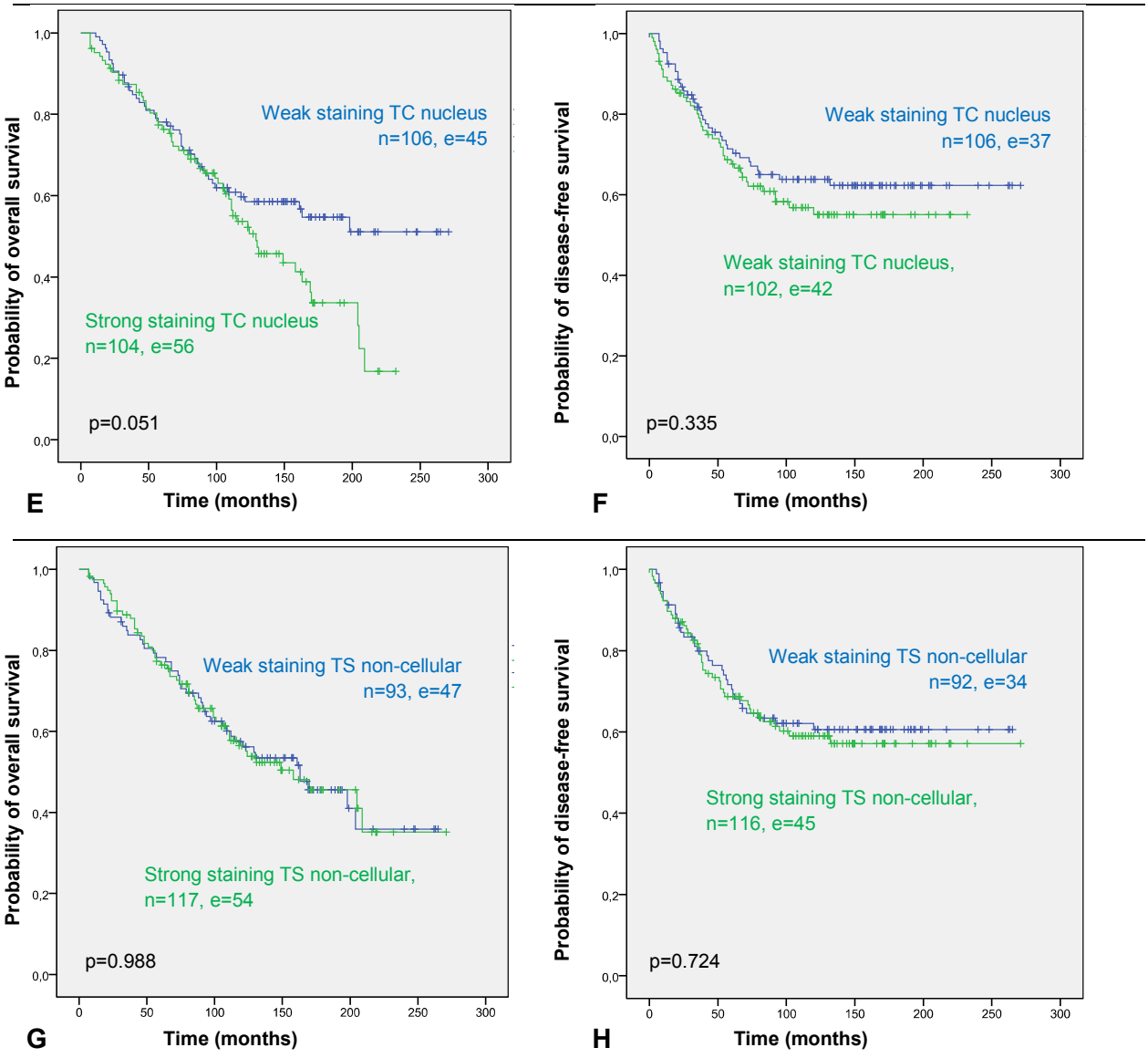


Figure 22 Kaplan-Meier curves show the impact of the PAI-1 staining intensity at four different areas on the probability of survival. All cases were stained immunohistochemically using automatic staining procedures with anti-PAI-1 mAb #3786 (American Diagnostica Inc., Greenwich, CT). **(A)** Probability of OS depending on staining of the cytoplasm of the tumor cells, p=0.032; **(B)** Probability of DFS depending on staining of the cytoplasm of the tumor cells, p=0.284; **(C)** Probability of OS depending on staining of the cells of the tumor stroma, p=0.016; **(D)** Probability of DFS depending on staining of the cells of the tumor stroma, p=0.002; **(E)** Probability of OS depending on staining of the nuclei of the tumor cells, p=0.051; **(F)** Probability of DFS depending on staining of the nuclei of the tumor cells, p=0.335; **(G)** Probability of OS depending on staining of the non-cellular part of the tumor stroma, p=0.988; **(H)** Probability of DFS depending on staining of non-cellular part of the tumor stroma, p=0.724; Significance is assessed by log-rank test. (n=number of patients, e=number of events) In two cases data of DFS is not available.

In **Figure 23** both areas (cytoplasm of the tumor cells and cells of the tumor stroma) are considered and four curves are calculated: breast tumors with weak cytoplasmatic staining but strong staining of the tumor stroma have the best survival rate (blue curve). In contrast patients with strong cytoplasmic staining and low staining of the cells of the tumor stroma have worst probability of survival

(beige curve **Figure 23**; representative staining examples see **Figure 20 C**). These patients which count about 40 % could be classified as “high-risk”, who would probably benefit from adjuvant therapy regimens.

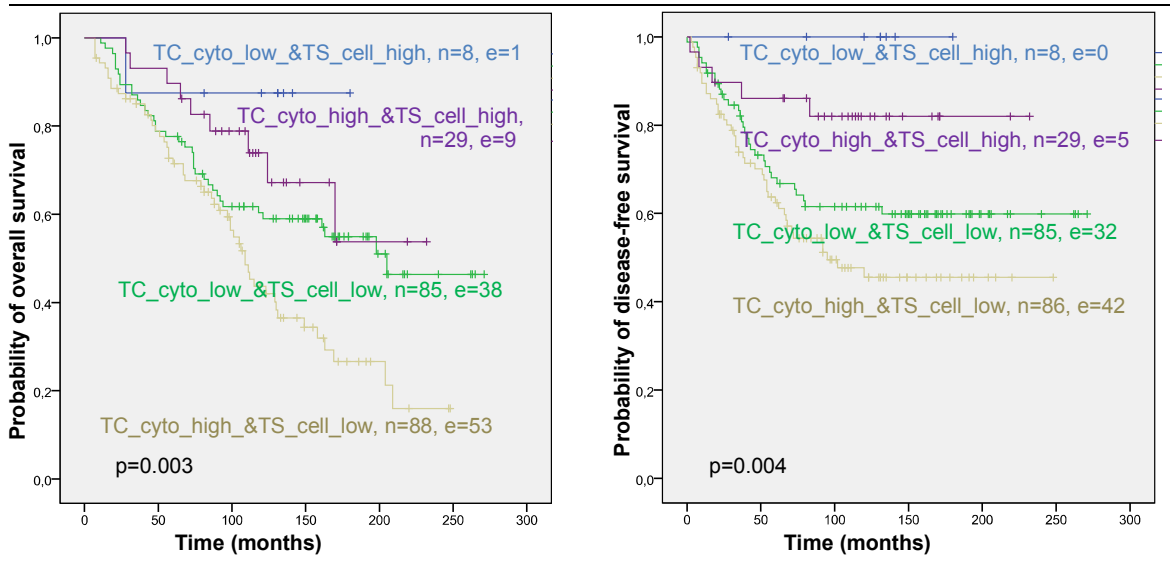


Figure 23 Kaplan-Meier curves show the probability of survival depending on the staining intensity of the cytoplasm of the tumor cells (TC_cyto) and of the cells of the tumor stroma (TS_cell). Four different groups are formed concerning staining intensity (high versus low). All cases are stained immunohistochemically using automatic staining procedures with anti-PAI-1 mAb #3786 (American Diagnostica Inc., Greenwich, CT). On the left the probability of OS is shown, whereas on the right the probability of DFS is shown. In two cases data of DFS is not available. Significance is assessed by log-rank test (n=number of patients, e=number of events).

5. Discussion

Today, breast cancer has become a curable disease for most of early-stage patients, as a result of effective loco-regional as well as systemic treatment options. However, more factors are urgently needed to better forecast the course of disease. With the aim to more precisely describe the course of the cancer disease and to personalize cancer therapy several different cancer biomarkers have been studied. But only a few, like for example the steroid hormone receptors and the oncoprotein HER2 have gained widespread clinical use for breast cancer.

A new pair of important biomarkers in breast cancer is uPA and PAI-1. Several lines of evidence suggest that the plasminogen activator system plays an important role in tumor-matrix degradation, promoting cancer invasion and metastasis in a variety of solid malignant tumor entities. There is a battery of studies showing the prognostic and predictive impact of uPA and PAI-1 *(e.g. Jänicke et al. 1989, 1990, 1991, 1994, 2001, Look et al. 2002, 2003, Harbeck et al. 2002b)*. As a result of all these studies, the two proteases were included in the 2007 Breast Cancer Treatment Guidelines of ASCO for clinical decision making in adjuvant breast cancer treatment options. Moreover, uPA and PAI-1 are the first and only breast cancer biomarkers which have reached the highest level of evidence, LOE-1 *(Schmitt et al. 2010)*.

To date, the clinical utility of uPA and PAI-1 as prognostic and predictive markers is still limited by the use of ELISA tests for their detection because this method depends on the use of fresh frozen tissue, which is rarely available in clinical routine. That's why alternative methods to determine uPA/PAI-1 expression in routinely prepared FFPE tissue material are needed.

A classical routinely available technique still is immunohistochemistry. In this study, reproducible standard operating procedures (SOPs) for assessment of uPA and PAI-1 by immunohistochemistry were developed and provided with the aim to bring this classical technique into future clinical routine use.

5.1 Assessment of uPA and PAI-1 by IHC

Many approaches have been done in order to find a practical and validated determination of uPA and PAI-1, other than by ELISA. Immunohistochemical expression of the plasminogen activator system has been investigated for several cancer entities including breast cancer *(e.g. Carriero et al. 1994, Christensen et al. 1996, Costantini et al. 1991, 1996, Damjanovich et al. 1994, Del Vecchio et al. 1993, Dublin et al. 2000, Jänicke et al. 1991, Jankun et al. 1993, Kennedy et al. 1998, Reilly et al. 1992, Sumiyoshi et al. 1991, Visscher et al. 1993, 1995)*. **Table 15** gives an overview of studies applying the same antibodies which have been used in this study. To date no studies have been found applying monoclonal antibodies #3786 and #ADG25 for immunohistochemical analyses of uPA and PAI-1.

Table 15 Studies using the same antibodies (#3689, #3785, #3786, #ADG25) as in this study. No literature was available for the anti-PAI-1 mAbs #3786 and #ADG25.

Antibody	Literature
Anti-uPA mAb #3689	*Jankun et al. 1993, De Vries et al. 1994, Schmalfeldt et al. 1995, Costantini et al. 1996, Christensen et al. 1996, Göhring et al. 1996, Ferrier et al. 1998, Zhao et al. 2002, Hildenbrand et al. 2004, Hurd et al. 2007*
Anti-PAI-1 mAb #3785	*Jankun et al. 1993, Bianchi et al. 1995, Schmalfeldt et al. 1995, Costantini et al. 1996, Christensen et al. 1996, Ferrier et al. 1998, Hildenbrand et al. 2004, Hurd et al. 2007*
Anti-PAI-1 mAb #3786	No literature available
Anti-PAI-1 mAb #ADG25	No literature available

To date, the identity of uPA and PAI-1 expressing cells in the tumor is discussed controversial: on the one hand, there is evidence that in general stromal cells play a major role in the generation and regulation of matrix degrading proteolytic activity and so in cancer invasion and metastasis *(Gregoire et al. 1995, Zipori 1990)*. Immunohistochemical studies of different types of human cancer, including breast cancer, have shown that stromal cells often contribute strongly to the overall levels of uPA and PAI-1 in the tumor tissue *(e.g. Bianchi et al. 1995, Pyke et al. 1991, Kennedy et al. 1998, Visscher et al. 1993, 1995)*.

In contrast, other groups report that the uPA and PAI-1 immunostaining is localized mainly in the cytoplasm of the cancer cells *(Duffy et al. 1990, Costantini et al.*

1991, 1996, Damjanovich et al. 1994, Del Vecchio et al. 1993, Jänicke et al. 1991, Jankun et al. 1993, Reilly et al. 1992, Sumiyoshi et al. 1991).

Other groups found substantial uPA and PAI-1 staining of both cancer cells and stromal cells *(Costantini et al. 1991, 1996, Carriero et al. 1994, Hubbard et al. 1995, Christensen et al. 1996, Umeda et al. 1997, Ferrier et al. 1999, Dublin et al. 2000, Zhao et al. 2002, Offersen et al. 2003, Castello et al. 2007)*. For example, in the study of *Carriero et al.* the cytoplasm and the membrane of epithelial tumor cells as well as stromal cells were stained with uPA-mAbs. *Christensen et al.* reported a strong staining intensity of uPA and PAI-1 in macrophages, mast cells, cells of the tumor stroma, and moderate staining in epithelial tumor cells of breast cancer tissue. According to *Offersen et al.* PAI-1 expression was seen in stromal as well as cancer cells and they found that the myofibroblast is the predominant PAI-1-expressing cell type in breast cancer. In addition *Castello et al.* demonstrated that uPA and PAI-1 were localized in stromal and cancer cells.

In this study staining of epithelial tumor cells as well as tumor stroma was scored in detail: our findings are in agreement with those of groups reporting staining of both tumor cells and tumor stroma, as positive uPA and PAI-1 staining was found as the follows: moderate to strong cytoplasmic staining of the tumor cells was found in most of the breast cancer cases. In about half of these cases weak to moderate staining of the tumor cell nuclei was found. Staining of the cells of the tumor stroma was roughly found in one third of the cases, while staining of the non-cellular part of the tumor stroma in more than half of the cases.

Causes for these conflicting results on the localization of uPA and PAI-1 in breast cancer are certainly complex. Possible factors contributing to these results might be varying criteria for scoring positivity, dissimilar cut-off points, the use of different antibodies with various specifities and diverse procedures concerning fixation conditions, proteolytic retrieval, and detergent wash. *Nielsen et al.* for example demonstrated that even prolonged formalin fixation leads to a lack of stromal cell staining. They reported that stromal cells were stained weakly when the tissue had been fixed for eight hours or more at room temperature, which was also the case in this study. Although fixation time is not specified in most of the publications not finding stromal uPA-staining, it is likely prolonged formalin fixation has been used, because it is part of the routine in pathology departments *(Nielsen et al. 2001)*. It is not possible to evaluate the influence of each of these parameters, as

sufficiently detailed information about the methods used is often lacking in the publications.

5.2 Comparison of the two different techniques to assess uPA and PAI-1: ELISA versus IHC

Enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) are techniques that provide information on protein expression in tissue samples. In several studies both methods have been used to investigate the impact of the plasminogen activation system in breast cancer *(Jänicke et al. 1990, Reilly et al. 1992, Hildenbrand et al. 1995, Christensen et al. 1996, Ferrier et al. 1999, Nielsen et al. 2001)*.

Jänicke et al. were the first in 1990 comparing the quantification of uPA by ELISA and its simultaneous localization by immunohistochemistry. In this study, tumors with strong immunohistochemical staining intensity revealed statistically significant higher uPA content than those with faint staining. In the study of *Reilly et al.* the intensity of the immunohistochemical staining of PAI-1 correlated with its respective ELISA values. Moreover, *Nielsen et al.* showed a close correlation between intensity of uPA immunostaining and uPA protein content measured by ELISA. In contrast *Ferrier et al.* did not find such correlation between ELISA values and IHC with respect to breast cancer.

In the present study antigen levels of uPA and PAI-1 in 210 breast cancer tissue extracts determined by ELISA were compared with immunohistochemical expression. Statistically significant correlation between ELISA and immunohistochemical staining intensity was found neither for the anti-uPA mAb nor for any of the three anti-PAI-1 mAbs. According to the findings of *Ferrier et al. 1999*, we showed that the two techniques are not directly exchangeable and that their clinical value may be different. One explication might be that the two techniques are not applied to adjacent parts of the same tissue sample in this study. In contrast, ELISA data has been performed independently in a routine lab which does not enable the comparison of results obtained on material of almost identical composition. In addition to this, different antibodies for IHC and ELISA were used, thus uniform epitope recognition cannot be achieved.

Another possible explanation for the discrepancies is that fractions of a component, as a result of divergent presentation of epitopes in tumor tissue extracts and in tissue sections, may be detected with different efficiencies by ELISA and IHC: new complexes between components could form while pre-existing complexes could dissociate for example. Besides, in IHC only one antibody selects the antigenic determinants, whereas both the catching and detecting antibodies in the ELISAs exert selection. Moreover, by using the ELISA method, discrimination of antigen level is possible among very high analyte concentrations, whereas estimation of protein expression levels above the level causing maximum staining is impossible *(Ferrier et al. 1999)*.

Comparing both techniques, ELISA and IHC both have their specific advantages. ELISA methods give an objective quantification of analyte levels, whereas IHC yields at best semi-quantitative information, but allows insight into tissue heterogeneity. The distribution of antigens over the different cell types, and therefore the clinical relevance of an antigen expression by a certain cell type, can only be studied using IHC. An important advantage of immohistochemistry is that this technique is quite simple, widely available, relatively inexpensive, and it can be performed on routinely processed paraffin-embedded tissue. On the other hand, interpretation of immunohistochemical staining is subjective and difficult to standardize *(Sturgeon et al. 2008)*.

To optimize comparison between the results of the two techniques following aspects should be considered: first, ELISA and IHC should be performed on consecutive tissue sections minimizing the effect of tissue heterogeneity. Secondly, IHC should be performed applying same antibodies that were used in the ELISA, which is advantageous to obtain good correlations because of uniform epitope recognition.

5.3 Clinical utility of uPA/PAI-1 staining results assessed by IHC

Different studies showed that uPA and PAI-1 are often overexpressed in cancer tissue, and that high levels of these proteases are associated with poor prognosis in several types of human cancer, including breast cancer *(Duffy et al. 1990, Jänicke et al. 1990, Sumiyoshi et al. 1991, Stephens et al. 1998, Têtu et al. 2001, Gupta et al. 2009*).

In the present study, we found uPA and PAI-1 expression assessed by IHC associated with different clinical and pathological features. Especially anti-PAI-1 mAb #3786 showed following statistically significant correlations: regarding PAI-1 expression in the cytoplasm of epithelial tumor cells, high staining intensity was statistically significant associated with advanced age, postmenopausal status, and positive lymph node involvement. PAI-1 staining of the nuclei of tumor cells showed to be connected with age, and postmenopausal status. PAI-1 staining in cells of tumor stroma was annexed to age, menopausal status, negative lymph node status, low UICC stadium, absence of recurrences and deaths. Regarding the non-cellular part of tumor stroma, statistically significant association of staining intensity using the anti-uPA mAb #3786 was shown for age, menopausal status, small tumor size, negative lymph node status, and low UICC stadium.

To sum up, it was demonstrated for the first time that high staining intensity of PAI-1 in tumor cells is associated with advanced tumor stage (e.g. positive lymph node involvement, and high UICC stadium) and consequently poor prognosis in general whereas PAI-1 expression in tumor stroma is associated with factors of better prognosis (e.g. negative lymph node status, lower UICC stadium). All three anti-PAI-1 mAbs illustrated that tendency, but mAb #3786 demonstrated most statistically significant associations.

Regarding other studies using IHC, most groups found high uPA/PAI-1 expression assessed by IHC associated with advanced tumor stage and poor prognosis in breast cancer *(Sumiyoshi et al. 1991, Hubbard et al. 1995, Umeda et al. 1997, Jahkola et al. 1999, Dublin et al. 2000, Têtu et al. 2001)*. Focusing on the part of the tumor (tumor cells versus tumor stroma), results are getting controversial: *Umeda et al.* showed that uPA expression in cancer cells was associated with poor prognosis, which is in accordance with our study. In contrast, *Dublin et al*. for example found that especially uPA/PAI-1 expression in stromal cells correlates with poor clinical

outcome, which is in accordance with the study of *Jahkola et al.* and *Têtu et al.* reporting an association between high staining of tumor stroma and increased risk of recurrence and so poor outcome.

To date, no clinical relevant data was published concerning the impact of immunohistochemical staining results of uPA and PAI-1 in breast cancer on OS and DFS. Regarding recent literature only a study concerning clinical impact of uPA and PAI-1 on outcome of prostate cancer patients published by *Gupta et al.* could be found. They showed uPA/PAI-1 expression assessed by IHC to be associated with pathologic features and to be predictive for aggressive disease recurrence in patients with prostate cancer.

In this study it was demonstrated that PAI-1 expression measured by IHC is correlated with disease-free and overall survival rate of breast cancer patients: according to the clinical factors, there is again a difference between epithelial cells and stromal cells of the tumor: while expression of PAI-1 in the cytoplasm of tumor cells is significantly associated with poor outcome (shorter DFS and OS), overexpression of PAI-1 in stromal cells is associated with a longer DFS and OS. This correlation is only statistically significant for the monoclonal antibody mAb #3786, whereas for the other two anti-PAI-1 monoclonal antibodies there is found same tendency.

When PAI-1 expression in both cell types is considered (see **Figure 23**), patients with high staining in cells of the tumor stroma and weak staining in the cytoplasm of the tumor cells have the highest probability of OS and DFS, followed by the cohort with high staining of the tumor cell cytoplasm and high staining of the cells of the tumor stroma. This underlines the hypothesis that especially the expression of PAI-1 in the tumor stroma cells contributes to good prognosis and longer survival. Tumors of patients with weak staining in the cytoplasm of tumor cells combined with weak staining in cells of the tumor stroma have a shorter OS and DFS rate, and patients with strong staining of the cytoplasm of the tumor cells pooled with weak staining of cells of the tumor stroma have the shortest OS and DFS. This result confirms the theory that the PAI-1 expressing cell type plays a key role for a breast cancer patients' outcome. According to the Kaplan-Meier-analyses in **Figure 23** about 40 % of the patients are classified consequently as high risk (beige curve). This means that measuring the PAI-1 expression by immunohistochemistry using monoclonal antibody #3786 would help to identify

people at risk of disease recurrence, which would probably benefit from alternative adjuvant therapy, e.g. particular chemotherapy. In contrast patients classified as “low-risk” do not benefit from adjuvant therapy and may be spared its toxic side-effects.

We have to admit that our patient sample is biased by the fact that patients have received adjuvant chemotherapy regimens which are obsolete today. Moreover relatively high positive lymph node rate is given. The cohort is therefore not directly representative of patients with breast cancer in most institutions today. Nevertheless, this study supports the theory that proteases produced by stromal cells and not only by tumor cells play a central role in tumor progression. A main difference between the results of this study and most of the previous reports is the fact that a difference in outcome regarding to the PAI-1-expressing cell type was found for the first time. Compared to other studies PAI-1 expression in stromal cells is associated with good prognosis, whereas PAI-1 expression in epithelial tumor cells is associated with poor prognosis. It is conceivable that PAI-1 can inhibit uPA in stroma preventing stroma degradation and hence tumor invasion and initiation of metastasis. In contrast, PAI-1 expressed by tumor cells cannot inhibit uPA leading to uncontrolled tumor progression and metastasis and hence poor prognosis.

5.4 Conclusions and outlook

In this study good reproducible staining procedures (standard operating procedures) with four specific primary antibodies were developed for manual as well as automated staining procedures. These SOPs show that – from the technical point of view – immunohistochemistry can be a good, quick and cost-effective method to determine uPA and PAI-1.

Unfortunately, no significant correlation was found between uPA/PAI-1 protein levels measured by ELISA and IHC staining results. One reason for this result might be the fact that different antibodies were used, which influences uniform epitope recognition. In addition, it was not possible to perform both methods on consecutive tissue sections what maximizes the effect of tissue heterogeneity. These results give an idea about the difficulty comparing protein

levels in “three-dimensional” tissue specimens to staining intensity of certain areas of “two-dimensional” tissue sections.

That’s why we tried to figure out whether there is, apart from the clinical impact of ELISA, a correlation between IHC staining results and prognostic impact regarding two different tumor areas: tumor cells and tumor stroma. In general, high PAI-1 staining intensity in epithelial tumor cells is significantly associated with advanced tumor stage (e.g. positive lymph node involvement, higher UICC stadium), whereas high PAI-1 staining intensity in the cells of the tumor stroma is significantly related to less advanced tumor stage (negative lymph node involvement, lower UICC stadium). Considering the prognostic impact of uPA/PAI-1 expression, it has been found for the first time that PAI-1 expression using anti-PAI-1 mAb #3786 is statistically significant associated with DFS and OS depending on the type of cell: whereas high staining intensity of tumor stroma is associated with longer disease-free and overall survival rate, high staining intensity of the cytoplasm of tumor cells is related to shorter disease-free and overall survival. With the use of Kaplan-Meier analyses, patients with strong staining of the cytoplasm of the tumor cells pooled with weak staining of cells of the tumor stroma have the shortest disease-free and overall survival and are consequently classified as high risk who would benefit from adjuvant therapy options, e.g. chemotherapy.

These results point to a multifunctional role of PAI-1 in tumors by regulating extracellular matrix, cell proliferation, adhesion, and migration. While in stromal cells the inhibiting function and hence the reduction of tumor growth and invasion is predominant, in epithelial tumor cells high expression of PAI-1 is associated with poor prognosis. As mentioned in the introduction, especially the imbalance of the components of the plasminogen activator system leads to cancer progression and metastasis.

To conclude, our good and reproducible SOPs and especially the results regarding monoclonal antibody anti-PAI-1 mAb #3786 are quite promising, though the routine clinical use of PAI-1 assessed by immunohistochemistry should await further confirmation by investigating a larger independent collective and later well controlled randomized studies to validate this method as an alternative to the ELISA.

Nevertheless, several limitations of this study need to be considered. As mentioned above, the cohort of this study is not directly representative of patients with breast cancer in most institutions today, as out-dated adjuvant therapy regimes were used and a high positive lymph node rate was given. The study is also limited by the fact that different antibodies were used for IHC and ELISA and that the two techniques are not applied to adjacent parts of the same tissue sample.

Besides, IHC is a very subjective method and has inherent limitations such as reproducibility and reliability. To improve this fact, TMAs allowing all tissues to be exposed to identical conditions, and automatic staining instruments increasing reproducibility, were used in this study. Even so IHC has become a standard assay in the pathology, standardization especially of slides evaluation is still missing. An approach for this standardization might be the use of quantitative methods like the digital image technology. Several image analysis applications for immunohistochemistry quantification have proved that automatic scoring by the application of algorithmic quantification software provides more reliable and uniform results than manual evaluation. These automated methods can be used as a computer aid for the immunohistochemical evaluation in order to increase observer reproducibility. The automated quantitative assessment of HER2 immunohistochemical expression in breast cancer published by *Masmoundi et al. 2009* is only one example.

Apart from their clinical utility, these factors are finally also promising targets for the development of novel tumor biologic therapeutic agents. Targeted therapies have the potential of attacking and eradicating tumor cells in particular. Therefore several strategies have been developed using e.g. antibodies to uPA or uPAR to prevent binding of naturally occurring uPA and uPA/PAI-1-complexes to uPAR in order to reduce tumor cell proliferation, invasion, and metastasis.

One example is WX-671 (MESUPRON®) developed by the Wilex AG, Munich, Germany, which is a promising orally available targeted drug candidate and currently being tested clinically in phase II in patients afflicted with breast or pancreatic cancer in combination with chemotherapeutic agents. WX-671 is a synthetic small-size serine protease inhibitor selective for uPA and plasmin *(Schmitt et al. 2008, www.wilex.de)*. Preclinical testing as well as Phase I/Ib/IIa monotherapy

and combination therapy clinical studies found the drug candidate to be safe and well-tolerated with no serious adverse effects *(Goldstein et al. 2008, 2010)*.

In women with epithelial ovarian cancer the Å6 peptide produced by Ångstrom Pharmaceutics (San Diego, CA) is another promising targeted drug candidate. This small synthetic uPA-derived peptide paralyzes cancer cells and so stops tumors from metastasizing. In a placebo-controlled phase II trial the disease-free survival was significantly doubled in patients receiving Å6 over that of placebo control *(Ghamande et al. 2008, Mengele et al. 2010, Schmitt et al. 2011)*.

Although further clinical trials are in progress and results are still pending, these examples do underline the enormous clinical potential of the cancer biomarker uPA and PAI-1, next to their predictive and prognostic value. That's why further investigations on the protein and gene level are indispensable to explore this huge potential of the two factors which are so far the only cancer biomarkers that have reached the highest level of evidence, especially for a heterogeneous disease like breast cancer.

6. Summary

In industrialized countries, breast cancer still is the most common malignancy in women. Due to effective loco-regional as well as systemic treatment options, breast cancer has become a curable disease for most of early-stage patients today. However, more factors are urgently needed to better forecast the course of disease. A pair of novel biomarkers predicting therapy response and course of disease in breast cancer is urokinase plasminogen activator (uPA) and its inhibitor PAI-1. So far, these factors are determined by standardized, quality assessed enzyme-linked immunosorbent assays (ELISAs). These tests are hampered by the fact that they have to be performed on extracts obtained from fresh-frozen tumor tissue specimen. As most breast care centers are not equipped for such analyses and as transport and storage of fresh-frozen tissue specimens are complex and often too expensive, more widely available determination techniques using formalin-fixed paraffin-embedded material such as immunohistochemistry (IHC) are favored for assessment of the biomarkers uPA and PAI-1.

The aim of the study therefore was to establish protocols (standard operating procedures, SOPs) for quantitative assessment of uPA and PAI-1 in formalin-fixed paraffin-embedded primary breast cancer tumor tissue by immunohistochemistry and to explore the clinical utility of this method as an alternative to the standardized ELISA.

Good and reproducible standard operating procedures were established for four different antibodies. These were worked out for manual staining and for two automated staining instruments, the Dako Autostainer and the VENTANA Benchmark® XT.

Tissue microarrays with tumor tissue of 210 breast cancer patients were stained using these SOPs. The stained slides were scored by pathologists considering different tumor areas: the epithelial tumor cells and the cells of the tumor stroma.

Staining results were correlated to ELISA values of the breast cancer patients. No significant correlations could be found between immunohistochemical staining intensity and ELISA values of uPA and PAI-1.

Considering clinical data and prognostic impact of uPA/PAI-1 expression detected by IHC, following associations could be observed: in general, there was a difference concerning the PAI-1 expressing cell type: high PAI-1 staining intensity in tumor cells was significantly associated with advanced tumor stage (e.g. positive lymph node involvement, higher tumor stadium), whereas high PAI-1 staining intensity in the cells of the tumor stroma was significantly related to less advanced tumor stage (e.g. negative lymph node involvement, lower tumor stadium).

Considering the prognostic impact of uPA/PAI-1 expression, we found that PAI-1 overexpression in the cells of the tumor stroma was associated with longer disease-free and overall survival, whereas PAI-1 overexpression in the cytoplasm of tumor cells was related to shorter disease-free and overall survival.

In brief, good, reproducible SOPs for uPA and PAI-1 were established serving from the technical point of view as an alternative to the standardized ELISA. Although results concerning association with clinical outcome in this patient cohort are quite promising, routine clinical use should await further confirmation by investigating larger collectives to validate this method.

7. Abbreviations

AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
BRCA 1/2	Breast Cancer 1/2 -gen
CEA	Carcinoembryotic Antigen
CMF	Cyclophosphamide/methotrexate/5-fluorouracil
CpG	Cytosine-phosphate-guanine
DAB	3,3‘- diaminobenzidine tetrahydrochloride
DCIS	Ductal carcinoma *in situ*
DFS	Disease-free survival
EC	Epirubicin/cyclophosphamide
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked Immunosorbent Assay
EORTC-RBG	European Organization for Research and Treatment of Cancer - Receptor and Biomarker Group
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor 2
HE stain	Hematoxylin and eosin stain
HIER	Heat Induced Epitope Retrieval
HMW	High molecular weight
Hour(s)	Hr(s)
HR	Hazard ratio
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
LCIS	Lobular carcinoma *in situ*

LN	Lymph node
LOE-1	Level of evidence 1
LSAB-Method	Labeled-Streptavidin-Biotin-Method
mAb	Monoclonal antibody
MINDACT-trial	Microarray In Node-Negative Disease May Avoid Chemotherapy-trial
Minutes(s)	Min(s)
MRI	Magnetic resonance imaging
NASBA	Nucleic acid sequence-based amplification
NBE	Normal breast epithelium
NBS	Normal breast stroma
NGS	Normal goat serum
NNBC-3-trial	Node-Negative Breast Cancer-3 trial
OS	Overall survival
PAI-1	Plasminogen activator inhibitor type-1
PAI-2	Plasminogen-activator inhibitor type-2
PFS	Progression-free survival
PIER	Proteolytic Induced Epitope Retrieval
PR	Progesterone receptor
RFS	Relapse-free survival
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
Serpins	Serin protease inhibitor
SOP	Standard operating procedure
SPSS	Statistical Package for the Social Sciences
TAILORx-trial	Trial Assigning Individualized Options for Treatment (Rx)- trial
TBS	Tris-buffered saline
TC	Tumor cells
TS	Tumor stroma
TNF-α	Tumor necrosis factor alpha
TMA	Tissue microarray
TMUGS	Tumor Marker Utility Grading System
tPA	Tissue-type plasminogen activator

UICC	Union internationale contre le cancer
uPA	Urokinase-type plasminogen activator
uPA-R	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VLDL-R	Very low-density lipoprotein receptor
WSG	West-German Study Group
WHO	World Health Organization

8. References

American Joint Committee on Cancer, AJCC Cancer Staging Manual, Springer, 7th edition (2009), 345-376.

Andreasen PA, Kjøller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 72 (1997), 1-22.

Andreasen PA, Eglund E, Petersen HH. The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci* 57 (2000), 25-40.

Annecke K, Schmitt M, Euler U, Zerm M, Paepke D, Paepke S, von Minckwitz G, Thomssen C, Harbeck N. uPA and PAI-1 in breast cancer: review of their clinical utility and current validation in the prospective NNBC-3 trial. *Adv Clin Chem* 45 (2008), 31-45.

Bajou K, Noël A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart, JM. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* 4(8) (1998), 923-928.

Becker KF, Schott C, Hipp S *et al.* Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. *J. Pathol* 211 (2007), 370-378.

Bevan P, Mala C: The role of uPA and uPA inhibitors in breast cancer. *Breast Care* 3(suppl2) (2008),1-2.

Beaufort N, Seweryn P, de Bentzmann S, Tang A, Kellermann J, Grebenchtchikov N, Schmitt M, Sommerhoff CP, Pidard D, Magdolen V. Activation of human pro-urokinase by unrelated proteases secreted by Pseudomonas aeruginosa. *Biochem J* 428 (2010), 473- 482.

Bernard-Marty C, Cardoso F, Piccart MJ. Facts and controversies in systemic treatment of metastatic breast cancer. *Oncologist* 9 (2004), 617-632.

Bianchi E, Cohen RL, Dai A, Thor AT, Shuman MA, Smith HS. Immunohistochemical localization of the plasminogen activator inhibitor-1 in breast cancer. *Int J Cancer* 60 (1995), 597-603.

Biermann JC, Holzscheiter L, Kotzsch M, Luther T, Kiechle-Bahat M, Sweep FCGJ, Span PN, Schmitt M, Magdolen V. Quantitative RT-PCR assays for the determination of urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 mRNA in primary tumor tissue of breast cancer patients: Comparison to antigen quantification by ELISA. *Int J Mol Med* 21 (2009), 251-259.

Bubendorf L, Nocito A, Moch H, Sauter G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. *J Pathol* 195 (2001), 72-79.

Carriero MV, Franco P, Del Vecchio S, Massa O, Botti G, D'Aiuto G, Stoppelli MP, Salvatore M. Tissue distribution of soluble and receptor-bound urokinase in human breast cancer using a panel of monoclonal antibodies. *Cancer Res* 54 (1994), 5445-5454.

Castello R, Landete JM, Espana F, Vazquez C, Fuster C, Almenar SM, Ramon LA, Radtke KP, Estelles A. Expression of plasminogen activator inhibitors type 1 and type 3 and urokinase plasminogen activator protein and mRNA in breast cancer. *Thromb Res* 120(5) (2007), 753-762.

Christensen L, Wiborg Simonsen AC, Heegaard CW, Moestrup SK, Andersen JA, Andreasen PA. Immunohistochemical localization of urokinase-type plasminogen activator, type-1 plasminogen-activator inhibitor, urokinase receptor and α_2-macroglobulin receptor in human breast carcinomas. *Int J cancer* 66 (1996), 441-452.

Costantini V, Zacharski LR, Memoli VA, Kudryk BJ, Rousseau SM, Stump DC. Occurrence of components of fibrinolysis pathways in situ in neoplastic and non-neoplastic human breast tissue. *Cancer Res* 51 (1991), 354-358.

Costantini V, Sidoni A, Deveglia R, Cazzato OA, Bellezza G, Ferri I, Bucciarelli E, Nenci GG. Combined overexpression of urokinase, urokinase receptor, and plasminogen activator inhibitor-1 is associated with breast cancer progression. *Cancer* 77 (1996), 1079-1088.

Conway C, Dobson L, O'Grady A, Kay E, Costello S, O'Shea D. Virtual microscopy as an enabler of automated/quantitative assessment of protein expression in TMAs. *Histochem Cell Biol* 130 (2008), 447-463.

Damjanovich L, Turzó C, Adány R. Factors involved in the plasminogen activation system in human breast tumours. *Thromb Haemost* 71 (1994), 684-691.

Degenhardt T, Gluz O, Thomssen C, Vetter M, Kates R, Warm M, Shak S, Augustin D, Kusche M, Untsch M, Paepke S, Uleer C, Dünnebacke J, Nitz U, Harbeck N. Risk group selection in primary breast cancer according to ASCO recommended biomarkers Oncotype DX and uPA/PAI-1. First experience from propective multicenter WSG Plan B trial. *Cancer Res.* 70 (2010), 209s (P2-09-05).

Del Vecchio S, Stoppelli MP, Carriero MV, Fonti R, Massa O, Li PY, Botti G, Cerra M, D'Aiuto G, Esposito G. Human urokinase receptor concentration in malignant and benign breast tumors by in vitro quantitative autoradiography: comparison with urokinase levels. *Cancer Res* 53 (1993), 3198-3206.

De Vries TJ, Quax PHA, Denijin M, Verrijp KN, Verheijen JH, Verspaget HW, Weidle UH, Ruiter DJ, van Muijen GNP. Plasminogen activators, their inhibitors, and urokinase receptor emerge in late stages of melanocytic tumor progression. *Am J Pathol* 144 (1994), 70-81.

Dublin E, Hanby A, Patel NK, Liebman R, Barnes D. Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma. Fibroblastic expression has strong associations with tumor pathology. *Am J Pathol* 157 (2000), 1219-1227.

Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fennelly JJ, Lijnen HJ. Urokinase-plasminogen activator, a marker for aggressive breast carcinomas. Preliminary report. *Cancer* 62(3) (1988a), 531-533.

Duffy MJ, O'Grady P, Devaney D, O'Siorain L, Fennelly JJ, Lijnen HR. Tissue-type plasminogen activator, a new prognostic marker in breast cancer. *Cancer Res* 48 (1988b), 1348-1349.

Duffy MJ, Duggan C, Mulcahy HE, McDermott EW, O'Higgins N. Urokinase plasminogen activator: a prognostic marker in breast cancer including patients with axillary node-negative disease. *Clin Chem* 44 (1998c), 1177-1183.

Duffy MJ, Reilly David, O'Sullivan Cathriona, O'Higgins Niall, Fennelly JJ, Andreasen P. Urokinase-plasminogen activator, a new and independent prognostic marker in breast cancer. *Cancer Res* 50 (1990), 6827-6829.

Duffy MJ. Proteases as prognostic markers in cancer. *Clin Cancer Res* 2 (1996), 613-618.

Duffy MJ. Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level I evidence studies. *Clin Chem* 48 (2002), 1194-1197.

Duggan C, Maguire T, McDermott E, O'Higgins N, Fennelly JJ, Duffy MJ. Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. *Int J Cancer* 61 (1995), 597-600.

Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DSA, Nobel AB, van't Veer LJ, Perou MC. Concordance among gene-expression-based predictors for breast cancer. *N Engl J Med* 355 (2006), 560-569.

Ferrier CM, van Geloof WL, de Witte HH, Kramer MD, Ruiter DJ, van Muijen GNP. Epitopes of components of the plasminogen activation system are re-exposed in formalin-fixed paraffin sections by different retrieval techniques. *J Histochem Cytochem* 46 (1998), 469-476.

Ferrier CM, de Witte HH, Straatman H, van Tienoven DH, van Geloof WL, Rietveld FJR, Sweep CGJ, Ruiter DJ, van Muijen GNP. Comparison of immunohistochemistry with immunoassay (ELISA) for the detection of components of the plasminogen activation system in human tumour tissue. *Br J Cancer* 79 (9/10) (1999), 1534-1541.

Foekens JA, Schmitt M, van Putten WLJ, Peters HA, Bontenbal M, Jänicke F, Klijn JGM. Prognostic value of urokinase-type plasminogen activator in 671 primary breast cancer patients. *Cancer Res* 52 (1992), 6101-6105.

Foekens JA, Schmitt M, van Putten WLJ, Peters HA, Jänicke F, Klijn JMG: Plasminogen activator inhibitor-1 and prognosis in primary breast cancer. J *Clin Oncol* 12 (1994), 1648-1658.

Foekens JA, Buessecker F, Peters HA, Krainick U, van Putten WLJ, Look MP, Klijn JGM, Kramer MD. Plasminogen activator inhibitor-2: prognostic relevance in 1012 patients with primary breast cancer. *Cancer Res* 55 (1995), 1423-1427.

Foekens JA, Peters HA, Look MP, Portengen H, Schmitt M, Kramer MD, Brünner N, Jänicke F, Meijer-van Gelder ME, Henzen-Logmans SC, van Putten WLJ, Klijn JGM. The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. *Cancer Res* 60 (2000), 636-643.

Fornier M, Hudis C. Adjuvant chemotherapy for primary breast cancer. *Curr Oncol Rep* 7 (2005), 18-22.

Ghamande SA, Silverman MH, Huh W, Behbakht K, Ball G, Cuasay L, Würtz SO, Brunner N, Gold MA. A phase 2, randomized, double-blind, placebo-controlled trial of clinical activity and safety of subcutaneous A6 in women with asymptomatic CA125 progression after first-line chemotherapy of epithelial ovarian cancer. *Gynecol. Oncol.* 111 (2008), 89-94.

Göhring UJ, Scharl A, Thelen U, Ahr A, Crombach G. Comparative prognostic value of Cathepsin D and urokinase plasminogen activator, detected by immunohistochemistry, in primary breast carcinoma. *Anticancer Res* 16 (1996), 1011-1018.

Goldhirsch A, Wood WC, Gelber RD, et al: Meeting highlights: updated international expert consensus of primary therapy of early breast cancer. *J Clin Oncol* 21 (2003), 3357-3365.

Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thürlimann B, Senn HJ *et al.* Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Ann Oncol* 18 (2007), 1133-1144 (2007).

Goldstein LJ: Experience in phase I trials and an upcoming phase II study with uPA inhibitors in metastatic breast cancer. *Breast Care* 3 (suppl 2) (2008), 25-28.

Goldstein LJ, Stemmer SM, Schmalfeldt B, Gottschalk N, Cardoso F, Dushkin H, Mala C, Uebler N, Bevan P, Harbeck N. Phase II, two-arm, double-blind multicenter, randomized study of the combination of oral WX-671 plus capecitabine versus capecitabine in first-line HER2-negative metastatic breast cancer (MBC). *J Clin Oncol* 28 (2010), 15s, suppl; abstract TPS131.

Grebenchtchikov N, Maguire TM, Riisbro R, Geurts-Moespot A, O'Donovan, Schmitt M, McGreal G, McDermott E, O'Higgins N, Bruenner N, Sweep CGJ, Duffy MJ. Measurement of plasmingen activator system components in plasma and tumor tissue extracts obtained from patients with breast cancer: an EORTC

Receptor and Biomarker Group (RBG) collaboration. *Oncol Rep* 14(1) (2005), 235-239.

Gregoire M, Lieubeau B. The role of fibroblasts in tumor behavior. *Cancer Metastasis Rev* 14 (1995), 338-350.

Grøndahl-Hansen J, Christensen IJ, Rosenquist C, Brünner N, Mouridsen HT, Danø K, Blichert-Toft M. High levels of urokinase-type plasminogen activator and its inhibitor PAI-1 in cytosolic extracts of breast carcinomas are associated with poor prognosis. *Cancer Res* 53 (1993), 2513-2521.

Grøndahl-Hansen J, Peters HA, van Putten WLJ, Look MP, Pappot H, Rønne E, Danø K, Klijn JGM, Brünner N, Foekens JA. Prognostic significance of the receptor for urokinase plasminogen activator in breast cancer. *Clin Cancer* Res 1 (1995), 1079-1987.

Gupta A, Lotan Y, Ashfaq R, Roehrborn CG, Raj GV, Aragaki CC, Montorsi F, Shariat SF. Predictive value of the differential expression of the urokinase plasminogen activaton axis in radical prostatectomy patients. *Eur Urol* 55 (2009), 1124-1133.

Harbeck N, Kates RE, Look MP, Meijer-van Gelder ME, Klijn JGM, Krüger A, Kiechle M, Jänicke F, Schmitt M, Foekens JA. Enhanced benefit from adjuvant chemotherapy in breast cancer patients classified high-risk according to urokinase.-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (n = 3424). *Cancer Res* 62 (2002a), 4617-4622.

Harbeck N, Schmitt M, Kates RE, Kiechle M, Zemzoum I, Jänicke F, Thomssen C. Clinical utility of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 determination in primary breast cancer tissue for individualized therapy concepts. *Clin Breast Cancer* 3 (2002b), 196-200.

Harbeck N, Kates RE, Schmitt M. Clinical relevance of invasion factors urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 for individualized therapy decisions in primary breast cancer is greatest when used in combination. *J Clin Oncol* 20 (2002c), 1000-1007.

Harbeck N, Kates RE, Schmitt M, Gauger K, Kiechle M, Jänicke F, Thomssen C, Look MP, Foekens JA: Urokinase-type plasminogen activator and its inhibitor type 1 predict disease outcome and therapy response in primary breast cancer. *Clin Breast Cancer* 5 (2004a), 348-352.

Harbeck N, Kates RE, Gauger K, Willems Amina, Kiechle M, Magdolen V, Schmitt M. Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1: novel tumor-derived factors with a high prognostic and predictive impact in breast cancer. *Thromb Haemost* 91 (2004b), 450-456.

Harbeck N, Schmitt M, Vetter M, Krol J, Paepke D, Uhlig S, Jänicke F, Geurts-Moespot A, von Minckwitz G, Sweep F, Thomssen C. Prospective biomarker trails

Chemo N0 and NNBC-3 Europe validate the clinical utility of invasion markers uPA and PAI-1 in node-negative breast cancer. *Breast Care* 3(suppl 2) (2008), 11-15.

Harbeck N, Salem M, Nitz U, Gluz O, Liedtke C. Personalized treatment of breast cancer: present concepts and future directions in early stage disease. *Cancer Treat. Rev.* 36(8) (2010), 584-594).

Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF, Bast RC Jr. American Society of Clincal Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol* 25 (2007), 5287-5312.

Hayes DF. Tumor markers for breast cancer. *Ann Oncol* 4 (1993), 807-819.

Hayes DF, Bast RC, Desch CE, Fritsche H, Kemeny NE, Jessup JM, Locker GY, Macdonald JS, Mennel RG, Norton L, Ravdin P, Taube S, Winn RJ. Tumor Marker Utility Grading System: a framework to evaluate clinical utility of tumor markers. *J Natl Cancer Inst*. 88(20) (1996), 1456-1466.

Hildenbrand R, Dilger I, Hörlin A, Stutte HJ. Urokinase plasminogen activator induces angiogenesis and tumor vessel invasion in breast cancer. *Pathol Res Pract* 191 (1995), 403-409.

Hildenbrand R, Arens N. Protein and mRNA expression of uPAR and PAI-1 in myoepithelial cells of early breast cancer lesions and normal breast tissue. *Br J Cancer* 91 (2004), 564-571.

Hubbard AL, Lauder J, Hawkins RA, Anderson TJ. Disregulation of urokinase plasminogen activator gene in breast cancer. *Europ J Cancer* 31 (1995), 103-107.

Hurd TC, Sait S, Kohga S, Winston J, Martinick M, Saxena R, Lankes H, Markus G, Harvey S, Gibbs JF. Plasminogen activator system localization in 60 cases of ductal carcinoma in situ. *Ann Surg Oncol* 14 (2007), 3117-3124.

Jänicke F, Schmitt M, Ulm K, Gössner W, Graeff H. Urokinase-type plasminogen activator antigen and early relapse in breast cancer. *Lancet* 2 (1989), 1049.

Jänicke F, Schmitt M, Hafter R, Hollrieder A, Babic R, Ulm K, Gössner W, Graeff H. Urokinase-type plasminogen activator (u-PA) antigen is a predictor of early relapse in breast cancer. *Fibrinolysis* 4 (1990), 69-78.

Jänicke F, Schmitt M, Graeff H: Clinical relevance of the urokinase- type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer. *Semin Thromb Hemostas* 17 (1991), 303-312.

Jänicke F, Schmitt M, Pache L, Ulm K, Harbeck N, Höfler H, Graeff H. Urokinase (uPA) and its inhibitor (PAI-1) are strong and independent prognostic factors in node-negative breast cancer. *Breast Cancer Res Treat* 24 (1993), 195-208.

Jänicke F, Pache L, Schmitt M, Ulm K, Thomssen C, Prechtl A, Graeff H. Both the cytosols and detergent extracts of breast cancer tissues are suited to evaluate the prognostic impact of the urokinase-type plasminogen activator and its inhibitor, plasminogen activator inhibitor type 1. *Cancer Res* 54 (1994), 2527-2530.

Jänicke F, Prechtl A, Thomssen C, Harbeck N, Meisner C, Untch M, Sweep CGJF, Selbmann HK, Graeff H, Schmitt M. Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1. *J Natl Cancer Inst.* 93 (2001), 913-920.

Jahkola T, Toivonen T, von Smitten K, Virtanen I, Wasenius V-M, Blomqvist C. Cathepsin-D, urokinase plasminogen activator and type-1 plasminogen activator inhibitor in early breast cancer: an immunohistochemical study of prognostic value and relations to tenascin-C and other factors. *Br J Cancer* 80 (1999), 167–174.

Jankun J, Merrick HW, Goldblatt PJ. Expression and localization of elements of the plasminogen activation system in benign breast disease and breast cancers. *J Cel Biochem* 53 (1993), 135-144.

Kämmerer U, Kapp M, Gassel AM, Richter T, Tank C, Dietl J, Ruck P. A new rapid immunohistochemical staining technique using the EnVision antibody complex. *J Histochem Cytochem* 49(5) (2001), 623-630.

Keates AC, Tummala S, Peek RM Jr et al. *Helicobacter pylori* infection stimulates plasminogen activator inhibitor 1 production by gastric epithelial cells. *Infect Immun* 76 (9) (2008), 3992-3999.

Kennedy S, Duffy MJ, Duggan C, Barnes C, Rafferty R, Kramer MD. Semi-quantitation of urokinase plasminogen activator and its receptor in breast carcinomas by immunohistochemistry. *Br J Cancer* 77 (1998), 1638-1641.

Kuhn W, Pache L, Schmalfeldt B, Dettmar P, Schmitt M, Jänicke F, Graeff H. Urokinase (uPA) and PAI-1 predict survival in advanced ovarian cancer patients (FIGO III) after radical surgery and platinum-based chemotherapy. *Gynecol Oncol* 55 (1994), 401-409.

Lamy PJ, Verjat T, Servanton AC, Paye M, Leissner P, Mougin B. Urokinase-type plasminogen activator and plasminogen activator inhibitor type-1 mRNA assessment in breast cancer by means of NASBA. Correlation with protein expression. *Am J Clin Pathol* 128 (2007), 404-413 (2007).

Leissner P, Verjat T, Bachelot T *et al.* Prognostic significance of urokinase plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in lymph node- and hormone receptor-positive breast cancer. *BMC Cancer* 6 (2006), 216.

Levi F, Lucchini F, Negri E, La Vecchia C. Continuing declines in cancer mortality in the European Union. *Ann Oncol* 18 (2007), 593-595.

Look MP, van Putten WLJ, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, Kates R, Spyratos F, Fernö M, Eppenberger-Castori S, Sweep CGJF, Ulm K, Peyrat JP, Martin PM, Magdelenat H, Brünner N, Duggan C, Lisboa BW, Bendahl PO, Quillien V, Daver A, Ricolleau G, Meijer-van Gelder ME, Manders P, Fiets WE, Blankenstein MA, Broët P, Romain S, Daxenbichler G, Windbichler G, Cufer T, Borstnar S, Kueng W, Beex LVAM, Klijn JGM, O'Higgins N, Eppenberger U, Jänicke F, Schmitt M, Foekens JA. Pooled Analysis of prognostic impact of urokinase-type plaminogen activator and its inhibitor PAI-1 in 8377 Breast Cancer Patients. *J Natl Cancer Inst* 94 (2002), 116-128.

Look MP, van Putten WLJ, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, Kates R, Spyratos F, Fernö M, Eppenberger-Castori S, Fred Sweep CG, Ulm K, Peyrat JP, Martin PM, Magdelenat H, Brünner N, Duggan C, Lisboa BW, Bendahl PO, Quillien V, Daver A, Ricolleau G, Meijer-van Gelder M, Manders P, Edward Fiets W, Blankenstein M, Broët P, Romain S, Daxenbichler G, Windbichler G, Cufer T, Borstnar S, Kueng W, Beex L, Klijn J, O'Higgins N, Eppenberger U, Jänicke F, Schmitt M, Foekens J. Pooled analysis of prognostic impact of uPA and PAI-1 in breast cancer patients. *Thromb Haemost* 90 (2003), 538-48.

Malinowsky K, Böllner C, Hipp S, Berg D, Schmitt M, Becker KF. uPA and PAI-1 analysis from fixed tissues - new perspectives for a known set of predictive markers. *Curr Med Chem* 17 (2010), 4370-4377.

Marchionni L, Wilson RF, Wolff AC, Marinopoulos S, Parmigiani G, Bass EB, Goodman SN. Systematic review: gene expression profiling assays in early-stage breast cancer. *Ann Intern Med* 148 (2008), 358-369.

Masmoundi H, Hewitt SM, Petrick N, Myers KJ, Gavrielides MA. Automated quantitative assessment of HER-2/neu immunohistochemical expression in breast cancer. *IEEE Trans Med Imaging* 28(6) (2009), 916-925.

Mengele K, Napieralski R, Magdolen V, Reuning U, Gkazepis A, Sweep F, Brünner N, Foekens J, Harbeck N, Schmitt M. Characteristics of the level-of-evidence-1 disease forecast cancer biomarkers uPA and its inhibitor PAI-1. *Expert Rev Mol Diagn* 10 (7) (2010), 947-962.

Myers J. Automated slide stainers for SS, IHC, and ISH: A review of current technologies and commercially available systems. *Med Lab Obs* 36 (2004), 28-30.

Nielsen BS, Sehested M, Duun S, Rank F, Timshel S, Rygaard J, Johnsen M, Danø K. Urokinase plasminogen activator is localized in stromal cells in ductal breast cancer. *Lab Invest* 81 (2001), 1485–1501.

Offersen BV, Nielsen BS, Høyer-Hansen G, Rank F, Hamilton-Dutoit S, Overgaard J, Andreasen PA. The myofibroblast ist the predominant plasminogen activator inhibitor-1-expressing cell type in human breast carcinomas. *Am J Pathol* 163 (2003), 1887-1899.

O'Grady P, Lijnen HR, Duffy MJ. Multiple forms of plasminogen activator in human breast tumors. *Cancer Res* 45 (1985), 6216-6218.

Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, Baehner FL, Walker MG, Watson D, Park T, Hiller W, Fisher ER, Wickerham DL, Bryant J, Wolmark N. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* 351 (2004), 2817-2826.

Pakneshan P, Tetu B, Rabbani SA. Demethylation of urokinase promoter as a prognostic marker in patients with breast carcinoma. *Clin Cancer Res* 10 (2004), 3035-3041.

Pyke C, Kristensen P, Ralfkiaer E, Grøndahl-Hansen J, Eriksen J, Blasi F, Danø K. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Amer J Pathol* 138 (1991), 1059-1067.

Reilly D, Christensen L, Duch M, Nolan N, Duffy MJ, Andreasen PA. Type-1 plasminogen activator inhibitor in human breast carcinomas. *Int J Cancer* 50 (1992), 208-214.

Rojo MG, Bueno G, Slodkowska J. Review of imaging solutions for integrated quantitative immunohistochemistry in the Pathology daily practice. *Folia Histochem Cytobiol* 4 (2009), 349-354.

Schmalfeldt B, Kuhn W, Reuning U, Pache L, Dettmar P, Schmitt M, Jänicke F, Höfler H, Graeff H. Primary tumor and metastasis in ovarian cancer differ in their content of urokinase-type plasminogen activator, its receptor, and inhibitors type 1 and 2. *Cancer Res* 55 (1995), 3958-3963.

Schmitt M, Harbeck N, Thomssen C, Wilhelm O, Magdolen V, Reuning U, Ulm K, Höfler H, Jänicke F, Graeff H. Clinical impact of the plasminogen actvation system in tumour invasion and metastasis: prognostic relevance and target for therapy. *Thromb Haemost.* 78 (1997), 285–296.

Schmitt M, Mengele K, Gkazepis A, Napieralski R, Magdolen V, Reuning U, Harbeck N. Assessment of urokinase-type plasminogen activator and its inhibitor PAI-1 in breast cancer tissue: Historical aspects and future prospects. B*reast Care* 3(Suppl. 2) (2008), 3-10.

Schmitt M, Mengele K, Napieralski R, Magdolen V, Reuning U, Gkazepis A, Sweep F, Brünner N, Foekens J, Harbeck N. Clinical utility of level-of-evidence-1 disease forecast cancer biomarkers uPA and its inhibitor PAI-1. *Expert Rev Mol Diagn* 10 (8) (2010), 1051-1067.

Schmitt M, Harbeck N, Brünner N, Jänicke F, Meisner C, Mühlenweg B, Jansen H, Dorn J, Nitz U, Kantelhardt EJ, Thomssen C. Cancer therapy trials employing level-of-evidence-1 disease forecast cancer biomarkers uPA and its inhibitor PAI-1. *Expert Rev Mol Diagn* 11 (6) (2011), 617-634.

Simon R, Mirlacher M, Sauter G. Tissue microarrays. *Methods Mol Med* 114 (2005), 257-268.

Stephens RW, Brünner N, Jänicke F, Schmitt M. The urokinase plasminogen activator system as a target for prognostic studies in breast cancer. *Breast Cancer Res Treat* 52 (1998), 99-111.

Sternlicht MD, Dunning AM, Moore DH, Pharoah PDP, Ginzinger DG, Chin K, Gray JW, Waldman FM, Ponder BAJ, Werb Z. Prognostic value of PAI-1 in invasive breast cancer: Evidence that tumor-specific factors are more important than genetic variation in regulating PAI-1 expression. *Cancer Epidemiol Biomarkers Prev* 15 (2006), 2107-2114.

Sturgeon CM, Duffy MJ, Stenman UH, Lilja H, Brünner N, Chan DW, Babaian R, Bast RC Jr, Dowell B, Esteva FJ, Haglund C, Harbeck N, Hayes DF, Holten-Andersen M, Klee GG, Lamerz R, Looijenga LH, Molina R, Nielsen HJ, Rittenhouse H, Semjonow A, Shih le- M, Sibley P, Sölétormos G, Stephan C, Sokoll L, Hoffman BR, Diamandis EP. National academy of clinical biochemistry laboratory Medicine practice guidelines for use of tumor markers in testicular, prostate, colorectal, breast, and ovarian cancers. *Clin Chem* 54(12) (2008), e11-e79.

Sparano JA. TAILORx: trial assigning individualized options for treatment (Rx). *Clin Breast Cancer* 7(4) (2006), 347-350.

Spyratos F, Bouchet C, Tozlu S, Labroquere M, Vignaud S, Becette V, Lidereau R, Bieche I: Prognostic value of uPA, PAI-1 and PAI-2 mRNA expression in primary breast cancer. *Anticancer Res* 22 (2002), 2997-3003.

Sumiyoshi K, Baba S, Sakaguchi S, Urano T, Takada Y, Takada A. Increase in levels of plasminogen activator and type-1 plasminogen activator inhibitor in human breast cancer: Possible roles in tumor progression and metastasis. *Thromb Res* 63 (1991), 59-71.

Tavasseo'li FA, Devillee P. Pathology and genetics. Tumours of the breast and female genital organs, IARC WHO Classification of Tumour (2003), 10-111.

Têtu B, Brisson J, Wang CS, Lapointe H, Beaudry G, Blanchette C. Expression of cathepsin D, stromelysin-3, and urokinase by reactive stromal cells on breast carcinoma prognosis. *Cancer* 92 (2001), 2957-2964.

Umeda T, Eguchi Y, Okino K, Kodama M, Hattori T. Cellular localization of urokinase-type plasminogen activator, its inhibitors, and their mRNAs in breast cancer tissues. *J Pathol* 183 (1997), 388-397.

Urban P, Vuaroqueaux V, Labuhn M, Delorenzi M, Wirapati P, Wight E, Senn HJ, Benz C, Eppenberger U, Eppenberger-Castori S. Increased expression of urokinase-type plasminogen activator mRNA determines adverse prognosis in erbB2-positive primary breast cancer. *J Clin Oncol* 24 (2006), 4245-4253.

Van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AAM, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH,

Bernards R. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347 (2002), 1999-2009.

van't Veer LJ, Dai H, van de Vijver MJ, Dai H, van de Vijver MJ, He YD *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 6 (2002), 415-530.

Visscher DW, Sarkar F, LoRusso P, Sakr W, Ottosen S, Wykes S, Crissman JD. Immunohistologic evaluation of invasion-associated proteases in breast carcinoma. *Mod Pathol* 6 (1993), 302-306.

Visscher DW, DeMattia F, Ottosen S, Sarkar FH, Crissman JD. Biologic and clinical significance of basic fibroblast growth factor immunostaining in breast carcinoma. *Mod Pathol* 8 (1995), 665-670.

Zemzoum I, Kates RE, Ross JS, Dettmar P, Dutta M, Henrichs C, Yurdseven S, Höfler H, Kiechle M, Schmitt M, Harbeck N. Invasion factors uPA/PAI-1 and HER2 status provide independent and complementary information on patient outcome in node-negative breast cancer. *J Clin Oncol* 21 (2003), 1022-1028.

Zhao H, Morimoto T, Sasa M, Tanaka T, Izumi K. Immunohistochemical expression of uPA, PAI-1, cathepsin D and apoptotic cells in ductal carcinoma in situ of the breast. *Breast Cancer* 9 (2002), 118-126.

Zipori D. Stromal cells in tumor growth and regression. *Cancer J* 3 (1990), 164-169.

Websites

American Diagnostica	www.american-diagnostica.de
Dako	www.dako.com
Manual Mammakarzinome 2009	www.tumorzentrum-muenchen.de
Robert-Koch Institute	www.rki.de
Tissue microarray construction	http://www.yalepath.org/YPTS/TMA.htm
Ventana	www.ventanamed.com
Wilex AG	www.wilex.de

9. Appendix

9.1 TMN staging system for breast cancer

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget's)	Paget's disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma associated with Paget's disease are categorized based on the size and characteristic of the parenchymal disease, although the presence of Paget's disease should still be noted.
T1	Tumor ≤ 20mm in greatest dimension
T1mi	Tumor ≤ 1mm in greatest dimension
T1a	Tumor > 1mm but ≤ 5mm in greatest dimension
T1b	Tumor > 5mm but ≤10mm in greatest dimension
T1c	Tumor > 10mm but ≤ 20mm in greatest dimension
T2	Tumor > 20mm but ≤ 50mm in greatest dimension
T3	Tumor > 50mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or the skin (ulceration or skin nodules)
T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma

Regional lymph nodes (N)	
pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastases identified histologically
pN1mi	Micrometastases (greater than 0.2 mm and/or more than 200 cells, but non greater than 2.0 mm)
pN1a	Metastases in 1-3 axillary lymph nodes, at least one metastasis greater than 2.0 mm
pN1b	Metastases in internal mammary nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN1c	Metastasis in 1-3 axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
N2a	Metastases in 4-9 axillary lymph nodes (at least one tumor deposit greater than 2.0 mm)
N2b	Metastases in clinically detected internal mammary lymph nodes in the

	absence of axillary lymph nodes metastases
N3a	Metastases in 10 or more axillary lymph nodes (at least one tumor deposit greater than 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
N3b	Metastasis in clinically detected ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
N3c	Metastasis in ipsilateral supraclavicular lymph nodes

Distant Metastasis (M)	
M0	No clinical or radiographic evidence of distant metastases
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2mm

Taken from American Joint Committee on Cancer, AJCC Cancer Staging Manual, Springer, 7th edition, 345-376 (2009).

9.2 UICC/AJCC breast cancer stadium

UICC/ AJCC Breast Cancer Stadium			
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0, T1	N1mi	M0
Stage IIA	T0, T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0, T1, T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	N0, N1, N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Taken from American Joint Committee on Cancer, AJCC Cancer Staging Manual, Springer, 7th edition, 345-376 (2009).

9.3 Histologic grade

GX	Grade cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Taken from American Joint Committee on Cancer, AJCC Cancer Staging Manual, Springer, 7th edition, 345-376 (2009).

9.4 WHO histological classification of tumors of the breast

Epithelial tumors

- Invasive breast carcinoma
  - Invasive ductal carcinoma
  - Invasive lobular carcinoma
  - Tubular carcinoma
  - Invasive cribriform carcinoma
  - Medullary carcinoma
  - Mucinous carcinoma
  - Neuroendocrine tumors
  - Invasive papillary carcinomas
  - Invasive micropapillary carcinomas
  - Apocrine carcinoma
  - Metaplastic carcinoma
  - Lipid-rich carcinoma
  - Secretory carcinoma
  - Oncocytic carcinoma
  - Adenoid cystic carcinoma
  - Acinic cell carcinoma
  - Glycogen-rich clear cell carcinoma
  - Sebaceous carcinoma
  - Inflammatory carcinoma

- Precursor lesions
  - Lobular neoplasia
    - Lobular carcinoma in situ
  - Intraductal proliferative lesions
    - Usual ductal hyperplasia
    - Flat epithelial atypia
    - Atypical papilloma
    - Ductal carcinoma in situ
  - Microinvasive carcinoma
  - Intraductal papillary neoplasms
    - Central papilloma
    - Peripheral papilloma
    - Atypical papilloma
    - Intraductal papillary carcinoma
    - Intracystic papillary carcinoma

- Benign epithelial proliferations
  - Adenosis including variants
  - Radial scar/ complex sclerosing lesion
  - Adenomas
    - Tubular adenoma
    - Lactating adenoma
    - Apocrine adenoma
    - Pleomorphic adenoma
    - Ductal adenoma

Myoepithelial lesions

- Myoepitheliosis
- Adenomyoepithelial adenosis
- Adenomyoepithelioma
- Malignant myoepithelioma

Mesenchymal tumors

- Haemangioma
- Angiomatosis
- Haemangiopericytoma
- Pseudoangiomatous strom. hyperplasia
- Myofibroblastoma
- Fibromatosis (aggressive)
- Inflammatory myofibroblastic tumor
- Lipoma
- Granular cell tumor
- Neurofibroma
- Schwannoma
- Angiosarcoma
- Liposarcoma
- Rhabdomyosarcoma
- Osteosarcoma
- Leiomyoma
- Leiomyosarcoma

Fibroepithelial tumors

- Fibroadenoma
- Phyllodes tumor
- Periductal stromal sarcoma, low grade
- Mammary hamartoma

Tumors of the nipple

- Nipple adenoma
- Syringomatous adenoma
- Paget disease of the nipple

Malignant lymphoma

- Diffuse large B-cell lymphoma
- Burkitt lymphoma
- Extranodal marginal-zone B-cell lymphoma of MALT type
- Follicular lymphoma

Metastatic tumors

Tumors of the male breast

Taken and modified from *Tavasseo'li et al. 2003*.

9.5 Definition of risk categories for patients with operated breast cancer and treatment allocation by therapeutic target and risk categories

HER2 gene overexpression and/or amplified		HER2-negative					HER2-positive				
Endocrine responsiveness		**Highly responsive**		**Incompletely responsive**		**Non-responsive**	**Highly responsive**		**Incompletely responsive**		**Non-responsive**
Menopausal status		**pre**	**post**	**pre**	**post**	**pre and post**	**pre**	**post**	**pre**	**post**	**pre and post**
Low	**Node-negative AND** **all** of the following features: pT ≤ 2 cm, Grade 1, no vascular invasion, HER2-negative, ER and/or PgR expressed, and age ≥ 35 years	E	E	E	E						
Intermediate Risk category	**Node-negative AND** **at least one** of the following features: pT > 2 cm, Grade 2-3, vascular invasion, ER and PgR absent, HER2-positive, age < 35 years	E C→E	E C→E	C→E E	C→E E	C	C→E +Tr	C→E +Tr	C→E +Tr	C→E +Tr	C +Tr
	Node-positive (1-3) AND ER and/or PgR expressed, AND HER2-negative	E C→E	E C→E	C→E E	C→E E						
High	**Node-positive (1-3) AND** ER and PgR absent, OR HER2-positive					C	C→E +Tr	C→E +Tr	C→E +Tr	C→E +Tr	C +Tr
	Node-positive (>4)	C→E	C→E	C→E	C→E	C	C→E +Tr	C→E +Tr	C→E +Tr	C→E +Tr	C +Tr

Treatment options in each cell are listed in the order of preference. C: chemotherapy; E: endocrine therapy (selected according to menopausal status); Tr: Trastuzumab.

Taken from *Goldhirsch et al. 2007*

9.6 Levels of evidence for grading clinical utility of tumor markers

Level	Type of evidence
I	Evidence from a single, high-powered, prospective, controlled study that is specifically designed to test marker or evidence from meta-analysis and/or overview of level II or III studies. In the former case, the study must be designed so that therapy and follow-up are dictated by protocol. Ideally, the study is a prospective, controlled randomized trial in which diagnostic and/or therapeutic clinical decisions in one arm are determined at least in part on the basis of marker results, and diagnostic and/or therapeutic clinical decisions in the control arm are made independently of makers results. However, study design may also include prospective but not randomized trials with marker data and clinical outcome as primary objective.
II	Evidence from study in which maker data are determined in relationship to prospective therapeutic trial that is performed to test therapeutic hypothesis but not specifically designed to test marker utility (i.e., marker study is secondary objective of protocol). However, specimen collection for marker study and statistical analysis are prospectively determined in protocol as secondary objectives.
III	Evidence from large but retrospective studies from which available numbers of samples are available or selected. Therapeutic aspects and follow-up of patient population may or may not have been prospectively dictated. Statistical analysis for tumor marker was not dictated prospectively at time of therapeutic trial design.
IV	Evidence from small retrospective studies that do not have propectively dictated therapy, follow-up, specimen selection, or statistical analysis. Study design may use matched case-controls, etc.
V	Evidence from small pilot studies designed to determine or estimate distribution of marker levels in sample population. Study design may include “correlation” with other known or investigational markers of outcome but is not designed to determine clinical utility.

Taken from *Hayes et al. 1996.*

9.7 Scoring results of core biopsies and respective primary tumors

A anti-uPA mAb #3689, n=10

Core biopsy

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	8	3	10	6
0.5	0	1	0	1
1	2	3	0	3
1.5	0	1	0	0
2	0	1	0	0
2.5	0	1	0	0
3	0	0	0	0

Primary tumor

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	7	1	10	1
0.5	0	0	0	1
1	2	5	0	8
1.5	0	1	0	0
2	1	3	0	0
2.5	0	0	0	0
3	0	0	0	0

B anti-PAI-1 mAb #3785, n=10

Core biopsy

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	6	1	10	3
0.5	0	0	0	0
1	4	3	0	7
1.5	0	2	0	0
2	0	3	0	0
2.5	0	1	0	0
3	0	0	0	0

Primary tumor

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	5	1	10	2
0.5	0	0	0	0
1	5	3	0	8
1.5	0	0	0	0
2	0	6	0	0
2.5	0	0	0	0
3	0	0	0	0

C anti-PAI-1 mAb #3786, n=10

Core biopsy

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	4	0	10	2
0.5	0	0	0	0
1	6	3	0	8
1.5	0	2	0	0
2	0	1	0	0
2.5	0	3	0	0
3	0	1	0	0

Primary tumor

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	4	0	10	0
0.5	0	0	0	0
1	6	2	0	10
1.5	0	2	0	0
2	0	4	0	0
2.5	0	2	0	0
3	0	0	0	0

D anti-PAI-1 mAb #ADG25, n=10

Core biopsy

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	6	0	10	1
0.5	0	0	0	0
1	4	5	0	9
1.5	0	1	0	0
2	0	4	0	0
2.5	0	0	0	0
3	0	0	0	0

Primary tumor

	Tumor cells nucleus	Tumor cells cytoplasm	Tumor stroma cellular	Tumor stroma non-cellular
0	6	0	10	2
0.5	0	2	0	0
1	4	1	0	8
1.5	0	3	0	0
2	0	3	0	0
2.5	0	1	0	0
3	0	0	0	0

9.8 Association of high uPA/PAI-1 expression in four different tumor areas with clinical and pathological factors

A Association of high uPA/PAI-1 expression in the cytoplasm of the tumor cells with clinical and pathological factors using Pearson's chi-square test. P is significant when <0.05; n=210. Statistically significant results are printed in bold.

		uPA high (IHC > 1.0)		**PAI-1 high #3785 (IHC > 1.0)**		**PAI-1 high #3786 (IHC > 1.0)**		**PAI-1 high #ADG25 (IHC > 1.0)**	
Parameter category	**n**	**n (%)**	**p-value**	**n (%)**	**p- value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Age (yrs)									
< 50	41	33 (80.5)	0.558	**8 (19.5)**	**<0.001**	**6 (14.6)**	**<0.001**	34 (82.9)	0.077
≥ 50	168	128 (76.2)		**102 (60.7)**		**111 (66.1)**		117 (69.2)	
Menopausal status									
Premenopausal	42	35 (83.3)	0.285	**9 (21.4)**	**<0.001**	**7 (16.7)**	**<0.001**	36 (85.7)	0.026
Postmenopausal	168	127 (75.6)		**102 (60.7)**		**110 (65.5)**		115 (68.5)	
Tumor size (cm)									
< 2 (x)	49	34 (69.4)	0.140	24 (49.0)	0.535	25 (51.0)	0.450	33 (67.3)	0.418
≥ 2 (x)	161	128 (79.5)		87 (54.0)		92 (57.1)		118 (73.3)	
pT									
1	54	37 (68.5)	0.206	25 (46.3)	0.117	**26 (48.1)**	**0.006**	35 (64.8)	0.549
2	101	84 (83.2)		52 (51.5)		**56 (55.4)**		76 (75.2)	
3	23	17 (73.9)		11 (47.8)		**9 (39.1)**		16 (69.6)	
4	32	24 (75.0)		23 (71.9)		**26 (81.3)**		24 (75.0)	
Lymph node status									
Negative (pN0)	54	**35 (64.8)**	**0.012**	**22 (40.7)**	**0.039**	29 (53.7)	0.730	38 (70.3)	0.771
Positive (pN1, pN2)	156	**127 (81.4)**		**89 (57.1)**		88 (56.4)		113 (72.4)	
Stadium UICC									
I	23	14 (60.9)	0.141	**8 (34.8)**	**0.030**	11 (47.8)	0.118	14 (60.9)	0.411
II	122	96 (78.7)		**61 (63.5)**		63 (51.6)		88 (72.1)	
III	65	52 (80.0)		**42 (64.6)**		43 (66.2)		49 (75.4)	
Grading									
1	7	4 (57.1)	0.070	3 (42.9)	0.840	4 (57.1)	0.906	4 (57.1)	0.060
2	105	76 (72.4)		55 (52.4)		60 (57.1)		69 (65.7)	
3	98	82 (83.7)		53 (54.1)		53 (54.1)		78 (79.6)	
Steroid hormone receptor status									
Positive	196	151 (77.0)	0.895	**110 (56.1)**	**<0.001**	**116 (59.2)**	**<0.001**	138 (70.4)	0.071
Negative	14	11 (78.6)		**1 (7.1)**		**1 (1.7)**		13 (92.9)	
HER2 status									
Positive	21	17 (81.0)	0.828	14 (66.7)	0.357	13 (61.9)	0.845	15 (71.4)	0.877
Negative	104	82 (78.8)		58 (55.8)		62 (59.6)		76 (73.1)	
Overall survival									
< Median 105.5 months	105	82 (78.1)	0.742	60 (57.1)	0.213	**66 (62.9)**	**0.037**	74 (70.5)	0.645
≥ Median 105.5 months	105	80 (76.2)		51 (48.6)		**51 (48.6)**		77 (73.3)	
Disease-free survival									
< Median 91.5	104	81 (77.9)	0.868	58 (55.8)	0.331	62 (59.6)	0.209	70 (67.3)	0.122
≥ Median 91.5	104	80 (76.9)		51 (49.0)		53 (51.0)		80 (76.9)	
Not available	2								
Progression-free survival									
< Median 21 months	40	34 (85.0)	0.762	21 (52.5)	0.653	23 (57.5)	0.648	31 (77.5)	0.762
≥ Median 21 months	40	33 (82.5)		23 (57.5)		25 (62.5)		30 (75.0)	
Recurrences									
No	129	95 (73.6)	0.157	66 (51.2)	0.472	68 (52.7)	0.505	90 (69.8)	0.685
Local	16	15 (93.8)		7 (43.8)		9 (56.3)		12 (75.0)	
Distant	65	52 (80.0)		38 (58.5)		40 (61.5)		49 (75.4)	
Deaths									
No	109	83 (76.1)	0.837	53 (48.6)	0.407	55 (50.5)	0.263	76 (69.7)	0.762
Breast cancer caused deaths	73	58 (79.4)		41 (56.2)		44 (60.3)		54 (74.0)	
Other caused deaths	28	21 (75.0)		17 (60.7)		18 (64.3)		21 (75.0)	

B Association of high uPA/PAI-1 expression in the cells of the tumor stroma with clinical and pathological factors using Pearson's chi-square test. P is significant when <0.05; n=210. Statistically significant results are printed in bold.

		uPA high (IHC ≥ 1.0)		**PAI-1 high #3785 (IHC ≥ 1.0)**		**PAI-1 high #3786 (IHC ≥ 1.0)**		**PAI-1 high #ADG25 (IHC ≥ 1.0)**	
Parameter category	**n**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p- value**
Age (yrs)									
< 50	41	14 (34.1)	0.071	0 (0.0)	0.066	**0 (0.0)**	**0.001**	**31 (75.6)**	**<0.001**
≥ 50	168	35 (20.8)		13 (77.4)		**37 (22.0)**		**51 (30.4)**	
Menopausal status									
Premenopausal	42	14 (33.3)	0.087	0 (0.0)	0.063	**0 (0.0)**	**0.001**	**32 (76.2)**	**<0.001**
Postmenopausal	168	35 (20.8)		13 (77.4)		**37 (22.0)**		**51 (30.4)**	
Tumor size (cm)									
< 2 (x)	49	12 (24.5)	0.827	3 (6.1)	0.982	12 (24.5)	0.149	21 (42.9)	0.586
≥ 2 (x)	161	37 (23.0)		10 (6.2)		25 (15.5)		62 (38.5)	
pT									
1	54	14 (25.9)	0.398	5 (9.3)	0.591	13 (24.1)	0.475	**25 (46.3)**	**<0.001**
2	101	24 (23.8)		5 (5.0)		17 (16.8)		**49 (48.5)**	
3	23	7 (30.4)		2 (8.7)		3 (13.0)		**7 (30.4)**	
4	32	4 (12.5)		1 (3.1)		4 (12.5)		**2 (6.3)**	
Lymph node status									
Negative (pN0)	54	16 (29.6)	0.204	9 (16.7)	**<0.001**	**23 (42.6)**	**<0.001**	**29 (53.7)**	**0.013**
Positive (pN1, pN2)	156	33 (21.2)		4 (2.6)		**14 (9.0)**		**54 (34.6)**	
Stadium UICC									**<0.001**
I	23	5 (21.7)	0.694	**5 (21.7)**	**0.005**	**9 (39.1)**	**0.005**	**13 (56.5)**	
II	122	31 (25.4)		**5 (4.1)**		**22 (18.0)**		**57 (46.7)**	
III	65	13 (20.0)		**3 (4.6)**		**6 (9.2)**		**13 (20.0)**	
Grading									
1	7	1 (14.3)	0.846	0 (0.0)	0.720	1 (14.3)	0.136	1 (14.3)	0.114
2	105	25 (23.8)		6 (5.7)		24 (22.9)		37 (35.2)	
3	98	23 (23.5)		7 (7.1)		12 (12.2)		45 (45.9)	
Steroid hormone receptor status									
Positive	196	45 (23.0)	0.631	13 (6.6)	0.320	37 (18.9)	0.073	**71 (36.2)**	**<0.001**
negative	14	4 (28.6)		0 (0.0)		0 (0.0)		**12 (85.7)**	
HER2 status									
Positive	21	3 (14.3)	0.193	1 (4.8)	0.993	3 (14.3)	0.724	8 (38.1)	0.893
Negative	104	29 (27.9)		5 (4.8)		12 (11.5)		38 (36.5)	
Overall survival									
< Median 105.5 months	105	**18 (17.1)**	**0.034**	**3 (2.9)**	**0.045**	14 (13.3)	0.103	**27 (25.7)**	**<0.001**
≥ Median 105.5 months	105	**31 (29.5)**		**10 (9.5)**		23 (21.9)		**56 (53.3)**	
Disease-free survival									
< Median 91.5	104	**17 (16.3)**	**0.014**	**3 (2.9)**	**0.045**	**12 (11.5)**	**0.018**	**30 (28.8)**	**0.001**
≥ Median 91.5	104	**32 (30.8)**		**10 (9.6)**		**25 (24.0)**		**53 (51.0)**	
Not available	2								
Progression-free survival									
< Median 21 months	40	8 (20.0)	1.000	3 (7.5)	0.077	1 (2.5)	0.090	13 (32.5)	0.356
≥ Median 21 months	40	8 (20.0)		0 (0.0)		5 (12.5)		17 (42.5)	
Recurrences									
No	129	33 (25.6)	0.535	10 (7.8)	0.392	**32 (24.8)**	**0.002**	54 (41.9)	0.294
Local	16	4 (25.0)		0 (0.0)		**0 (0.0)**		8 (50.0)	
distant	65	12 (18.5)		3 (4.6)		**5 (7.7)**		21 (32.3)	
Deaths									
No deaths	109	29 (26.6)	0.379	9 (8.3)	0.432	**27 (24.8)**	**0.014**	51 (46.8)	0.076
Breast cancer caused deaths	73	13 (17.8)		3 (4.1)		**6 (8.2)**		24 (32.9)	
Other caused deaths	28	7 (25.0)		1 (3.6)		**4 (14.3)**		3 (10.7)	

C Association of high uPA/PAI-1 expression in the nuclei of the tumor cells with other clinical and pathological factors using Pearson's chi-square test. P is significant when <0.05; n=210. Statistically significant results are printed in bold.

		uPA high (IHC ≥ 1.0)		**PAI-1 high #3785 (IHC ≥ 1.0)**		**PAI-1 high #3786 (IHC ≥ 1.0)**		**PAI-1 high #ADG25 (IHC ≥ 1.0)**	
Parameter category	**n**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Age (yrs)									
< 50	41	30 (73.2)	0.348	**2 (4.9)**	**<0.001**	**3 (7.3)**	**<0.001**	32 (78.0)	0.081
≥ 50	168	110 (65.5)		**78 (46.4)**		**101 (60.1)**		107 (63.7)	
Menopausal status									
Premenopausal	42	32 (76.2)	0.163	**3 (7.1)**	**<0.001**	**4 (9.5)**	**<0.001**	**34 (81.0)**	**0.028**
Postmenopausal	168	109 (64.9)		**77 (45.8)**		**100 (59.5)**		**106 (63.1)**	
Tumor size (cm)									
< 2 (x)	49	29 (59.2)	0.176	16 (32.7)	0.370	22 (44.9)	0.460	30 (61.2)	0.356
≥ 2 (x)	161	112 (69.6)		64 (39.8)		82 (50.9)		110 (68.3)	
pT									
1	54	32 (59.3)	0.445	17 (31.5)	0.075	23 (42.6)	0.050	33 (61.1)	0.574
2	101	70 (69.3)		39 (38.6)		51 (50.5)		72 (71.3)	
3	23	15 (65.2)		6 (26.1)		8 (34.8)		15 (65.2)	
4	32	24 (75.0)		18 (56.3)		22 (68.8)		20 (62.5)	
Lymph node status									
Negative (pN0)	54	28 (51.9)	0.006	16 (29.6)	0.137	27 (50.0)	0.935	36 (66.7)	1.000
Positive (pN1, pN2)	156	113 (72.4)		64 (41.0)		77 (49.4)		104 (66.7)	
Stadium UICC									
I	23	11 (47.8)	0.074	6 (26.1)	0.182	11 (47.8)	0.352	15 (65.2)	0.977
II	122	82 (67.2)		44 (36.1)		56 (45.9)		82 (67.2)	
III	65	48 (73.8)		30 (46.2)		37 (56.9)		43 (66.2)	
Grading									
1	7	3 (42.9)	0.062	2 (28.6)	0.648	4 (57.1)	0.307	3 (42.9)	0.276
2	105	65 (61.9)		43 (41.0)		57 (54.3)		68 (64.8)	
3	98	73 (74.5)		35 (35.7)		43 (43.9)		69 (70.4)	
Steroid hormone receptor status									
Positive	196	131 (66.8)	0.724	**80 (40.8)**	**0.002**	**104 (53.1)**	**<0.001**	129 (65.8)	0.328
negative	14	10 (71.4)		**0 (0.0)**		**0 (0.0)**		11 (78.6)	
HER2 status									
Positive	21	14 (66.7)	0.429	8 (38.1)	0.662	9 (42.9)	0.448	14 (66.7)	0.749
Negative	104	78 (75.0)		45 (43.3)		54 (51.9)		73 (70.2)	
Overall survival									
< Median 105.5 months	105	71 (67.6)	0.883	45 (42.9)	0.155	57 (54.3)	0.168	65 (61.9)	0.143
≥ Median 105.5 months	105	70 (66.7)		35 (33.3)		47 (44.8)		75 (71.4)	
Disease-free survival									
< Median 91.5	104	70 (67.3)	0.883	42 (40.4)	0.390	55 (52.9)	0.267	64 (61.5)	0.142
≥ Median 91.5	104	69 (66.3)		36 (34.6)		47 (45.2)		74 (71.2)	
Not available	2								
Progression-free survival									
< Median 21 months	40	28 (70.0)	0.617	14 (35.0)	0.644	21 (52.5)	0.823	27 (67.5)	0.459
≥ Median 21 months	40	30 (75.0)		16 (40.0)		22 (55.0)		30 (75.0)	
Recurrences									
No	129	82 (63.6)	0.371	49 (38.0)	0.811	60 (46.5)	0.537	82 (63.6)	0.461
Local	16	12 (75.0)		5 (31.3)		9 (56.3)		12 (75.0)	
distant	65	47 (72.3)		26 (40.0)		35 (53.8)		46 (70.8)	
Deaths									
No deaths	109	72 (66.1)	0.567	39 (36.8)	0.741	48 (44.0)	0.250	71 (65.1)	0.536
Breast cancer caused deaths	73	52 (71.2)		29 (39.7)		40 (54.8)		52 (71.2)	
Other caused deaths	28	17 (60.7)		12 (42.9)		16 (57.1)		17 (60.7)	

D Association of high uPA/PAI-1 expression in the non-cellular part of the tumor stroma with clinical and pathological factors using Pearson's chi-square test. P is significant when <0.05; n=210. Statistically significant results are printed in bold.

		uPA high (IHC ≥ 1.0)		**PAI-1 high #3785 (IHC ≥ 1.0)**		**PAI-1 high #3786 (IHC ≥ 1.0)**		**PAI-1 high #ADG25 (IHC ≥ 1.0)**	
Parameter category	**n**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**	**n (%)**	**p-value**
Age (yrs)									
< 50	41	29 (70.7)	0.834	**18 (43.9)**	**0.014**	**15 (36.6)**	**0.005**	32 (78.0)	0.286
≥ 50	168	116 (69.0)		**109 (64.9)**		**102 (60.7)**		117 (69.6)	
Menopausal status									
Premenopausal	42	29 (69.0)	0.940	**18 (42.9)**	**0.009**	**15 (35.7)**	**0.004**	33 (78.6)	0.252
Postmenopausal	168	117 (69.6)		**109 (64.9)**		**102 (60.7)**		117 (69.9)	
Tumor size (cm)									
< 2 (x)	49	36 (73.5)	0.493	34 (69.4)	0.145	**35 (71.4)**	**0.011**	39 (79.6)	0.149
≥ 2 (x)	161	110 (68.3)		93 (57.8)		**82 (50.9)**		111 (68.9)	
pT									
1	54	39 (72.2)	0.193	36 (66.7)	0.474	34 (63.0)	0.485	41 (75.9)	0.602
2	101	75 (74.3)		60 (59.4)		51 (50.5)		73 (72.3)	
3	23	14 (60.9)		11 (47.8)		13 (56.5)		16 (69.6)	
4	32	18 (56.3)		20 (62.5)		19 (59.4)		20 (62.5)	
Lymph node status									
Negative (pN0)	54	48 (88.9)	<0.001	34 (63.0)	0.665	**41 (85.4)**	**0.001**	**51 (94.4)**	**<0.001**
Positive (pN1, pN2)	156	98 (62.8)		93 (59.6)		**76 (48.7)**		**99 (63.5)**	
Stadium UICC									
I	23	22 (95.7)	0.009	18 (78.3)	0.170	**20 (87.0)**	**0.005**	**22 (95.7)**	**0.024**
II	122	84 (68.9)		70 (57.4)		**61 (50.0)**		**84 (68.9)**	
III	65	40 (61.5)		39 (60.0)		**36 (55.4)**		**44 (67.7)**	
Grading									
1	7	5 (71.4)	0.666	4 (57.1)	0.977	5 (71.4)	0.249	4 (57.1)	0.383
2	105	70 (66.7)		64 (61.0)		63 (60.0)		72 (68.6)	
3	98	71 (72.4)		59 (60.2)		49 (50.0)		74 (75.5)	
Steroid hormone receptor status									
Positive	196	133 (67.9)	0.050	120 (61.2)	0.407	111 (56.6)	0.316	**136 (69.4)**	**0.014**
negative	14	13 (92.9)		7 (50.0)		6 (42.9)		**14 (100.0)**	
HER2 status									
Positive	21	14 (66.7)	0.658	11 (52.4)	0.654	13 (61.9)	0.084	15 (71.4)	0.391
Negative	104	64 (61.5)		60 (57.7)		43 (41.3)		64 (61.5)	
Overall survival									
< Median 105.5 months	105	67 (68.8)	0.072	63 (60.0)	0.888	63 (60.0)	0.211	69 (65.7)	0.067
≥ Median 105.5 months	105	79 (75.2)		64 (61.0)		54 (51.4)		81 (77.1)	
Disease-free survival									
< Median 91.5	104	65 (62.5)	0.035	65 (62.5)	0.479	61 (58.7)	0.402	69 (66.3)	0.126
≥ Median 91.5	104	79 (76.0)		60 (57.7)		55 (52.9)		79 (76.0)	
Not available	2								
Progression-free survival									
< Median 21 months	40	22 (55.0)	0.104	20 (50.0)	0.112	21 (52.5)	0.366	27 (67.5)	0.626
≥ Median 21 months	40	29 (72.5)		27 (67.5)		25 (62.5)		29 (72.5)	
Recurrences									
No	129	94 (72.9)	0.307	79 (61.2)	0.924	71 (55.0)	0.254	93 (72.1)	0.952
Local	16	9 (56.3)		9 (56.3)		12 (18.5)		11 (68.8)	
distant	65	43 (66.2)		39 (60.0)		34 (52.3)		46 (70.8)	
Deaths									
No deaths	109	78 (71.6)	0.799	63 (57.8)	0.109	63 (57.8)	0.555	77 (70.6)	0.527
Breast cancer caused deaths	73	49 (67.1)		42 (57.5)		41 (56.2)		55 (75.3)	
Other caused deaths	28	19 (67.9)		22 (78.6)		13 (46.4)		18 (64.3)	

10. Acknowledgement

I would like to express my gratitude to:
Prof. Dr. med. Marion Kiechle for the opportunity to accomplish this thesis at the Department of Obstetrics and Gynecology of the Klinikum rechts der Isar, Technical University of Munich.
My doctoral father, Prof. Dr. rer. nat. Dr. med. habil. Manfred Schmitt for his great support, interesting discussions and his patience in guiding the thesis to its end.
Dr. med. Julia Dorn for helpful advices and for the time spending on critical reading my manuscript.
All members of the Clinical Research Unit, namely Dr. rer. nat. Rudolf Napieralski, Lina Seiz, Karin Mengele, Daniela Hellmann, Rosi Bräuer, Anita Welk, and Claudia Beutner for instructing and supporting me during my work and for providing a pleasant working atmosphere.
Peter Strzelczyk, laboratory technician at the routine lab of the Institute of Pathology for his kind help working with the automated staining instruments, even at the weekend.
Dr. med. Julia Slotta-Huspenina of the Institute of Pathology of the Technical University of Munich for excellent cooperation and for scoring numerous stained slides.
Dr. med. Corinna Propping for her help with the follow-up.
Dr. rer. nat. Viktoria Kehl of the Institute for Medical Statistics and Epidemiology, Technical University of Munich, for assistance in statistics.
Dr. Erk Mennenga-Klopp and Dipl.-Phys. Frank Olsenberg for the possibility to use the Nanozoomer in Herrsching am Ammersee and their kind assistance during scanning process.

Finally, I like to cordially thank my family and friends for their constant, infinite encouragement and support.