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Quantification of Urokinase Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor Type 1 (PAI-1) mRNA in Breast Cancer Tissue

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Index

1 INTRODUCTION

1.1 Summary

Urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) play a key role in tumor-associated processes such as the degradation of extracellular matrix proteins, tissue remodeling, cell adhesion, migration, and invasion. High antigen levels of uPA and PAI-1 in tumor tissue of various solid malignant tumors, including breast cancer, are associated with poor patient prognosis. This work primarily examines whether analysis of uPA and PAI-1 mRNA expression in breast cancer represents an alternative to the measurement of the respective antigen levels. Highly sensitive quantitative real-time PCR (QPCR) assays, based on the LightCycler technology, were established to quantify uPA and PAI-1 mRNA expression in different cell lines as well as in tumor tissue of breast cancer patients. The mRNA concentrations were normalized to the housekeeping genes G6PDH, ß-actin, or PBGD (thereby evaluating their applicability for QPCR assays). The respective uPA and PAI-1 antigen concentrations were determined by established ELISA formats. In the cell lines, uPA and PAI-1 mRNA and antigen values were highly correlated. In contrast, correlations between uPA/PAI-1 mRNA and protein in the breast cancer samples were found to be distinctly weaker or not significant. Thus, quantitative determination of mRNA expression for both factors does not mirror exactly antigen levels in breast cancer tissue. Except for nodal status being inversely correlated with uPA mRNA levels in our Dutch cohort, no significant interrelations were observed between uPA or PAI-1 mRNA expression and clinicopathological parameters when using G6PDH as housekeeping gene. On the protein level, elevated uPA and PAI-1 values were associated with a negative steroid hormone receptor status in the Dutch cohort. Summing up, the implementation of mRNA quantification of uPA and PAI-1 in breast tumors is unable to serve as a one-to-one substitution for antigen determination by ELISA. Furthermore, mRNA was extracted from 28 formalin-fixed, paraffin-embedded tissue samples with existing corresponding fresh-frozen tissue extracts. uPA and PAI-1 mRNA levels were successfully quantified in these samples. Normalized to G6PDH, the values determined by both methods correlated significantly.

1.2 Background: Breast Cancer Epidemiology

The mammary carcinoma is the most frequent malignant tumor of women in the western countries (about 28% of female malignancies, followed by lung and bowel cancer), and, according to the American cancer statistics of 2008, behind lung cancer the second most frequent cause of death from cancer in the USA [181]. In Germany, breast cancer has the highest mortality, followed by bowel and lung cancer. Due to the present lack of a comparable nationwide registration of cancer incidence in Germany, incidence numbers have to be estimated based on regional register data. Calculations indicate that more than 57,000 women are diagnosed with breast cancer every year. The mean onset age is 63 years, 6 years earlier than all combined malignancies [201]. In an analysis of cancer mortality data of 50 countries in 2002, Germany showed to have the ninth highest breast cancer death rate in females, Denmark and the Netherlands being the countries with the highest mortality [101]. Approximately every tenth woman develops breast cancer in her lifetime. There are numerous factors increasing the risk of disease; examples are obesity, early menarche and late menopause, advanced age at first pregnancy, nullipara, postmenopausal hormone-replacement therapy, ionizing radiation, and genetic disposition (BRCA1/2-gene mutation). American cancer statistics of 2008 describe a decrease of breast cancer incidence by 3.5% per year from 2001 to 2004 after having increased since 1980. On the one hand, this is attributed to a saturation effect in screening mammography, on the other hand, the decreased use of hormone replacement therapy among postmenopausal women is held responsible for this tendency (Figure 1). According to an analysis of Munich Cancer Registry data, survival rates of patients with metastasized breast cancer have remained stagnant during the last two decades [296]. Nevertheless, estimates based on German statistical resources for the first time reported a slight drop in breast cancer incidence since the middle of the 1990's, supposedly due to a recent decline in hormone therapy and enhanced adjuvant treatment [54,201]. Most women diagnosed with breast cancer nowadays have more therapeutic options and a better chance of long-term survival than ever before. In Germany, an improved nationwide screening system is currently being established for all women between 50 and 69 years of age to further reduce the percentage of metastatic tumors. At present, the 5-year survival rate in Germany is about 81% [201]. A definitive cure of breast cancer - which can only be spoken of between 20 to 40 years after diagnosis - is accomplished in about 50% of all cases.

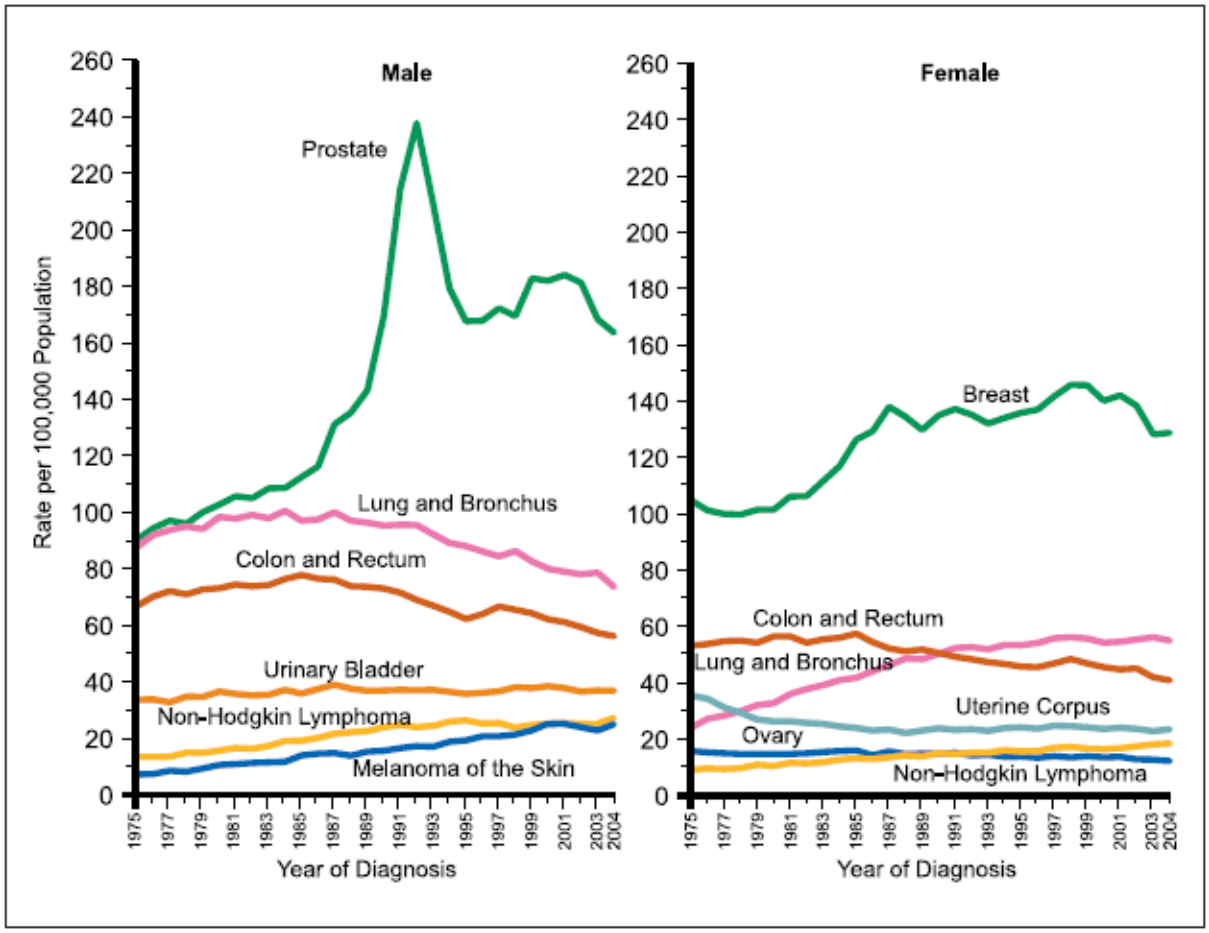


Figure 1: Annual age-adjusted* cancer incidence rates among males and females for selected cancers by sex, United States, 1975 to 2004 (*Rates are age-adjusted to the 2000 US standard population and adjusted for delays in reporting) [181]. Source: Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov). Delay-Adjusted Incidence database: "SEER Incidence Delay-Adjusted Rates, 9 Registries, 1975–2004." National Cancer Institute, DCCPS, Surveillance Research Program, Statistical Research and Applications Branch, released April 2007, based on the November 2006 SEER data submission.

1.3 Breast Cancer Classification and Prognostic Parameters

Clearly, prognosis for patients varies strongly depending on several individual risk factors, for example tumor stage, invasiveness, and nodal involvement. The classical factors used to decide if or which kind of adjuvant therapy is to be administered are lymph node status, tumor size and grade (TNM classification), patient age, menopausal status, and steroid receptor status. Over the last decades, several additional markers have been found that might help estimate the proliferation rate and invasiveness of breast tumors, the most valuable markers being those directly involved in tumor development. Of these to date the serine protease urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) have been shown to be the most promising factors [151,299,348]. In current breast cancer trials, hospitals are already measuring uPA and PAI-1 levels in primary breast cancer tissue by enzyme-linked immunosorbent assay (ELISA) [177]. Both factors have been extensively validated in preclinical and clinical studies. In the NNBC-3 Europe trial, participating centers opt to either perform risk estimation by clinicopathological factors or by the uPA and PAI-1 concentration in tumor tissue biopsies [257]. In fact, uPA and PAI-1 are the first tumor-biological markers to be validated at the highest level of evidence (LOE I) with respect to their clinical utility in breast cancer management [149]. The resulting data is being added to the pool of factors used for therapy decisions.

This section gives an overview of the tumor features that currently play a role for the establishment of individual breast cancer treatment, and presents an outlook on potential further improvement of these schemes by introduction of new tumor biomarkers. Provided that preliminary results are validated by a number of well-designed clinical studies, some of these may soon be analyzed routinely for determination of the optimum therapeutic concept or may even be used as targets for newly developed drugs.

1.3.1 Histopathological Classification

The histological origin of the breast cancer patient's primary tumor is one of the most decisive components of his individual prognosis. The doubling time of the tumor cells, the extension of the tumor - multi-centric or bilateral -, and the general liability of the tumor to metastasize depends on the histological phenotype. These characteristics of a tumor type are influenced by cell differentiation, frequency of mitosis and cell-core structure (see in 'Tumor Grading Criteria' below). The mammary carcinomas derived from ductal or ductular epithelia are diagnosed most frequently with a percentage of 85 to 90. The remaining tumors originate from lobular epithelia, except the very rare and especially aggressive inflammatory carcinoma - characterized by undifferentiated tumor cells and a lymphangiosis carcinomatosa of the skin -, and the mammary sarcoma (incidence less than 1%; Table 1). Mammary carcinomas are divided into non-invasive and invasive tumors. In the past, the non-invasive forms - comprising the ductal carcinoma *in situ* (DCIS) and the carcinoma lobulare *in situ* (CLIS) - which are considered to be early forms of invasive breast cancer were diagnosed only in about 5% of all documented cases. Through extended mammography screenings and the overall improvement of the sensitivity of the applied diagnostic methods it was possible to elevate this rate to approximately 20%. If the tumor has been resected successfully by breast-preserving surgery and the *in situ* nature has been confirmed histopathologically, subsequent radiation of the area and/or tamoxifen therapy (see below) is usually sufficient without axillary dissection [65,94]. In case of a CLIS, radiation may not be necessary. No adjuvant therapy is required, unless the tumor is especially large or displays multifocal growth. While *in situ* carcinomas can only be identified by apparative diagnostic measures, the majority of breast cancers is still discovered by the patients themselves, implicating that the tumor stage is already advanced by the time of diagnosis. The invasive ductal carcinoma is the most frequent breast carcinoma; it also includes various special histological forms with own growth patterns, clinical features, and thus, prognosis. For patients with invasive tumors without infested axillary lymph nodes a good chance of cure does exist, although the applied adjuvant therapy schemes vary widely. The mode and aggressiveness of an adjuvant treatment depends on defined prognostic factors determined in the tumor tissue which will be described in the following, since one essential aim of cancer research is the improvement of methods that allow prediction of unfavorable courses of the disease.

Table 1: WHO classification of breast tumor histopathologies [365]. The table includes benign as well as malignant tumors.

Invasive breast carcinomas

- Invasive ductal carcinoma
  - Most are "not otherwise specified"
  - Remaining subtypes:
    - Mixed type carcinoma
    - Pleomorphic carcinoma
    - Carcinoma with osteoclastic giant cells
    - Carcinoma with choriocarcinomatous features
    - Carcinoma with melanotic features
- Invasive lobular carcinoma
- Tubular carcinoma
- Invasive cribriform carcinoma
- Medullary carcinoma
- Mucinous carcinoma and other tumors with abundant mucin
  - Mucinous carcinoma
  - Cystadenocarcinoma and columnar cell mucinous carcinoma
  - Signet ring cell carcinoma
- Neuroendocrine tumors
  - Solid neuroendocrine carcinoma (carcinoid of the breast)
  - Atypical carcinoid tumor
  - Small cell/oat cell carcinoma
  - Large cell neuroendocrine carcioma
- Invasive papillary carcinoma
- Invasive micropapillary carcinoma
- Apocrine carcinoma
- Metaplastic carcinomas
  - Pure epithelial metaplastic carcinomas
    - Squamous cell carcinoma
    - Adenocarcinoma with spindle cell metaplasia
    - Adenosquamous carcinoma
    - Mucoepidermoid carcinoma
  - Mixed epithelial/mesenchymal metaplastic carcinomas
- Lipid-rich carcinoma
- Secretory carcinoma
- Oncocytic carcinoma
- Adenoid cystic carcinoma
- Acinic cell carcinoma
- Glycogen-rich clear cell carcinoma
- Sebaceous carcinoma
- Inflammatory carcinoma
- Bilateral breast carcinoma

Mesenchymal tumors (including sarcoma)

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

Precursor lesions

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

Benign epithelial lesions

- Adenosis, including variants
  - Sclerosing adenosis
  - Apocrine adenosis
  - Blunt duct adenosis
  - Microglandular adenosis
  - Adenomyoepithelial adenosis
- Radial scar/complex sclerosing lesion
- Adenomas
  - Tubular adenoma
  - Lactating adenoma
  - Apocrine adenoma
  - Pleomorphic adenoma
  - Ductal adenoma

Myoepithelial lesions

- Myoepitheliosis
- Adenomyoepithelial adenosis
- Adenomyoepithelioma
- Malignant myoepithelioma

Fibroepithelial tumors

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

Tumors of the nipple

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

Malignant lymphoma

Metastatic tumors

Tumors of the male breast

- Gynecomastia
- Carcinoma
  - *In situ*
  - Invasive

1.3.2 Tumor Size and Lymph Node Status

Tumor size and axillary lymph node status are two classical parameters describing the primary extension and presumed prognosis of a malignant breast tumor. Since several decades, studies have described the associations between large primary tumors and a positive lymph node status on the one hand, and a large tumor and shorter disease-free and overall survival on the other [104]. Until the 1980's, the lymph node status was actually the only parameter deciding about further treatment of a patient. However, the prognostic value of the tumor size is questionable. It is supposed that this parameter indicates a certain stage in tumor progression rather than predicting the liability of a tumor to cause a relapse. Tumor aggressiveness is reflected much more accurately in the tumor tissue's histological phenotype. The determination of the axillary lymph node status is still one of the most important diagnostic means of identifying breast cancer patients with higher risk of tumor recurrence. For this purpose, at least ten lymph nodes of the levels I and II must be excised during surgery [202]. In the past, node-positive patients were classified as 'high-risk' and usually received and profited from adjuvant systemic treatment. The recent St. Gallen consensus recommendations more specifically define three risk groups - 'low', 'intermediate', and 'high' - based on endocrine responsiveness, HER2/*neu*-status (see below) and menopausal status (Table 2) [124]. Chemotherapy alone is applied in cases of endocrine receptor- and HER2-negativity. Combinations of chemotherapy and trastuzumab may be given patients with HER2-positive disease or in cases in which the use of endocrine therapy alone is uncertain. Concerning node-negative patients classification and therapeutic procedure remain heavily disputed, as there is a substantial percentage - approximately 30% - at risk of suffering a relapse. In fact, momentarily the risk group is not the main decision criterion for treatment selection. Today, following the National Institutes of Health (NIH) [91] and St. Gallen consensus guidelines, up to 90% of node-negative patients receive adjuvant chemotherapeutic treatment. Evidently, there is a considerable rate of overtreated patients who could have been spared from the unpleasant side-effects of chemotherapy. Additional prognostic factors are necessary to identify the group of patients with increased risk of relapse and thereby to help in finding the best possible treatment. For node-positive patients predictive factors, *i.e.* parameters that can predict the response to a particular adjuvant therapy, may be useful to find alternative

therapeutical approaches. Figure 2 shows an excerpt of the criteria for the classification of breast tumors into TNM stages.

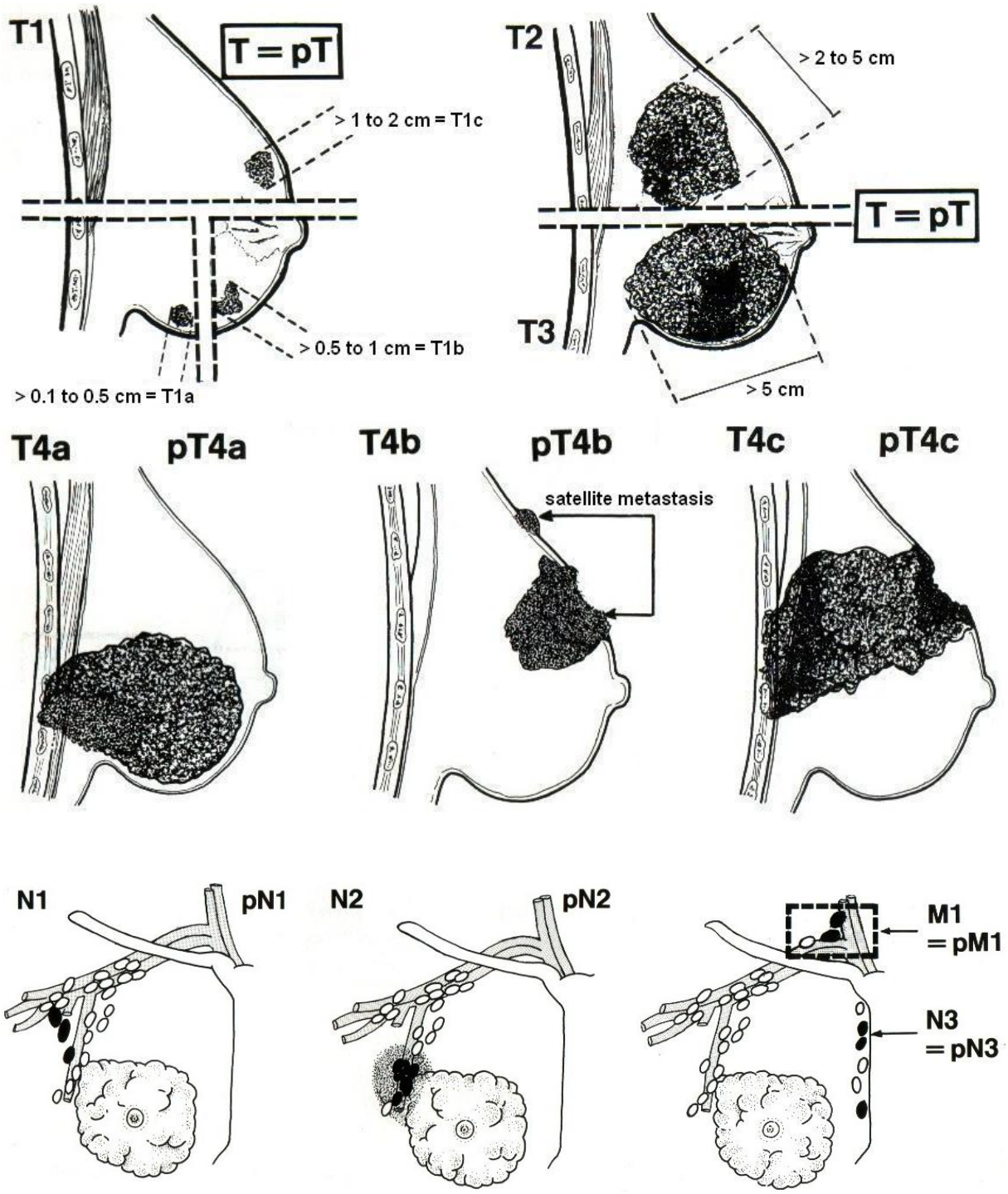


Figure 2: Breast cancer stages [367]. Tumor size and localization determine its classification into tumor stages ranging from T1a to T4c. Not shown are *in situ* carcinomas, Paget's disease, T1mic (microinvasion; tumor size of 0.1 cm or less) - which precede T1a -, and T4d (inflammatory breast carcinoma). N1: metastasis to movable regional axillary lymph nodes on the same side as the affected breast; N2: metastasis to fixed regional axillary lymph nodes or metastasis to the internal

mammary lymph nodes, on the same side as the affected breast; N3: metastasis to supraclavicular lymph nodes or infraclavicular lymph nodes or metastasis to the internal mammary lymph nodes with metastasis to the axillary lymph nodes. M1 indicates the presence of breast cancer cells in locations other than the breast, including distant metastasis (*e.g.* bone, brain, lung). TX/0, NX/0 and MX/0 would indicate no assessment, or evidence, respectively, of the primary tumor, affected lymph nodes, and metastasis. Pathologic staging (pT/pN/pM) adds information gained by a pathologist's microscopical examination.

1.3.3 Tumor Grading Criteria by Scarff, Bloom, and Richardson

The Scarff-Bloom-Richardson (SBR) system is the most commonly used type of cancer grading system. Generally, three characteristic features of a breast tumor - degree of glandular formation (percentage of carcinoma composed of tubular structures), nuclear pleomorphism, and mitotic rate - are examined and evaluated by a pathologist and assigned to a total score ranging from three to nine points. The tumor grades 1, 2, and 3, distinguishing well differentiated from poorly differentiated tumors, are deduced from this score [23,295]. In the past decades, the different criteria contributing to the tumor score of the components have gradually been specified, and new grading systems originating from SBR have evolved. Some studies suggest the mitotic index alone to be of sufficient prognostic value [55]. In 1989, Le Doussal *et al.* [213] described a modified SBR (MSBR) grading that is able to identify additional risk subgroups of invasive ductal breast carcinoma patients for metastasis-free survival (MFS), with an especially high predictive value for lymph node-negative patients. This modified form, that was applied in the present study, was created by eliminating the factor of ductoglandular formation which, in a separate MFS analysis, had shown to be the SBR system's component with the least predictive capacity. In invasive ductal carcinomas, the SBR grading has proven to be valuable as a marker of chemosensitivity for neoadjuvant treatment: SBR grade 3 tumors showed a significantly better response to chemotherapy than SBR grade 1 tumors [6]. Nuclear grading by Black *et al.* [21] also excludes the tubular formation as a component of the grading system. In Europe, today the Elston-Ellis modification (Nottingham grading system) is preferred. By exactly defining the three original system components and assessing the field area dimension used for mitotic count, this modification attempts to further standardize tumor grading. In addition, this grading classification, along

with tumor size and lymph node status, forms the Nottingham prognostic index which may contribute to individual therapy decisions [92,93].

1.3.4 Steroid Hormone Receptors

It has been long since known that some breast tumors require steroid hormones for their continuous growth, others develop in the postmenopause after decrease of hormonal influence. The prognostic relevance of hormone receptors has been a field of research for more than 30 years [52,59,122,181]. The presence of estrogen and progesterone receptors is favorable for the outcome in breast, ovarian, and endometrial cancer. For hormone-positive patients there exist diverse possibilities of endocrine treatment, such as the application of anti-hormones - including aromatase inhibitors (AI), selective estrogen receptor modulators (SERM) like tamoxifen [90], and estrogen receptor downregulators (ERD) - or the ablative endocrine therapy. Yet, estrogen and progesterone receptor presence does not necessarily ensure a positive response to endocrine therapy, the rate of resistant tumors ranging between 30 and 40%. Just as little does a negative hormone receptor status in the tumor tissue rule out any response. For decision-making whether to apply endocrine therapy, three groups of endocrine responsiveness have been defined: (1) highly endocrine responsive, (2) incompletely endocrine responsive, (3) endocrine non-responsive. For example, patients' tumors adhering to the first category with low risk may be treated with endocrine therapy alone [124]. The effectiveness of advances in chemotherapy may also depend on the estrogen receptor status [17]. Resistance to endocrine therapy, although endocrine responsive, has been attributed to steroid receptor polymorphism [320], altered transcription of progesterone receptors [274], presence of amplified EGFs, and altered apoptosis, leading to a resistance to AIs [9]. The identification of receptor variants has become a new area of interest in breast cancer research. Other hormone receptors that can be determined for identification of endocrine responsiveness are LH-RH and somatostatin receptors [100,332]. These could potentially also be targeted by analogous agents.

1.3.5 New Prognostic and Predictive Factors

Proliferation Parameters

Today, advances in flow cytometry and thymidine labeling of biopsies enable the determination of proliferation parameters, such as the number of cells composing the S-phase fraction (SPF), presence of the nuclear antigen Ki-67, the ploidy of the tumor, and the thymidine labeling index (TLI). Low-risk breast cancer patients with low SPF were shown to have significantly longer disease-free survival (DFS) rates than patients with high SPF [53]. The cell core-associated proliferation antigen Ki-67 is another parameter found in breast tumors that can be detected by immunostaining with the monoclonal antibody MIB-1 [118,140]. Different authors have described the predictive value of SPF and Ki-67 for the response to chemotherapy; tumors with high rates of either of the factors were described to display a better response [25,48,276]. The occurrence of diploid tumors has been associated with a better prognosis and less involvement of axillary lymph nodes in comparison to aneuploid tumors [157]. The thymidine labeling index is acknowledged as an independent prognostic factor for local and distant metastasis in breast cancer, and also seems valuable as a predictor of response to polychemotherapy [5]. Cyclins, as cyclin D and E that regulate cell cycle progression [333], and the nuclear enzyme topoisomerase II α [198] are further proliferation factors being analyzed with respect to breast cancer prognosis and tumor responsiveness.

Growth Factors

Growth factors have been shown to be involved in cell proliferation and angiogenesis processes. Contrary to steroid receptors, overexpression of the EGF receptor (EGFR) is an indicator of poor prognosis in breast cancer [250,290]. EGFR, also known as erbB-1/HER-1, is a member of the erbB family and, simultaneously, of the type I receptor tyrosine kinase family. As a novel therapeutic approach, growth factors and receptors are being tested as targets for antibodies and tyrosine kinase inhibitors which are believed to have an acceptable toxicity profile and less side-effects as compared to conventional cytotoxic agents [251]. Other growth factors of prognostic relevance are the transforming growth factor-ß (TGF-ß) - described both as tumor suppressor and promoter [244,307] -, vascular endothelial growth factors (VEGF) - associated with a poor prognosis and capable of predicting endocrine responsiveness of advanced breast cancer [232] -, insuline-like growth factors (IGF),

and associated receptors. IGF-1, by interaction with IGF binding proteins (IGFBP) and estrogens, is known to increase the risk of developing breast cancer, especially for premenopausal women [200,220]. Its functions are mediated by the IGF-1 receptor which has shown to be elevated in breast cancer and related to a longer disease-free and overall survival (OS) [26,258].

Oncogenes

The oncogene c-erbB-2 (HER-2/*neu*), originally isolated from rat neuroblastomas, encodes a surface glycoprotein named 'p185 *neu*', structurally resembles the EGF receptor, and has been shown to be present also in human breast cancer. High expression has been associated to a poor prognosis and is found in about 20 to 30% of all mammary carcinomas [219,252]. Since 2005, these patients are a target group for treatment with the monoclonal anti-HER-2/*neu* antibody trastuzumab. Recent publications have revealed this antibody - particularly in combination or in series with adjuvant chemotherapy - to provide a benefit to most patients, also to those with metastasic disease [265,286,334,370]. Besides HER-2/*neu*, several other proto-oncogenes are being analyzed in respect of their prognostic and predictive significance. Mutations of the tumor suppressor gene p53 occuring in breast cancer mostly have been found to lead to a poorer disease-free and overall survival [264], similar to the proto-oncogene c-myc [75]. The expression of the Bcl-2 gene, a suppressor of apoptosis, has been positively correlated to patient survival [195]. But seemingly, none of these factors can as yet compete with HER-2/*neu* and its protein product in its functions as a marker as well as a therapeutic target.

Proteolytic Factors/Members of the Plasminogen Activation System

Since 1989, extensive evidence has proven the estrogen-induced lysosomal aspartyl protease cathepsin D to be linked to an unfavorable outcome for breast cancer patients [110,329,344,349]. Additional proteases spotted as prognostic markers in breast cancer are the cysteine proteases cathepsins B and L, also correlated to a poor prognosis [109,346], and the serine protease uPA, uPAR, and PAI-1 which will be thoroughly described in the following chapter. A characteristic of cathepsin L is its prognostic relevance in lymph node-positive patients which have received adjuvant therapy, while the prognostic potential of the other proteases is diminished in adjuvantly treated patients. This indicates that through cathepsin L a group of

patients may be identified that does not respond to standard adjuvant therapy regimens [144,172]. Combinations of different proteolytic factors for enhancement of their prognostic information have been discussed, for instance uPA/PAI-1 [146,177], PAI-1/cathepsin L [347], or PAI-1/cathepsin D [144,208], and will surely be subject to future investigations.

Proteomics

Proteomic analysis is based on the idea that different clinical states, also cancer, are represented by distinct protein patterns or signatures, potentially consisting of completely differing proteins, truncated peptide fragments, or post-translationally modified proteins. The most widely studied methods involve identification of proteomic profiles as peaks on mass spectrometric analysis with precise charge-to-mass ratios. Despite a large number of breast cancer studies over the last 10 years, analyzing serum, secreted fluids, and cancerous tissue, the research is too preliminary as to be able to deduce areas of clinical application [151]. Up to now, prognostic breast cancer studies are few and lack comparability [1,171].

Gene Expression Profiles

Recently, studies with Affymetrix genechip arrays have shown a strong sensitivity in distinguishing between different risk groups of breast cancer patients on the basis of gene signatures [362]. For example, significantly differing gene profiles were found in tamoxifen-responsive compared to tamoxifen-resistant tumors [179]. Analogous to determination of selected proteolytic factors, gene expression profiling, amongst others, aims at identifying low-risk node-negative patients to help avoid unnecessary adjuvant systemic treatment. Oncotype Dx and MammaPrint are tests that may help discriminate if chemotherapy is to be applied in addition to endocrine therapy. Still, in the 2007 St. Gallen consensus meeting, these tools have not yet been considered sufficiently reliable for defining risk categories. Prospective clinical trials are on-going [24,325].

Other Prognostic Markers

CA 15-3 and CA 27.29 blood tests allow the detection of circulating MUC-1 antigen and have shown to display prognostic value in breast cancer [234]. Levels of carcinoembryonic antigen (CEA) are less commonly elevated; increase of this tumor

marker may provide supplementary information in case of negative CA 15-3 and CA 27.29 [135]. The applicability of these tumor markers for therapy decisions in early-stage cancer remains to be further evaluated [240]. Bone-marrow micrometastases, defined as an elevated number of (potentially malignant) epithelial cells in the bone marrow in tumor patients [32], and the number of circulating tumor cells (CTCs), meaning cells detected in the blood that possess antigenic or genetic characteristics of a specific tumor type [61], have also been discussed as potential tumor markers for breast cancer. A further example of a potential new breast cancer marker is the estrogen-related pS2 protein, occurrence of which is related to a favorable prognostic effect and predicts an improved response to endocrine therapy [13,105]. Eventually, heat-shock proteins (HSP), molecular chaperones, have been reported to function as protectors of malignant transformed cells against apoptosis or adjuvant therapy [51].

Concluding Remarks on Clinical Utility

In the American Society of Clinical Oncology 2007 Update of Recommendations for the Use of Tumor Markers in Breast Cancer, the following categories were recommended for application in clinical practice: CA 15-3, CA 27.29, and CEA (only as components for therapeutic decisions in metastasized breast cancer), estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, uPA, PAI-1, and certain multiparameter gene expression assays. No sufficient evidence for introduction into routine use were stated for DNA flow cytometry-based parameters, p53, cathepsin D, cyclin E, proteomics, certain multiparameter assays, detection of bone marrow micrometastases, and CTCs [151].

Table 2: Treatment allocation by therapeutic target and risk categories [124]. Treatment options in each cell are listed in the order of preference. b: Endocrine therapy is effective for prevention of DCIS and therefore might be considered even for very low risk invasive breast cancer. C: chemotherapy; E: endocrine therapy (selected according to menopausal status); Tr: trastuzumab (note 1: trastuzumab should not be viewed as a standard treatment in women with a primary tumor <1 cm of size and with no axillary node involvement. This is particularly true in patients with highly and perhaps also incompletely endocrine responsive disease; note 2: trastuzumab should be given concurrently and after chemotherapy or following completion of all chemotherapy according to clinical trial evidence available at present, though a majority of the Panel agreed that trastuzumab without prior or concurrent chemotherapy may become appropriate for some patients in the future).

HER2/*neu* 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
Risk category	Low	Node negative and all of the following features: pT ≤2 cm, Grade 1, no vascular invasion, HER2(−), ER and/or PgR expressed, Age ≥35 years	E[b]	E[b]	E[b]	E[b]						
	Intermediate	Node negative and at least one of the following features: pT >2 cm, Grade 2−3, vascular invasion, HER2(+), ER and PgR absent, 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
		1–3 nodes positive AND ER and/or PgR expressed and HER2(−)	E C → E	E C → E	C → E E	C → E E						
	High	1–3 nodes positive AND ER and PgR absent OR HER2(+)					C	C → E + Tr	C → E + Tr	C → E + Tr	C → E + Tr	C + Tr
		>4 nodes positive	C → E	C → E	C → E	C → E	C	C → E + Tr	C → E + Tr	C → E + Tr	C → E + Tr	C + Tr

1.4 The Plasminogen Activation System

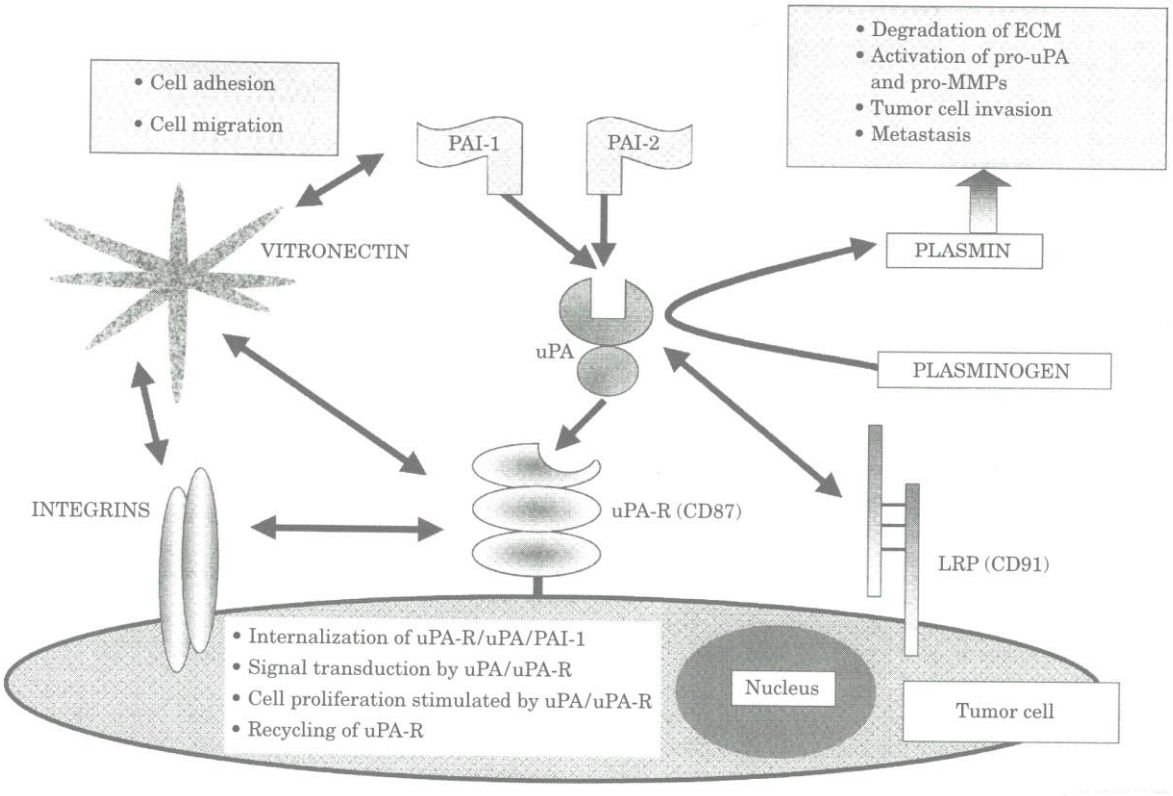


Figure 3: Interactions of components of the plasminogen activation system (uPA, uPAR, PAI-1, PAI-2, plasmin(ogen)), integrins, vitronectin, and LRP, and influences on tumor cell invasion and metastasis [301].

Invasive tumor growth and metastasis are the result of complex interactions between pericellular proteolytic enzymatic systems, adhesive proteins, integrins, growth factors, and steroid hormones. Typical features of malignant tumor cells are an altered gene expression, abnormal signal transduction pathways, loss of cell cycle control, neovascularization, degradation of extracellular structures, and cell dedifferentiation. A tumor's aggressiveness depends on the ability of the malignant cells to control the biological processes within and surrounding a neoplasia. An imbalance in different proteolytic enzyme systems can cause changes in the structure of the extracellular matrix (ECM) that facilitate tumor cell spread and invasion into the vascular system. Proteases involved in such extracellular remodeling processes, which are modulated by their specific receptors and inhibitors, are metalloproteinases, cysteine proteinases, aspartyl proteases, and serine

proteases. These factors have been shown not only to be elevated in various carcinomas, expression levels of certain proteases can also be of prognostic relevance for cancer patients. Pericellular proteolytic systems are influenced by various growth factors, among them epidermal growth factor (EGF), transforming growth factors α and ß (TGF-α/-ß), basic fibroblast growth factor, and platelet-derived growth factor [188]. Thrombin for example, an important serine protease of the blood coagulation system, has been shown to stimulate cell proliferation and chemotaxis [360], and, through generation of fibrin conglomerates around tumor cells, contributes to tumorigenesis, along with fibrin itself [82,89,246]. A major role in invasive processes is further ascribed to the serine protease uPA and other members of the plasminogen activation system [8,66,68,85,139,145,148,149,233,279,298,299,300], although several of these factors inhibit fibrin formation. Procoagulant and fibrinolytic systems are equally present in tumors, and composition as well as interaction determine potential further tumor progression.

Although a role in various malignancies has long since been assumed, researchers examining members of the plasminogen activation system, first described by Astrup and Permin in 1947 [10], mostly concentrated on hematological aspects, *i.e.* intravascular degradation of fibrin deposits by plasmin and regulation of hemostasis. In the 1980's and 90's, through a better understanding of the numerous functions of the involved proteins and the examination of transgenic mouse models, it became evident that the system also played a major role in extravascular cell regulation and tissue formation.

Plasmin is a serine protease evolving from the inactive zymogen plasminogen through catalysis by the serine proteases uPA (urokinase-type plasminogen activator) and tPA (tissue-type plasminogen activator). Pro-uPA is activated by the cysteine proteases cathepsin B and L, or by plasmin through a positive feedback mechanism [299,301]. uPA and uPA:PAI-1 or uPA:PAI-2 complexes bind to the membrane-anchored uPA receptor (uPAR; CD87). Through inhibition of plasmin, α_2-antiplasmin plays an essential role in fibrinolysis and also participates in the degradation of various other proteins. Besides α_2-antiplasmin, there are several further inhibitors of plasminogen activation, for example PAI-1, -2, and protease nexin 1 (PN-1), belonging to the serpin (serine proteinase inhibitor) family [56]. Maspin

is a newly discovered serpin that inhibits cell surface-associated uPA and fibrinogen-bound tPA [19].

In murinal models, surprisingly, the deficiency of plasminogen, uPAR, uPA, tPA, and PAI-1, respectively, is compatible with development and reproduction [34,35,42,43,44,284]. Combined uPA/tPA-deficiency, however, leads to severe thrombosis with a considerable impact on life expectancy and fertility [37,44].

This section gives a description of single components of the plasminogen activation system, focusing on uPA and PAI-1. The first paragraph of each of these chapters displays a selection of biochemical features. In the following paragraphs, their biological roles, various interactions, and their particular significance for the pathogenesis of diseases, including carcinogenic effects, are shown. In the final three chapters of this section, the importance of the plasminogen activation system in cancer is summarized, scientific and clinical approaches to further explore and find application areas in cancer therapy are depicted. The description of uPA/PAI-1 quantification methods leads to the main topic of this doctoral thesis.

1.4.1 Plasmin

The serine endopeptidase plasmin (M_r ~ 90 kDa) consists of two disulfide bond-linked polypeptide chains. The C-terminal B chain encompasses a typical serine protease domain responsible for its catalytic activity. Peptide bonds that are localized on the C-terminal side of lysine (Lys) and arginine (Arg) residues are hydrolyzed under plasmin catalysis. The zymogen plasminogen consists of only one chain and its catalytic activity lies about 10^4- to 10^6-fold lower than that of plasmin. Plasminogen (Plg), secreted by the liver as a single chain glycoprotein, is localized intra- and extravascularly. Activation of plasminogen takes place by cleavage of the single peptide bond Arg561-Val562. Angiostatin, which is known as an inhibitor of angiogenesis, is a fragment of plasmin. By autoproteolysis of plasmin in endothelial cells, angiostatin can be generated and is able to display antimigratory effects [8].

Plasmin has a very broad spectrum of substrates. In the blood, plasmin, also referred to as fibrinolysin, dissolves fibrin clots and degrades various other proteins including

fibrinogen and the coagulation factors V and VII. Extravascular plasmin is able to degrade fibrin and other ECM proteins, activates latent TGF-ß, and leads to detachment of the basic fibroblast growth factor from its ECM-binding sites [238,282]. Additionally, plasmin catalyzes the activation of matrix metalloproteinases (MMP), a family of zinc-dependent enzymes which also degrade various components of the ECM proteolytically [154,216]. The primary inhibitor of plasmin is the serpin α_2-antiplasmin.

Plasminogen-deficiency in mice leads to severe thrombosis and thrombotic lesions in several organs and a high mortality, but does not impair general development or fertility. Surprisingly, levels of uPA measured in urine were not reduced in Plg-deficient mice [34,284]. This suggests plasminogen not to be essential for uPA activation and supports the idea of functions of uPA independent of plasminogen activation. By simultaneously inducing fibrinogen-deficiency it was possible to prevent the mice from the thrombogenic effects caused by the lack of plasmin [36]. From these results it was deduced that the physiological role of plasminogen may more or less be restricted to fibrinolysis processes. Coherent to functions of the plasminogen activation system in inflammatory cell recruitment, Ploplis *et al.* [267] showed an impaired response to inflammation in Plg-/- mice. The induction of Lewis lung carcinoma in Plg-/- mice was characterized by a slight decrease in tumor growth and longer survival time in comparison to the Plg+/+ control group [38]. Moreover, the development of metastasis in induced mammary tumors was promoted by plasminogen, pointing out a crucial role in tumor biology [39].

1.4.2 The uPA Receptor (uPAR)

uPAR (M_r ~ 55-65 kDa) is a cysteine-rich glycoprotein which is synthesized as a single polypeptide chain of 313 amino acid residues, preceded by a 21-residue signal peptide. Post-translationally, the last 30 C-terminal residues are cleaved and a glycophosphatidylinositol (GPI) tail is attached to Gly283. Mature uPAR consists of three homologous, independently folded domains which are members of the Ly-6/uPAR/α-neurotoxin protein domain family. Domain 3 (D3) is the C-terminal domain that anchors the molecule to the cell membrane through the GPI tail. The three domains form a central cavity that recognizes the growth factor domain of uPA. uPA

and the somatomedin B (SMB) domain of vitronectin can simultaneously bind to the N-terminal domain 1 (D1) [363]. All three domains are required for high affinity binding. While uPA occupies the central cavity, other ligands, for example vitronectin, bind at the D1 domain and the D1-D2 linker region on the outer side of the receptor [167,218] (Figure 4). uPAR can be cleaved by uPA and other proteases resulting in truncated forms, for example without domain 1, unable to bind uPA or vitronectin. Depending on the localization of the cleavage site, the truncated forms are able to exert varying functions. Until now, several molecular forms, glycosylation variants, and splice variants of uPAR have been identified that are widely expressed *in vitro* and *in vivo*. Soluble forms (suPAR) have been detected in body fluids, for instance human plasma, urine, and ovarian cystic fluids [241,269,359]. Conformational changes, as induced by uPA, have been shown to cause the appearance of novel binding sites for vitronectin [311], thrombospondin [160], uPAR-associated protein [14], and the disappearance of binding sites for the α2-macroglobulin receptor [160].

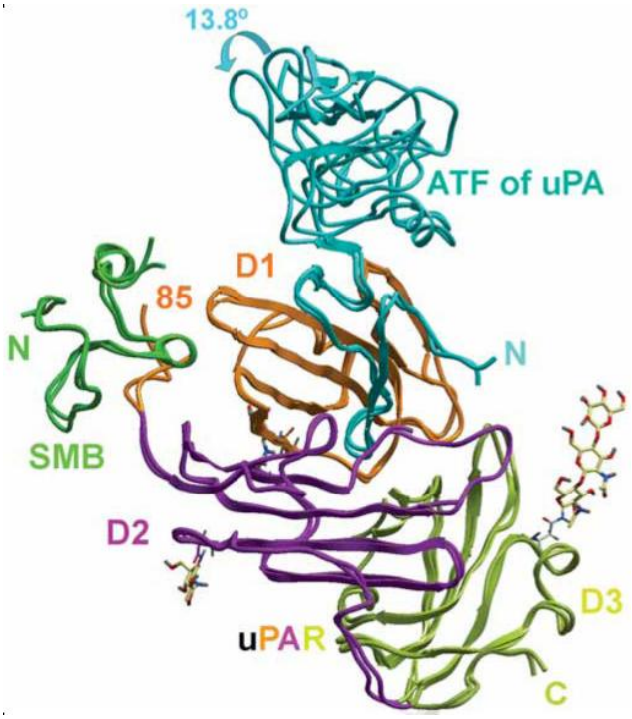


Figure 4: Recognition of both the uPA N-terminal fragment (ATF) and the vitronectin SMB domain by uPAR [167]. Stereoview of the crystal strucure of a suPAR-ATF-SMB complex.

The uPA receptor is essential for the cell-surface associated plasminogen activation, thereby promoting uPA-catalyzed degradation of ECM which can lead to a spread of cancer cells into vascular or lymphatic systems. Additionally, it is believed to influence cell adhesion, proliferation, differentiation, and migration by non-proteolytic mechanisms. These mechanisms imply the interaction with vitronectin and the regulation of the activity state of integrins [22,269]. The localization of uPAR has shown to be dependent on its binding to uPA. While unoccupied uPAR is quite mobile, it is concentrated at cell contact sites upon interaction with uPA [245]. The uPA:uPAR complex at the cell surface triggers signaling cascades [357] and focusses uPA to the leading edge of migrating cells, thereby facilitating local proteolysis and directional invasion [96]. This effect has also been shown to promote wound healing by re-epithelialization of keratinocytes [283]. Partitioning into membrane lipid rafts causes modulation of the functions of GPI-linked uPAR [63,316]. Truncated soluble variants have been shown to be potent chemoattractants for monocyte-like cells, thus imitating the chemotaxis-promoting effects modulated by the uPA:uPAR complex [277]. *In vitro*, the G protein-coupled receptor FPRL1/LXA4R that interacts with an active soluble cleaved form of the uPA receptor ($D2D3_{88-274}$) has been found - as uPAR - to be necessary for the chemotactic activity of uPA [278]. As mentioned above, uPAR also binds to integrins and to vitronectin, this ligation being stimulated by pro-uPA, uPA, the amino-terminal fragment of uPA (ATF; see below), and the uPA:PAI-1 complex, but inhibited by PAI-1 alone [46,76,183,281,363]. Despite no apparent direct contact between ATF of uPA and SMB of vitronectin upon simultaneous binding to uPAR, the suPAR-vitronectin binding affinity is reduced considerably in absence of uPA [167,313] (Figure 4). uPA-stimulated uPAR-vitronectin binding indicates uPAR's function as an adhesion receptor. Agonistic and antagonistic interactions between integrins and uPAR *in vitro* with effects on adhesion processes suggest a role of uPAR and uPA:uPAR complexes, respectively, also in the regulation of cell adhesion [8,269]. As uPAR lacks a transmembrane domain, the signaling cascade is believed to comprise additional transmembrane adapters helping to connect to diverse binding partners, such as intra- and extracellular tyrosine kinases [99,277]; integrin receptor families have been discussed in this respect [364]. Further interactors of uPAR include EGF and its receptor, the mannose-6-phosphate receptor, and the family of low-density lipoprotein receptors (LDLR). Upon inhibition of uPAR-bound uPA by PAI-1 or PAI-2, the complex is endocytosed after binding to members of the LDLR family. While the

plasminogen activator and its inhibitor are degraded in lysosomes, uPAR recirculates to the cell surface [22,58,254]. uPA-uPAR interaction in tumor and endothelial cells, through interaction with integrins and vitronectin, activates the mitogen-activated protein kinases (MAPK) extracellular regulated kinases (ERK) 1 and 2 [345]. Downregulation of uPAR has been reported to lead to cancer cell dormancy and reduction of the MAPK extracellular signal regulated kinase kinase (MEK) pathway activation [3].

Disruption of the uPAR gene alone does not affect fertility, development or hemostasis in mice. Although activated peritoneal macrophages from uPAR-deficient mice did not promote plasminogen activation *in vitro*, no considerable inhibitory effect on uPA activation was seen [35,78]. Dual deficiency of uPAR and tPA caused sinusoidal fibrin deposits in the liver, proving uPAR's physiological relevance in fibrinolysis, complementary to tPA. Still, the consequences for the development of the animals were not nearly as grave as those for mice with combined uPA/tPA-deficiency. uPA supposedly can independently compensate for some of the lacking fibrinolytic capacity of uPAR and tPA [37].

uPAR was first described as a membrane receptor for urokinase on human monocytes and cells of the promyelocyte leukemia cell line U937 by Vassalli *et al.* [355], and is also expressed by neutrophils, T lymphocytes [253,266], fibroblasts, keratinocytes, and a variety of cancer cells. Overexpression of uPAR in tumor cells has shown to lead to increased tumor invasion and growth [67,87,369]. Application of synthetic peptide antagonists of the uPA-uPAR interaction impaired the dissemination of tumor cells *in vitro* and *in vivo* [230,268,294]. The uPA receptor has been found to be upregulated in HIV-1 infections [113,312,328] and in a variety of malignancies like lung [260], gastric [158], colorectal [337], pancreas [41], kidney [162], breast [18,87,98,131,189,199,227], and ovarian cancer [315,359], mostly together with its ligand. Membrane-bound and soluble, full-length, cleaved, and spliced variants have been examined, and some were significantly related to a poor outcome. The significance of the different uPAR variants remains to be further explored.

1.4.3 uPA and tPA

uPA (M_r ~ 55 kDa) consists of two disulfide bond-linked polypeptide chains. The N-terminal A chain contains a growth factor domain, a kringle domain and an interdomain linker region. The C-terminal B chain harbors the serine protease domain. By proteolytic cleavage of a single peptide bond, Lys158-Ile159 in human uPA, the single-chain zymogen form pro-uPA is converted into uPA which has an at least 250-fold higher activity. This process can be catalyzed by plasmin or other proteases, such as kallikreins, nerve growth factor-γ, cathepsins B and L, or Factor XIIa [8,125,193,279,356]. By proteolytic cleavage within the linker region of the A chain, high-molecular-weight uPA (HMW-uPA) can be split up into a so-called amino-terminal fragment (ATF), consisting of the growth factor and the kringle domain, and low-molecular-weight uPA (LMW-uPA) forms, consisting of a part of the linker and the serine protease domain. LMW-uPA, though proteolytically active, can not bind uPAR. uPA binds to the uPA receptor through its growth factor domain. Pro-uPA and active uPA have the same high affinity to uPAR [339]. Lacking the catalytic domain, ATF can bind to uPAR, but cannot cleave plasminogen to generate plasmin (Figure 5).

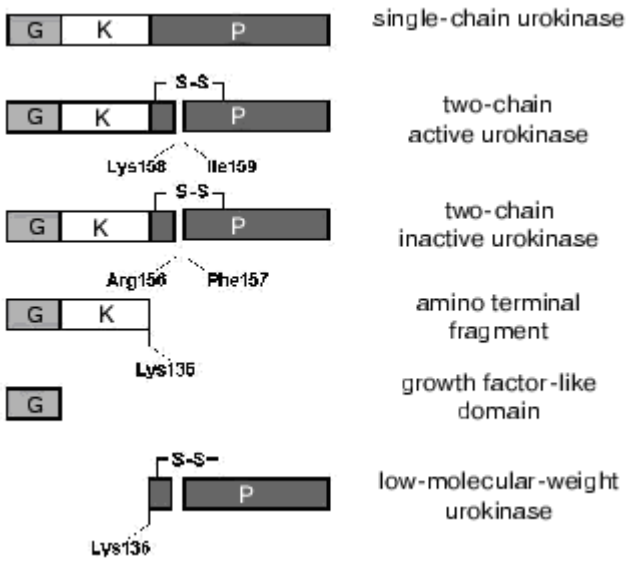


Figure 5: Urokinase fragments generated upon proteolytic processing of urokinase on the cell surface [335]. G: growth factor-like domain; K: kringle domain; P: protease domain.

tPA (M_r ~ 70 kDa) is also composed of two chains and activated originating from a one-chain form by cleavage of a single polypeptide bond, *i.e.* Arg 275-Ile276 in human tPA. The single-chain form's activity lies 10- to 50- fold beneath that of the two-chain form. The N-terminal A chain consists of a fibronectin type II domain, a growth factor domain, and two kringle domains with the C-terminal B chain holding the serine protease domain. Both are connected by a disulfide bond. tPA's and its proform's activity are accelerated by fibrin [8].

tPA's activity is mainly restricted to plasmin conversion in fibrinolytic processes within blood vessels, continuously being secreted by the endothelium. By concomitant binding of tPA and plasminogen to fibrin, tPA-directed plasminogen activation is accelerated [44,57].

In addition to its thrombolytic functions, uPA elicits matrix-degrading processes, influences mitosis, tumor growth, and tumor tissue remodeling. These functions imply fibroblast proliferation and ECM protein synthesis. Findings of positive correlations of uPA levels and microvessel density support its assumed role as a stimulator of angiogenesis and angioinvasion [161]. Some of these tumor promoting functions are independent of plasmin generation. The uPA:uPAR complex is able to activate cellular responses independent of any pericellular proteolytic activity. Chemotaxis, for example, is stimulated by non-proteolytic uPA through activation of Src-type tyrosine kinase p56/p59hck, protein kinase C, and extracellular signal-regulated kinase (ERK) [71,249,277]. Additionally, there are studies showing that the attachment of uPA to uPAR accelerates plasmin's activation of certain matrix metalloproteinases (MMP-2/gelatinase A and MMP-9/gelatinase B) which in turn degrade various ECM proteins, for example collagen types 1 and 4, denatured collagens, fibronectin and laminin [235]. But uPA has also shown to be able to activate MMP-9 directly [372], among other members of the MMP family. There are studies indicating that the presence of the uPA receptor is indispensible for the function of uPA as a growth factor, contributing strongly to cell proliferation enhancement, independent of its enzymatic activity [272,366]. Still, some studies observed growth functions without binding to uPAR [184], in others mitogenic effects were seen only in presence of fully intact uPA [155]. Concerning uPA's chemotactic effects, interaction with uPAR seems essential, uPAR in fact has chemotaxis-promoting functions independent of its ligand [137,277]. Cell

migration triggered by uPA includes movement of tumor cells as well as leukocytes in inflammation processes [74,136,137]. uPA further influences cell migration by direct cleavage of ECM proteins like fibronectin and activation of TGF-ß. In addition, uPA catalyzes the conversion of the inactive proforms of the hepatocyte growth factor/scatter factor (HGF/SF) and of the macrophage-stimulating protein (MSP) into their active forms. HGF/SF and MSP structurally resemble plasmin, but lack protease activity [20,277].

Similar to plasminogen-/- mice models, uPA-/- mice developed hepatic fibrin sediments and ischemic rectal ulcerations. Surprisingly, considering tPA's emphasized role in fibrinolysis, tPA-deficiency did not lead to thrombotic deposits in multiple organs, except for rare fibrin clots in the liver. Nevertheless, combined uPA/tPA-deficiency caused a severe impairment of wound repair mechanisms as well as reduced body weight, fertility, and life expectancy [44]. It may be concluded that uPA and tPA have complementary functions in vascular and extravascular fibrinolytic processes. uPA is involved in wound healing processes and is capable of fibrin clearance independent of tPA and its binding to uPAR. However, the healing in mice with combined uPA/tPA-deficiency was less impaired than compared to mice with Plg-deficiency, suggesting the existence of a third Plg activator that contributes to wound healing, potentially plasma kallikrein [226]. A study examining the relevance of uPA protease activity in inflammation showed a significant decrease of pulmonary inflammatory response to Cryptococcus neoformans in uPA-/- transgenic mice when compared to uPA+/+ mice [138]. Murinal tumor models have underlined uPA's role in tumor invasion [4,289,308].

uPA is secreted by numerous normal - among them monocytes-macrophages [354], capillary endothelial cells [263], and implanting trophoblastic cells [210,291,340] - and malignant cells [66]. It was originally isolated from human urine and named 'urokinase' by Sobel *et al.* in 1952 [321]. Therapeutically, uPA and tPA are used as thrombolytic agents. In breast cancer as in other cancers, high uPA expression levels have been related to significantly poorer prognoses than low levels, while elevated levels of tPA have been associated with a favorable prognosis in cancer [86,190,288].

1.4.4 PAI-1 and PAI-2

PAI-1 (M_r ~ 50 kDa) is a single-chain glycoprotein that consists of 379 amino acids. It is present in three forms with differing conformation: an active (Figure 6), a latent, and a non-functional substrate form. While in latent PAI-1 the reactive center loop (RCL) near the C-terminal end is withdrawn by insertion into ß-sheet A, the reactive center is exposed on the surface in the active form. During inhibition, the surface-exposed reactive center loop (RCL) of PAI-1 is cleaved between R346 and M347 (P1-P1' bond) by the target protease, an acylenzyme intermediate complex is formed, and the N-terminal part of the RCL is inserted into ß-sheet A as additional strand 4A (s4A), translocating the protease across the plane of ß-sheet A. In the subsequent complex, the structure of the active site of the serine protease is disorderd and, thus, its enzymatic activity is inhibited [305]. PAI-1 reacts only once with its target protease. PAI-1 is synthesized as an active molecule which is rapidly inactivated in free solution, but this conversion can be delayed by binding to vitronectin. Reactivation of the latent form can be achieved by exposure to chaotropic agents or phospholipids [45,119,336,356]. Sites for binding of vitronectin are the α-helices E/F and the ß-strand 1A [212,256,352].

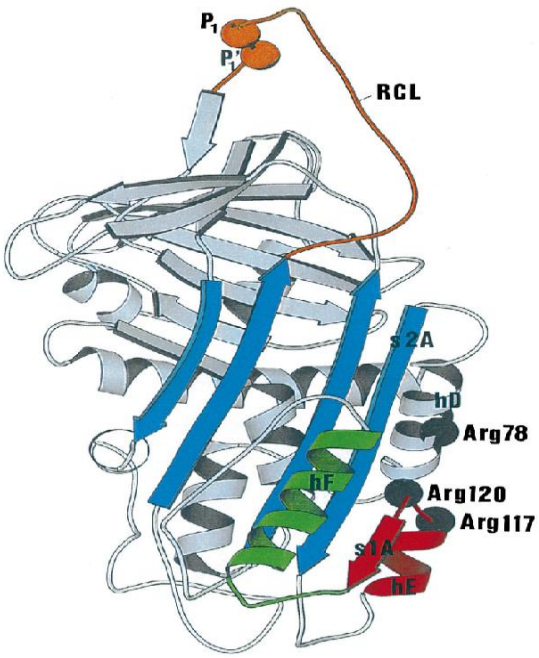


Figure 6: Model of the three-dimensional structure of active PAI-1 [8]. In the center, the large ß-sheet A (s2A with ß-strand 1A (s1A)) is visible. Besides ß-strand 1A, α-helix F (hF) and α-helix E (hE) are regions implicated in the binding to vitronectin.

PAI-2 exists in two forms: one is located intracellularly (M_r ~ 47 kDa), the other is a glycolsylated secreted form (M_r ~ 60 kDa); both contain 415 amino acids and are encoded by a single mRNA [15,116]. In solution, unlike PAI-1, secreted PAI-2 does not lose its activity spontaneously [336]. PAI-1 and PAI-2 only have 24% of their amino acid sequence in common and belong to different serpin classes. However, their structure - containing three ß-sheets, 8-9 α-helices and the RCL - is quite similar. A structural feature unique to PAI-2 is a sequence of 33 amino acids between α-helices C and D known as the C-D loop (Ala65-Glu96) [204].

PAI-1 is synthesized by numerous healthy as well as tumorous cells, and its expression is influenced by different cytokines, hormones, growth factors, and endotoxins. The better part of intravascular PAI-1 is stored in α-granules of platelets [203]. It forms an inactive complex with tPA in the vasculary system, thereby preventing early fibrinolysis by tPA [81]. Furthermore, PAI-1 inhibits uPA: by forming a stable complex with active uPA, it exerts a negative feedback control with consequent inhibition of plasmin formation. Binding of PAI-1 to uPAR-bound uPA results in inactive PAI-1:uPA:uPAR complexes that are internalized by endocytosis receptors of the LDLR family, such as LRP and the very-low-density lipoprotein receptor (VLDLR), while sustaining mitogenic signaling events. Thus, PAI-1 can regulate uPA/uPAR levels on the cell surface and their signaling activity. A high-affinity site for the VLDLR within the PAI-1 moiety has been discovered which is not present in PAI-2 - thereby not affecting cell proliferation - and may explain the differences between their functional implications in cancer [62], as described below. PAI-1 can also, for instance *via* direct interaction with LRP, stimulate cell migration independently of uPA, tPA, and vitronectin [72]. Through its various interactions PAI-1 plays a role in signal transduction and has been shown to facilitate tumor invasion in compliance with uPA and uPAR. Similar to uPAR, PAI-1 interacts with integrins and, as mentioned above, with the adhesive ECM glycoprotein vitronectin, the primary PAI-1 binding protein. PAI-1 and uPAR compete for the same vitronectin binding site, and, thus, PAI-1 causes detachment of cells by disruption of uPAR-vitronectin and integrin-vitronectin interactions [223]. PAI-1, moreover, binds to and disrupts uPA:uPAR:integrin complexes; several other cells of the ECM are detached from fibronectin and collagen type 1 [64]. Further binding partners of PAI-1 are fibrin and heparin. By binding heparin or vitronectin, PAI-1's substrate specificity is altered and

permits inhibition of an additional serine protease: thrombin. These often contradicting functions may explain the simultaneous stimulatory and inhibitory effects on tumor invasion modulated by PAI-1. PAI-1 and associated proteins are also active in embryological processes, such as the regulation of trophoblast invasion [210,229,255].

PAI-2 also inhibits uPA and tPA, but the reaction takes place at a lower pace than the inhibition by PAI-1. While PAI-1 inhibits all active forms of uPA and tPA, PAI-2 only recognizes and inhibits the two-chain forms of the proteases. It is not known to bind to further intra- or extracellular sites apart from uPA and tPA [229].

Generation of PAI-1-deficient mice by homologous recombination in embryonic stem cells revealed that the animals were fertile and showed no abnormalities in their development to adulthood [42], despite the various regulative functions ascribed to PAI-1 in embryology. Disruption of PAI-1 did not impair hemostasis considerably, but caused just a mild hyperfibrinolytic state. This may be explained by the compensation of PAI-1's inhibitory functions through other factors such as PAI-2, α_2-antiplasmin, α_2-macroglobulin, C_1-esterase inhibitor, or α_1-antitrypsin [43].

PAI-1 was first purified from endothelial cells in 1984 [353]. The highest concentrations in the human body are found in the liver and the spleen [336]. Concerning tumor biology the role of PAI-1 has to be further explored. In analogy to uPA, PAI-1 is assumed to promote angiogenesis and cancer invasiveness. In a murinal trial with transplantation of malignant keratinocytes, Bajou *et al.* [11] found a decrease of invasiveness and tumor vascularization in absence of PAI-1. In another study, deletion of PAI-1 in mice prevented subretinal choroidal angiogenesis induced by laser photocoagulation [211]. Liu *et al.* [217] stated that PAI-1 *in vitro* promoted the invasion of human lung cancer cells in presence of uPA and uPAR. Other studies observed inhibitory effects of PAI-1 on tumor progression [33,178,322]. For instance, PAI-1 inhibited uPA/uPAR-modulated invasiveness of human A549 and Calu-1 lung carcinoma cells [33]. Intravenous injection of PAI-1 into SCID mice with xenografts of human prostate carcinoma cells led to a considerable size reduction of the xenografts [178]. In spite of these deviating results, for breast cancer diagnostics PAI-1 is today regarded as one of the most valuable prognostic factors [86].

PAI-2 was first purified from the placenta, where it is secreted by the trophoblastic epithelium [186], and is additionally synthesized by macrophages, and by monocyte and other cell lines [116,368]. In the course of the evaluation of the tumorbiological role of PAI-2, induction of malignant tumor growth with addition of PAI-2 in rodents was followed by significantly less tumor progression and occurrence of metastatic lesions than in the comparison groups [97,243]. In correspondence to these results, PAI-2 levels have been inversely correlated to lymph node involvement as well as to the metastasis-promoting factors uPA, uPAR, and PAI-1 [169]. Furthermore, high PAI-2 levels have been related to a positive outcome for breast cancer patients [28,29,88,108,350], whereas low levels were associated with metastasis of various carcinomas, including breast cancer [86,111,204].

1.4.5 The Plasminogen Activation System and Cancer

Since several decades, the plasminogen activation system is being put in the context of tumor growth and metastasis [66]. The components of the system have been found in stromal fibroblast cells, endothelial cells, and tumor cells, indicating their importance in stroma remodeling and desmoplasia, angiogenesis, and tumor invasion. In large part, the understanding of the system's functions in cell migration and ECM formation was gained from cell culture studies, and the system's components were thought to be indispensable for regulation of tissue organization, including under physiological conditions. But murinal studies with plasminogen, uPA-, tPA-, PAI-1-, and uPAR-deficiencies surprisingly found no significant delay in development. The exact locations and quantities of the components have extensively been analyzed by immunohistochemistry and *in situ* hybridization. In different tumors, a great diversity of expression patterns was found, depending on tumor type and tumor stage or grade. Members of the uPA system can potentially be produced by a large number of various cell types [27,50,77], and it has been hypothesized that different tumor cells collaborate in producing members of the system [67]. Basically, the processes modulated by the plasminogen activation system are also existent in non-cancer cells. But these cells are also capable of contributing to cancer development by reorganization of tissue in proximity to tumor cells [350]. For example, in a breast cancer study analyzing the expression of uPA, uPAR, and PAI-1 in tumor cells and fibroblasts, their presence in fibroblasts seemed to be more decisive for tumor

invasion than their localization in the tumor cells themselves [83]. There are indications that the promoting effects of the uPA system on tumor growth are due to an imbalance of its components [7,11,12,217,317].

uPA was first associated with breast cancer development by Duffy *et al.* [84], the predictive value of PAI-1 was described for the first time by Jänicke *et al.* [174]. In different studies, analysis of uPA and PAI-1 expression revealed these factors to be independent prognostic markers in numerous malignancies apart from mammary cancer, such as cervical [194], ovarian [205,206], endometrial, bladder [86], renal cell [162], prostate [239], pulmonary [259,260], gastric [158,247], esophageal [248], and colorectal [114,292] cancer. High antigen concentrations in the primary tumor correlated significantly with tumor progression and, in total, with a poor prognosis. These effects were also shown in patients with oral squamous cell carcinomas [168]. By examination of the development of human keratinocyte cell lines of differing malignancy in PAI-1-deficient mice its stage-dependent significance was described. Only early tumor stages profited from PAI-1-deletion, while aggressive tumors kept growing [231]. Elevated uPA and PAI-1 contents were also found in metastatic lesions of different tumors [297,314]. High uPAR levels in malignant breast tumors have been associated with a poor prognosis, but mostly with a less strong prognostic impact than uPA and PAI-1 [87,98,131,189,199,227]. Its value as a prognostic marker therefore remains uncertain. tPA and PAI-2 showed to be associated with a favorable prognosis for breast cancer patients [28,29,88,108,190,288,350]. The multifunctional roles of the individual components of the plasminogen activation system reflect the great complexity of their influence on tumor growth and vary depending on the respective tumor type.

1.4.6 Clinical Application of uPA System Research in Breast Cancer Diagnostics and Treatment

Since the 1980's, numerous research groups have focused on the importance of the uPA system in breast cancer, providing evidence of the prognostic relevance of uPA and/or PAI-1 in lymph node-negative as well as in node-positive patients [29,30,48,84,95,107,111,130,131,133,134,141,142,143,174,175,177,190,192,208,215,222,242,262,288,298,331,347,371], PAI-1 frequently displaying a higher prognostic significance than uPA. In an analysis of the prognostic strength of uPA, PAI-1, and the steroid receptor status at different

times of follow-up, Schmitt *et al.* [300] found a time-variation of their prognostic power and differences between the two factors. While there was a continuous decrease of prognostic impact of the uPA status over the first three years, the prognostic value of PAI-1 improved over time. Harbeck *et al.* [146] were able to demonstrate that the greatest clinical relevance of uPA and PAI-1 for breast cancer patients is reached when evaluated in combination, *i.e.* divided into two groups: both low (defined as negative) or either or both high (defined as positive). In a German prospective randomized adjuvant chemotherapy trial to examine the predictive value of uPA and its inhibitor, high-risk axillary node-negative breast cancer patients were identified by the uPA and PAI-1 levels, and split up into a group with application of adjuvant therapy and an observation group. As a preliminary result there was a significantly lower rate of recurrences in the chemotherapy group [177]. The response to hormone therapy, however, seems to be weak in patients with high uPA and PAI-1 values [86,139]. In metastasized tumors with high uPA/PAI-1 in the primary tumor tissue, palliative endocrine therapy turned out to be less effective [148]. These results may indicate that high uPA/PAI-1 expression levels are present in especially aggressive tumors that are urgently to be treated by adjuvant systemic therapy. On the other hand, uPA/PAI-1 measurements can help identify low-risk patients to avoid unnecessary adjuvant treatment [177]. Thus, in the future, the uPA system may become a decisive component of individualized breast cancer management.

Taking the diverse importance of the plasminogen activation system in cancer development into consideration, its components may, furthermore, serve as potential targets for anti-invasive and anti-metastatic therapy [180,319]. For instance, peptide sequences have been found, forming the basis for the development of a selective uPA inhibitor that barely inhibits tPA and thereby fibrinolysis [187]. The binding process of uPA to uPAR may be a potential target [7,280,285,327], as well as the PAI-1-vitronectin binding site [304]. Moreover, the ability of PAI-1 to inhibit binding of uPAR and integrins to vitronectin [8] may be used for therapeutic purposes. Antisense oligonucleotide therapy represents an alternative approach that inhibits biosynthesis of the plasminogen activation system components [299]. Another option is the application of angiostatin as an inhibitor of angiogenesis [150,293].

1.4.7 Quantification Methods for uPA and PAI-1

Available methods for measuring uPA system components in cancer patients' tumors are receptor/ligand assays, enzymometric assays, immunohistochemical methods, ELISA, Western blot analysis, and PCR. The ELISA is the most commonly applied technique, with tumor samples being provided as cytosol fractions or detergent extracts. Today, several standardized ELISA methods, such as the Femtelle assay (American Diagnostica, Stamford, USA), are commercially available. Immunohistochemical staining of uPA-/PAI-1-positive cells has yielded likewise results [50,102], but not with the same high predictive capacity as ELISA assays [151]. Apart from this, advantages of the ELISA method compared to immunohistochemistry (IHC) for measurement of plasminogen system components imply the use of standardized methods, parallel analysis on a large number of specimens, and a higher sensitivity allowing assessment of smaller amounts of tissue. Disadvantages of ELISA include quantification errors due to cross-reactions between the respective antibodies, a lack of sensitivity or specificity for different forms of uPA and its complexes with inhibitors depending on the ELISA-kit used, influences of differing reaction conditions on the antigen/antibody interaction, and the restricted application only to cryostat, not to paraffin sections [102,147,303,304,342,343].

More and more malignant breast tumors are diagnosed at earlier stages of the disease, making it necessary to acquire tissue from increasingly small-sized primary tumors. In this situation, the extraction of RNA for quantitation by PCR may be a solution or even the detection in plasma or serum; the latter approach would avoid sampling bias due to tissue heterogeneity. The system's components have already been measured in the blood of healthy donors and breast cancer patients by ELISA [129,358]. More specific new ELISAs were established for detection of soluble uPA:PAI-1 and uPA:uPAR complexes in blood plasma and showed these to be present much more frequently in the cancer groups [79,261]. But more research on this item will be necessary as the complexes to some extent were also detectable in healthy women. Furthermore, Grebenchtchikov *et al.* found no significant correlations between the expression of uPA, PAI-1, and uPA:PAI-1 complexes in plasma and tumor tissue, and plasma levels were not significantly elevated in malignant breast cancer [127]. There has been an increase in studies using semiquantitative PCR assays for detection of uPA system members [169,227,318], and recently, quantitative real-time PCR

assays have been introduced [40,47,48,98,215,274,331,338,351]. But up to now, systematic studies with high patient numbers in order to compare uPA/PAI-1 mRNA expression values with antigen expression values determined by ELISA are rarely found.

A major problem in the application of biomarkers is the frequent lack of sufficient validation data before clinical use. Varying results of different study groups on the same subject can theoretically be due to differing detection methods. For uPA and PAI-1, *e.g.*, the comparison of different extraction methods for their determination by ELISA led to varying conclusions. Mostly high correlations were found, but some described the best prognostic significances in samples extracted by Triton X [176], others yielded better results from non-detergent extraction [131]. The Receptor and Biomarker Group (RBG) of the European Organisation for Research and Treatment of Cancer (EORTC) plays an important role in the determination of standards for the assessment of new biomarkers and supervises External Quality Assessment (EQA) schemes [304,342,343]. The evaluated standards have been applied to uPA and PAI-1 in the breast cancer trial by Jänicke *et al.* [177] and the pooled analysis by Look *et al.* [222], validating uPA and PAI-1 as significant breast cancer biomarkers in lymph node-negative as well as in lymph node-positive patients.

1.5 Objectives

The subject of this work is the establishment of quantitative real-time LightCycler PCR assays for the detection of the proteolytic factors uPA and PAI-1 in mRNA isolated from malignant breast tumor tissue. The aim is the evaluation of the detection of uPA/PAI-1 mRNA by QPCR as a potential alternative to the antigen determination by ELISA. This work further evaluates the applicability of preserved tissue extracts for determination of uPA and PAI-1. The featured detection method is designed for utilization in very small amounts of tissue specimens for potential future application in early stage cancer diagnosis. The setup of the study includes the following procedures:

- Establishment of the quantitative RT-PCR assays.
- Detection of uPA and PAI-1 by the established assays in breast and ovarian cancer cell lines, in a melanoma and a keratinocyte cell line.
- Application of the quantification method in two breast cancer collectives and statistical evaluation utilizing the available patient and histomorphological data.
- Separate analysis of the QPCR results obtained from our Munich breast cancer cohort: here the samples were available in duplicate - one part of the mRNA was isolated from fresh-frozen (pulverized) tumor tissue, the other from tissue preserved in paraffin and formalin (FFPE).
- Evaluation of the quantified housekeeping genes: in addition to G6PDH - which was available for all samples - ß-actin was quantified in Nijmegen, PBGD in Munich.

In each of the examined cohorts, the quantitative RT-PCR results for uPA and PAI-1 are compared to the available matching antigen concentrations measured by ELISA. In case of availability of the respective data, the housekeeping genes G6PDH and ß-actin, and G6PDH and PBGD, respectively, are statistically related to each other.

2 MATERIALS AND METHODS

2.1 The LightCycler Method

The decision to use the LightCycler Real-Time PCR (Roche Diagnostics, Penzberg, Germany; Software Version 3.5) for this study was made because of its advantages concerning speed, high sensitivity, and the possibility of using extremely small amounts of tumor tissue. In a normal amplification run, the measurement of a sample only takes about 20 milliseconds.

The LightCycler Apparatus

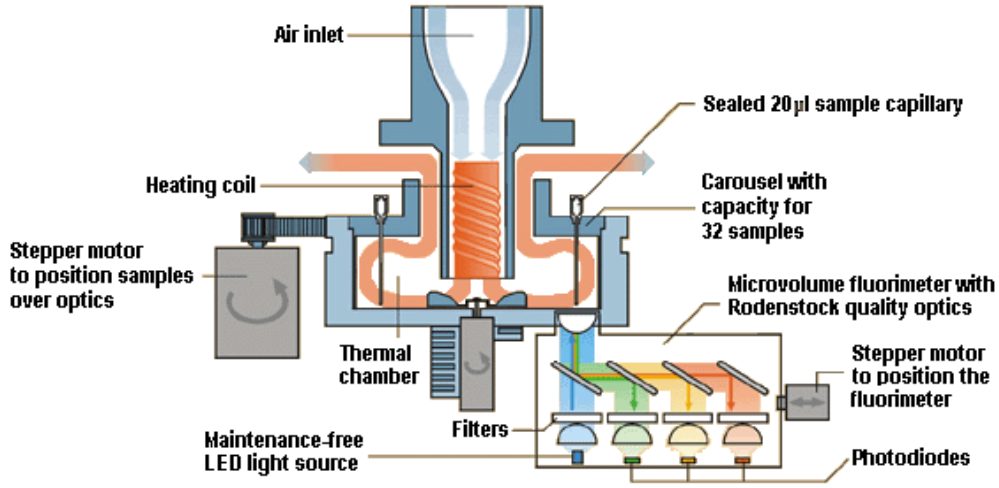


Figure 7: Sectional view of a LightCycler [373]**.** The center of the construction harbors the rotor-like sample carousel which is easily removable for the loading of the samples. Two stepper motors work together to achieve optimal positioning of the capillaries into the focus of the fluorimeter. By the arrangement of the glass capillaries, most of the emitted light is focused on the tip of the capillary. A complete turn of the rotor takes about 5 seconds. For heating and cooling of the samples, ambient air is drawn into the machine by a small fan and heated up by a heating coil. A ramp rate of about 20°C per second can be reached. Air has a very low thermal capacity, and the samples are located in small glass capillaries with a very high surface-to-volume ratio. Heating and cooling in the LightCycler system occurs about ten times faster than in a normal thermal cycler. An amplification cycle requires only 30 to 60 seconds. The amplification products are quantified by fluorescence measurement using a single microvolume fluorimeter. For fluorescence excitation, the LightCycler system is equipped with a blue LED light source which emits light with a wavelength of 470 nm. The fluorimeter provides three channels which detect emitted light with wavelengths of 530 nm, 640 nm, and 710 nm.

Hybridization Probes

In this work, the 'LightCycler FastStart DNA Master Hybridization Probes' Kit (Roche Diagnostics) was used. In addition to forward and reverse primers, template-specific hybridization probes have to be produced as detection format. These hybridization probes are two oligonucleotides that hybridize to adjacent internal sequences of the amplicon. The donor oligonucleotide has a fluorescein label at its 3' end. The second oligonucleotide, the acceptor, has the fluorophore LightCycler Red 640 at its 5' end. The two probes come in close proximity when they hybridize to the template DNA, resulting in the so-called fluorescence resonance energy transfer (FRET) between the two fluorophores. During FRET, the donor fluorophore is excited by the light source of the LightCycler instrument, and part of the excitation energy is transferred to the acceptor fluorophore. The fluorescence emitted by the acceptor fluorophore is then measured by the LightCycler instrument (Figure 8).

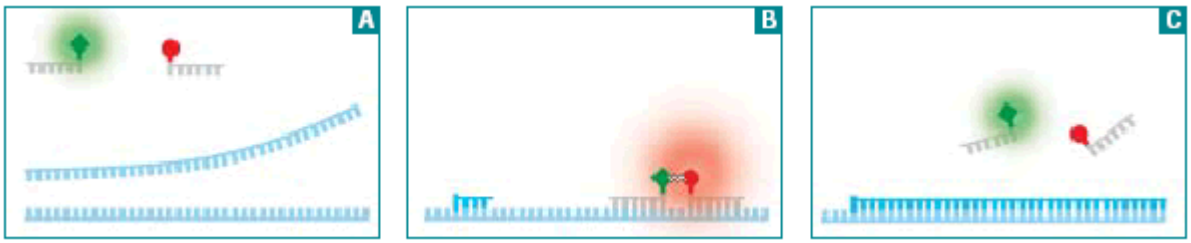


Figure 8: Annealing process of the hybridization probes [373]. A: The acceptor probe on the right side is labed with LightCycler Red at the 5' end. Hybridization does not take place during the denaturation phase of PCR, and the distance between the dyes is too large to allow energy transfer to occur. B: During the annealing phase, the probes hybridize to the amplified DNA fragment in a close head-to-tail arrangement. When fluorescein is excited by the light from the LED, it emits green fluorescent light, transferring the energy to the acceptor probe which then emits red fluorescent light. The red fluorescence is measured at the end of each annealing step when the fluorescence intensity is highest. C: After annealing, the temperature is raised and the hybridization probes are displaced during elongation. At the end of this step, the PCR product is double-stranded and the displaced probes are again too far apart to allow FRET to occur.

Quantitative RT-PCR Procedure and Calculation of Results by Standard Calibration

In each LightCycler PCR run, 2 µl (about 5 ng) of template cDNA and 2 µl of the 'Hot Start' reaction mix per capillary were added, the final volume was 20 µl. The total capacity of the LightCycler carousel is 32 capillaries. The amplification program starts with pre-denaturation at 95°C for 10 minutes, followed by 45 cycles of amplification, temperatures differing slightly according to the respective amplicon lengths: in the runs measuring the houskeeping genes, denaturation took place for 10 seconds at 95°C, annealing for 15 seconds at 55°C, and elongation for 15 seconds at 72°C. For amplification of uPA, 10 seconds of heating at 95°C were followed by 10 seconds at 62°C and 5 seconds at 72°C. For PAI-1, 10 seconds at 95°C, 10 seconds at 63°C, and 5 seconds at 72°C were programmed. Finally, the amplification products were cooled down to 40°C for 30 seconds. In order to be able to calculate the absolute copy numbers of the amplicons, eight plasmid-coated standard capillaries - containing 10, 20, 50, 100, 1,000, 5,000, 10,000, and 100,000 copies of the amplicon to be detected - were produced. For this purpose, the linearized plasmids pUC18-uPA and pMelBacA-PAI-1, which harbored the complete cDNA sequence of uPA and PAI-1, respectively, had been calibrated by HPLC and coated to the capillaries (Roboscreen, Leipzig, Germany) [196]. In the course of further uPA/PAI-1 detection in different collectives, multiparameter standard capillaries coated with uPA and PAI-1 DNA were used. Log-phase analysis was applied to calculate the amount of template cDNA measured in every cycle. By the second derivative maximum method the so-called crossing point (CP) - the measuring point of the fluorescence curve - was determined for each sample. This method works as follows: an algorithm of the LightCycler software identifies the first turning point of the fluorescence curve's second derivative which is defined as the crossing point. A calibration curve is constructed by plotting the logarithm of the copy number (x-axis) against the cycle number (y-axis). The regression graph runs through the standards' crossing points, numbers of the samples are determined from this straight line (Figure 9, Figure 10). By contrast, conventional PCR uses end-point analysis which measures only at the end of amplification for calculation of the result. Its accuracy depends on the initial template concentration and PCR efficiency.

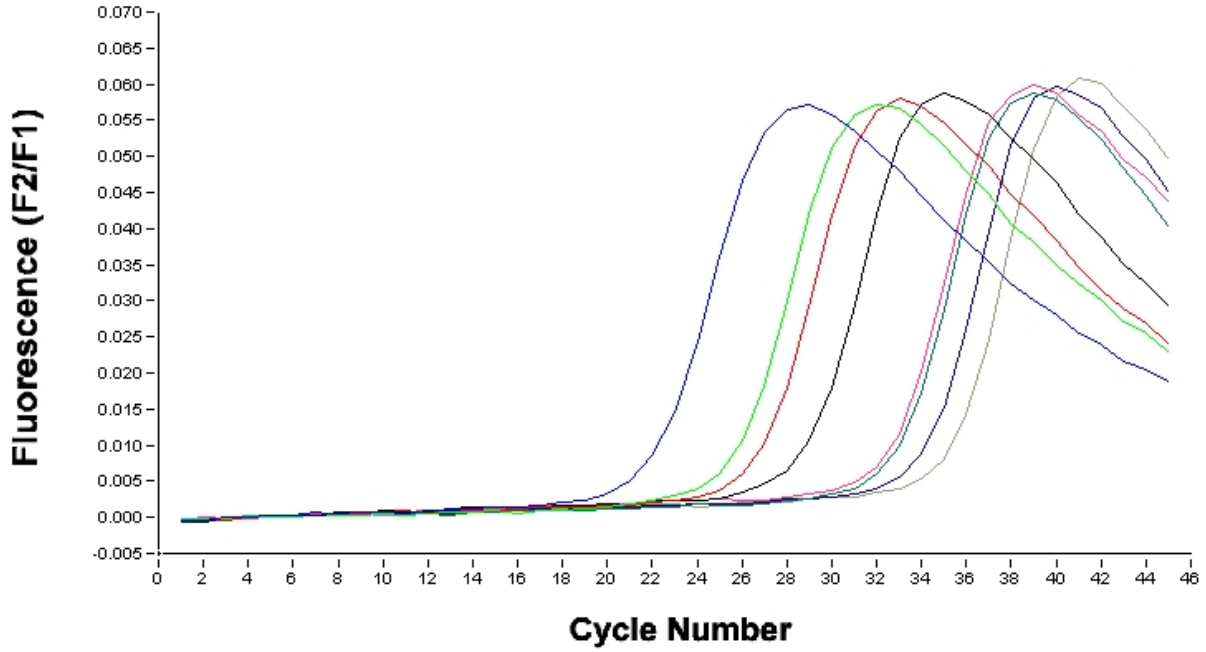


Figure 9: Example of a fluorescence curve diagram (PAI-1 standards). The 8 shown curves represent 100,000, 10,000, 5,000, 1,000, 100, 50, 20, and 10 copies of the amplicon; emission of fluorescence during an earlier cycle indicating a higher copy number.

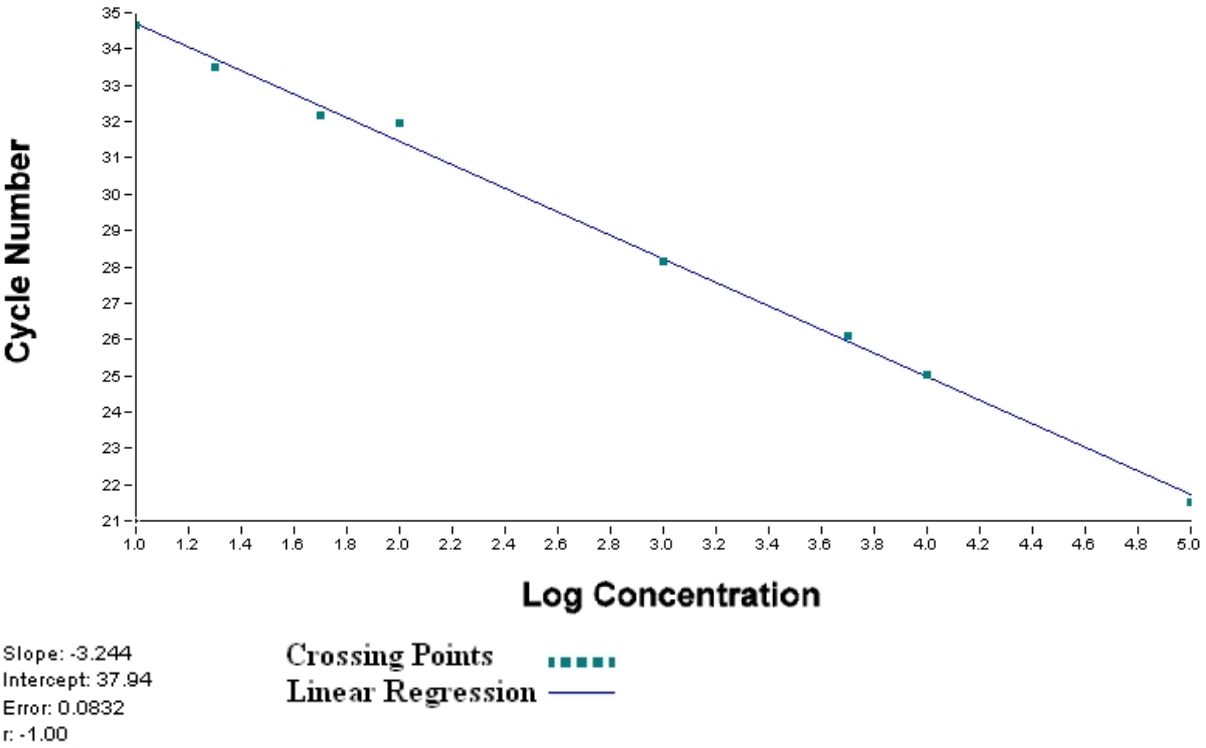


Figure 10: PAI-1 log-regression calibration graph. This is an example of a log-regression graph through the crossing points of PAI-1 standards which is used to calculate the molecule numbers of the samples. All calibration curves showed correlation coefficients of $r < -0.99$, indicating a precise log-linear relationship.

Housekeeping Genes

The measured values were normalized to the housekeeping genes G6PDH (glucose-6-phosphate-dehydrogenase) and PBGD (porphobilinogen deaminase) [197], at first by using the respective housekeeping gene sets of Roche Diagnostics. In these assays, the sample numbers were calculated using five standards with 500, 5,000, 50,000, 500,000, and 5,000,000 copies of the respective housekeeping gene. The kit detecting G6PDH produces amplicons with a length of 123 base pairs. G6PDH has already been established as a housekeeping gene in a breast cancer study by Farthmann *et al.* [98]. Later, for detection of PBGD, eight plasmid-coated standard capillaries with defined copy numbers were produced in analogy to uPA and PAI-1. The PBGD amplicon comprised 150 base pairs. For the Nijmegen collective, our collaborating laboratory in the Netherlands additionally measured ß-actin as housekeeping gene (amplicon length: 294 base pairs). The standard capillaries for ß-actin which were used for generation of the calibration curves contained between 100 and 10,000,000 molecules. The evaluation and statistical analyses of the data were carried through using the relative mRNA expression ratios: zmol target gene/amol housekeeping gene - in case of ß-actin: zmol target gene/fmol housekeeping gene.

Assay Quality Control

To ensure stability and comparability of the calculated values, the standard crossing points of all runs within each assay were observed. With arrival of each new standard capillary delivery, one set of old and new standards were compared in a single LightCycler run. Intra- and inter-assay precision of the sample results were proportionally determined by the variation coefficient (VC). Dilution series of the cDNA being measured were conducted at frequent intervals on condition that the copy numbers were high enough (Table 5). Correlation coefficients of the calibration curves of uPA, PAI-1, and housekeeping gene standards were recorded in every single LightCycler run. The specificity of the method was examined by the regular use of negative controls (without uPA or PAI-1 expression).

2.2 Cell Lines

Cell Line Selection and Treatment

The following cultured cells were used in the present work: (1) The mammary carcinoma cell lines MDA-MB-231 (adenocarcinoma cell line) and MDA-MB-231 BAG (subline of MDA-MB-231, stably transfected with the bacterial lacZ gene) were obtained from the Institute of Experimental Oncology and Therapy Research, Technical University of Munich, Munich, Germany. The adenocarcinoma cell line MCF-7, and aMCF-7, an adriamycine-resistant subline of MCF-7, were provided by the Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany. (2) The MDA-MB-435 cell line was also supplied by the Institute of Experimental Oncology and Therapy Research of the Technical University of Munich. Formerly believed to represent a breast cancer cell line, MDA-MB-435 was officially redefined as derivative of the M14 melanoma cell line in 2007 [115,273]. (3) The ovarian cystadenocarcinoma cell lines OV-MZ-6 and OV-MZ-10 were obtained from the Städtische Kliniken Frankfurt a.M.-Höchst, Frankfurt a.M.-Höchst, Germany. (4) The non-malignant human keratinocyte cell line HaCaT was provided by the Department of Dermatology of the Dresden University of Technology, Dresden, Germany.

The cells were first cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Invitrogen), 1% penicilline-streptomycine (Biochrom, Berlin, Germany), 1% arginine-asparagine (Sigma, Deisenhofen, Germany), and 1% HEPES buffer (Invitrogen). The cells were harvested from monolayer dishes after two days [98].

Antigen Determination

For uPA and PAI-1 antigen determination by ELISA, the culture supernatant was collected, cleared by centrifugation, and stored at -20 °C until use. uPA and PAI-1 antigen levels in the supernatants were expressed as ng analyte per 10^6 cells after 48 hours of cultivation.

For uPA, the ‘IMUBIND uPA ELISA Kit’, Product No. 894, was used, for the PAI-1-ELISA, the ‘IMUBIND Tissue PAI-1 ELISA Kit’, Product No. 821 (American Diagnostica, Stamford, USA). With these ELISA kits uPA and PAI-1 can be determined both from cell culture supernatants and from tissue extracts. The

‘IMUBIND ELISA Kits’ use murine monoclonal antibodies against human uPA and PAI-1, respectively. The samples incubate in precoated microtest wells, and a second biotinylated antibody recognizes the bound uPA or PAI-1 molecules. The addition of streptavidin-conjugated horseradish peroxidase (HRP) completes the formation of the antibody-enzyme detection complex. Then perborate/3,3’,3,5’ - tetramethylbenzidine (TMB) substrate is added which, through reaction with HRP, creates a blue-colored solution. Sensitivity is increased by addition of a sulfuric acid stop solution, yielding a yellow color. The respective antigen levels are quantified by measurement of solution absorbances at 450 nm and comparison of the values with those of a standard curve.

RNA Isolation and cDNA Synthesis

In total, three independent mRNA preparations of these cell lines were produced. For RNA isolation, ‘Trizol Reagent’ (Invitrogen) was used according to the manufacturer’s instructions. mRNA concentrations were determined spectrophotometrically. The quality of the RNA was evaluated by the 260/280 nm quotient. Approximately 1 µg of each RNA sample was then reversely transcribed into complementary DNA using random hexamer primers with the ‘AMV cDNA First Strand Synthesis Kit’ (Roche Diagnostics). The steps consisted in 10 minutes of RNA denaturation at 65°C, 10 minutes of annealing of the random primer at 25°C, after that, one hour of cDNA synthesis at 42°C, and 5 minutes of enzyme denaturation at 99°C. Then the PCR product was cooled down rapidly to 4°C. The cDNA was diluted in water 1:20, and stored at -20°C.

RT-PCR

Finally, uPA und PAI-1 contents were quantified in the LightCycler instrument. Additional measurements of the target genes in negative control samples were done. As housekeeping gene G6PDH was measured. The gene contents of each cDNA were obtained from double measurements in several LightCycler runs, the number of runs depending on the extent of result divergence. Assays were repeated in case of an intra- and inter-assay variability of > 20% and > 40%, respectively.

2.3 Patient Cohorts

2.3.1 Dutch Collective (Nijmegen)

Tissue specimens from a population-based cohort of 105 primary breast cancer patients, having undergone breast cancer surgery between 1989 and 1996 in participating hospitals of the Comprehensive Cancer Center East in the Netherlands, were included in the study. The local ethical committees officially permitted the utilization of the samples. After surgical resection of the primary tumor, representative tumor tissue specimens were selected macroscopically by a pathologist and immediately snap-frozen in liquid nitrogen (Department of Chemical Endocrinology, University Medical Center Nijmegen, the Netherlands). The clinical data were collected retrospectively. The histological grade of the tumors was determined according to the criteria established by Scarff, Bloom, and Richardson [23,295]. Tumor stages were classified according to the TNM classification system.

Patient Selection

Patients who had received neo-adjuvant treatment or had had a previous diagnosis of cancer or a carcinoma *in situ*, as well as patients with recurrent disease within one month after surgery, or with distant metastases at the time of diagnosis were excluded from the study. The patients' age at diagnosis ranged from 30 to 88 years, with a median of 58 years. The patients had undergone modified radical mastectomy (n = 84) or breast-preserving lumpectomy (n = 21), combined with axillary lymph node dissection. Postoperative locoregional radiotherapy of the breast was carried out in 68 cases (64.8%), after incomplete resection or breast-preserving treatment, with one patient's information missing. Axillary or supraclavicular regions were irradiated depending on the degree of nodal involvement. Lymph node metastases were detected in 54 patients (51.4%), 44 patients (41.9%) were lymph node-negative. Information on lymph node involvement could not be retrieved in 7 cases. Adjuvant systemic treatment was administered according to respective consensus recommendations at the time.

Of the 57 patients with whose data a survival analysis was conducted 29 patients received no further treatment - mostly patients without axillary lymph-node involvement -, 17 patients were treated with endocrine therapy after tumor resection,

7 were given chemotherapy alone, and 4 patients received both chemotherapy and endocrine therapy. Premenopausal patients received chemotherapy, an additional endocrine tamoxifen therapy was administered in estrogen and/or progesterone receptor-positive patients. Postmenopausal node-positive patients with estrogen and/or progesterone receptor-positive tumors received adjuvant endocrine tamoxifen therapy for two years. If the primary tumor of node-positive patients was hormone receptor-negative, no adjuvant therapy was administered. In the absence of complaints or suspicion of relapse, patients were routinely checked once every three months during the first two years, once every six months for five years, and thereafter once a year. Follow-up information was available with a median follow-up time of 68 months and a maximum follow-up period of 122 months. During that time, 24 patients (42.1%) had a recurrence and 18 patients (31.6%) died.

Antigen Determination

Cytosol fractions were prepared from 105 pulverized deep-frozen primary breast cancer tissue specimens obtained from participating hospitals of the Comprehensive Cancer Center East, Nijmegen, the Netherlands. uPA and PAI-1 antigen contents were determined in the high-speed supernatant (cytosol fraction) by published in-house ELISA formats [16,128,342]: the tumor tissues were also pulverized in the frozen state, but then - in contrast to the German samples - homogenized in a buffer lacking the non-ionic detergent Triton X-100 (so-called EORTC buffer, but without monothioglycerol or glycerol). Protein contents of the breast cancer cytosol fractions were measured as modified by Lowry [224], using BSA as standard.

RNA Isolation and cDNA Synthesis

For RNA isolation from about 20 mg of tissue powder, the ‘RNeasy Mini Kit’ (Qiagen, Hilden, Germany) with on-column DNase-I treatment was used. RNA concentrations were measured spectrophotometrically at 260 nm using ‘Genequant’ (Amersham, Eindhoven, the Netherlands). The purified total RNA (1 µg) was denatured for 10 minutes at 70°C and immediately cooled on ice. Reverse transcription was performed with the ‘Reverse Transcription System’ (Promega Benelux BV, Leiden, the Netherlands) following the instructions of the manufacturer’s protocol. After the annealing phase (10 min at 20°C) - during which the random hexamer binds to the RNA -, cDNA synthesis proceeded for 60 minutes at 42°C, and, thereafter, 5 minutes

of enzyme denaturation at 95°C took place. The cDNA samples were diluted three-fold [324].

RT-PCR

The applied quantitative RT-PCR assay followed the procedure as described above. As housekeeping genes G6PDH and ß-actin mRNA expressions were measured in all 105 samples. QPCR runs of cDNA probes were performed at least twice, the frequency depending on the extent of inter-run result deviation.

2.3.2 German Collective (Dresden and Munich)

Patient Selection

The German cohort comprised 74 breast cancer patients who were treated in the University Medical Center Dresden or in the Klinikum rechts der Isar in Munich between 1993 and 2003. The study adhered to the respective national regulations on ethical issues and was approved by local ethical committees. At the time of surgery the patients' age ranged from 33 to 84 years, with a median age of 58 years. The patients had either undergone modified radical mastectomy (n = 21) or breast preserving lumpectomy (n = 30; surgical method of 23 patients unknown), combined with axillary lymph node dissection. In the majority of patients of whom informations on postoperative treatment were available, a postoperative locoregional radiotherapy of the breast was administered (79.5%); axillary or supraclavicular regions were irradiated depending on the degree of nodal involvement. In 29 cases (39.2%), tumor cells were detected in the lymph nodes; 39 patients (52.7%) were lymph node-negative; the lymph node status of 6 patients (8.1%) was unknown.

Antigen Determination

Protein concentrations were measured by ELISA. Detergent extracts (not cytosol fractions as in case of the Dutch samples) were prepared from 74 primary breast cancer tissue specimens: deep-frozen tumor tissue specimens were first pulverized and then suspended in TRIS buffer containing the non-ionic detergent Triton X-100; after separation of cell debris by ultra-centrifugation uPA and PAI-1 antigen concentrations were assessed in the supernatant by commercially available ELISA kits ('IMUBIND uPA ELISA Kit' # 894 and 'IMUBIND PAI-1 ELISA Kit' # 821; American Diagnostica, Stamford, USA) [199,237,304]. The ELISA procedure

corresponded to that described for the cell lines (see above). Protein contents of the detergent-released fractions were measured using the 'BCA Protein Kit' (Pierce, Rockford, USA), using BSA as standard. Data were available for all tumor samples, and results were expressed in ng analyte per mg of total protein.

RNA Isolation and cDNA Synthesis

mRNA of the samples from Munich was isolated from frozen tissue (microdismembrator pellet; amount: one scalpel tip) and the corresponding paraffin-embedded, formalin-fixed tissue (3*10 µm sections) of the breast carcinomas. The examined paraffin sections were obtained from paraffin blocks originally used for determination of HER2/*neu*. For each block HE-stained sections are available.

Prior to the RNA extraction procedure, the paraffin sections were solubilized in xylol and thereby deparaffinized. mRNA of the corresponding tissue specimens was isolated with the 'High Pure RNA Paraffin Kit' of Roche Diagnostics according to the manufacturer's protocol. mRNA concentrations were determined spectrophotometrically. The quality of the mRNA was evaluated by the 260/280 nm quotient. Approximately 1 µg of each RNA sample was again reverse transcribed into complementary DNA using random primers with the 'AMV cDNA First Strand Synthesis Kit' (Roche Diagnostics). In some cases, mRNA concentrations were not high enough for the photometer to determine the sample volume containing 1 µg of RNA. Here, in divergence to the standard procedure, the maximum volume of the respective mRNA was put into the reverse transcription reaction (8.2 µl RNA + 11.8 µl reverse transcription master mix = 20 µl total volume).

Extraction of the mRNA from Dresden was conducted using the 'RNeasy Mini Kit' (Qiagen, Hilden, Germany). Reverse transcription of the mRNA was performed using the 'cDNA-Cycle Kit' (Invitrogen, Karlsruhe, Germany).

The reverse transcription steps in the cohort from Munich corresponded to those administered to the mRNA extracted from cell lines (see above). The PCR product was immediately cooled down to 4°C and diluted 1:2 (Dresden samples: four-fold), resulting in a total volume of 40 µl. Then the cDNA samples were stored at -20°C.

RT-PCR

Eventually, uPA and PAI-1 mRNA contents were quantified by LightCycler PCR. For the tumor collective from Munich, the housekeeping gene PBGD was measured in addition to G6PDH. Only samples with a G6PDH expression of at least 500 molecules (corresponding to the lowest G6PDH standard) were considered valid, the remaining samples were excluded from further analysis. If only one of the two corresponding tumor samples had a G6PDH expression above 500, this sample was deleted as well. Thus, of the 38 matched pairs of mRNA originally extracted, 28 corresponding tumor samples remained for evaluation. The specificity of template detection was affirmed in the LightCycler by uPA/PAI-1 measurements in negative controls. Similar to the collectives from Nijmegen and Dresden runs were repeated at least once, additional measurements were conducted in case of an inter-assay variability of > 40%.

2.4 Statistical Analysis

The results measured by QPCR and other data of the breast cancer patients composed of continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test; uPA and PAI-1 mRNA and antigen expressions did not follow a Gaussian distribution. In view of lacking normally distributed data, associations between uPA and PAI-1 expression values and clinical and histomorphological parameters were determined using nonparametric tests (Mann-Whitney U or Kruskal-Wallis tests). The levels of significance in correlations between continuous variables were calculated with the Spearman rank correlation (r_s). For survival analysis, the disease-free survival time was defined as the time from surgery until diagnosis of recurrent disease or death (combined DFS). The association of the mRNA expression levels of uPA/PAI-1 as well as of other clinical and histomorphological factors with combined DFS was analyzed using the Cox univariate and multivariate proportional hazard regression models [60]. Survival curves were generated by univariate Kaplan-Meier estimation using the log rank regression model [185]. P-values ≤ 0.05 were considered statistically significant. Calculations were performed using the SPSS statistical package, release 13.0 (SPSS Inc., Chicago, USA).

3 RESULTS

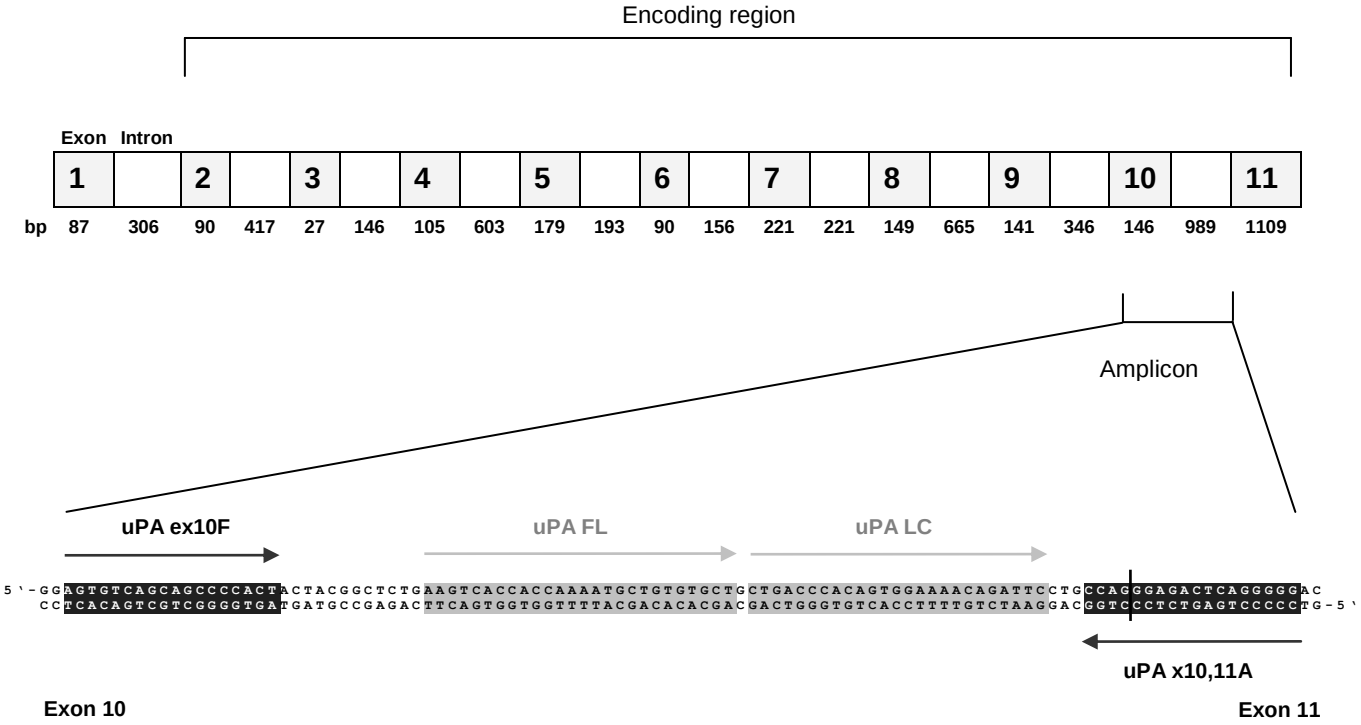
3.1 Establishment of the Quantification Method

3.1.1 Primer Design

Amplicon lengths of the QPCR assays were chosen as small as possible to be suitable for the analysis of samples from fresh tissue and cryo-preserved tissue, as well as from formalin-fixed, paraffin-embedded (FFPE) tissue. Preferably, the amplicons should encompass between 100 and 150 base pairs, considering that the hybridization probes necessary for detection anneal within the amplicon. According to these instructions and based on the cDNA sequences of uPA (NM_002658) and PAI-1 (NM_000602), Tib MolBiol (Berlin, Germany) proposed and delivered primers and hybridization probes.

First, different primer combinations were to be tested. For this process, uPA and PAI-1 plasmids were isolated from the bacteria pUC18-uPA and pMelBacA-PAI-1, respectively, and then linearized. Subsequently, a conventional qualitative PCR was done using the different primer pairs, and the amplification products were plotted onto an electrophoresis gel. In parallel to this step, the optimal plasmid concentrations for the following PCR runs were tested. For all primer combinations intense bands became visible. In order to minimize the risk of amplification of genomic DNA, it was decided that at least one primer in each assay should overlap an exon boundary, *i.e.* bind to two exons. The forward primer for uPA annealed within exon 10, the reverse primer overlapped the boundary between exon 10 and 11. These exons are separated by an intron of 989 base pairs in the genomic DNA. The forward primer of PAI-1 overlapped the exons 5 and 6, the reverse primer overlapped the exons 6 and 7, the intervening regions comprising 1592 and 120 base pairs, respectively. The amplicon length of the definite uPA assay consisted of 103 base pairs, the PAI-1 amplicon was 132 base pairs long (Figure 11, Table 3).

a)


Encoding region
Exon Intron
1 2 3 4 5 6 7 8 9 10 11
bp 87 306 90 417 27 146 105 603 179 193 90 156 221 221 149 665 141 346 146 989 1109
Amplicon
uPA ex10F
uPA FL
uPA LC
5`-GGAGTGTCAGCAGCCCCACTACTACGGCTCTGAAGTCACCACCAAAATGCTGTGTGCTGCTGACCCACAGTGGAAAACAGATTCCTGCCAGGGAGACTCAGGGGGAC
CCTCACAGTCGTCGGGGGTGATGATGCCGAGACTTCAGTGGTGGTTTTACGACACACGACGACTGGGTGTCACCTTTTGTCTAAGGACGGTCCCTCTGAGTCCCCCTG-5`
uPA x10,11A
Exon 10
Exon 11

b)

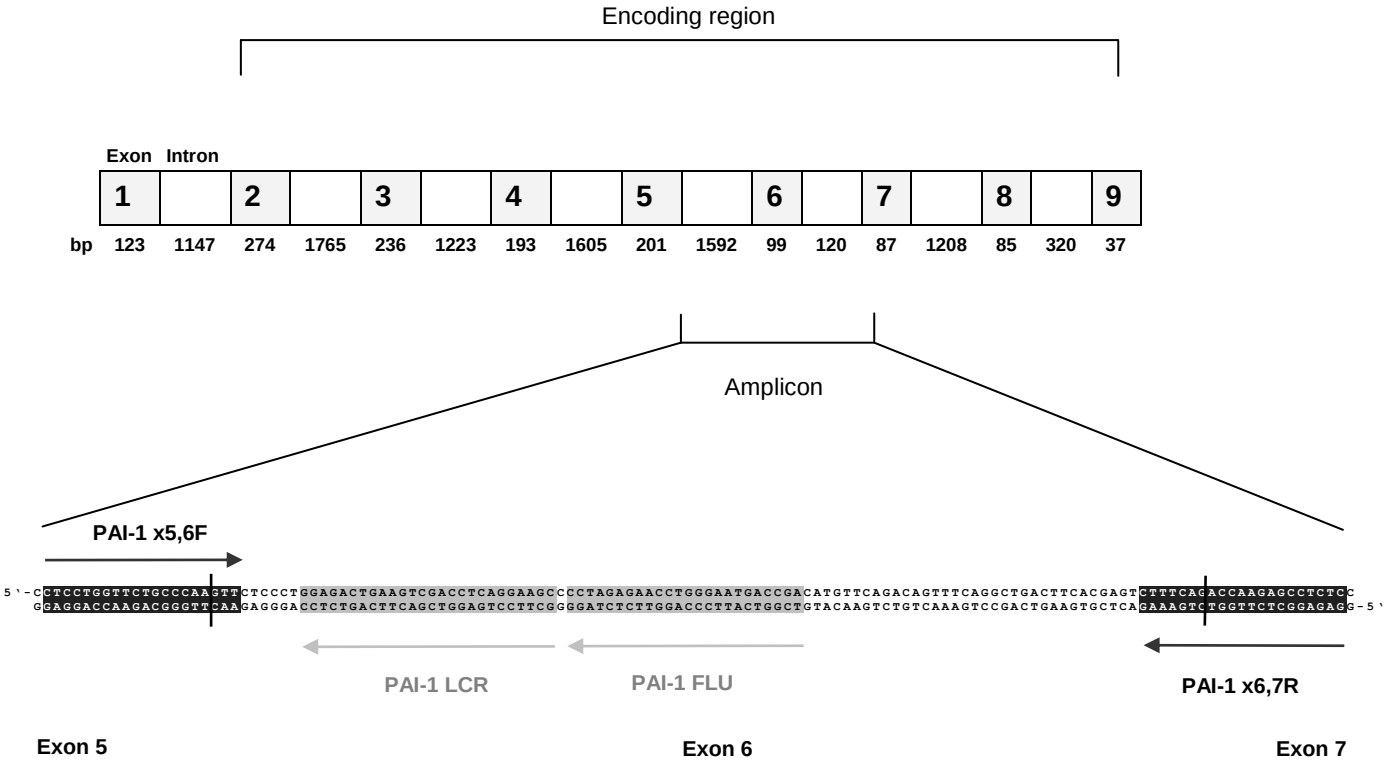


Figure 11: uPA and PAI-1 genes, and amplicon localizations in the QPCR assays. The uPA amplicon (a) encompasses 103, the PAI-1 amplicon (b) 132 base pairs. The detection of the correct amplicons was confirmed by sequencing (Figure 14). The localization and orientation of the primers is indicated by arrows; exon/exon boundaries by vertical bars.

Table 3: Sequences of primers and hybridization probes for uPA and PAI-1 applied in the LightCycler QPCR assay.

		Oligonucleotide sequence (5'→ 3')	Fragment size (bp)
uPA	**Forward Primer (uPA ex10F)**	**AGT GTC AGC AGC CCC ACT**	**103**
	Reverse Primer (uPA x10,11A)	**CCC CCT GAG TCT CCC TGG**	
	Hyb Probe 1 (uPA FL)	**AAG TCA CCA CCA AAA TGC TGT GTG CT**	
	Hyb Probe 2 (uPA LC)	**CTG ACC CAC AGT GGA AAA CAG ATT C**	
PAI-1	**Forward Primer (PAI-1 x5,6F)**	**CTC CTG GTT CTG CCC AAG TT**	**132**
	Reverse Primer (PAI-1 x6,7R)	**GAG AGG CTC TTG GTC TGA AAG**	
	Hyb Probe 1 (PAI-1 FLU)	**TCG GTC ATT CCC AGG TTC TCT AGG**	
	Hyb Probe 2 (PAI-1 LCR)	**GCT TCC TGA GGT CGA CTT CAG TCT CC**	

In addition to the visualization of single bands for each amplicon by gel-electrophoresis (1.5% agarose gel; Figure 13), the QPCR products were extracted and sent to the Eurofins Medigenomix GmbH (Martinsried, Germany) for sequencing to ensure detection of the correct amplicons (Figure 14). Primers and hybridization probes were used in concentrations of 0.5 µM and 0.2 µM, respectively.

3.1.2 Optimization of RT-PCR Conditions

As prerequisite for the application of the chosen hybridization probes and primers in the Lightcycler the optimal magnesium concentration in the master mix had to be determined. For each assay a magnesium titration series with varying amounts of $MgCl_2$ was performed which was evaluated according to the respective amplification level and the fluorescence curves generated by the LightCycler software. $MgCl_2$-concentrations ranging from 1 to 7 mM were tested. uPA plasmid was added in a concentration of about 5 pg/µl, the concentration of the PAI-1 plasmid was approximately 2 pg/µl. Samples yielding the highest copy numbers of the respective amplicons were preferred, the optimum amplification curve conformations, displaying steep gradients, were chosen. Consecutively, the amplification products were brought onto an electrophoresis gel to verify amplification of the correct amplicons. Eventually, $MgCl_2$-concentrations of 5 mM for uPA and of 3.5 mM for PAI-1 were chosen, since these concentrations yielded the best amplification results.

3.2 Assay Quality Control

The log-linear calibration curves produced in each run by the QPCR method all had correlation coefficients of below r = -0.99 as indicators of precise amplicon detection, including areas of low target gene expression (Figure 10). Crossing point deviations of the standard samples did not exceed two cycles within each sample collective. Plots of measured *versus* theoretical transcript numbers of uPA and PAI-1 for the capillaries coated with 10 to 100,000 standard template copies in both assays, generated from 36 and 33 independent QPCR runs (Dutch and German cohorts), respectively, are shown in Figure 12. The correlations of mRNA values were highly significant with $r_s = 0.99$ ($p < 0.001$) in both QPCR assays.

As mentioned earlier, for evaluation of intra-assay precision the cell line samples were measured in duplicate within each run. The inter-assay variability was determined from at least two measurements. The three preparations of cell line mRNA measured by QPCR resulted in comparable sample contents, mostly with variation coefficients of distinctly below 40% (Table 4).

The mRNA contents of the breast cancer samples were obtained from repeated LightCycler measurements, the number of runs depending on inter- and intra-assay divergence. The inter-assay variation coefficients ranged from 0 to 43% (uPA) and from 0 to 23% (PAI-1), with means of 11% and 8%, respectively.

Most assay procedures demand dilution of the samples for detectability of the analyte's contents within a specific concentration range. Dilution series revealed a good stability of the measured values (Table 5).

After electrophoresis in agarose gel, the QPCR products were visualized by ethidium-bromide staining as single bands of the expected amplicon lengths (Figure 13). Amplification of the correct amplicons was verified by sequencing (Figure 14). In non-template negative controls no fluorescence was observed.

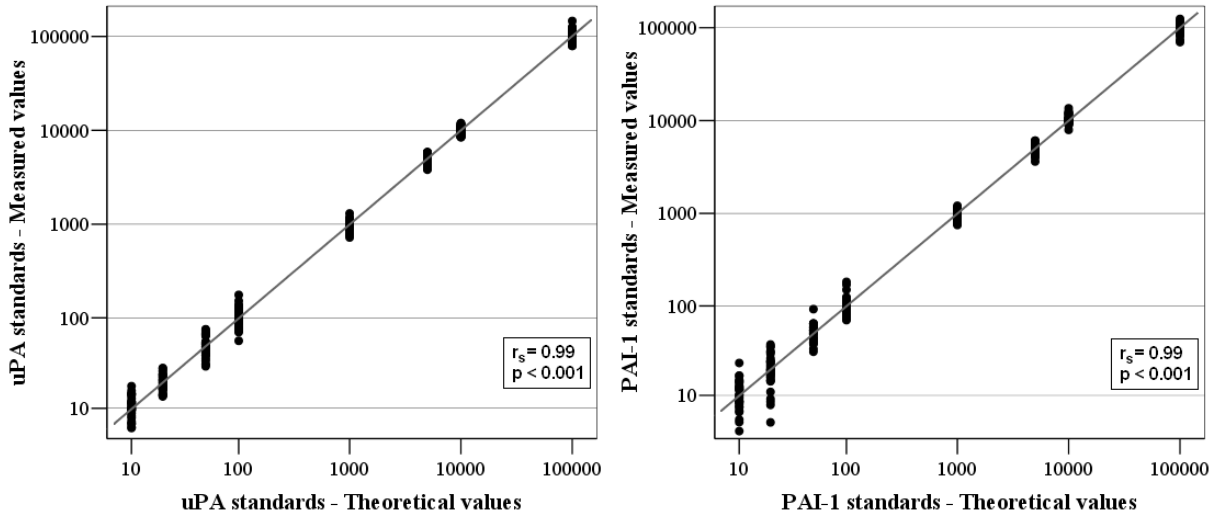


Figure 12: uPA and PAI-1 mRNA standard measurements by LightCycler QPCR. The results were obtained from 36 (uPA) and 33 (PAI-1) LightCycler PCR runs, respectively.

Table 4: uPA mRNA quantification by LightCycler QPCR in cell lines. The mean mRNA transcript levels, originating from three independent preparations, were normalized to G6PDH. The standard deviation (SD) and the coefficient of variation (VC) show to which extent the uPA amounts varied in the three runs.

	uPA mRNA (zmol uPA/amol G6PDH)					
	1st	2nd	3rd	Mean	SD	VC (%)
Mammary carcinoma						
MDA-MB-231	159.62	99.12	130.96	129.90	30	23
MDA-MB-231 BAG	150.34	117.77	136.36	134.82	16	12
MCF-7	0.18	0.12	0.18	0.16	0	22
aMCF-7	2.43	1.69	1.13	1.75	1	37
Ovarian carcinoma						
OVMZ-6	72.17	84.05	98.38	84.87	13	15
OVMZ-10	1452.85	1219.77	1556.68	1409.77	173	12
Melanoma						
MDA-MB-435	0.11	-	0.08	0.09	0	20
Keratinocytes						
HaCaT	70.33	74.60	136.51	93.81	37	39

Table 5: LightCycler QPCR dilution series. The cell lines OV-MZ-10 and MDA-MB-231 serve as examples. The mRNA transcript levels were normalized to G6PDH.

	uPA		**PAI-1**			
	OV-MZ-10		**OV-MZ-10**		**MDA-MB-231**	
Dilution	**Measured value**	***Theoretical calculation***	**Measured value**	***Theoretical calculation***	**Measured value**	***Theoretical calculation***
Undiluted	**90455**	***90455***	**111500**	***111500***	**60660**	***60660***
1:2	**41585**	***45228***	**47480**	***55750***	**33560**	***30330***
1:4	**20155**	***22614***	**24860**	***27875***	**16870**	***15165***
1:8	**9242**	***11307***	**13250**	***13938***	**8408**	***7583***
1:16	**4441**	***5653***	**7108**	***6969***	**4244**	***3791***
1:32	**2209**	***2827***	**3889**	***3484***	**2272**	***1896***
1:64	**1233**	***1413***	**1815**	***1742***	**1628**	***948***

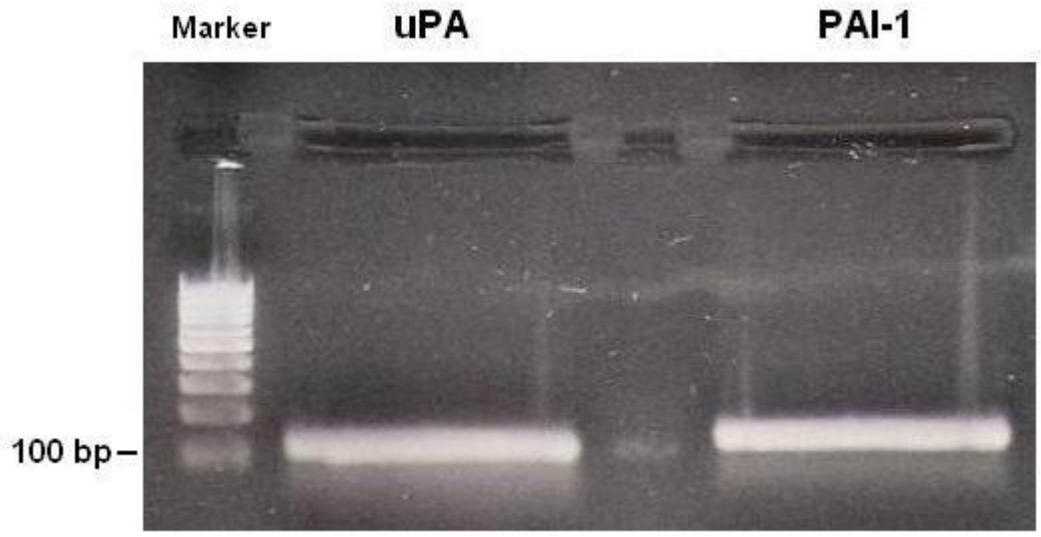
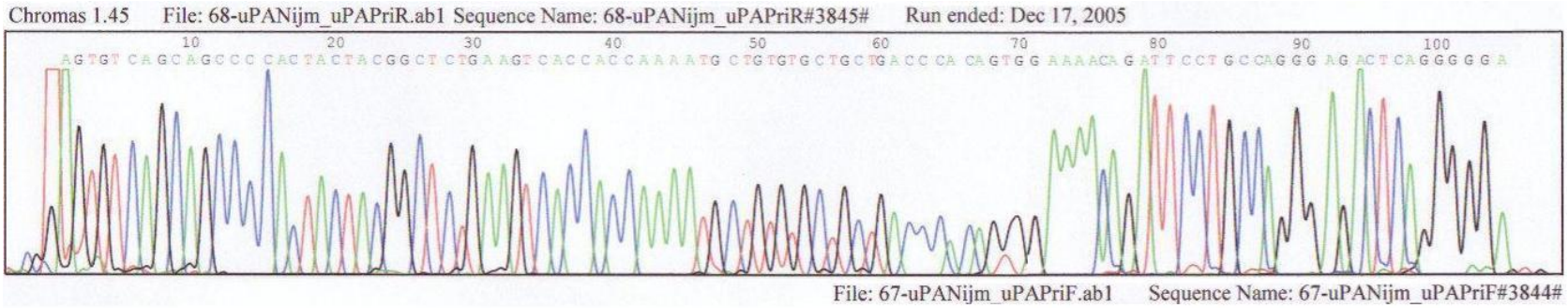


Figure 13: Examples of uPA and PAI-1 QPCR products visualized by a 1.5% agarose gel-electrophoresis. QPCR products of the cell line analysis and the breast cancer collectives with sufficiently high concentrations of the target genes were applied to gel electrophoresis, purified, and then subjected to sequencing (Figure 14).

a)



b)

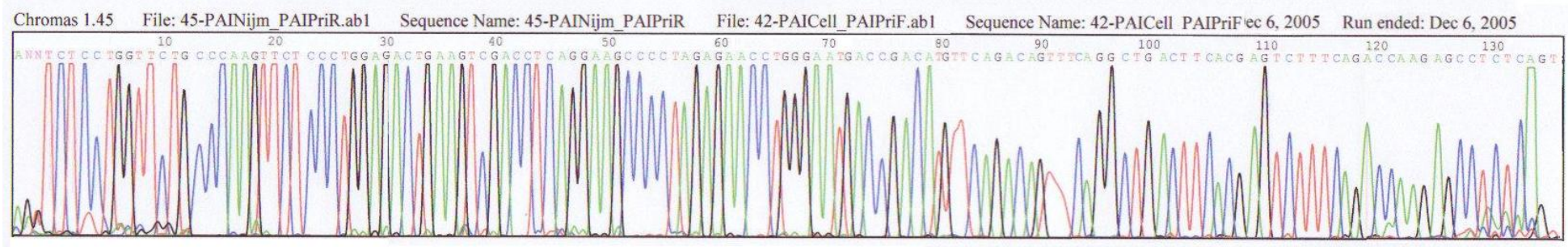


Figure 14: Sequencing results. LightCycler QPCR products from the quantification of uPA and PAI-1 in cell lines and breast cancer samples were extracted and sent to the Eurofins Medigenomix GmbH (Martinsried, Germany) for sequencing to ensure detection of the correct amplicons. a) uPA amplicon sequence; b) PAI-1 amplicon sequence.

3.3 Application of the Quantification Method

After establishment of the assay systems, the first step was to test uPA and PAI-1 expressions in cell lines, and to see if protein expressions - measured by specific ELISA from cell supernatants - correlated to the respective mRNA concentrations. Secondly, the assays were applied to two breast tumor collectives, one from The Netherlands (Nijmegen), and one from Germany (Dresden and Munich). These were statistically analyzed, particularly comparing antigen and mRNA expression of uPA and PAI-1 measured by ELISA and QPCR, respectively. Another analysis was done with respect to clinical and histomorphological parameters and to the prognosis of the patients in the Dutch cohort. The tumor samples from Munich, provided by the Gynecological Department of the Klinikum rechts der Isar, were available in duplicate. One of each tumor specimen was stored as pulverized cryopowder, the other was embedded in paraffin and fixed in formalin. The uPA and PAI-1 expression patterns in these different tissue preservations were compared. In addition, the applicability of different housekeeping genes was tested.

3.3.1 Cell Line Analysis

The analysis of the eight cell lines showed an about five times higher expression of PAI-1 than of uPA, when measured by ELISA. By LightCycler QPCR quantification, uPA expression was more than 3 times higher than PAI-1 expression (Table 6). mRNA concentrations ranged from 0.09 to 1409.77 zmol uPA/amol G6PDH (median 89.34) and from 0.16 to 837.44 zmol PAI-1/amol G6PDH (median 28.26). uPA antigen expression in the cell lines ranged from 0.01 to 582.76 ng/10^6 cells per 48 hours (median 4.38), PAI-1 antigen expression was measured in concentrations between 0.06 and 1883.80 ng/10^6 cells per 48 hours (median 27.62).

Table 6: uPA and PAI-1 in cell lines - median antigen and mRNA expressions (with inter-quartile ranges (IR)). The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH (zmol uPA or PAI-1/amol G6PDH). Antigen levels of uPA and PAI-1 were measured using the IMUBIND uPA # 894 and the IMUBIND PAI-1 # 821 ELISA kits.

	uPA median (IR)	PAI-1 median (IR)
ELISA [ng/10^6 cells per 48h]	**4.38 (50.25)**	**27.62 (817.83)**
QPCR [normalized to G6PDH]	**89.34 (133.04)**	**28.26 (271.14)**

Correlations between uPA and PAI-1 ELISA and QPCR

Antigen expression levels measured by ELISA were in accordance with mRNA levels measured by QPCR. Table 7 shows the data divided in high, medium, and low expression levels. Significant correlations were found of $r_s = 0.95$ ($p < 0.001$) between uPA ELISA and QPCR, and also of $r_s = 0.95$ ($p < 0.001$) between PAI-1 ELISA and QPCR (Table 8). Figure 15 shows bi-logarithmic regression graphs demonstrating the relationship between the results of the two methods. There were also distinctly significant correlations between uPA and PAI-1 measured by both methods, with the strongest correlation coefficient of $r_s = 0.98$ ($p < 0.001$) between uPA and PAI-1 ELISA results and the same correlation between uPA QPCR and PAI-1 ELISA results (Table 8).

Table 7: uPA and PAI-1 mRNA expression and antigen levels in the cell lines. The mRNA transcript levels were quantified by LightCycler QPCR and normalized to G6PDH. Antigen levels of uPA and PAI-1 were determined in cell culture supernatants by use of the IMUBIND uPA # 894 and the IMUBIND PAI-1 # 821 ELISA kits.

	uPA			PAI-1	
	mRNA [zmol uPA/ amol G6PDH]	Antigen [ng/10^6cells per 48 h]		mRNA [zmol uPA/ amol G6PDH]	Antigen [ng/10^6cells per 48 h]
High expression:					
OV-MZ-10	1409.77	582.76	OV-MZ-10	837.44	1883.80
			MDA-MB-231	320.76	894.85
			MDA-MB-231 BAG	137.11	610.42
Medium expression:					
MDA-MB-231 BAG	134.82	30.11	OV-MZ-6	35.74	21.70
MDA-MB-231	129.90	57.06	HaCaT	20.77	33.53
HaCaT	93.81	4.52	aMCF-7	13.60	20.29
OV-MZ-6	84.87	4.24			
Low expression:					
aMCF-7	1.75	0.01	MDA-MB-435	0.41	0.06
MCF-7	0.16	0.26	MCF-7	0.16	1.12
MDA-MB-435	0.09	0.01			

Table 8: Spearman correlations between uPA and PAI-1 ELISA and QPCR. Significant p-values are underlined.

	uPA/G6PDH	uPA protein	PAI-1/G6PDH
uPA protein	r_S = 0.952 p < 0.001		
PAI-1/G6PDH	r_S = 0.929 p = 0.001	r_S = 0.905 p = 0.002	
PAI-1 protein	r_S = 0.976 p < 0.001	r_S = 0.976 p < 0.001	r_S = 0.952 p < 0.001

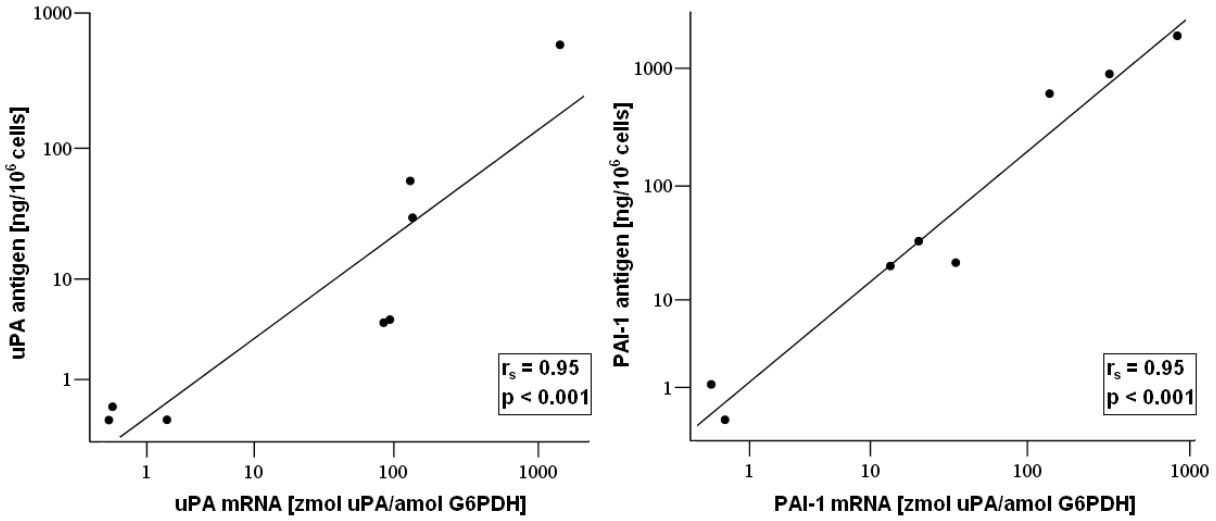


Figure 15: uPA and PAI-1 in cell lines: bi-logarithmic regression graphs demonstrating correlations between antigen and mRNA values. The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH (zmol uPA or PAI-1/amol G6PDH). Antigen levels of uPA and PAI-1 were measured using the IMUBIND uPA # 894 and the IMUBIND PAI-1 # 821 ELISA kits.

3.3.2 Breast Cancer Samples

To analyze uPA and PAI-1 mRNA and protein expression in breast cancer cytosols or detergent-released fractions, two different patient cohorts were used, the Dutch cohort with 105 primary tissue samples and the German primary tumor tissue cohort encompassing 74 samples. Whereas in both cases mRNA isolation was achieved employing comparable technical approaches, different methods of uPA and PAI-1 protein extraction and antigen determination were used (as described in the 'Materials and Methods'). Therefore, the expression levels of the antigen measurements by the Dutch and German laboratories cannot be compared one-to-one (Table 9).

Table 9: uPA and PAI-1 determined in breast cancer collectives – QPCR and ELISA results. The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH (ratio: zmol/amol) or ß-actin (ratio: zmol/fmol). Antigen levels of uPA and PAI-1 were measured using either published in-house ELISA formats (cytosol fraction - Dutch samples) or IMUBIND uPA and PAI-1 ELISA kits (detergent extracts - German samples).

	Dutch collective (Nijmegen)				German collective (Dresden and Munich)		
	Patient no.	QPCR [G6PDH]	QPCR [ß-actin]	ELISA [ng/mg protein]	Patient no.	QPCR [G6PDH]	ELISA [ng/mg protein]
uPA median (IR)	105	57.16 (81.55)	52.53 (64.19)	0.28 (0.38)	74	166.50 (161.54)	3.49 (4.18)
PAI-1 median (IR)	104	136.40 (182.43)	138.00 (156.67)	1.36 (1.84)	71	189.50 (219.97)	18.80 (20.62)

3.3.2.1 Dutch Collective

The collective comprised a total number of 105 patients. The housekeeping genes G6PDH and ß-actin were measured by LightCycler PCR along with uPA and its inhibitor. G6PDH was primarily applied for interpretation of the results. However, for the sake of completeness, the ß-actin values and ratios are listed in each table as well. The uPA and PAI-1 mRNA concentrations, normalized to G6PDH, ranged from 27.13 to 108.68 zmol uPA/amol G6PDH (median 57.16) and from 72.84 to 255.28 zmol PAI-1/amol G6PDH (median 136.40), respectively. uPA and PAI-1 antigen levels in the cytosol extracts ranged from 0.18 to 0.56 ng uPA/mg total protein (median 0.28) and from 0.81 to 2.64 ng PAI-1/mg total protein (median 1.36), respectively (Table 9).

Correlations between uPA and PAI-1 ELISA and QPCR Results

In the Dutch cohort (*i.e.* cytosolic extraction method), there were significant correlations between uPA ELISA results and uPA measured by QPCR of $r_s = 0.35$ ($p < 0.001$). Correlations between PAI-1 ELISA results and PAI-1 mRNA quantification by the LightCycler were also significant with correlation coefficients of $r_s = 0.20$ ($p = 0.045$), as shown in Figure 16 and Table 10.

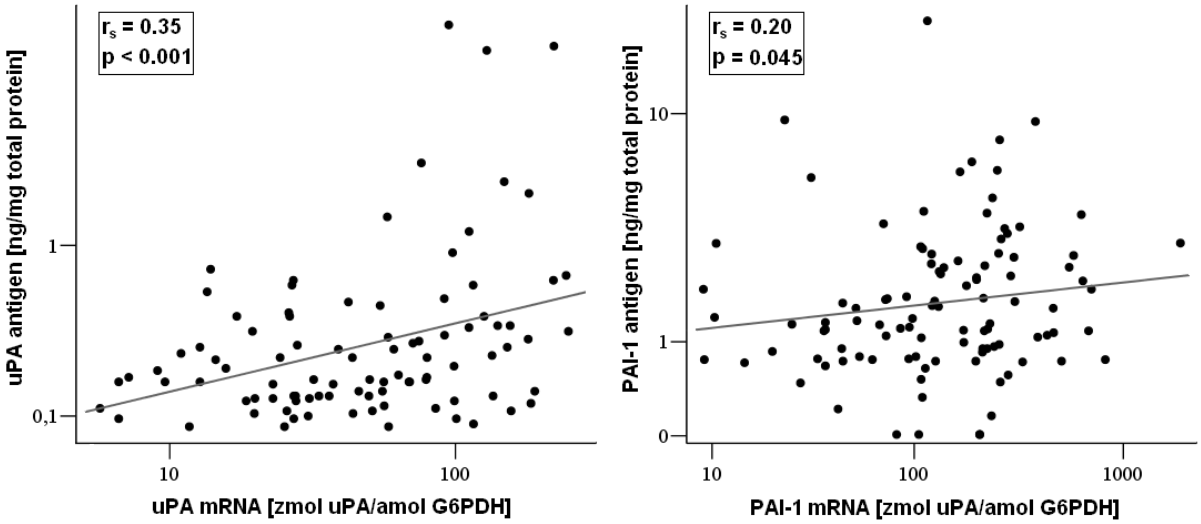


Figure 16: Dutch samples: Correlation of uPA (n = 105) and PAI-1 (n = 104) mRNA with antigen levels determined by ELISA from cytosolic extracts normalized to G6PDH. mRNA transcript levels were quantified by LightCycler QPCR. uPA and PAI-1 antigen contents were determined in the high-speed supernatant by published in-house ELISA formats.

Other Significant Correlations

Apart from the results above, significant correlations between uPA and PAI antigen ($r_s = 0.44$; $p < 0.001$), and uPA and PAI-1 mRNA ($r_s = 0.61$; $p < 0.001$), respectively, became apparent. These correlations are stronger than correlations between uPA antigen and mRNA, as well as between PAI-1 antigen and mRNA. Additionally, significant correlations are visible between uPA QPCR and PAI-1 ELISA results of $r_s = 0.20$ ($p = 0.041$; Table 10).

Table 10: Dutch samples: correlations beween all measured variables. Significant p-values are underlined.

	uPA protein	uPA/G6PDH	uPA/ß-actin	PAI-1 protein	PAI-1/G6PDH
uPA/G6PDH (n = 105)	**r_S = 0.346** **<u>p < 0.001</u>**				
uPA/ß-actin (n = 105)	**r_S = 0.405** **<u>p < 0.001</u>**	**r_S = 0.562** **<u>p < 0.001</u>**			
PAI-1 protein (n = 104)	**r_S = 0.435** **<u>p < 0.001</u>**	**r_S = 0.200** **<u>p = 0.041</u>**	**r_S = 0.470** **<u>p < 0.001</u>**		
PAI-1/G6PDH (n = 104)	**r_S = 0.028** **p = 0.777**	**r_S = 0.611** **<u>p < 0.001</u>**	**r_S = 0.186** **<u>p = 0.058</u>**	**r_S = 0.197** **<u>p = 0.045</u>**	
PAI-1/ß-actin (n = 104)	**r_S = 0.032** **p = 0.749**	**r_S = 0.196** **<u>p = 0.046</u>**	**r_S = 0.519** **<u>p < 0.001</u>**	**r_S = 0.414** **<u>p < 0.001</u>**	**r_S = 0.625** **<u>p < 0.001</u>**

Relationship between uPA/PAI-1 Expression and Clinical and Histomorphological Parameters

Before analyzing the measurement results with respect to different clinical and histomorphological factors of the patient collective, the normality of all continuously scaled data was tested applying Kolmogorov-Smirnov analysis. Here, no normal distribution was found for any of the variables. Subsequently, all available clinical and histomorphological data, including menopausal status, lymph node (LN) status, tumor size (pT), tumor grade (Scarff-Bloom-Richardson), estrogen receptor (ER) status, progesterone receptor (PR) status, the application of adjuvant therapy after surgery, and the age of the patients (categorized as ≤ 60 years versus > 60 years), were tested regarding uPA and PAI-1 expression levels (antigen and mRNA content), applying the nonparametric Mann-Whitney-U and Kruskal-Wallis tests.

The level of uPA and PAI-1 mRNA expression did not differ significantly between tumors in relation to clinical and histomorphological parameters, except for the lymph node status which was inversely correlated with uPA mRNA expression ($p = 0.020$). Regarding PAI-1 protein expression, significantly higher PAI-1 antigen levels were found in ER-negative ($p = 0.009$) as well as in PgR-negative tumors ($p = 0.001$). Significantly elevated uPA antigen levels were only found in ER-negative patients ($p = 0.015$). The uPA protein level was also elevated in PgR-negative tumors, but the difference only approached statistical significance ($p = 0.062$). There was no significant association of uPA/PAI-1 antigen levels, neither with menopausal status, LN status, tumor size, tumor grade, nor with the age of the breast cancer patients (Table 11, Table 12).

Table 11: Dutch breast cancer samples: uPA mRNA and antigen expression levels related to clinical or histomorphological parameters (n = 105). The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH (or ß-actin). uPA antigen levels were quantified by in-house ELISA formats from cytosolic extracts. Significant p-values are underlined.

Variable	Patient no. (%)	uPA QPCR				uPA ELISA[f]	
		G6PDH[d]		ß-actin[e]			
		Median (IR[c])	P-value	Median (IR[c])	P-value	Median (IR[c])	P-value
Menopausal status[a]	**105**		**0.879**		**0.290**		**0.736**
pre/peri	28 (26.7)	56.1 (70.4)		41.8 (52.1)		0.31 (0.34)	
post	77 (73.3)	58.5 (86.0)		54.0 (66.5)		0.27 (0.40)	
Lymph node status[a]	**98**		**<u>0.020</u>**		**0.105**		**0.192**
negative	44 (44.9)	74.0 (69.1)		55.1 (86.8)		0.26 (0.31)	
positive	54 (55.1)	41.3 (72.9)		48.3 (55.7)		0.33 (0.46)	
Tumor size (pT)[b]	**104**		**0.228**		**0.155**		**0.138**
1	22 (21.2)	65.5 (73.3)		51.8 (67.2)		0.24 (0.22)	
2	67 (64.4)	57.2 (87.3)		52.8 (61.8)		0.36 (0.46)	
3/4	15 (14.4)	32.6 (78.9)		31.4 (66.6)		0.29 (0.45)	
Grade (Bloom-Richardson)[a]	**62**		**0.782**		**0.926**		**0.306**
1/2	35 (56.5)	56.8 (65.2)		55.4 (61.1)		0.26 (0.27)	
3	27 (43.5)	52.0 (116.8)		55.3 (65.8)		0.37 (0.41)	
Estrogen receptor status[a]	**103**		**0.811**		**<u>0.038</u>**		**<u>0.015</u>**
negative	38 (36.9)	58.7 (135.2)		75.5 (79.2)		0.42 (0.53)	
positive	65 (63.1)	56.8 (68.3)		44.7 (55.0)		0.24 (0.30)	
Progesterone receptor status[a]	**104**		**0.776**		**0.170**		**0.062**
negative	42 (40.4)	53.9 (124.2)		74.0 (78.2)		0.39 (0.53)	
positive	62 (59.6)	58.0 (66.2)		47.7 (53.6)		0.25 (0.30)	
Age category	**105**		**0.625**		**0.625**		**0.167**
≤ 60 years	56 (53.3)	59.0 (82.0)		48.9 (62.6)		0.25 (0.38)	
> 60 years	49 (46.7)	56.9 (78.5)		54.9 (65.1)		0.39 (0.37)	

a **Mann-Whitney-U Test**
b **Kruskal-Wallis Test**
c **Inter-quartile range (IR)**
d **zmol target gene/amol G6PDH**
e **zmol target gene/fmol ß-actin**
f **ng antigen/mg total protein**

Table 12: Dutch breast cancer samples: PAI-1 mRNA and antigen expression levels related to clinical or histomorphological parameters (n = 104). The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH (or ß-actin). PAI-1 antigen levels were quantified by in-house ELISA formats from cytosolic extracts. Significant p-values are underlined.

Variable	Patient no. (%)	PAI-1 QPCR				PAI-1 ELISA[f]	
		G6PDH[d]		ß-actin[e]			
		Median (IR[c])	P-value	Median (IR[c])	P-value	Median (IR[c])	P-value
Menopausal status[a]	**104**		**0.837**		**0.781**		**0.126**
pre/peri	28 (26.9)	148.9 (154.7)		157.4 (173.1)		1.03 (1.26)	
post	76 (73.1)	136.4 (192.1)		134.7 (149.2)		1.49 (1.93)	
Lymph node status[a]	**97**		**0.056**		**0.190**		**0.540**
negative	44 (45.4)	198.2 (199.5)		153.1 (192.7)		1.77 (1.66)	
positive	53 (54.6)	125.4 (185.1)		113.6 (150.4)		1.23 (1.97)	
Tumor size (pT)[b]	**103**		**0.607**		**0.370**		**0.249**
1	22 (21.3)	172.7 (175.2)		172.6 (260.0)		1.20 (1.90)	
2	66 (64.1)	136.4 (182.9)		172.9 (157.1)		1.61 (1.76)	
3/4	15 (14.6)	107.4 (224.8)		113.6 (121.3)		0.85 (1.65)	
Grade (Bloom-Richardson)[a]	**61**		**0.642**		**0.760**		**0.380**
1/2	34 (55.7)	132.4 (143.0)		126.2 (151.0)		1.98 (1.99)	
3	27 (44.3)	111.1 (227.3)		140.1 (172.0)		1.40 (2.32)	
Estrogen receptor status[a]	**102**		**0.502**		**0.816**		**<u>0.009</u>**
negative	37 (36.3)	130.9 (229.5)		140.1 (206.0)		1.96 (2.66)	
positive	65 (63.7)	138.8 (147.2)		120.3 (145.3)		1.21 (1.45)	
Progesterone receptor status[a]	**103**		**0.757**		**0.618**		**<u>0.001</u>**
negative	41 (39.8)	134.0 (213.9)		140.1 (219.0)		2.27 (2.76)	
positive	62 (60.2)	135.3 (161.5)		112.3 (146.7)		1.18 (1.32)	
Age category	**104**		**0.091**		**0.372**		**0.700**
≤ 60 years	56 (53.8)	188.5 (150.7)		143.0 (144.0)		1.37 (1.93)	
> 60 years	48 (46.2)	110.5 (193.8)		116.8 (180.5)		1.34 (1.82)	

a Mann-Whitney-U Test
b Kruskal-Wallis Test
c Inter-quartile range (IR)
d zmol target gene/amol G6PDH
e zmol target gene/fmol ß-actin
f ng antigen/mg total protein

Survival Analysis

In this survival analysis (Table 13), the combined disease-free survival (DFS), an event being defined as either recurrent disease or death, was used. Differing patient numbers were available, depending on the quantification method or on the respective housekeeping gene applied in the QPCR assay. The data, including ELISA results, were only complete for a number of 57 patients of the Dutch cohort. In this group of breast cancer patients, it was found in univariate ($p = 0.015$) as well as in multivariate ($p = 0.010$) Cox regression analysis that - as expected - a positive lymph node status is significantly associated with an unfavorable outcome for the patients. Apart from the lymph node status only PAI-1 mRNA expression (normalized to G6PDH) showed to be significantly linked to relapse occurrence; low PAI-1 expression predicted a longer recurrence-free survival in both univariate ($p = 0.014$) and multivariate ($p = 0.029$) Cox regression analysis. Univariate Cox analysis indicated adjuvantly treated patients to have a poorer prognosis, the borderline significance ($p = 0.052$) could not be confirmed by multivariate analysis.

In a recent study [164], we evaluated expression of KLK7 mRNA, encoding the serine protease human tissue kallikrein-related peptidase 7 in tumor specimens of 155 breast cancer patients. High KLK7 mRNA expression was found to be significantly associated with a better outcome for the patients, according to both univariate and multivariate Cox survival analysis. The same patient cohort was also used to quantify the expression of uPA and PAI-1 mRNA, respectively. Unfortunately, only for a part of this cohort uPA and PAI-1 antigen values were available. In these 155 samples, in contrast, neither uPA nor PAI-1 mRNA values were associated with patient prognosis (data not shown).

Table 13: Univariate and multivariate Cox regression analyses – ELISA and QPCR results (n = 57). The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH. Significant p-values are underlined.

Variable	Patient no.	EF[c] (%)	Univariate analysis		Multivariate analysis[f]	
			HR[d] (95% CI[e])	P-value	HR[d] (95% CI[e])	P-value
uPA						
- **ELISA**	**57**	**24 (42.1)**	**1.17 (0.66-2.07)**	**0.582**	**1.14 (0.30-4.27)**	**0.685**
- **QPCR[a]**	**57**	**24 (42.1)**	**1.00 (1.00-1.01)**	**0.362**	**1.00 (0.99-1.01)**	**0.685**
PAI-1						
- **ELISA**	**57**	**24 (42.1)**	**1.03 (0.93-1.14)**	**0.574**	**0.99 (0.83-1.17)**	**0.865**
- **QPCR[a]**	**57**	**24 (42.1)**	**>1.00 (1.00-1.00)**	**<u>0.014</u>**	**1.00 (1.00-1.01)**	**<u>0.029</u>**
Menopausal status			**1.03 (0.43-2.50)**	**0.942**	**0.85 (0.83-1.17)**	**0.851**
pre/peri	**16**	**7 (43.8)**				
post	**41**	**17 (41.5)**				
Lymph node status			**3.24 (1.25-8.39)**	**<u>0.015</u>**	**9.84 (1.72-56.16)**	**<u>0.010</u>**
negative	**23**	**6 (26.1)**				
positive	**34**	**18 (52.9)**				
Tumor size (pT)			**1.26 (0.60-2.65)**	**0.538**	**1.43 (0.61-3.38)**	**0.411**
1	**12**	**5 (41.7)**				
2	**41**	**17 (41.6)**				
3/4	**4**	**2 (50.0)**				
Grade (Bloom-Richardson)			**1.13 (0.50-2.56)**	**0.772**	**1.21 (0.44-3.35)**	**0.710**
1/2	**33**	**14 (43.8)**				
3	**25**	**10 (40.0)**				
Estrogen receptor status			**1.56 (0.65-3.77)**	**0.322**	**3.61 (0.89-14.73)**	**0.073**
negative	**23**	**7 (30.4)**				
positive	**34**	**17 (50.0)**				
Progesterone receptor status			**1.23 (0.53-2.82)**	**0.632**	**1.11 (0.36-3.40)**	**0.860**
negative	**25**	**9 (36.0)**				
positive	**32**	**15 (46.9)**				
Adjuvant treatment			**2.30 (0.99-5.33)**	**0.052**	**0.44 (0.09-2.17)**	**0.310**
no	**29**	**9 (31.0)**				
yes[b]	**28**	**15 (53.6)**				
Age	**57**	**24 (42.1)**	**1.00 (0.97-1.03)**	**0.964**	**0.99 (0.93-1.05)**	**0.729**

a zmol target gene/amol G6PDH
b Endocrine therapy (n = 17), chemotherapy (n = 7), both (n = 4)
c Event frequency (EF)
d Hazard ratio (HR) estimated from Cox proportional hazard regression model
e Confidence interval (CI) of the estimated HR
f Multivariate analysis includes uPA and PAI-1 measured by ELISA and QPCR/G6PDH (continuous), menopausal status, lymph node status, tumor size, grade, estrogen and progesterone receptor status, adjuvant treatment, and age (continuous)

3.3.2.2 German Collectives

The uPA and PAI-1 mRNA concentrations of the German cohort ranged from 23.28 to 695.04 zmol uPA/amol G6PDH (median 166.50) and from 9.83 to 1633.80 zmol PAI-1/amol G6PDH (median 189.50). Antigen levels in the detergent extracts ranged from 0.37 to 12.73 ng uPA/mg total protein (median 3.49) and from 4.45 to 147.00 ng PAI-1/mg total protein (median 18.80).

Correlations between uPA and PAI-1 ELISA and QPCR Results

As depicted in Figure 17 and Table 14, in the German breast cancer samples (detergent extracts), a significant, but weak correlation (r_s = 0.48; p < 0.001) between mRNA and antigen values was seen for uPA expression, but not in case of PAI-1 (r_s = 0.06; p = 0.613).

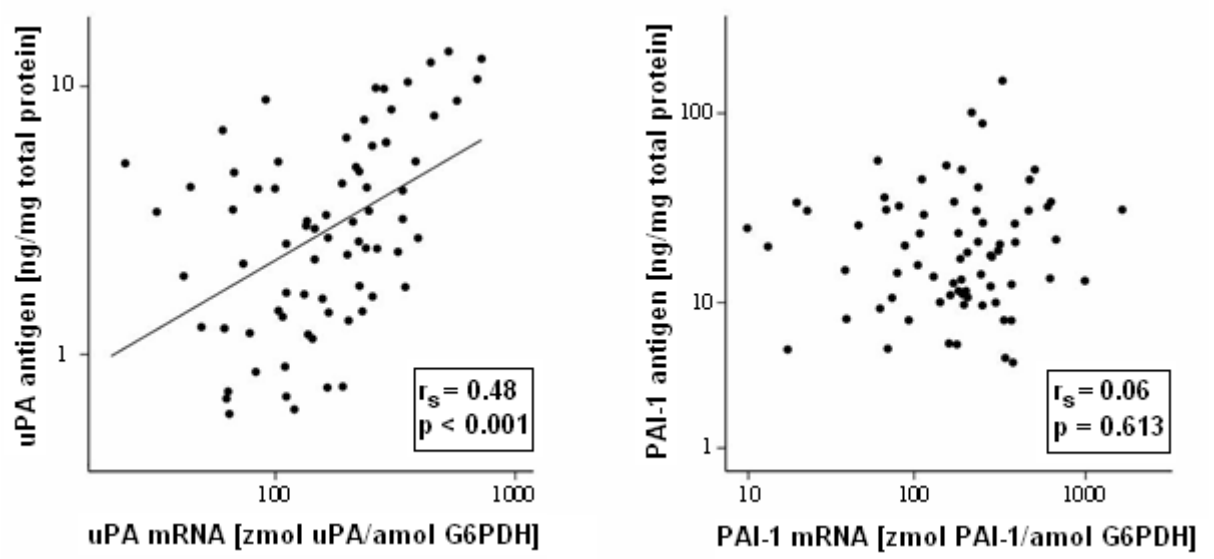


Figure 17: German samples: correlation of uPA (n = 74) and PAI-1 (n = 71) mRNA with antigen levels determined by ELISA from detergent extracts. mRNA transcript levels were quantified by LightCycler QPCR. uPA and PAI-1 antigen levels were determined in detergent extracts of breast cancer tissues by use of the 'IMUBIND uPA ELISA Kit' # 894 (uPA), and the 'IMUBIND PAI-1 ELISA Kit' # 821 (PAI-1).

Other Significant Correlations

In the whole German cohort, the highest correlation was observed between uPA and PAI-1 protein (r_S = 0.49; p < 0.001), followed by uPA protein and uPA mRNA, as described above, and uPA and PAI-1 mRNA (r_S = 0.38; p = 0.001; Table 14).

Table 14: German samples: correlations (Spearman) between all measured variables. Significant p-values are underlined.

	uPA/G6PDH	**uPA protein**	**PAI-1/G6PDH**
uPA protein (n = 74)	**r_S = 0.478** **p < 0.001**		
PAI-1/G6PDH (n = 74)	**r_S = 0.379** **p = 0.001**	**r_S = 0.010** **p = 0.935**	
PAI-1 protein (n = 71)	**r_S = 0.101** **p = 0.402**	**r_S = 0.494** **p < 0.001**	**r_S = 0.061** **p = 0.613**

Relationship between uPA/PAI-1 Expression and Clinical and Histomorphological parameters

Regarding the German breast cancer samples (detergent extracts), no significant associations were found with clinical or histomorphological data, neither for uPA nor PAI-1 mRNA, or antigen expression (Table 15, Table 16).

Table 15: German breast cancer samples: uPA mRNA and antigen expression levels related to clinical or histomorphological parameters (n = 74). The mRNA transcript levels were quantified by LightCycler PCR and normalized to G6PDH. uPA antigen levels were quantified by the 'IMUBIND uPA ELISA Kit' # 894 from detergent extracts. Significant p-values are underlined.

Variable	Patient no. (%)	uPA QPCR[d]		uPA ELISA[e]	
		Median (IR[c])	P-value	Median (IR[c])	P-value
Menopausal status[a]	**74**		**0.134**		**0.119**
pre/peri	20 (27.0)	135 (141)		3.2 (2.5)	
post	54 (73.0)	195 (184)		3.9 (4.8)	
Lymph node status[a]	**68**		**0.581**		**0.356**
negative	39 (57.4)	166 (135)		3.0 (3.8)	
positive	29 (42.6)	198 (220)		4.0 (5.8)	
Tumor size (pT)[b]	**73**		**0.285**		**0.222**
1	32 (43.8)	156 (143)		3.0 (3.1)	
2	32 (43.8)	200 (160)		4.0 (4.9)	
3/4	9 (12.4)	91 (186)		1.4 (6.6)	
Grade (Bloom-Richardson)[a]	**73**		**0.208**		**0.520**
1/2	40 (54.8)	184 (162)		3.1 (4.8)	
3	33 (45.2)	163 (174)		4.0 (3.4)	
Estrogen receptor status[a]	**74**		**0.762**		**0.082**
negative	19 (25.7)	223 (188)		4.8 (4.4)	
positive	55 (74.3)	165 (152)		3.2 (4.0)	
Progesterone receptor status[a]	**73**		**0.860**		**0.307**
negative	28 (38.4)	207 (250)		4.3 (5.8)	
positive	45 (61.6)	165 (127)		3.2 (3.5)	
Age category	**74**		**0.839**		**0.440**
≤ 60 years	43 (58.1)	167 (166)		3.7 (4.9)	
> 60 years	31 (41.9)	163 (155)		2.8 (3.9)	

a Mann-Whitney-U Test
b Kruskal-Wallis Test
c Inter-quartile range (IR)
d zmol uPA/amol G6PDH
e ng uPA/mg total protein

Table 16: German breast cancer samples: PAI-1 mRNA and antigen expression levels related to clinical or histomorphological parameters (n = 71). The mRNA transcript levels were quantified by LightCycler QPCR and normalized to G6PDH. PAI-1 antigen levels were quantified by the 'IMUBIND PAI-1 ELISA Kit' # 821 from detergent extracts. Significant p-values are underlined.

Variable	Patient no. (%)	PAI-1 QPCR[d]		PAI-1 ELISA[e]	
		Median (IR[c])	P-value	Median (IR[c])	P-value
Menopausal status[a]	**71**		**0.421**		**0.488**
pre/peri	19 (26.8)	181.7 (169.2)		16 (22)	
post	52 (73.2)	211.8 (241.5)		19 (21)	
Lymph node status[a]	**65**		**0.432**		**0.701**
negative	38 (58.5)	184.5 (195.6)		19 (22)	
positive	27 (41.5)	206.4 (267.3)		18 (21)	
Tumor size (pT)[b]	**70**		**0.775**		**0.257**
1	30 (42.9)	213.9 (222.8)		15 (18)	
2	31 (44.3)	200.8 (285.3)		21 (22)	
3/4	9 (12.9)	189.4 (208.3)		14 (18)	
Grade (Bloom-Richardson)[a]	**70**		**0.683**	14 (22)	**0.137**
1/2	39 (55.7)	296.3 (204.9)		21 (19)	
3	31 (44.3)	189.4 (296.8)			
Estrogen receptor status[a]	**71**		**0.937**		**0.350**
negative	18 (25.4)	241.7 (224.0)		20 (22)	
positive	53 (74.6)	189.6 (227.0)		17 (21)	
Progesterone receptor status[a]	**70**		**0.884**		**0.181**
negative	26 (37.1)	203.4 (236.5)		21 (22)	
positive	44 (62.9)	191.1 (207.6)		15 (19)	
Age category	**71**		**0.658**		**0.831**
≤ 60 years	41 (57.7)	189.6 (240.1)		19 (21)	
> 60 years	30 (42.3)	209.0 (229.5)		16 (22)	

a **Mann-Whitney-U Test**
b **Kruskal-Wallis Test**
c **Inter-quartile range (IR)**
d **zmol PAI-1/amol G6PDH**
e **ng PAI-1/mg total protein**

3.4 Cryopowder *versus* Paraffin Extracts (Samples from Munich)

After exclusion of mRNA with low G6PDH expression (< 500 copies) the collective comprised a total number of 28 tumor samples - each from fresh as well as from paraffin-embedded, formalin-fixed tumor tissue. ELISA results were available of all of these tumor samples.

The results and inter-quartile ranges of uPA and PAI-1 mRNA quantification by LightCycler PCR and of uPA and PAI-1 protein quantification by ELISA are shown below in Table 17. Antigen detection revealed an almost 10 times higher expression of PAI-1 than of uPA. The antigen levels ranged from 0.51 to 9.88 ng uPA/mg total protein (median 3.05) and from 5.35 to 147.00 ng PAI-1/mg total protein (median 21.08). Normalized to G6PDH, the mRNA concentrations in the cryopowder samples ranged from 31.45 to 460.08 zmol uPA/amol G6PDH (median 122.11 zmol uPA/amol G6PDH) and from 9.83 to 1633.80 zmol PAI-1/amol G6PDH (median 132.58 zmol PAI-1/amol G6PDH). The mRNA concentrations in the paraffin-preserved samples ranged from 36.69 to 490.25 zmol uPA/amol G6PDH (median 226.13 zmol uPA/amol G6PDH) and from 20.91 to 1096.88 zmol PAI-1/amol G6PDH (median 85.06 zmol PAI-1/amol G6PDH).

Non-parametric correlation analysis between LightCycler quantification of uPA and PAI-1 and their quantification by ELISA revealed no significances (Table 18).

Table 17: Munich breast cancer samples (n = 28): uPA and PAI-1 median values measured by ELISA and QPCR.

	uPA median (IR)		PAI-1 median (IR)	
ELISA [ng antigen/total protein]	3.05 (3.28)		21.08 (16.95)	
Housekeeping gene	G6PDH	PBGD	G6PDH	PBGD
Cryopowder	122.11 (147.24)	2306.66 (2644.97)	132.58 (173.54)	1869.18 (3119.80)
Paraffin sections	226.63 (131.65)	4034.29 (3021.80)	85.06 (84.30)	812.38 (1334.55)

Table 18: Munich breast cancer samples (n = 28): Spearman correlations between QPCR and ELISA results.

		Quantitative LightCycler PCR			
		Cryopowder samples, results normalized to …		Paraffin- and formalin-preserved samples, results normalized to…	
		G6PDH	PBGD	G6PDH	PBGD
		uPA			
ELISA	uPA	0.29 (ns)	0.21 (ns)	0.27 (ns)	0.03 (ns)
		PAI-1			
	PAI-1	0.25 (ns)	0.26 (ns)	0.01 (ns)	-0.18 (ns)

Correlations between uPA expression in pulverized and FFPE tissue were $r_s = 0.51$ ($p = 0.006$) when normalized to G6PDH (Figure 18, a)) and $r_s = 0.18$ (ns) when normalized to PBGD. Correlation coefficients describing the connection between PAI-1 expression in pulverized tissue and paraffin-embedded tissue were $r_s = 0.51$ ($p = 0.005$) when normalized to G6PDH (Figure 18, b)) and $r_s = 0.15$ (ns) when normalized to PBGD.

Figure 19 shows uPA and PAI-1 expression levels, directly comparing cryopowder and paraffinized tumor samples. When using G6PDH as housekeeping gene, the majority of samples originating from the same tumor had similar uPA/PAI-1 expression levels.

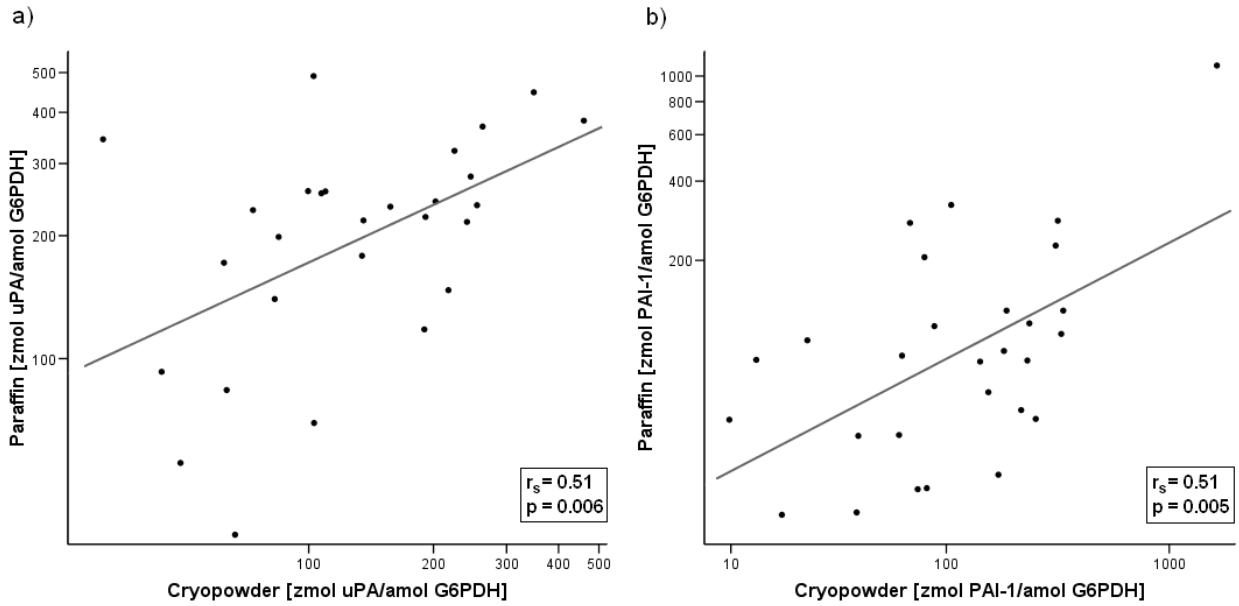
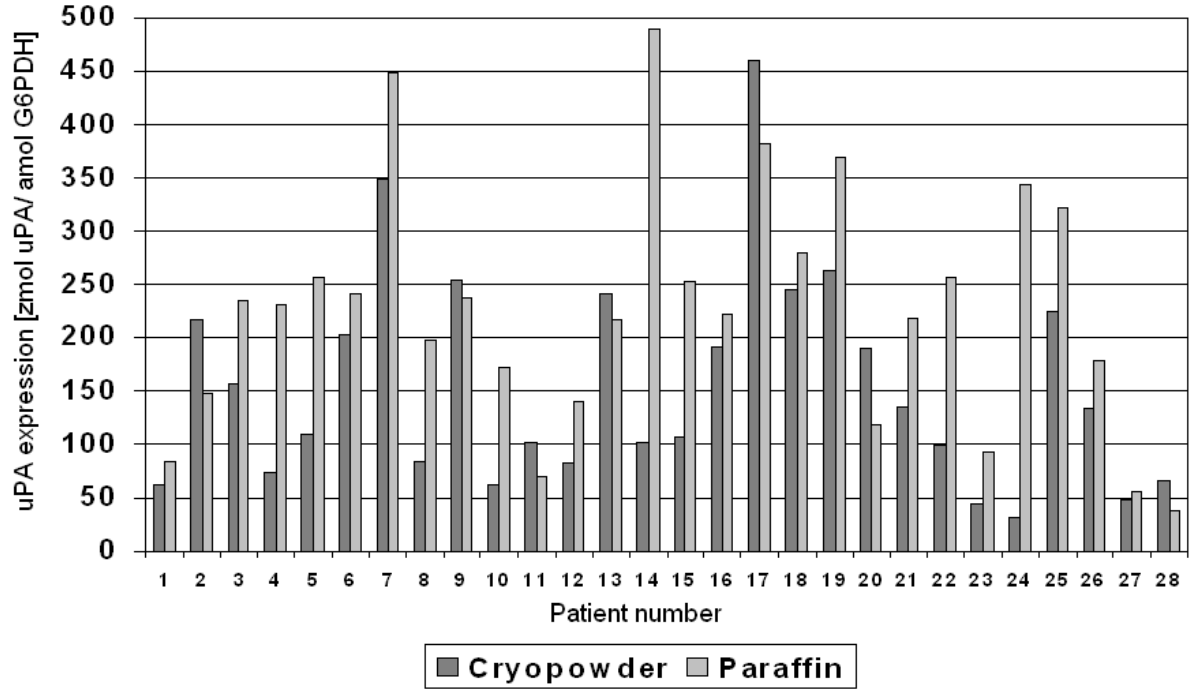


Figure 18: Scatterplots showing the relationship between uPA and PAI-1 contents (normalized to G6PDH) in cryopowder and paraffin-embedded tissue. The mRNA transcript levels were quantified by LightCycler PCR. a) uPA; b) PAI-1.

a)



b)

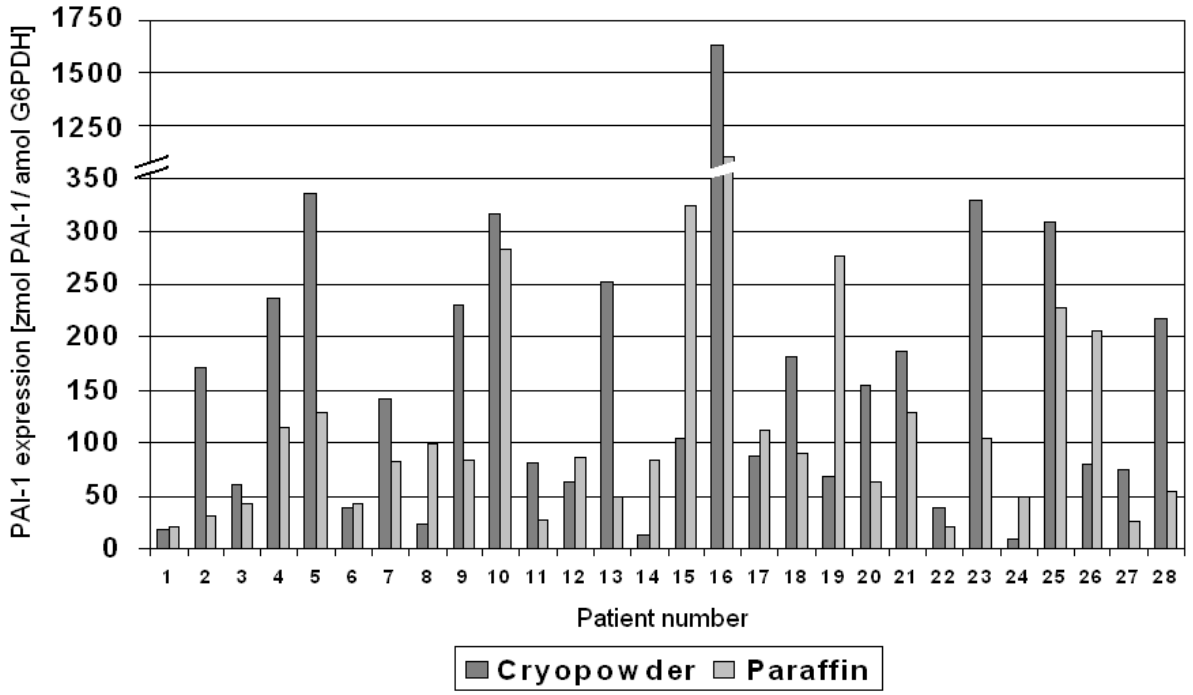


Figure 19: uPA (a) and PAI-1 (b) mRNA normalized to G6PDH, isolated from cryopowder and paraffin-embedded breast cancer samples, respectively.

3.5 Housekeeping Gene Analysis (Samples from Munich and Nijmegen)

Dutch Collective: Correlations of the Housekeeping Genes G6PDH and ß-actin

For housekeeping correlation analysis, data of 105 (uPA)/104 (PAI-1) patients for both G6PDH and ß-actin were available. Correlations between the absolute values of G6PDH and ß-actin were $r_s = 0.64$ ($p < 0.001$; Figure 20). Analysis of the relative values resulted in correlations between uPA/G6PDH and uPA/ß-actin of $r_s = 0.56$ ($p < 0.001$), and between PAI-1/G6PDH and PAI-1/ß-actin of $r_s = 0.63$ ($p < 0.001$; Figure 21).

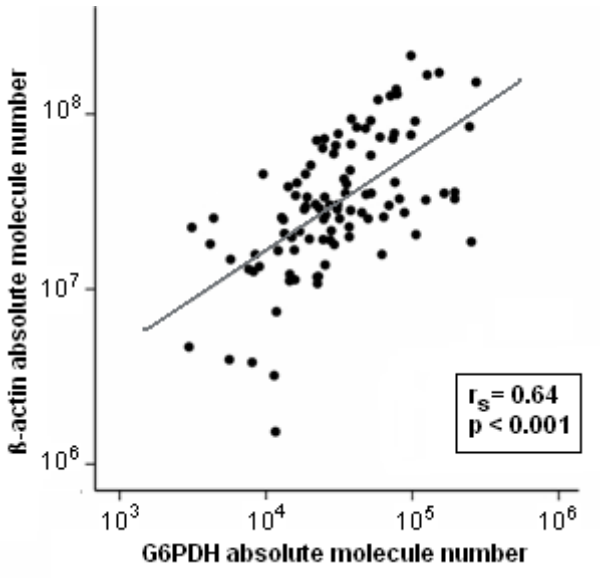


Figure 20: Absolute values - G6PDH *versus* ß-actin (n = 105). The mRNA transcript levels were quantified by LightCycler PCR.

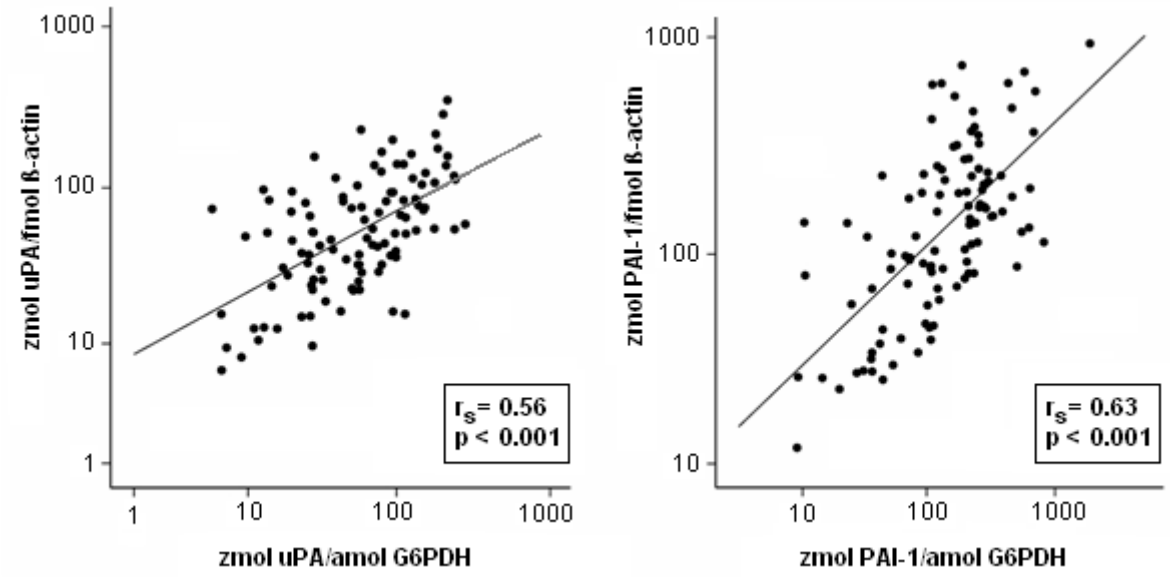


Figure 21: Relative values - G6PDH *versus* ß-actin (uPA: n = 105; PAI-1: n = 104). The mRNA transcript levels were quantified by LightCycler PCR.

Munich Collective: Correlations of the Housekeeping Genes G6PDH and PBGD

The relationships of the housekeeping gene quantities of mRNA isolated from cryopowder and FFPE tissue of corresponding tumors were about 9 to 1 for G6PDH, and 7 to 1 for PBGD. Normally, cryopowder yielded more mRNA than paraffinized tissue. In average, PBGD was expressed at a 20 times lower level than G6PDH (Table 19, Figure 22). The wide distribution of the absolute values is probably due to the varying concentrations of mRNA used for reverse transcription into cDNA. In case that the measurement of G6PDH resulted in a molecule number below 500 in either a paraffin or a cryopowder probe, a second cDNA was synthesized from the isolated mRNA. Sometimes the new cDNA displayed similarly low molecule numbers, indicating the mRNA to be of poor quality. If sufficient tumor tissue was still available, the mRNA isolation was repeated, and in several cases a higher yield was attained.

Table 19: Medians and interquartal ranges of the housekeeping genes' absolute values in cryopowder and paraffin sections. The mRNA transcript levels were quantified by LightCycler PCR.

	Cryopowder median (IR)	Paraffin sections median (IR)
G6PDH	**7512.33 (14393.28)**	**1847.95 (1937.20)**
PBGD	**332.45 (787.35)**	**102.15 (94.63)**

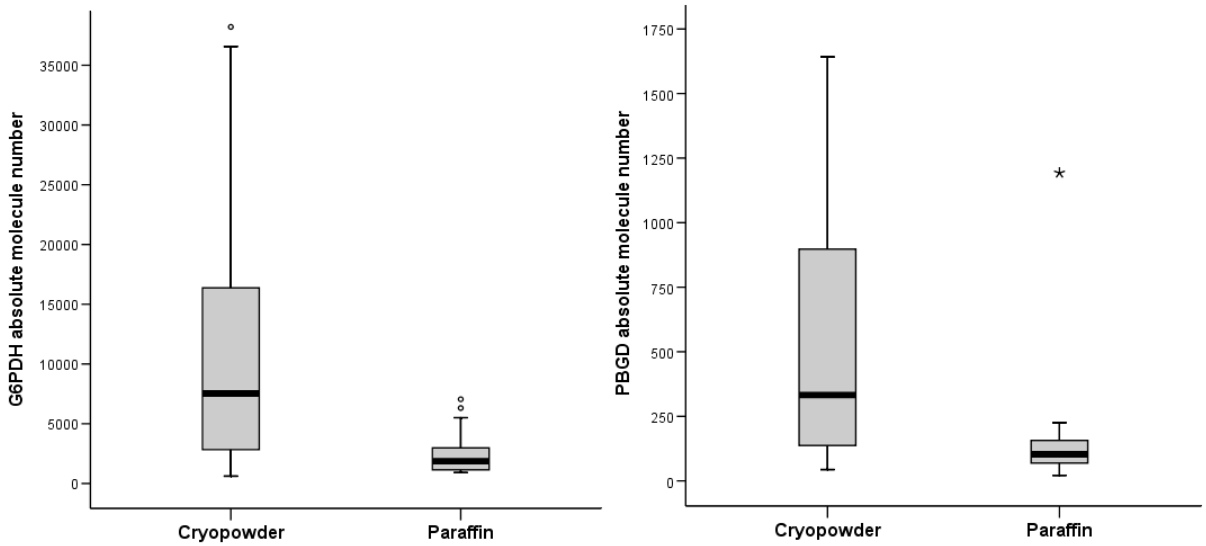


Figure 22: Comparison of G6PDH and PBGD expression in cryopowder and paraffin-embedded tissue. The mRNA transcript levels were quantified by LightCycler PCR.

The correlation between the absolute values of the two housekeeping genes was high, with a correlation coefficient of $r_s = 0.84$ ($p < 0.001$). The regression graph is plotted bi-logarithmically for visualization of the whole range of values (Figure 23). When comparing the relative uPA values measured in the same tissue type with different housekeeping genes, there were significant correlations between G6PDH and PBGD of $r_s = 0.66$ ($p < 0.001$) in cryopowder and of $r_s = 0.71$ ($p < 0.001$) in FFPE tissue. PAI-1 normalized to both housekeeping genes showed highly significant correlations between G6PDH and PBGD of $r_s = 0.89$ ($p < 0.001$) in cryopowder and of $r_s = 0.77$ ($p < 0.001$) in paraffin-embedded tissue (not shown). When combining

the samples, the correlation between G6PDH and PBGD was $r_s = 0.70$ ($p < 0.001$) for uPA and $r_s = 0.81$ ($p < 0.001$) for PAI-1 (Figure 24).

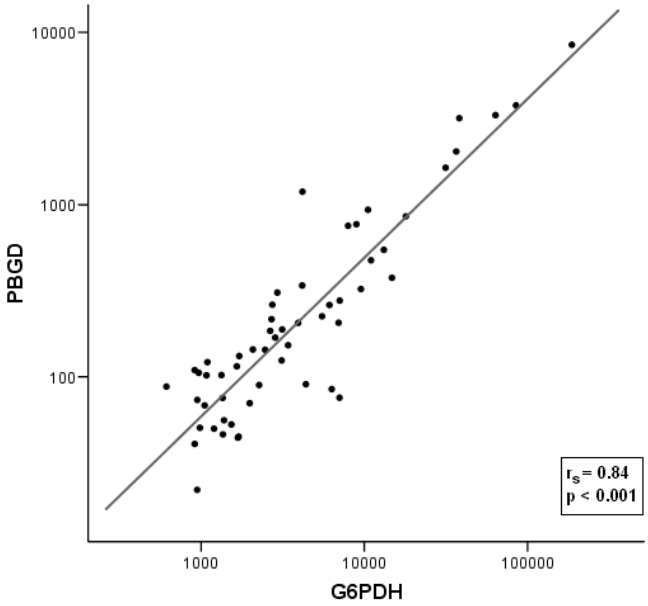


Figure 23: Scatterplot showing the relationship between absolute values of the two housekeeping genes (n = 56). The mRNA transcript levels were quantified by LightCycler PCR.

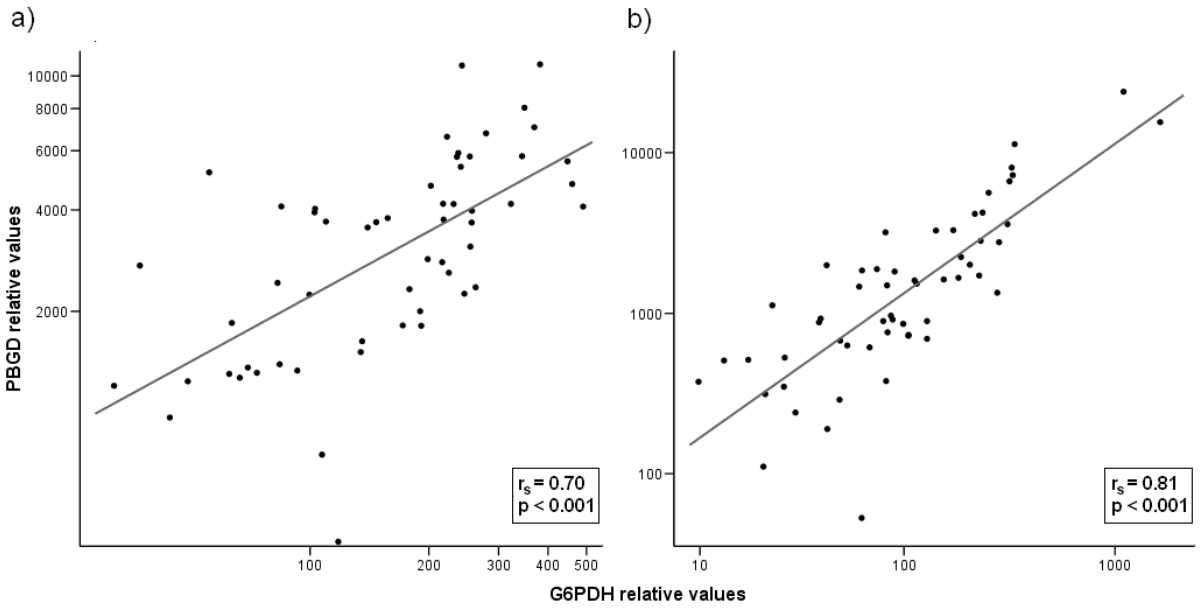


Figure 24: Relationships between relative values of the housekeeping genes G6PDH and PBGD in the combined cryopowder and paraffin-embedded, formalin-fixed samples (n = 56). The mRNA transcript levels were quantified by LightCycler PCR. a) uPA; b) PAI-1.

4 DISCUSSION

4.1 ELISA *versus* QPCR

4.1.1 uPA and PAI-1 Antigen Quantification

The first analysis of enzymatic uPA activity in breast cancer cytosols with respect to prognosis was performed by Duffy *et al.* [84]. Numerous studies followed showing the prognostic impact of members of the plasminogen activation system in different malignancies by application of either detergent-extracted tissues or cytosol fractions [106,130,173,174,175,330,341]. In our applied ELISA formats, the assays were designed to detect all latent and active, as well as complexed and receptor-bound forms of uPA (both single chain and HMW-uPA) and PAI-1.

uPA contents in breast cancer tissue of higher than 2.97 ng/mg protein, PAI-1 contents of greater than 14 ng/mg protein in detergent extracts [142,175], and of above 1.15 ng uPA/mg protein in routinely prepared cytosols [106] are known to indicate high risk tumors and a shortened life expectancy. The prognostic value of PAI-1 was also shown to prevail in tumor extracts from routinely prepared cytosols [107,176].

When comparing both methods in 247 cases of breast cancer patients, Jänicke *et al.* [176] found the strongest prognostic impact of uPA when using Triton X-100-extracted tissues. Additionally, the uPA antigen yield was much higher in these tissues, which was not the case for PAI-1. The two methods were highly correlated. Rosenquist *et al.* [287] found the greatest efficiency of uPA extracted by a pH 4.2 buffer containing the non-ionic detergent Triton X-100; a non-detergent extraction method for cytosol extracts was less efficient. De Witte *et al.* [80], who also found significant correlations for uPA and PAI-1 between cytosolic and detergent extracts retrieved from centrifuged pellets, detected the highest prognostic impact of uPA and PAI-1 when using cytosolic extracts.

In the breast cancer samples presented in this thesis, we found a higher yield of both uPA and PAI-1 when using detergent extracts (German samples), with the proviso

that there are, of course, differences regarding tissue selection and ELISA methodology between the two cohorts. Eventually, both detergent and cytosol extracts seem valuable for antigen quantification of members of the uPA system, always depending on the factor being detected. The differing results in search of the optimum extraction method demonstrate the need for standardization of uPA and PAI-1 detection methodologies to be able to include the factors as new markers in routine breast cancer diagnostics. A number of available uPA and PAI-1 kits have been compared to each other in technical papers, often resulting in substantial variation of antigen levels. When using the same type of ELISA kit, there were acceptable coefficients of variation between laboratories, but other influencing factors, such as differences in quality assessment and laboratory experience, remain. Therefore, strict requirements for newly introduced markers are mandatory, detection assays and procedures should be standardized. For this purpose, biomarker evaluation guidelines have been proposed [120,152,153,236,342,343].

4.1.2 Correlations of mRNA Quantification and Antigen Detection by ELISA

The analysis of uPA and PAI-1 expression in the eight cell lines - including four breast cancer cell lines, two ovary cancer cell lines, a keratinocyte, and a melanoma cell line - showed high correlations between uPA and PAI-1 in both antigen and mRNA quantification (which was confirmed in the breast cohorts). Furthermore, high correlations between ELISA and the QPCR method of above $r_s = 0.9$ for both uPA and PAI-1, normalized to G6PDH, became apparent.

Of the cell lines mentioned above, the two cell lines most common in murinal studies - the ER-negative breast cancer cell line MDA-MB-231 and the melanoma cell line MDA-MB-435 - tend to develop aggressively in mice, especially the latter which is able to produce lung and lymph metastases. Tumors obtained from ER-positive breast cancer cell lines - represented by MCF-7 -, on the contrary, are rarely invasive, and tumor growth requires estrogenic supplementation. The above cell lines are often used to analyze molecular details of breast cancer progression. MDA-MB-231, for example, can be used to represent advanced, MCF-7 earlier stages of breast

cancer. OV-MZ-6 and -10 are tumorigenic and invasive ovarian cell lines known to express uPA and PAI-1 among other members of the plasminogen activation system. In our examination, highest antigen concentrations of both uPA and PAI-1 were seen in OV-MZ-10, MDA-MB-231, and MDA-MB-231 BAG, whereas the lowest levels were measured in MCF-7 and MDA-MB-435. Holst-Hansen *et al.* [163] also measured high uPA and PAI-1 concentrations by immuno-assays in the MDA-MB-231 BAG cell line, and distinctly low levels in MDA-MB-435 BAG and MCF-7 cell lines. In that study, MDA-MB-231 BAG cells - in contrast to the two other cell lines - showed a high activity in mediating plasmin formation and cell invasion; the uPA system was assumed to be actively involved in the invasive process. In a study focusing on the importance of PN-1 in breast cancer, additionally uPA and PAI-1 were measured by QPCR in MDA-MB-231 and MCF-7 cell lines, and were found to be distinctly increased in cultured MDA-MB-231 cells [40].

Given the accordance of ELISA and QPCR results, it was hypothesized that high correlations may also be seen by comparing the two quantification methods in breast cancer samples. However, cell lines often lack representativeness as breast cancer *in vivo* displays a greater heterogeneity than breast cancer cell lines [209]. Alterations of the pheno- or genotype may occur depending on culturing conditions. Furthermore, due to posttranscriptional regulation processes which have been previously described for components of the plasminogen activation system including uPA and PAI-1 [156,159,225,309,310,361], mRNA levels do not entirely image the respective antigen quantification by ELISA. It is assumed that there are control mechanisms by short-lived proteins which are altered at a specific stage during tumor progression, thereby influencing stability or processing of nuclear transcripts [159].

Two patient cohorts from the Netherlands and Germany consisting in 105 patients and 74 patients, respectively, were analyzed with respect to the inter-relation between ELISA and QPCR. Again, significant correlations were found between the two methods, but the Spearman correlation coefficients were low, ranging between 0.34 and 0.48 (uPA), and between 0.19 and 0.41 (PAI-1), depending on the applied housekeeping gene. In the German cohort, PAI-1 protein and mRNA values did not correlate at all. Separate evaluation of the 28 breast cancer samples from Munich, in

fact, did not show any significant correlations between the ELISA and the QPCR results, neither when isolated from fresh-frozen nor from preserved tissues.

Our results are in line with observations by Spyratos *et al.* [331] who found uPA, PAI-1 (and PAI-2) protein and mRNA levels to be significantly correlated, but in part with only borderline significance. This was only examined in a very small cohort (n = 21). In another study, no significant correlations between protein and mRNA measurements were found in specimens of 54 breast cancer patients [47]. Recently, however, the same research group found significant correlations between mRNA and antigen results for uPA and PAI-1 in tumor tissue of 70 breast cancer patients [48]. Still, there are only few groups so far which have analyzed correlations between uPA and PAI-1 mRNA, and antigen levels, larger patient numbers would be desirable.

In the study by Spyratos *et al.* [331], corresponding to our results, positive correlations between the uPA and PAI-1 mRNA values were observed as well. Several other groups found correlations between uPA and PAI-1 antigen levels [29,30,69,107,111,131,168,175,222]. These findings are not surprising when considering the various interactions of the members of the plasminogen activation system and the synergic effects ascribed to uPA and PAI-1 in tumor growth and metastasis.

4.2 uPA/PAI-1 Expression and Clinical and Histomorphological Parameters, and Survival Analysis

Our findings in the Dutch cohort that uPA and PAI-1 antigen levels were significantly higher in ER-negative and in case of PAI-1 also in PgR-negative patients as compared to the receptor-positive patient samples are supported by a number of further studies [107,111,222]. This association with the ER-status was also confirmed for uPA mRNA that was normalized to ß-actin.

In the study by Castello *et al.* [47], a positive correlation between uPA and PAI-1 antigen and mRNA values with tumor severity (tumor grade), as well as between uPA and PAI-1 antigen levels and the lymph node status, was found. Look *et al.* [222] found positive correlations between antigen levels of PAI-1 and the lymph node status, and uPA and PAI-1 antigen values also correlated significantly with the histologic grade. These results could not be supported by our findings which showed a negative association of uPA mRNA expression - normalized to G6PDH - and lymph node status (Dutch cohort)**.** Publications on relationships between uPA, PAI-1, and histopathological factors, however, are often inconsistent. Alltogether, there were no relevant associations in this work between uPA/PAI-1 levels and clinicopathological variables, neither when quantifying mRNA nor antigen amounts.

Up to now, there are only few published prognostic studies with relatively small patient numbers in which uPA and PAI-1 have been measured by application of QPCR assays [48,215,331,351]. In the study of Spyratos *et al.* [331], high uPA and PAI-1 mRNA expression was significantly associated with shorter disease-free survival in a population of 130 primary breast cancers, independent of hormone receptor or lymph node status. Leissner *et al.* [215], who included 87 patients, all node-positive/hormone receptor-positive, observed that high PAI-1 mRNA expression was significantly associated with a shorter metastasis-free survival (MFS), whereas uPA mRNA levels were not of prognostic relevance. In a subset of ErbB2-positive breast cancer patients Urban *et al.* [351] identified uPA by quantitative PCR among 60 other genes as the most significant marker associated with MFS.

In several studies, uPA antigen or mRNA contents in malignant breast tumors also showed prognostic relevance, but in most cases only PAI-1 correlated significantly with relapse-free or overall survival in multivariate Cox regression analysis, along with other strong established prognostic factors such as lymph node status [48,107,133,142,262,288]. Also in this work, a significant association was seen between PAI-1 mRNA levels and recurrence-free survival in both univariate and multivariate Cox regression analyses, high levels indicating an unfavorable outcome (Dutch cohort). Apart from PAI-1, only the lymph node status of the patients had comparable predictive capacity.

Antigen levels, in part, showed quite different results compared to mRNA levels when analyzing for coherences with histomorphological factors and prognostic information. Varying results may be in part ascribable to tumor heterogeneity: tumor tissue, apart from neoplastic cells and subclones, consists of numerous cells (*e.g.* stromal cells, macrophages), and there are influencing factors (cytokines, growth factors, hormones - partially by cAMP regulation) that could affect expression of certain genes. For future projects, it would therefore be reasonable to select tissue under microscopic control and analyze different areas of a tumor. And, as described above, transcriptional and posttranscriptional regulation processes causing degradation of nuclear or cytoplasmic precursor or mature mRNA with special occurrence in malignant tissues may constitute differences in expression of proteolytic factors, a deeper understanding of which would necessitate further investigations. Yet, both methods have meanwhile independently rendered valuable information on the relevance of the uPA system in breast cancer.

Unfortunately, in most clinical studies described above, there are no corresponding ELISA results available in order to directly compare the prognostic strength of uPA and PAI-1 in protein versus mRNA determination. Such studies would be essential for paving the way for the introduction of the highly sensitive, reproducible, and fast QPCR assays for uPA and PAI-1, which depend on small amounts of tumor tissue, to clinical routine.

4.3 Cryopowder *versus* Formalin-Fixed, Paraffin-Embedded Tissue

In the past, there have been limitations to RT-PCR studies as they depended on the availability of fresh-frozen samples. Formalin-fixed paraffin-embedded archives worldwide would provide a rich source of histomorphological and clinical data for gene expression analyses, allowing extensive retrospective and prospective studies in addition to the tissues' present routine use for genetical and immunohistochemical analyses. But there have been reports of extensive RNA degradation in paraffin-preserved tissues and varying mRNA detection depending on the applied fixative [112]. Today, highly sensitive quantitative RT-PCR assays and improved RNA isolation techniques provide the opportunity to amplify very small RNA strands, despite its partially extensive fragmentation by this kind of preservation. There have been studies describing successful RT-PCRs of mRNA from FFPE tissue [2,121,170,214,326]. Godfrey [121] even found paraffinized tissues - in contrast to frozen tissues - to be independent of pre-fixation time. Abrahamsen [2], however, described a strong decrease of mRNA levels (of from 85 to 99%) when matching the samples to frozen tissue, in particular when amplifying long sequences. When using small amplicons, however, the mRNA yield was much better (up to 100-fold), prolonged preservation did not affect the amount of amplified PCR product. But also the RNA yield in fresh-frozen samples was higher when using smaller amplicons, showing long amplicons in general to be more susceptible to degradation. This indicates that it is necessary to minimize the amplicon length of target and housekeeping genes as far as possible, as pursued in the present work (amplicon lengths lying between 103 (uPA) and 150 (PBGD) base pairs).

Our comparison of expression levels of QPCR quantification from fresh as well as from paraffin-embedded, formalin-fixed tumor tissue revealed that much less target and housekeeping gene mRNA could be isolated from paraffin-embedded than from cryo-preserved tumor tissue which corresponds to previous studies. Nevertheless, when using G6PDH as housekeeping gene, there was a significant correlation of uPA and PAI-1 contents, respectively, between both tissues.

Further investigations concerning the choice of amplicon length, optimization of reference genes, and RNA extraction procedure might contribute to the establishment of a standardized mRNA retrieval technique for application in archival cancer studies. Our results confirm, in line with other QPCR studies, the applicability of formalin-fixed, paraffin-embedded tissue for mRNA extraction. Prior to routine clinical application, however, there is a need for further methodological studies encompassing larger patient cohorts.

4.4 Housekeeping Genes

In order to be able to compare expression levels of genes in different tissue samples, it is necessary to normalize the data to so-called housekeeping genes when applying quantitative RT-PCR assays. Housekeeping genes are genes which are ideally expressed in all cells at a comparable expression level, they encode molecules that are necessary for basic maintenance and essential cellular functions. ß-actin encodes for components of the cytoskeleton. Porphobilinogen Deaminase (PBGD) is a key enzyme of the heme synthesis pathway. One transcript of the PBGD gene is solely expressed in erythroid tissues, the other transcript is present in all tissues and represents the housekeeping variant [126]. Glucose-6-phophatedehydrogenase (G6PDH) catalyzes the dehydrogenation of D-glucose-6-phosphate to 6-phosphogluconolacton and NADPH in the phosphogluconate metabolic pathway. Although ubiquitously expressed, it has been shown to display varying basal activity from one tissue to another. In a number of tissues - like proliferating and adipose cells, the liver, and the lung -, the cellular level of G6PDH is regulated by various external stimuli, such as hormones, growth factors, nutrients, and oxidant stress [191]. In this study, G6PDH was applied in the first place, since it has already been proven an appropriate housekeeping gene in a previous breast cancer study [98]. For the choice of an appropriate reference gene further prerequisites are mandatory: (1) there should be no known regulation of the housekeeping gene expression in the analyzed tissue; (2) specific primers and hybridization probes - binding to exon/intron boundaries - should be used to avoid amplification of pseudogenes/traces of DNA; (3) the expression levels of housekeeping gene and analyzed target gene should be similar.

Until now, no optimum housekeeping gene has been identified. The numerous influencing factors - potentially including neoplastic growth - that may influence the expression of the housekeeping gene have to be taken into consideration as this may have substantial impact on the interpretation of the respective results.

Only few studies so far have tried to investigate variations of housekeeping gene expression in different cancerous tissues [70,73,103,117,123,207]. A Dutch QPCR study comparing 13 different housekeeping genes, including ß-actin and PBGD, stated that

there is no single housekeeping gene that shows invariable expression between different cell types. It is recommended to use mean expression of multiple housekeeping genes for normalization. Of the examined genes, HPRT (hypoxanthine ribosyltransferase) showed to be the best single reference gene as it most accurately reflected the mean expression of the other analyzed genes [73].

In this work, the collectives with two housekeeping genes being available revealed high correlations between their expression levels, in particular between the absolute values, which underlines the reliability of the values measured by the LightCycler as well as the exchangeability of the respective two housekeeping genes. The relative values of uPA and PAI-1 in the samples from Munich revealed higher correlation coefficients when using G6PDH as housekeeping gene which may be ascribable to the distinctly lower expression level of PBGD (PBGD was expressed at an about 20 times lower level) and thereby a higher variability of its results. ß-actin has the highest expression of the three examined housekeeping genes. G6PDH is known to have a medium, PBGD a low expression level, which we also observed in our samples. G6PDH and PBGD correlated stronger than G6PDH and ß-actin.

For future QPCR investigations, the choice of a certain reference gene depends on further research results comparing the use of certain genes in the respective tissue and on target gene expression. In small tumor extracts with high target gene expression, it may be useful to apply a highly expressing housekeeping gene like ß-actin. For instance, in our case, PBGD does not seem valuable when detecting uPA and PAI-1 mRNA that was extracted from paraffin-embedded breast tumor tissue.

4.5 Concluding Remarks

High antigen contents of uPA and PAI-1 in malignant breast tumors have been shown to indicate an unfavorable prognosis for the patients, and, consequently, quantification of these factors gives valuable information on prognosis and, even more important, can help decide whether adjuvant treatment is to be administered. With reference to the National Institutes of Health (NIH) [91] and St. Gallen consensus guidelines [124], up to 90% of node-negative breast cancer patients are eligible to receive adjuvant chemotherapeutic treatment. By use of uPA and PAI-1 as additional prognostic factors for identification of high risk patients - aside from the established TNM criteria, tumor grading, and steroid hormone receptor status - a considerable number of patients could be spared from the exposure to toxic chemotherapy. In fact, elevated antigen levels of the serine protease uPA and its inhibitor PAI-1 in primary breast cancer tissue determined by ELISA have already entered clinical practice as indicators of poor breast cancer prognosis [149,257]. However, in general, the ELISA method requires relatively large quantities of fresh-frozen tumor material, the procedure is time-consuming and elaborate, and requires an adequate capacity for the storage of tumor samples. As the tumor material, due to earlier diagnosis of cancer, becomes more and more limited and is obtained from cryostat sections, fine needle aspirates, or core biopsies [302], there is a need for alternative more sensitive and less material-consuming methods for quantitative determination of prognostic factors in tumor material. In the present study, quantitative real-time PCR assays for uPA and PAI-1 - applying the LightCycler technology - were established.

Among the first to evaluate uPA and PAI-1 mRNA by QPCR were Castello *et al.* [47] who - as most researchers applying quantitative RT-PCR - used SYBR Green I for template detection and ß-actin as housekeeping gene. In their work, the amplicon lengths were considerably larger than in our study (ranging from 341 to 481 base pairs). SYBR Green is a fluorescent dye which intercalates with double-stranded DNA molecules, melting-point analysis has to be performed in order to distinguish amplification products from primer dimers. In contrast, the FRET method, applying hybridization probes, is more precise and specific. Moreover, to our knowledge, our QPCR assays, with target genes ranging from 103 to 132 base pairs in length (normalization to G6PDH), represent the smallest QPCR assays for uPA and PAI-1

described so far.

In order to recapitulate this work, the following conclusions can be made.

- The assays that were designed in this work differ from other published assays in their abililty to detect very small fractions of the target genes. They display high sensitivity (especially due to the selection of amplicons that overlap exon boundaries and the additional binding of hybridization probes to the amplicon), and the technique is reliable and simple.

- Correlations between uPA and PAI-1 antigen and mRNA determination were significant, albeit less strong in the breast cancer collectives than in the selected cell lines. Differences, also in the relationship with patient tumor characteristics, indicate limitations to the one-to-one transferability of the antigen expression of members of the plasminogen activation system to the respective quantification of mRNA copies.

- In the Dutch cohort, PAI-1 mRNA was confirmed as a negative prognostic factor in both univariate and multivariate analysis. Apart from PAI-1 only the lymph node status was significantly linked to overall survival; but there was only a small number of patients in the available Dutch cohort with complete prognostic information (n = 57).

- mRNA extraction and subsequent quantification from formalin-fixed, paraffin-embedded tissue by QPCR was successful. By selection of an appropriate housekeeping gene corresponding to the expression height of a target gene and expansion of patient numbers, the applicability of similar assays could be further explored. This may give way to extensive retrospective studies using the multitude of archived tissue material held in pathological departments around the world.

In summary, these findings indicate that it is not as yet possible to exchange antigen quantification by ELISA of uPA and PAI-1 with mRNA detection and measurement

via quantitative RT-PCR in clinical practice. The detailed mechanisms of mRNA processing prior to translation into protein products have to be further understood. But the method can provide valuable - including prognostic - information on the importance of uPA, PAI-1, and other parameters with tumor-promoting capacities. The sensitive and fast assays we have designed and applied by quantifying uPA and PAI-1 mRNA in a number of cell lines and two breast cancer cohorts have set an example for studies in which only very small amounts of tissue, potentially archived FFPE tissues, are available.

5 APPENDIX

Abbreviations

AI	aromatase inhibitor
Ala	alanine
Arg	arginine
ATF	amino-terminal fragment
bp	base pairs
BRCA	breast cancer gene
BSA	bovine serum albumin
CA	cancer antigen
CD	cluster of differentiation
(c)DNA	(complementary) deoxyribonucleic acid
CEA	carcinoembryonic antigen
CI	confidence interval
CLIS	carcinoma lobulare in situ
CP	crossing point
CTC	circulating tumor cell
D	domain
DCIS	ductal carcinoma in situ
DFS	disease-free survival
DMEM	Dulbecco's modified eagle medium
ECM	extra-cellular matrix
EF	event frequency
EGF(R)	epidermal growth factor (receptor)
ELISA	enzyme-linked immunosorbent assay
ERD	estrogen receptor downregulator
ERK	extracellular regulated kinase
exon	expressed region
FFPE	formalin-fixed, paraffin-embedded
FPRL1/LXA4R	FPR-like receptor-1/lipoxin A4 receptor
FRET	fluorescence resonance energy transfer
Gly	glycine
GPI	glycosylphosphatidylinositol
(h-)G6PDH	(human) glucose-6-phosphate-dehydrogenase
HE	hematoxylin-eosin

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HER-2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HIV-1	human immunodeficiency virus 1
HMW-uPA	high molecular weight uPA
HR	hazard ratio
HRP	horseradish peroxidase
HPLC	high performance liquid chromatography
HPRT	hypoxanthine ribosyltransferase
HSP	heat-shock protein
IGF(BP)	insuline-like growth factor (binding protein)
Ile	isoleucine
IHC	immunohistochemistry
intron	intervening region
IR	interquartile range
LDLR	low density lipoprotein receptor
LED	light-emitting diode
LH-RH	luteinizing hormone-releasing hormone
LMW-uPA	low molecular weight uPA
LRP	LDLR-related protein
Lys	lysine
MAPK	mitogen-activated protein kinase
MEK	MAPK extracellular signal regulated kinase kinase
MFS	metastasis-free survival
MMP	matrix metalloproteinase
(M)SBR	(modified) Scarff-Bloom-Richardson
MSP	macrophage-stimulating protein
(m)RNA	(messenger) ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NIH	National Institutes of Health
NNBC	node negative breast cancer
ns	non-significant
OS	overall survival
PAI	plasminogen activator inhibitor
PBGD	porphobilinogen deaminase
Plg	plasminogen
PN-1	protease nexin 1

(p)TNM	(pathologic) tumor, node, metastasis
QPCR	quantitative PCR
(RT)PCR	(reverse transcriptase) polymerase chain reaction
RCL	reactive center loop
r_S	Spearman rank correlation
SCID	severe combined immunodeficiency
SD	standard deviation
Ser	serine
SERM	selective estrogen receptor modulator
serpin	serine proteinase inhibitor
SF	scatter factor
SMB	somatomedin B
SPF	S-phase fraction
(s)uPAR	(soluble) urokinase-type plasminogen activator receptor
TGF	transforming growth factor
TLI	thymidine labeling index
TMB	tetramethylbenzidine
tPA	tissue-type plasminogen activator
Tris	trishydroxymethylaminomethane
uPA	urokinase-type plasminogen activator
Val	valine
VC	coefficient of variation
VEGF	vascular endothelial growth factor
VLDLR	very-low-density lipoprotein receptor

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