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Determination of uPA and PAI-1 by ELISA in Small Amounts of Breast Cancer Tissue: Clinical Evaluation in Pre- and Post-Primary Systemic Therapy Specimens

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Abbreviation Index

α_2AP	α_2-anti-plasmin
A-ABB	Automated-assisted breast biopsy
Ab	Antibody
ABB	Assisted breast biopsy
AC	Adriamycin/Cyclophosphamid
ACS	American Cancer Society
ADI	American Diagnostics Incorporated
Ag	Antigen
AGO	Arbeitsgemeinschaft Gynäkologische Onkologie (Organ Commission Mamma)
ASCO	American Society of Clinical Oncologists
AST	Adjuvant systemic therapy
ATF	Aminoterminal fragment
BCA	Bicinchoninic acid assay (Smith)
BCT	Breast-conserving therapy
BI-RADS	Breast Imaging Reporting and Data System
BIOMED	Biomedicine and Health Program of the European Union (1996 - 1999)
BSA	Bovine serum albumin
CD	Cluster of differentiation
CE	Communauté Européenne (European Community)
cf.	confer
CMF	Cyclophosphamid/ Methotrexate/ 5-Fluorouracil
CR	Complete response
CT	Computed tomography
CV	Coefficients of variation
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
EB	Excisional biopsy
EBCTG	Early Breast Cancer Trialists' Collaborative Group
EBM	Evidence-based medicine
ECD	Extracellular domain
ECM	Extracellular matrix
ECTO	European Cooperative Trial in Operable Breast Cancer
e.g.	Abbr. of Latin 'exempli gratia'
ELISA	Enzyme-linked immunosorbent assay
EORTC	European Organization for Research and Treatment of Cancer
EPI	Epirubicin
ER	Estrogen receptors
EU	European Union
EUSOMA	European Society of Mastology
FCS	Fetal calf serum
FDA	Food and Drug Administration
FEC	5-Fluorouracil (syn. 5FU), Epirubicin and Cyclophosphamid
FISH	Fluorescent in situ hybridization
FNA / FNB	Fine-needle aspiration / -biopsy
GBG	German Breast Group
GeparDuo	Study: Doxorubicin with Cyclophosphamid followed by Docetaxel
GeparTrio	Study: Docetaxel, Doxorubicin and Cyclophosphamid (TAC) vs. Vinorelbin and Capecitabine (NX)
GIT	Gastro-intestinal tract
HER-2 HER-2/neu	Human epidermal growth factor receptor respectively measurable correlate in the blood
HMW	High molecular weight
HRP	Horseradish peroxidase
IB	Incisional biopsy
ICC	Immunocytochemistry
ICH	International Committee of Harmonization
ICH-GCP	ICH harmonized tripartite guideline "Note for Guidance on Good Clinical Practice"
i.e.	Abbr. of Latin 'id est'
IHC	Immunohistochemistry
Ki67	Proliferation marker Ki67

LABC	Locally advanced breast cancer
LCNB	Large-core needle biopsy
LMW	Low molecular weight
LOBC	Large operable breast cancer
LOE	Level of evidence
(m)	Multivariate
MaCA	Mamma Carcinoma Database
µg / µl	Microgram / Microliters
mg / ml	Milligram / Milliliter
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MRM	Magnetic resonance mammography
MRT	Magnetic resonance tomography
N+	Lymph node-positive
N_0/N0	Lymph node-negative
n/N=	Number of cases (patients)
NC	No change (stable disease)
NCI	National Cancer Institute
ng	Nanogram
nm	Nanometer
NOAH	Paclitaxel containing chemotherapy (AT&T) followed by CMF versus the same chemotherapy plus Herceptin
NPV	Negative predictive value
NSABP	National Surgical Adjuvant Breast and Bowel Project
NSE	Neoadjuvant Systemic Endocrine Therapy
NST	Neoadjuvant systemic therapy (Primary systemic therapy, induction therapy)
OS	Overall survival
Pat ID	Patient identification number
PCR	Polymerase chain reaction
pCR	Complete histopathologic remission
PD	Progressive disease
PET	Positron emissions tomography
PAI-1 / -2	Plasminogen activator inhibitor-1 / -2
PR	Partial response
PREPARE	Preoperative Epirubicin-Paclitaxel-Aranesp-Study
PSE	Primary Systemic Endocrine Therapy
PST	Primary systemic therapy (neoadjuvant therapy, induction therapy)
PPV	Positive predictive value
RBG	Receptor and Biomarker Group
RECIST	Response Evaluation Criteria in Solid Tumors criteria
resp.	respectively
RFS	recurrence free survival
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROC ROC AUC	Receiver Operating Characteristic curve analysis resp. area under the curve statistic
SL	Storage life
SOP	Standard operating procedure
syn.	synonymously
TBS	Tris-Buffered-Saline
TECHNO	Taxol Epirubicin Cyclophosphamid Herceptin Neoadjuvant Studie
TLI	Thymidine labeling index
TMB	Tetramethylbenzidine
TMUGS	Tumor marker utility grading system
(u)	Univariate
UICC	Union Internationale Contre le Cancer
uPA	Urokinase-type plasminogen activator
uPA-R	Urokinase-type plasminogen activator receptor
US(A)	United States (of America)
UT	Urinary tract
V-ABB	Vacuum-assisted breast biopsy
vs.	versus
w/w	Weight per weight
WHO	World Health Organization
WX-UK1	Molecule inhibitor of the uPA system
X-ray	Radiography

1 Summary

One of the most thoroughly studied systems with respect to prognostic relevance in patients with breast cancer is the **plasminogen activation system**. Among others, it comprises the urokinase-type plasminogen activator (uPA) and its main inhibitor, the so-called plasminogen activator inhibitor-1 (PAI-1). Clinically, uPA and PAI-1 found in breast cancer tissue extracts are used to identify patients at risk to experience disease recurrence, metastasis, or early death. Elevated levels of uPA and PAI-1 can be found when comparing breast carcinoma tissue to normal breast tissue. Studies revealed a strong correlation between high uPA and PAI-1 levels and a bad prognosis. Prognostic impact of uPA and PAI-1 on disease free survival (DFS) and overall survival (OS) was confirmed by several studies as well as by a meta-analysis, applying biochemical assays. Data show that the combined assessment of uPA and PAI-1 is superior to either factor taken alone and outperforms established prognostic factors such as tumor size, grade, hormone receptor status, or menopausal status. Methodologically, **biochemical measurement** of uPA and PAI-1 levels surpasses any other method and is considered as method of choice in routine hospital practice. **Enzyme-linked immunosorbent assay (ELISA)** is used as regular technical approach to clinical relevant results on uPA and PAI-1 in breast cancer. However, the practical value of uPA and PAI-1 might increase further, if uPA and PAI-1 could be detected simpler, e.g. by using micro ELISA or immunohistochemistry (IHC) on surgical tumor specimen or tissue obtained by needle biopsy.

This thesis comprises a methodical and a clinical part. Methodically, we developed a new micro determination procedure to analyze the uPA and PAI-1 level within small tumor specimens. We investigated the possibility to use cryostat sections as an example for small amounts of tumor tissue in order to test biochemically for uPA and PAI-1 content by means of standardized quality approved ELISA (ADI, American Diagnostica, Stamford, CT, USA).

In completion to the methodological part, we were interested to transfer our findings into the clinical setting. We therefore **clinically** conducted a retrospective analysis. We analyzed uPA and PAI-1 levels in small amounts of tumor tissue obtained by large-core needle biopsy (LCNB) specimens prior to primary systemic therapy (pre-PST) as well as in conventional biopsy specimens after primary systemic therapy (post-PST). We put emphasis on the distribution of uPA and PAI-1 levels, the change of uPA and PAI-1 levels under PST, the correlation of uPA and PAI-1 between pre- and post-PST, and the correlation between uPA and PAI-1 with respect to response to PST.

Methodically, we demonstrated that by the use of commercially available standardized ELISA (micro ELISA) the reliable determination of uPA and PAI-1 in small amounts of breast cancer tumor tissue such as 90µm cryostat sections or core biopsies is feasible. This technique allows rapid and reproducible quantifiable determination of uPA and PAI-1 levels even in small tumor specimen. The determination of uPA and PAI-1 in core biopsy seems therefore possible even in preoperative settings.

Clinically, we were able to show that PAI-1 might have a discriminative function after PST and may function as a "**surrogate marker of response**". PAI-1 thus does not only have predictive character in the adjuvant setting, but as well as in the already treated (or post-PST) setting.

2 Introduction

With approximately 1 million new cases annually, breast cancer is the **most common malignant neoplasia in women** in the industrialized world (Coleman, 1999) covering 60-80% of all malignant tumors. According to the Union Internationale Contre le Cancer (UICC), in 1996 more than 910,000 women worldwide (9% of all cancers) were diagnosed with breast cancer. Breast cancer mortality[1] is estimated at 390,000 amounting to about 5.5% of all cancer deaths. With similar incidences for Europe, the US and Canada, approximately one in nine women will develop breast cancer within her lifetime. At present, 321,000 new cases of breast cancer are diagnosed in Europe each year being associated with 124,000 deaths (EUSOMA, 2002). Current numbers for Germany estimate one in ten women to develop breast cancer within her lifetime. In 2000, in Germany 47,517 women were diagnosed with breast cancer with 17,814 deaths resulting thereof (Engel, Schubert-Fritschle, and Hölzel, 2005).

Consequently, further analysis and a systematic approach with respect to early diagnosis of breast cancer are needed. With the new concept of “individualized treatment and targeted therapies” the categorization of any breast cancer tumor specimen is a **primary task** and tumor-associated biomarkers located within the tumor tissue and the blood have been given a new role (Murphy, Millar, and Lee, 2005). Tumor tissue-associated biomarkers may facilitate stratifying patients in addition to categorizing specimens precisely. This enables risk assessment and treatment response identification (Cross and Burmester, 2004; Dowsett, 2004; Smeds et al., 2005). So far, uPA and PAI-1 are the only novel tumor-biological prognostic factors fulfilling such expectations. Breast cancer patients with elevated antigen levels in their tumor tissue are likely to experience disease recurrence, metastasis, and/or early death. In breast cancer, **clinical utility of uPA and PAI-1** has been validated at the **highest level of evidence [LOE I, +]** (Harbeck et al., 2002d). To determine uPA and PAI-1 levels, ELISA has been proven a feasible standardized quality-assured method (Benraad et al., 1996; Schmitt et al., 2002).

More efficient and earlier detection of small breast tumors (Cady, 1997; Hayes, 2005; Thomssen and Janicke, 2000) and the advent of proteome analysis (Schmitt et al., 2002; Sweep et al., 2003) set a **second task** for the clinician: Tumor masses at first time diagnosis tend to be smaller. The increase in incidence of invasive breast cancer is associated with low-stage tumors to some extent. Therefore, there is an ever-increasing demand to measure uPA and PAI-1 in smaller pieces of breast cancer tumor specimens obtained by adjusted diagnostic methods including fine needle aspirates, core biopsies, and cryostat sections. **Minimal invasive techniques** have been proven feasible according to the LOE score in general assessment of breast symptoms or lesions (LOE Ic/A+) and in subsequent pretherapeutic assessments of lesion extension (LOE Ic/A++)[2] as well as in the Carcinoma Ductale in situ (DCIS) and Carcinoma Lobulare in situ (LCIS), resp. Atypical Ductal Hyperplasia (ADH) and Atypical Lobular Hyperplasia (ALH) preoperative diagnostics and the preoperative diagnostic work-up [LOE IIb/B++ and LOE IIb/B+] (AGO, Gynecologic Oncology, and ARO, 2005c).

A **third task,** among others, is the evaluation of therapeutical approaches such as **PST**, which for we examined a possible change of uPA and PAI-1 tissue levels under PST.

[1] Defined as number per 100,000 dying of a disease during a given year.

[2] If clinical examination, mammography and sonography (e.g. plus MRI) do not allow assessement of lesion extension.

2.1 Breast Cancer Epidemiology

There is a large variation in the incidence of breast cancer between different regions, among differing ethnic groups, and within different age ranges as depicted by the National Cancer Institute Surveillance, Epidemiology, and End Results Program, Racial/Ethnic Patterns of Cancer in the United States 1988-1992 (Kelsey, Gammon, and John, 1993; Seer and Surveillance, 2001). Beyond the age of 25, the incidence rises steadily and peaks around the age range of 40 to 55 years, where it is the most common cause of death amongst women. After a decrease, the number of cases rises again with the aging process (Engel, Schubert-Fritschle, and Hölzel, 2005; EUSOMA, 2002). In the USA, Canada, Great Britain, and Germany the number of incidences has risen between 1967 and 1987, while a leveling off was recognized since 1988 (Blanks et al., 2000; Engel, Schubert-Fritschle, and Hölzel, 2005). Reasons causing the stagnation are not entirely understood. It may be ascribed to changes in age distribution as well as to earlier diagnosis caused by screening programs introduced in the late 1980s and early 1990s (EUSOMA, 2002). In the USA, the leveling off is attributed partially to the adjuvant systemic therapy [AST] (Engel, Schubert-Fritschle, and Hölzel, 2005). German findings may be ascribed to **indirect screening,** habitually realized by declaring suspicious areas having to be evaluated. This ensures coverage by the public health insurances (Barth, 2003).

Findings from the 2005 Early Breast Cancer Trialists' Collaborative Group (EBCTG) illustrate, that established types of chemotherapy and endocrine therapy have much greater effects on 15-year than on 5-year survival. This might explain why breast cancer death rates have been falling rapidly in the UK, the USA, and some other countries ever since the early 1990s (Chia, Bryce, and Gelmon, 2005; Clarke et al., 2005; EUSOMA, 2002).

Apart from improved therapy schemes and improved survival rates, a **rise in the incidence of smaller tumors** below 2 cm has been described (Cady, 1997; Harris et al., 1992). Emphasized by Harbeck et al. (2001), the **rise in incidence of smaller tumors** below 2 cm **is not correlated to less aggressiveness**.

Consequently, a **criterion for improved specialized and individualized therapy concepts** is needed in order to allow early categorization of breast cancer patients. Subsequently outlined, **uPA and PAI-1** are a promising approach with predictive and prognostic impact in different therapy settings and their role in the spread of cancer has been thoroughly examined.

2.2 Breast Cancer as Systemic Disease

A main focus in any therapy regime to treat breast cancer is the type of disease. While breast cancer was mainly considered a localized disease until the mid 1950s, it is now a potential systemic disease (Jatoi, 1997). Historically, three approaches reflect the increasing understanding of breast cancer:

I. The Halsted Theory

II. The Systemic Theory

III. The Spectrum Theory

The ***Halsted theory*** was postulated primarily around 1894 as a model of "contiguous" development of metastases, rising from a localized disease which, when left untreated, spread throughout the lymphatic system first to nearby lymph nodes and subsequently to other organs in the body. Halsted, inventor of the Halsted radical mas-

tectomy, articulated this approach the first time. Thus it is known as the Halsted theory[3] (Fisher, 1999). In 1980 Fisher et al. (1980) postulated a new ***systemic theory:*** Metastasis of any significance was thought of as already existent by the time the tumor is diagnosed by palpation or mammography. Subsequently, two paradigms became obvious (Fisher, 1999): (a) how could the fact be explained, that controlling localized disease with radiation after mastectomy improves OS (e.g., that the site from which "secondary dissemination" could have occurred got eradicated by radiotherapy) favoring the Halstedian point of view, and (b) controlling distant disease with chemotherapy and/or Tamoxifen again improves OS, implying that the disease had already disseminated or was systemic in the first place, and therefore supporting the systemic-approach. This led to a third hypothesis (Hellman, 1994): The ***Spectrum Theory.*** Hellmann (1994) considered both the Halsted and the systemic hypotheses as too limiting: Breast cancer does not only metastasize contiguously and a small tumor is not necessarily an early manifestation of a systemic and metastized disease. And, any tumor size has a proportion of patients with distant metastasis. Subsuming, breast cancer is heterogenous.

At present, we may regard breast carcinoma being a rapidly progressive disease (Tabar et al., 1999), most likely **systemic at the time of diagnosis** (Chia, Bryce, and Gelmon, 2005; Harbeck, 2001; Heiss et al., 1995a; Samarasekera, 2005; Solomayer et al., 1997). This point of view is supported by the fact, that although patients still appear to be free of metastasis clinically and mechanically, by the time of primary surgery an increasing rate of 23% to 40% of them have tumor cells in the bone marrow identified immunohistochemically (Harbeck, 2001; Heiss et al., 1995a; Lebeau et al., 2005; Solomayer et al., 1997). Andreasen estimated about 50% of the patients to have a disseminated disease when diagnosed for breast cancer (Andreasen et al., 1997). And, even after application of PST the rate of immunohistochemical identified bone-marrow metastasis remains higher compared to the AST setting, which Solomayer et al. (2003) explained by a possible resection of the primary tumor as a source of metastasis. Accordingly, breast cancer has to be **treated as being systemic by the time of diagnosis** as emphasized by Harbeck (2001).

2.3 Tumor Invasion and Metastasis

Since metastasis is the primary cause of mortality in cancer patients, predicting the likelihood of a tumor to metastasize is tantamount. Basically, the theory of cancer metastasis refers to a three-step process: (1) The degradation of extracellular matrix (ECM) is followed by (2) tumor cell invasion and (3) successive metastasis. The most prominent feature of malignant tumor cells is their ability to invade tissues actively (infiltration) as a prerequisite for subsequent metastasizes (resp. spread), thus enabling the cells to move to a distant spot and form metastasis.

Tumor cell detachment from the primary neoplasm facilitates invasiveness, which can be characterized as a several-step process requiring the coordinated and temporal regulation of a series of adhesive, proteolytic and migratory events such as **intravasations, extravasations[4], and cell migrations [locomotion]** (Andreasen et al., 1997; Blasi, 1993; Schmitt et al., 2000). In fact, invasiveness, intravasation, and extravasation are very similar processes. They can be summarized as a three-step pattern (figure 1) consisting of attachment, matrix degradation, and locomotion.

[3] Respectively Halsted hypothesis, Halsted paradigm, Halsted model, or "Halstedian view".

[4] *Intravasation* is considered an entrance of externally formed matter into vessels. *Extravasation* is defined as the leakage of intravenous fluids into the interstitial tissue.

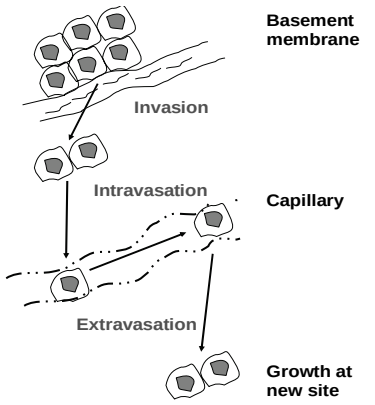


Figure 1 Process of metastasis

An innate loss in intercellular adhesion enables malignant tumor cells to dissociate from their organized tumor cell complex. Intravasation plays a key role connecting the first stage of invading the basement membrane (**invasion**) and the second step of filtering through the endothelial layer of the blood vessel (**intravasation**). The tumor cells gain access to the inner vessel by penetrating this second basement membrane barrier and the layer of endothelial cells forming the vessel's inner lining. However, growth and spread of neoplasm depends on the establishment of adequate blood supply. Thus, tumor-related stimulating angiogenesis plays a critical role in tumor growth and metastasis. Consequently, newly formed blood capillaries in the tumor are readily available for intravasation. This process is facilitated by the fact, that tumor vessels often hold defective and therefore more permeable endothelium tissue susceptible to tumor invasion. In healthy tissue, angiogenesis inhibiting factors dominate, whereas balance is biased towards invasion in rapidly dividing tissue. However, successful intravasation and circulation of the tumor cell alone do not constitute metastasis. Once in circulation, the cell must avoid tumoricidal recognition by the immune system surveillance. Besides, the malignant cells still have to migrate (**extravasate**) from inside the vessel towards the outside in order to invade the target organ. This process involves three steps: (1) attachment to the endothelial lining, (2) retraction of the endothelial cells followed by tumor cell attachment to exposed glycoproteins as well as destruction of the basement membrane, and finally (3) migration into the surrounding stroma. Subsequently, the tumor cells still have to migrate to their new location.

A characteristic feature is overproduction of proteases (e.g., pro-uPA) in response to multiple hormonal, cytokine or growth factor signals (Dano et al., 1985). After **binding** to their **corresponding receptor [e.g., urokinase plasminogen activator receptor (uPA-R)],** these **proteases (e.g., uPA) are activated (pro-uPA → uPA) They cause limited proteolysis of plasminogen to plasmin thus enabling destruction of the ECM** giving **tumor cells their metastatic and invasive character** (Duffy, 1993; Graeff et al., 1992; Schmitt et al., 2002). Additionally, tumor cells are attracted by proteolysis-associated peptides serving as chemotactic agents. Accordingly, proteolysis functions as a key process in metastasis and its connected events. Altogether, cathepsins, matrix metalloproteinases (MMPs), and serine proteases of the plasminogen activation system are involved in the proteolytic process. Although they occur in malignancy-associated processes such as metastasis, there remains a wide range of benign processes, e.g. pregnancy (disruption of the ovarian follicle during ovulation and blastocyst implantation), angionesis, and tissue healing (Andreasen et al., 1997; Duffy, 1996; Schmitt et al., 1997a; Schmitt et al., 2000; Schmitt, Janicke, and Graeff H, 1992). Finally, proliferation of the tumor cells in the recipient tissue is initially confined to a small region around the new blood vessel; only here perfusion to oxygenate is sufficient (Boecker, Denk, and Heitz, 1997).

2.4 Level of Evidence (LOE) Classification and Clinical Utility of uPA and PAI-1

Evaluation guidelines for possible new markers in breast cancer	
1.	Availability of a biological model supporting the possible role of a factor
2.	Simple and validated method of factor determination
3.	Statistical planning of analysis
4.	Cross-check with respect to correlation with established factors
5.	Optimized threshold level for differentiation in low and high-risk group
6.	Univariate analysis (DFS and OS)
7.	Multivariate analysis (independency and validity of factors)
8.	Result confirmation and validation using independent patient cohort
9.	Prospective clinical trial in order to verify the prognostic impact
10.	Transfer into clinical procedure

Table 1 Modified from Clark (1992, 1994), Graeff, Janicke, and Schmitt, (1991), Harbeck (2001 and 2001b), and McGuire (1991)

Quality assurance is of crucial importance in biomarker research and the subsequent implementation of biomarkers in clinical routine use (Schrohl et al., 2003). Unfortunately, different assay formats and different procedures (e.g., collection, storage, and sample processing) may yield different results. To avoid this case, assays and procedures have to be standardized and standard operating procedures (SOPs) should be developed for each type of sample and assay format (Schrohl et al., 2003). Further tools to decide which biomarkers to incorporate into treatment concepts may be the LOE, the grade of recommendation, or the effectiveness. This commonly referred to as evidence- based medicine (EBM) approach, allows quality assured diagnostic and treatment. On a European level the European Organization for Research and Treatment of Cancer (EORTC) is a coordinating group, while in Germany the AGO Breast Commission issues evidence based guidelines. Final objective is the multimodality, resp. multidisciplinary launch of highly effective treatment plans, permitting swift attendance to cancer patients (EORTC, 2002).

Evaluation criteria concerning staging and scoring systems are realized and updated by the tumor-node-metastasis (TNM) staging classification at regular intervals. Assessment of response used to be realized according to the 1979 World Health Organization (WHO) guidelines (Therasse et al., 2000). The EORTC issued adapted guidelines together with the National Cancer Institutes of the United States and Canada (2000), in order to suit rising needs by extending the 1979 WHO criteria. Daily clinical research practice in Europe has been adapted from the US “Good Clinical Practice Standards and Specific National Regulations”. In 1990, the EORTC modified, renamed and extended these standards to comply with the needs of the EU. The according standards are published as “Good Clinical Practice Standards for Trials on Medicinal Products in the European Community“ (EORTC, 2002). Moreover, the International Committee of Harmonization (ICH) issued guidelines to establish a unified standard for the European Community, Japan, and the United States. The ICH harmonized tripartite guideline “Note for Guidance on Good Clinical Practice” is referred to within the EORTC as “ICH-GCP”, allowing a standardized approach facilitating comparison of results (EMEA, 2002). With respect to standardization within Germany, the AGO focuses among other topics on the evaluation of “evidence based recommendations on primary treatment of carcinomas of the breast” (von Minckwitz et al., 2002) as outlined in the “2001 Gravenbruch Consensus on Treatment of Primary Breast Cancer” (Brunnert et al., 2001). Basic principle is the definition of a LOE, considering current literature in combination with data-quality, and consistency of findings. The term “level of evidence” refers to EBM as defined by Sackett (1996). Definition of the LOE and the grade of recommendation are based on the suggestion of the Oxford Centre for Evidence Based Medicine (Phillips et al., 2003). The highest level of evidence for tumor markers is reached by a successful prospective clinical therapy trial or metaanalysis (Hayes et al., 1996).

Therapeutic relevance with respect to the corresponding “Level of Evidence (LOE)” and the “Grade of Recom-

mendation" on behalf of the AGO can be found using the AGO guidelines (2003; 2005c). Additional literature as well as current recommendations are available on the internet page of the AGO (www.ago-online.org).

Aiming to comply with the 1991 published „Breast Cancer Prognostic Factors: Evaluation Guidelines" by McGuire (1991), proposals by Clark (1992), and the 1996 tumor marker utility grading system (TMUGS) as well as the LOEs by Hayes et al. (1996) and recognizing the difficulty in one single laboratory performing all tasks necessary to establish new markers, **a stepwise multicentre evaluation of new prognostic indicators was proposed** (table 1)**.** The Receptor and Biomarker Group (RBG)[5] of the EORTC is the quality-assuring organ for comparable and reproducible measurements of uPA and PAI-1. Furthermore, the EORTC-RBG issues guidelines and evaluates methods used to identify and to measure diagnostically valuable parameters, and compares and assesses the most suitable and reliable method for various types of cancer and establishes quality control programs (Benraad et al., 1996; Sweep et al., 1998). **Prerequisites are summarized using the example of uPA and PAI-1** (table 2).

Prerequisites for prognostic factors using the example of uPA and PAI-1

	Preliminary	*uPA and PAI-1*	*Source*
1.	Suitable biological model.	Tumor associated proteolyses, invasion, migration, adhesion, migration.	(Andreasen et al., 1997)
2.	Easy, convenient and reliable determination method, quality approved.	ELISA method, similar to earlier hormone receptor analysis (needs deep frozen tissue), reproducibility with low variation coefficient proven in multicentre trials.	(Janicke et al., 1994a; Sweep et al., 1998)
3-6.	Prospective, controlled study; marker data and clinical outcome as primary objective.	Retrospective and prospective exploration studies with correlations and prognosis analysis have been conducted.	(Harbeck et al., 2002d; Prechtl et al., 2000)
7-8.	Validation of clinical significance according to level of evidence [LOE] (table 1).	Cut-off optimizing realized and validated.	(Janicke et al., 1994a)
9-10.	Prospective clinical trial, therapy decision will be of clinical relevance, transfer to clinical practice.	Homogenous results in multicentre prospective and retrospective unicenter explorative studies; results in prospective multicentric confirmative studies and in meta-analysis validated. Pooled analysis conducted. Optimal chemotherapy for high-risk N_0 patients with high uPA/PAI-1.	(Harbeck et al., 2001c; Harbeck et al., 2002b; Janicke et al., 2001; Look et al., 2002; NNBC-3 Europe Studie, 2006; Prechtl et al., 2000)

Table 2 Modified from Thomssen and Harbeck (2002) and von Minckwitz et al. (2002)

	Parameter		Oxford		AGO
			LOE	Grade	
Prognostic factors for N_0 breast cancer	Grading		IIb	B	++
	Tumor size		IIb	B	+
	Age		IIb	B	+
	uPA and PAI-1		Ia	A	+
	Proliferation	S-phase; mitotic index; Ki67; Mib-1	IIb	B	+/-
		TLI	Ib	B	+/-

Table 3 Modified from Phillips (2003) and AGO (2003 and 2005c)

Tables 3 and 4 summarize information on prognostic and predictive factors as well as their utility as provided by the "Gravenbruch Consensus 2001, AGO - State of the Art Meeting on Therapy of Primary Breast Cancer" (Brunnert et al., 2001; von Minckwitz et al., 2002) and the 2003 and 2005 AGO Guidelines (AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005c).

[5] Formerly known as the EORTC Receptor Study Group, now EORTC Pathobiology Group.

Tumor biological factors uPA and PAI-1 and level of evidence (LOE)						
Parameter	**Subgroup**	**Comments**	**ASCO LOE**	**Oxford LOE**	**Grade**	**AGO**
uPA and PAI-1	uPA lower or equal vs. above 3ng/mg Protein PAI-1 lower or equal vs. above 14ng/mg Protein	Adjuvant chemotherapy at node-negative breast cancer to identify patients not needing therapy	I	Ia	A	+

Table 4 Modified from AGO (2005c), Brunnert (2001), and von Minckwitz (2002)

2.5 Preclinical Relevance of the Urokinase-Plasminogen-Activation System

Among other factors, the plasminogen activation system consists of serine protease plasmin, uPA, the specific uPA receptor (uPA-R, CD 87), and PAI-1 (Reuning et al., 1998; Schmitt et al., 2000) [figures 2 and 3]. Two different types of plasminogen activators can be distinguished: The urokinase type (uPA) and the tissue type (tPA). Figures 2 and 3 demonstrate that in the event of internalization, the plasminogen activator is capable of catalyzing the conversion of the inactive zymogene plasminogen to the active proteinase plasmin, while leading to degradation of most extra cellular proteins. Whereas uPA enables plasmin to be involved in ECM degradation (e.g., wound healing and cell migration), tPA appears to be mainly related to thrombolysis and the initial solubilization of fibrin clots (Ferno et al., 1996).

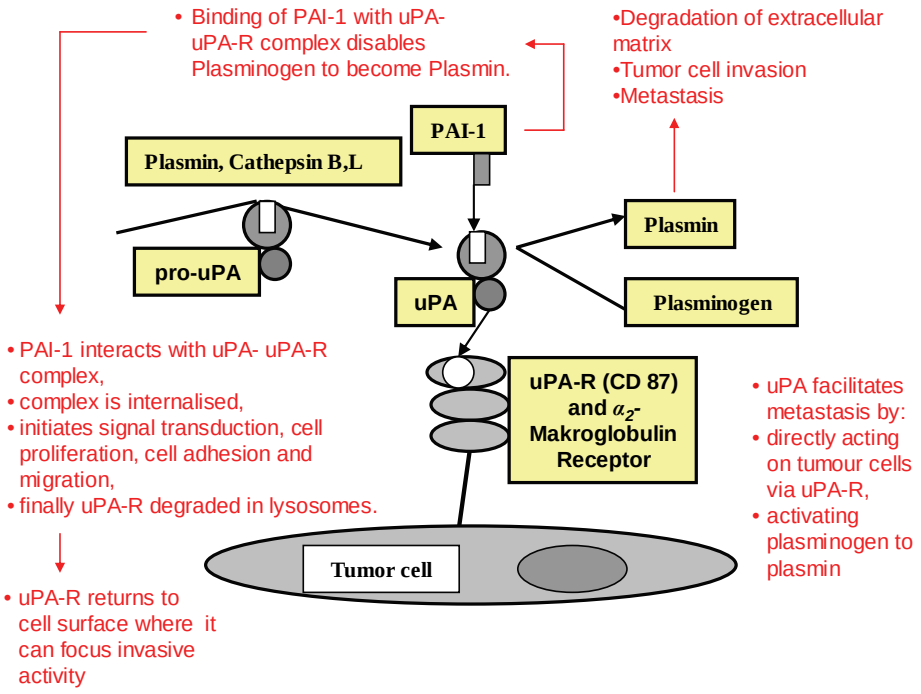


Figure 2 **Components and functional cascade of uPA and PAI-1 interaction,** modified from Schmitt (2002)

Moreover, we can differentiate between two main inhibitors of plasminogen activators: PAI-1 and PAI-2. Plasmin itself is inhibited by α_2-anti-plasmin (α_2AP). The specific uPA receptor (uPA-R, CD 87) is a cell membrane-anchored binding protein for uPA causing plasminogen activation activity at cell surfaces (Andreasen et al., 1997).

Figure 2 shows that zymogene[6] pro-uPA, which is produced by numerous normal and tumor cells, is activated by

[6] *Zymogene*: Inactive pro form of an enzyme (Enzymogen, Proenzyme) until activated by Kinases (Phosphotransferases: transfer end high energy phosphate of a nucleotidetriphosphat to a substrate).

limited proteolysis[7] via cysteine proteases cathepsin B and L or plasmin (Goretzki et al., 1992; Kobayashi et al., 1991). The evolving enzymatic active form uPA then transforms the zymogene plasminogen to serine protease plasmin (figure 3), finally enabling the ECM degradation (Dano et al., 1985; Schmitt et al., 1997a; Schmitt et al., 2000; Schmitt, Janicke, and Graeff H, 1992).

Once high levels of plasminogen activation system components are present, a "cancer cell-directed tissue remodeling process" is initialized, alleviating and triggering signal transduction, cell proliferation, adhesion as well as migration. Interaction between the uPA/uPA-R complex and PAI-1 leads to a ternary complex internalized by the cell [figure 2] (Andreasen et al., 1997; Reuning et al., 1998; Schmitt et al., 1997a; Schmitt et al., 2000).

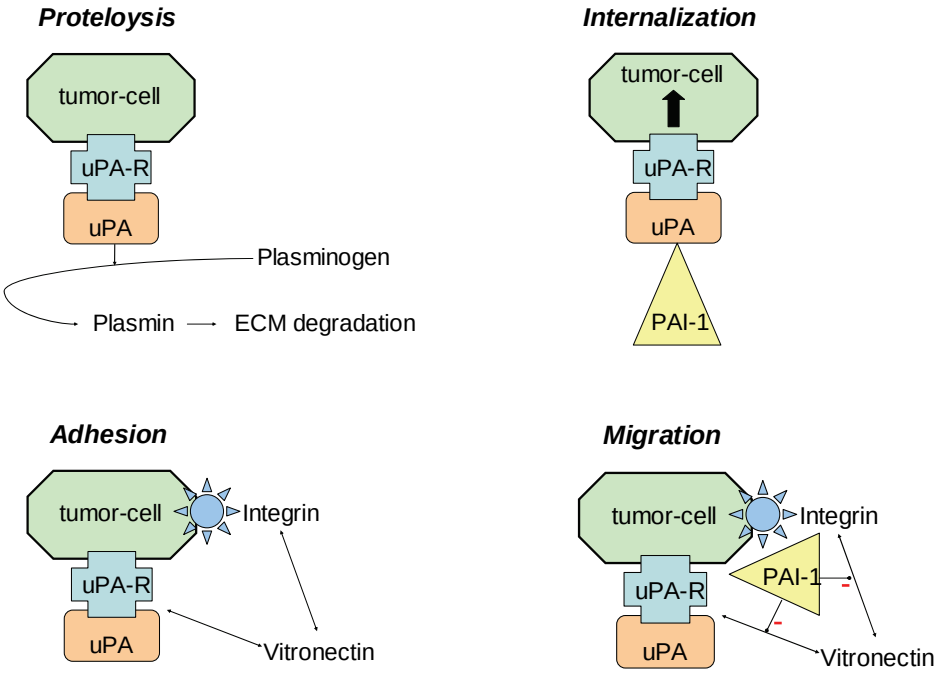


Figure 3 **Components and detailed function of uPA and PAI-1**, modified from Harbeck (2001)

2.6 Clinical Relevance of the Urokinase-Plasminogen-Activation System

In 1988, the first relevant data on the possible prognostic impact of uPA in breast cancer was published (Duffy et al., 1988). The cause of cell malignancy constituted the main focus, before uPA or even PAI-1 were associated with metastasis. Dano et al. (1985) proposed a novel approach, in which a "combination of non-specific properties" accumulated in a cell was assumed responsible for the potential malignancy. The combination of the aforementioned non-specific properties was assumed to lead to a malignant development within the cell. Dano suggested a functional approach, focusing on specific biochemical processes to cause the malignant transformations such as tissue degradation and subsequent invasion. This theory proved viable.

The currently favored theory with respect to tumor metastasis encompasses a three-step process, basically consisting of (1) Degradation of the ECM, (2) tumor cell invasion, finally leading to (3) metastasis.

Historically, it was based on the **identification of the degradation causing factor.** Initial findings within tissue cultures showed a consistent proteolytic degradation with dissolution of plasma clots used as their agars (Duffy, 1987). Degradation was believed to occur due to a "lytic agent", possibly causing activation of an inactive proenzyme (Fischer, 1946). Fisher (1946) argued proteolysis of cultured tumor cells was caused by the activation of an inactive proenzyme. Goldhaber et al. (1947) identified this proenzyme as a cancer cell-released enzyme capable

[7] *Limited proteolysis*: Enzymatic or hydrolytic dissolution of peptide fragments.

of turning profibrinolysin into fibrinolysin (e.g., plasminogen to plasmin). Between 1953 and 1973, reports by several authors emphasized the relation between cancer and fibrinolysis. At the same time, the discovery of proteolytic activity was reported as being unspecific for cancer tissue, while present in benign tumors and normal tissue. With no apparent connection of plasminogen activators in neoplasia and a malignant state, research was cut down until the 1970s. Interest arose again with the observation of an increase in extracellular proteolytic activity in previously cultured and transformed cells triggered by the release of plasminogen activators (Duffy, 1987). Astedt et al. (1976) identified uPA as a key determinant of tumor invasiveness immunologically, which finally led to the:

Proteases-inhibitor model for cancer-metastasis. In the 1980s, both Mullins/Rohrlich and Duffy focused on proteases and their role in invasion as well as in cellular metastasis (Duffy, 1987; Mullins and Rohrlich, 1983). Duffy (1987) outlined **three main functions of proteases or their inhibitor**: (1) “Common” destructive events such as mammary gland involution after lactation or prostate gland involution after castration, (2) model tumor systems in which correlation between protease activity and metastatic potential could be proven, and (3) protease inhibitors preventing metastasis in experimental animals.

In 1988, Duffy (Duffy et al., 1988) reported **uPA to correlate with tumor size:** He found highly elevated levels of uPA activity in invading tumors and presented a significant correlation of uPA activity in breast carcinomas with a worse outcome.

The prognostic value of uPA levels determined by means of biochemical analysis was confirmed by several independent groups in the following years (Duffy et al., 1990; Ferno et al., 1996; Foekens et al., 1994; Grondahl-Hansen et al., 1993; Janicke et al., 1989; Spyratos et al., 1992). Additionally, Janicke et al. (1991 and 1993) for the first time were able to describe these findings for uPA and PAI-1 both concerning node-negative and node-positive breast cancer patients.

Duffy’s 1988 study and Jänicke´s studies (1989, 1991), confirming and extending the results to PAI-1, are today reported to be the first studies highlighting the role of uPA and/or PAI-1 with respect to breast cancer. To resolve the question, whether uPA and PAI are factors with prognostic impact, several authors focused on breast cancer within the following years and demonstrated the prognostic relevance of uPA and/or PAI-1 (table 5).

Proving their role as important prognostic factors in cancer, uPA, uPA-R, and PAI-1 have not only been found elevated in tumor tissue and blood, but also in numerous types of solid cancers compared to corresponding benign lesions. Supporting data is available on cancer of the breast (Schmitt et al., 1997a; Schmitt et al., 2000; Schmitt, Janicke, and Graeff H, 1992), the cervix (Kobayashi, Fujishiro, and Terao, 1994), the ovary (Gleeson et al., 1996; Kuhn et al., 1994; Kuhn et al., 1999), the lung (Pedersen et al., 1994), the brain (Arai et al., 1998; Mohanam et al., 1997), the gastro-intestinal tract (Ganesh et al., 1994; Heiss et al., 1995b; Nekarda et al., 1998; Verspaget, 1995), the urinary tract (Hofmann et al., 1996; Miyake et al., 1999), the prostate (Crowley et al., 1993), the oropharynx (Hundsdorfer et al., 2004), along with pediatric malignant bone-tumors (Nowak-Goettl et al., 1999).

Author	Year	Country	Assay (1)	Cut-off	Patients		Follow-up (month)	Prognostic impact (2)	Reference
					All	N_0 (6)			
Duffy (7)	1988	Ireland	Activity (cytosol)	Median	52	25	17	yes	(Duffy et al., 1988)
Janicke (3)	1991	Germany	ELISA ADI	Optimized	115	53	25	yes(u,m)	(Janicke, Schmitt, and Graeff, 1991)
Janicke (3)	1993	Germany	ELISA ADI	Optimized	247	101	30	yes(u,m)	(Janicke et al., 1993)
Grohndahl-H. (3)	1993	Denmark	ELISA MONO	Median	191	23	102	yes(u,m)	(Grondahl-Hansen et al., 1993)
Foekens (3)	1994	Netherlands	ELISA ADI	Optimized	657	273	48	yes(u,m)	(Foekens et al., 1994)
Grohndahl-H. (5)	1995	Denmark	ELISA in house	Median	505	193	54	yes(u,m)	(Grondahl-Hansen et al., 1995)
Fernö (7)	1996	Sweden	LIA (4)	Median	688	265	42	yes(m)	(Ferno et al., 1996)
Eppenberger (3)	1998	Switzerland	ELISA ADI	Optimized	305	159	37	yes(u)	(Eppenberger et al., 1998)
Kim (3)	1998	Japan	ELISA Bio	Optimized	130	130	53	yes(u)	(Kim et al., 1998)
Kute(3)	1998	USA	ELISA MONO	Median	168	168	58	yes(u,m)	(Kute et al., 1998)
Knoop (3)	1998	Denmark	ELISA MONO	Median	429	178	61	yes(u,m)	(Knoop et al., 1998)
Harbeck (3)	1999	Germany	ELISA ADI	Optimized	125	125	76	yes(u,m)	(Harbeck et al., 1999a)
Bouchet (3)	1999	France	ELISA ADI	Quartiles	499	233	72	yes(u,m)	(Bouchet et al., 1999)
Foekens (3)	2000	Netherlands	ELISA ADI	Optimized	2780	1405	88	yes(u,m)	(Foekens et al., 2000)
Harbeck(3)	2000	Germany	ELISA ADI	Optimized	276	130	109	yes(u,m)	(Harbeck et al., 2000)
Konecny (1),(3)	2001	USA/Germany	ELISA ADI	Optimized	587	283	26	yes(m)	(Konecny et al., 2001)
Janicke (3)	2001	Germany	ELISA ADI	Optimized	374	374	32	yes(u,m)	(Janicke et al., 2001)
Harbeck (1),(3)	2002	Germany	ELISA ADI	Optimized	761	269	60	yes(u,m)	(Harbeck, Kates, and Schmitt M, 2002)
Look, Harbeck (3),(4)	2002	Europe (EORTC)	Different assays (1),(4)	Median	8377	4,676	79	yes(u,m)	(Look et al., 2002)
Hansen	2003	Denmark	ELISA in house	Median	228	124	150	yes(u,m)	(Hansen et al., 2003)
Manders	2004	Netherlands	ELISA in house	Median	1119	594	2 - 267	yes(u,m)	(Manders et al., 2004)

(1) ELISA Assays (commercially available): *ADI* (American Diagnostica, Stamford, CT, USA); *Bio* (Biopool, Umea, Sweden); *Mono* (Monozyme, Horsholm, Denmark); *Santec* (Bromma, Sweden).

(2) Univariate (u) and/or multivariate (m) analysis.

(3) As determined by ELISA.

(4) Levels of uPA and PAI were determined in (either) cytosolic tumor extracts and/or Triton X-100-treated tumor extracts; *ELISA*: cf. (1); *LIA*: Santec, Bromma, Sweden; *in house*: in house assay; *Protein Assays:* Bio-Rad, Hercules, CA, USA; Pierce, Rockford, IL, USA.

(5) Levels of uPA and PAI were determined in (either) cytosolic tumor extracts and/or Triton X-100-treated tumor extracts.

(6) N_0= node-negative patients.

(7) uPA determination only.

Table 5 **Selected references demonstrating prognostic relevance of uPA and/or PAI-1 in primary breast cancer and method of determination**, modified from Harbeck (2001b)

Findings by Sier (1994) and Schmalfeldt (1995) emphasized the crucial role of components of the fibrinolytic system (the enhanced urokinase cascade resp.) causing the cells´ aggressive potential, re-implantation, and consolidation of a new tumor stroma. Moreover, an initial disturbance of the critical balance between uPA and its inhibitor PAI-1 was identified as a prerequisite for optimal invasiveness, leading to efficient local proteolysis, ECM degradation, migration, and subsequent tumor cell invasion and metastasis (Liu, Shuman, and Cohen, 1995). Bajou et al. (1998, 2000) found a deficient PAI-1 and a lack of plasminogen being responsible for reduced tumor vascularization and invasion. **Altogether, these findings implied that the biological role of PAI-1 goes be-**

yond that of a simple protease inhibitor.

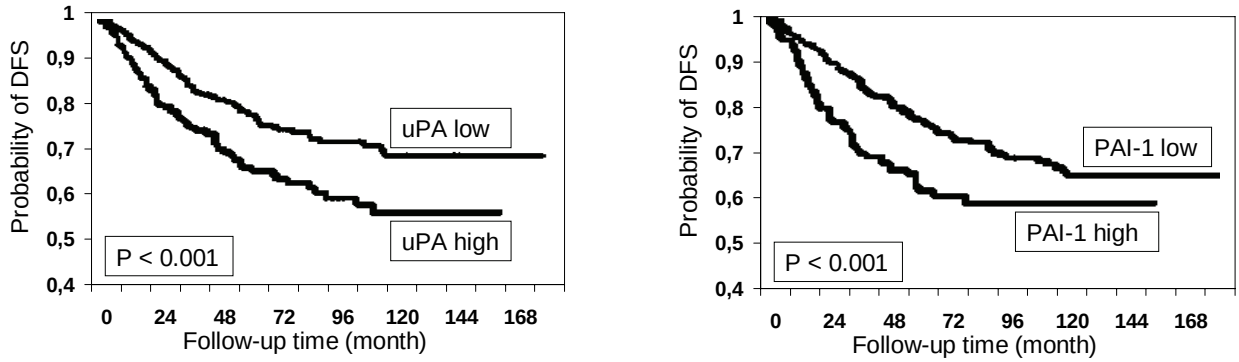
Schmitt et al. (1991) and Harbeck (2001) clarified high levels of PAI-1 to predict worse outcome for the patients: Binding of PAI-1 to uPA on top of uPA-R inhibits uPA transforming plasminogen to plasmin. Then, the complex of uPA/uPA-R/PAI-1 is internalized, thereby promoting intracellular signal transduction (e.g., proliferation). After internalization uPA may migrate to the cell surface, focusing continuous invasive activity (Andreasen et al., 1997). Just as PAI-1, uPA is a critical mediator of metastatic development (Duffy, 2002). Additionally, PAI-1 is able to inhibit cell adhesion to the ECM component vitronectin by blocking the joining together of the surface antigen $\alpha_v\beta_3$ and vitronectin (Stefansson and Lawrence, 1996; Wei et al., 1996). Binding of uPA to PAI-1 stops this cycle enabling adhesion again. This alternation facilitates cell migration (Lauffenburger, 1996). **A critical balance between uPA and PAI-1** as well as the cell surface receptor **uPA-R**, CD 87, are **requirements for efficient local proteolysis, adhesion, and migration, causing "optimal" tumor cell invasion and subsequent metastasis** (Schmitt et al., 1997a). Consequently, plasmin proteolysis can be considered an essential and strictly controlled process allowing vessel stabilization and maturation, thus impeding angiogenesis (Andreasen et al., 1997; Harbeck et al., 2002d; Schmitt et al., 2000).

Within the past decade, different international research teams worked on the correlation of high levels of uPA and PAI-1 with both short-term disease-free survival (DFS) and OS in node-negative (N_0) as well as node-positive (N+) breast cancer. The goal consisted of substantiating the potential use of **uPA/PAI-1 as prognostic factors and enabling subgroup selection** (Harbeck et al., 1998b). Janicke et al. (1991 and 1993) was the first group to describe such findings in a paper on uPA and PAI-1 both concerning N_0 and N+ breast cancer patients (Janicke et al., 1993). Supporting data has also been reported by others (Bouchet et al., 2000; Duffy et al., 1998; Ferno et al., 1996; Foekens et al., 1992; Grondahl-Hansen et al., 1993; Harbeck et al., 1999b). Harbeck et al. employed log-rank statistics to validate cut-off levels for uPA at 3ng/mg protein and for PAI-1 at 14ng/mg protein: **While high-risk patients show levels of uPA above 3ng/mg protein and/or PAI-1 above 14ng/mg protein in their primary tumor, low-risk patients exhibit levels of both uPA lower or equal 3ng/mg protein and PAI-1 lower or equal 14ng/mg protein in their primary tumor tissue.**

Patients belonging to the low-risk group have an excellent prognosis, with a probability of relapse of less than 5% after five years (Harbeck et al., 1998a; Harbeck et al., 1999a). The combination of both uPA and PAI-1 is superior to the use of either factor alone as well as to established prognostic factors such as tumor size, grade, hormone receptor status, or menopausal status (Harbeck et al., 1999a). While in the total collective, the prognostic significance of uPA and PAI-1 is only surpassed by nodal-state, in N_0 patients uPA/PAI-1 are the strongest prognostic factor (Harbeck, Kates, and Schmitt M, 2002). The impact of PAI-1 may increase with time and remains a strong prognostic factor for relapse-free time in N_0 breast cancer with a median follow up exceeding 6 years. The impact of uPA seems to be most pronounced during the first two years after primary therapy (Harbeck et al., 1999a; Schmitt et al., 1997b). Furthermore, uPA/PAI-1 outperforms additional tumor biological factors such as cathepsins B, D, tumor suppressor protein p53, S-phase, proliferation measured by MIB1, or DNA ploidity with respect to prognostic relevance (Harbeck et al., 1999a; Harbeck et al., 2001a). As for the human epidermal growth factor receptor Her2/neu (syn. ErbB-2) and uPA/PAI-1, these reveal independent prognostic information in primary breast cancer (Konecny et al., 2001). With respect to the previously mentioned subgroup characterization, long-term follow-up data appear to support using the combined values of uPA and PAI-1. Patients with either one or both factors high appear to have a substantially higher risk of recurrence than patients with both factors low. While Knoop et al. (1998) did not observe a considerable impact on loco-regional recurrence, Cufer et

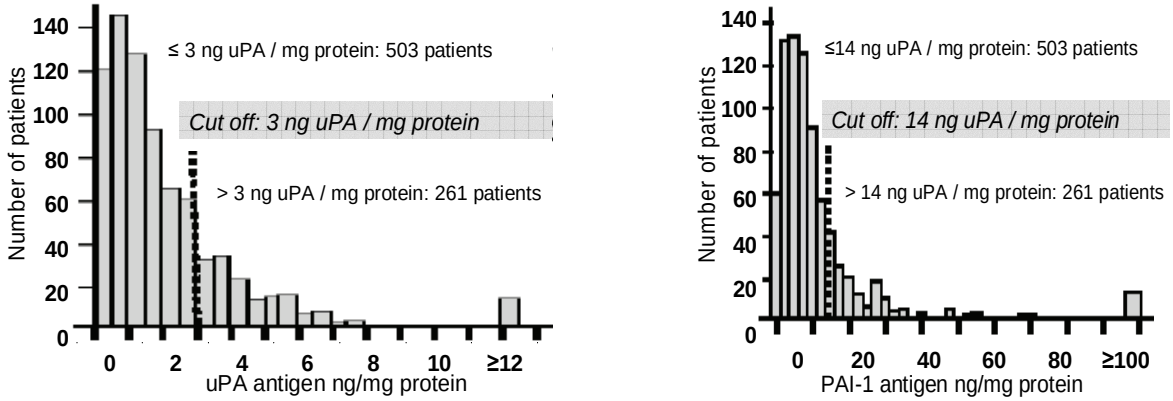
al. (2002) described PAI-1 levels in primary tumor tissue above the median to be correlated significantly with an increased risk for loco-regional relapse.

Both reports by Harbeck et al. (1998a and 1999a) depicted a clear cut risk group determination and have not been contradicted to this point, neither for traditional factors nor for any other tumor-biological factors. This emphasizes the need for risk group discrimination using prognostic factors prior to therapeutic recommendations.



Disease-free survival of patients with high tumor antigen levels of either factor vs. patients with low levels using previously optimized and re-evaluated cutoffs of 3ng uPA and 14ng PAI-1 per mg tumor tissue extract protein.

Figure 4 **Clinical relevance of uPA and PAI-1 in primary breast cancer median time of follow-up: 5 years). Follow up time (0 to 14 years) and probability of DFS (0.4 to 1)** (Janicke et al., 2001), modified from Schmitt (2002)



Distribution of uPA and PAI-1 antigen levels in tumor tissue extracts of primary breast cancer patients (n=764). Levels of the analytes are expressed in ng per mg tumor tissue extract protein. uPA: Range 0.04 to 66.0; mean 3.1 median 2.06. PAI-1: Range 0.06 to 247.2; mean 14.7; median 9.1.

Figure 5 **Clinical relevance of uPA and PAI-1 in primary breast cancer (median time of follow-up: 5 years). Antigen distribution and number of patients** (Janicke et al., 2001), modified from Schmitt (2002)

Published in a meantime by Schmitt et al. (2002), figures 4 and 5 show the distribution of uPA and PAI-1 and Kaplan-Meier curves, demonstrating the course of the disease (DFS) for n=764 patients. uPA levels in this **adjuvant setting** ranged from 0.04 to 66.0 (mean 3.1; median 2.06; spread 65.96) and PAI-1 levels from 0.06 to 247.2 (mean 14.7; median 9.1; spread 247.12) ng per mg tumor tissue extract protein. Patients with high tumor antigen levels of either factor had a significantly shorter DFS than patients with low levels. Low uPA levels were found in n=503 with 106 relapses, while high uPA levels were noted in n=261 with 85 relapses. Low PAI-1 levels found in n=551 were associated with 125 relapses with high PAI-1 values in n=213 with 66 relapses.

The clinical relevance and reliability of uPA and PAI-1 was determined in an explorative manner at the department of Obstetrics and Gynecology of the Technische Universität, München, Germany. As a consequence to an affirming unicenter study by Harbeck et al. (1999b), a prospective multicentre trial for the German N_0 Study

Group was started in June 1993, comprising fourteen departments of obstetrics and gynecology or departments of surgery: Until December 1998, a total of 689 N_0 patients without distance metastasis were enrolled. Patients with low uPA and PAI-1 values (lower or equal 3ng uPA and 14ng PAI-1 per mg of tumor tissue protein) were observed only. In contrast, patients with elevated uPA and/or PAI-1 (above 3ng uPA or 14ng PAI-1 per mg of tumor tissue protein) in their primary tumors were randomized to observation or to six treatment-cycles of Cyclophosphamid/ Methotrexate/ 5-Fluorouracil [CMF] (Janicke et al., 1994b). The first interim analysis 4.5 years after the trial launch showed a strong significant statistical difference in DFS with a 2.83-fold higher risk of disease recurrence for patients with high uPA and/or PAI-1 levels compared to those with low uPA and PAI-1 levels (Janicke et al., 2001). A second interim analysis confirmed the results of the first follow-up, substantiating the prognostic impact of uPA and PAI-1 on DSF and extending it to OS (Harbeck et al., 2001c).

Complying with the technical principles for ELISAs and in accordance with strict criteria for evaluation of new prognostic markers according to the EORTC-RBG, **the Chemo N_0 multicentre study can be interpreted as a validation of the strong prognostic significance of uPA and PAI-1** levels for patients with lymph node-negative breast cancer at the highest level of evidence. What's more, an **EORTC validation** by a pooled analysis comprising 8377 primary breast cancer patients (Look et al., 2002) confirmed these findings. uPA and PAI-1 were recognized as being the strongest prognostic indicators for DFS and OS next to the nodal status, while in untreated N_0 patients (n=3362), uPA and PAI-1 were the strongest **predictors** of OS and DFS. The **EORTC validation** provided level I evidence **(LOE I, +)** supporting the prognostic role of these molecular markers and suggested they should be used routinely to guide adjuvant therapy in women with N_0 breast cancer (Mokbel and Elkak, 2001). Further publications proved uPA and PAI-1 to be the strongest parameters for DFS and OS both in univariate and multivariate analysis emphasizing their capability to outperform the Nottingham prognostic index[8] or HER-2 for risk assessment in N_0 breast cancer (Janicke et al., 2001; von Minckwitz et al., 2002; Zemzoum et al., 2003).

While uPA and PAI-1 as **prognostic factors** allow prediction of the course of the disease and assessment of the individual risks of recurrence as well as of mortality, uPA and PAI-1 as **predictive factors** enable to predict response or resistance to a specific therapy (Cianfrocca and Goldstein, 2004; Hayes et al., 1996; Hayes, Isaacs, and Stearns, 2001). This predictive capacity would then enable the clinician to estimate the probability of response or non-response and thus to shape a more individualized therapy concept (Harbeck et al., 2002a). In addition to the prognostic impact of uPA and PAI-1 on DFS and OS, Harbeck et al. (2003) were able to prove for the first time a **predictive impact of uPA and PAI-1** related to response to adjuvant chemotherapy. This was honored with the Schmidt-Matthiesen-Award in 2002 (Harbeck et al., 2002c). Their findings were confirmed by Manders (2004).

In summary, the invasion factors **uPA** and its inhibitor **PAI-1** are the **first novel tumor-biological factors** in breast cancer with **the prognostic and the predictive impact** validated at the highest level of evidence and all evaluation criteria for transfer into clinical practice fulfilled (Harbeck et al., 2002d; Harbeck et al., 2004a). uPA and PAI-1 may help to **resolve the question, whether or not to apply adjuvant chemotherapy** (Harbeck, Kates, and Schmitt M, 2002; Janicke et al., 2001). Nearly half of the patients with lymph N_0 breast cancer, char-

[8] *Nottingham prognostic index*: combination of three prognostic factors comprising (1) tumor size (cm x 0.2), (2) lymph node stage (1= lymph node-negative, 2= 1-3 metastatic nodes, 3= above or equal 4 metastatic nodes) and (3) histological grade (1-3, good, moderate, poor). Alternatively lymph nodes can be classified according to level of involvement. A prognostic index < 3.4 implies a good prognosis, 3.4 to 5.4 a moderately good one and over 5.4 a poor prognosis.

acterized by **low uPA/PAI-1 levels** as **low-risk patients,** might **avoid adjuvant chemotherapy** due to their good prognosis (Harbeck et al., 2002d), while **N_0 patients** with **high uPA/PAI-1 levels** are **at an increased risk of relapse** comparable to patients with above or equal 3 involved axillary lymph nodes (Harbeck et al., 2002d). The latter benefit significantly from adjuvant chemotherapy (Harbeck and Thomssen, 2003), which was confirmed by Manders et al. (2004) independently. In the 2005 St. Gallen (Switzerland) Consensus Recommendations on the Primary Therapy of Early Breast Cancer, uPA and PAI-1 were discussed again (Goldhirsch et al., 2005): High levels (as measured on tissue extracts using ELISAs) indicate a poor prognosis (Harbeck et al., 2004a), while patients with low uPA/PAI-1 and positive estrogen receptors (ER) show a particularly good prognosis (Harbeck and EORTC RBG, 2005).

However, **recommendations for therapy concepts taking into account high and low uPA and PAI-1 levels and the risk of recurrence** remain under debate and require further investigation (Harbeck et al., 2002d; Harbeck and Thomssen, 2003). Recent studies are the NNBC-3 trial in N_0 breast cancer (Harbeck et al., 2002d; NNBC-3 Europe Studie, 2006; Paepke et al., 2006) and the ADEBAR trial in N+ breast cancer. Yet, there is a need of facilitated measurement and further prospective studies using methods such as micro ELISAs (Hayes, 2005) as follows.

2.7 Measurement of uPA and PAI-1

Biochemical measurement of uPA and PAI-1 levels outperforms any other assay methodologically and is considered the optimal in clinical-daily-procedure (Benraad et al., 1996; Harbeck et al., 2002d; Janicke et al., 1993; Janicke et al., 1994a; Schmitt et al., 2002)**.** For the most part, **ELISAs** have been used as technical approaches for obtaining clinical relevant results for uPA and PAI-1 in breast cancer (Schmitt et al., 2000). Since uPA and PAI-1 are both strong and statistically independent prognostic factors, **simultaneous determination of both** is recommended to yield optimal prognostic information in breast cancer patients (Janicke et al., 1994b; Janicke et al., 1993; Janicke et al., 1994a; Janicke, Schmitt, and Graeff, 1995; Schmitt et al., 2002).

uPA and PAI-1 antigen determination is generally conducted using approved (Benraad et al., 1996) commercially available ELISA tests by ADI (American Diagnostica, Stamford, CT, USA) in detergent extracted (Triton X-100) tissue samples (Janicke et al., 1994a; Schmitt et al., 2002). Levels of uPA and PAI-1 are given as ng per ml for fluids resp. ng per mg for tissue and cells. The amount of protein is determined using a bicinchoninic acid protein assay kit (BCA) by Pierce Biotechnology (Rockford, IL, USA). Tissue disintegration with 1% (w/w) non-ionic detergent Triton X-100 by Sigma-Aldrich [Munich, Germany] including Tris-Buffered-Saline (TBS) extracts additional membrane and intracellular bound uPA[9] and generates a tissue lysate with the majority of cellular matrix proteins retrievable (Duggan et al., 1995; Janicke et al., 1994a; Romain et al., 1995; Schmitt et al., 2002).

So far, no scientific reports have been published contradicting the prognostic impact of uPA and PAI-1 measured by ELISA in any kind of tumor (Schmitt et al., 2002). This may be attributed both to the strong biological role of uPA and PAI-1 in tumor spread and metastasis and the quality control of the EORTC-RBG (Harbeck et al., 2002d; Schmitt et al., 2002).

As outlined by Sweep et al. (2003), immunoassay methods can be divided according to the type of analysis (quantitative, semi-quantitative, or qualitative), the assay system, and the assay conditions (liquid phase, solid-

[9] Additional PAI-1 is not released by this technique.

liquid phase assays, equilibrium vs. nonequilibrium assays, manual vs. automated assays). Two systems might be distinguished: (1) the non-labeled methods allowing the antigen-antibody complex being detected without markers and (2) the labeled methods, subdivided according to the type or marker used to expose the antigen-antibody complex and the reaction design.

Whereas the term immunoassay refers to a competitive method [e.g., Radioimmunoassay (RIA)[10]], the term immunometric assay specifies a non-competitive method (e.g., ELISA). **ELISA** is a frequently applied type of immunometric enzyme immunoassay in clinical research and as an antigenic test it gives a quantitative measurement of the analyte antigen (Goldsby et al., 2003; Sweep et al., 2003). The antigen reacts in a noncompetitive manner to an excess amount of solid-phase coupled antibody and subsequently an excess of marker labeled antibody binds to another site of the antigen. It measures active and latent forms of analyte. There are several different forms using fetal calf serum (FCS), bovine serum albumin (BSA), or milk proteins for blocking nonspecific binding, alkaline phosphatase or peroxidase conjugated secondary antisera for primary antibody detection, and colored, fluorescent, or radioactive substrates for endpoint detection. The basic principle of ELISA (figure 6) is using an enzyme to detect the binding of antigen (Ag) and antibody (Ab). The enzyme converts a colorless substrate (chromogen) into a colored product, indicating the presence of an Ag:Ab-binding. This principle is an easy applicable and strong method to estimate ng to pg per ml resp. mg ordered materials in the solution, such as serum, urine, culture supernatant, pharmacological substances, hormones, and proteins such as tumor markers uPA and PAI-1.

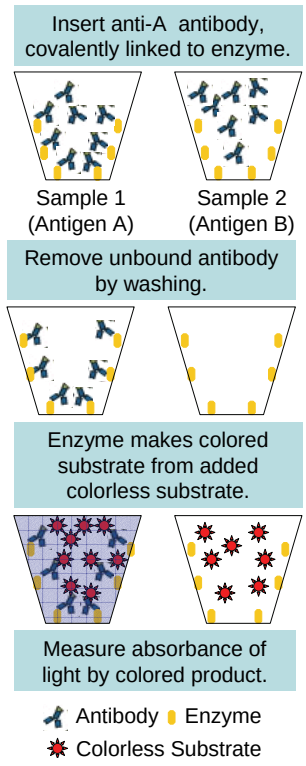


Figure 6 **ELISA,** modified from Goldsby (2003)

In detail, the specific Ab against the Ag to be measured is firmly bound chemically either to the wall of the probe container or to polymer-pellets. The Ag in the probe to be searched for connects with the bound Ab. Any supernatant is washed away. Although plates can be hand washed, most reproducible data are generated with automatic 96-well plate washers. Ab towards first step bound Ag - associated with a detector enzyme - is added. Enzyme activity of Ab-Ag-Ab (sandwich technique) is measured photometrically after adding the proper substrate.

To measure an undefined amount of protein, a **protein assay** is required in which measurable quantity is related to protein concentration. Preferably, this should be in direct proportion. The "**Lowry two-step protein assay"** was the method of the choice for some time (Lowry et al., 1951). Basic principle of the Lowry assay is an alkaline condition in which the divalent copper ion forms a complex with peptide bonds thereafter reduced to monovalent ion. The monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with a folin reagent in order to produce an unstable product subsequently getting reduced to molybdenum/tungsten-blue. Extended by **Hatree**, the "Hatree version" uses fewer agents, augments the sensitivity with certain proteins, is not as often incompatible with certain salt solutions, and supplies a further linear response, while the change of saturation is diminished. Comprising all the benefits of the "Lowry-Hatree assay", the "modified Lowry assay" is conducted entirely at room temperature (Caprette, 2000; Pierce Biotechnology, 2002b). The original "Lowry as-

[10] *RIA – Radioimmunosaasy*: competitive method for determining antigen concentrations with a limited amount of antibody insufficient to bind all antigens.

say" is nowadays replaced by a single step **BCA assay** (e.g., by Pierce Biotechnology, Rockford, IL, USA), a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantification of the protein total. It is completed within 10 instead of 40 minutes, functions with alkaline stable reagent, and is less sensitive to interfering agents. The BCA is based on the reduction of divalent copper ion to monovalent ion under alkaline conditions (Biuret reaction). And, it combines the reduction of the Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by means of a specific reagent containing BCA. Due to the alkaline stable condition, it can be used within the copper solution to allow a one step procedure. The specific reaction product of this assay is formed by chelating of two molecules BCA with one cuprous ion (Pierce Biotechnology, 2002a) which is then visible as a purple color (molybdenum/tungsten blue). Since this water-soluble complex exhibits a strong absorbance at 562nm linear to increasing protein concentrations over a broad concentration range (20 - 2,000 µg/ml), it subsequently is read at 562nm. By increasing incubation time, the assay sensitivity might be amplified. Since the BCA method is not a true end-point method, the final color continues to develop. However, following incubation the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed within a single run. Although the assay might be performed at room temperature, it is recommended to make use of a constant temperature to minimize variability among proteins, which could diminish the assay sensitivity (Caprette, 2000).

Two main **assay procedures** can be distinguished. On the one hand the larger protein volume requiring (100µl) a "**test tube procedure**"; on the other hand, the smaller protein volume (10-25µl) requiring a "**microplate procedure**". Since the test tube procedure uses a sample to working reagent ratio of 1:20, the effect of interfering substances is minimized. In contrast, the microplate procedures´ sample to working reagent ratio is 1:8 resulting in less flexibility in overcoming interfering substance concentrations and obtaining low detection levels (Pierce Biotechnology, 2002a). However, use of the **BCA assay was confirmed to be microtiter applicable** (Stoscheck, 1990) and **together** with **ELISA kits by ADI** (American Diagnostica, Stamford, CT, USA) both can be recommended as **reliable** [tables 16 and 17] (Schmitt et al., 2002).

The "**Bradford assay"** as an alternative method uses a similar amount of protein as needed for the "Lowry assay". It is quite reliable and out of range samples might be reinvestigated within minutes. The Bradford method is suggested for wide-ranging use, especially for validation of protein content of cell fractions and assessment of protein concentrations for Gel electrophoresis. Moreover, because of the extinction coefficient of a dye-albumin complex solution being constant over a 10-fold concentration range, it is widely applicable. Applying the standard procedure, the assay is sensitive for approximately 20 to 200mg protein. The required microtiter plate protocols are outlined in the kit-accompanying leaflet. The basic principle rests on binding to a protein resulting in a change of the absorbance maximum for an acidic solution of Coomassie blue G-250 from 465nm to 595nm. The anionic form of the dye is stabilized by hydrophobic and ionic interactions, resulting in an observable color change. Detailed assay procedures can be assessed from Caprette (2000).

Protein concentrations are generally determined and reported with reference to standards of a common protein such as BSA. After preparing the assay, a **standard curve of absorbance** vs. micrograms protein (or reversed) is set up, followed by determining the amounts of protein from the curve. Comparing absorbances of samples with known amounts of protein to those of the unknown allows estimating the unknown quantities. By addition of 1M NaOH, the solubilization of membrane proteins is enabled and protein-to-protein variation in color yield is reduced (Hartree, 1972; Oosta, Mathewson, and Catravas, 1978; Pierce Biotechnology, 2002a; Stoscheck, 1990).

For protein determination in this thesis we used the Bradford and Pierce method.

2.8 Tissue Sampling

Following the European Society of Mastology [EUSOMA] (2002) guidelines for diagnosis of breast cancer lesions, diagnosis is based on the assessment of physical examination, bilateral mammography in two projections, ultrasound, and minimal invasive technique. Trying to reduce open biopsies and to facilitate individualized therapy planning (Smyczek-Gargya et al., 2002) at a superior level of quality and at reduced financial expenses (Groenewoud et al., 2004), the minimal invasive biopsy technique is a feasible and accurate method (McIlhenny et al., 2002; Rutgers, 2001) with good evidence (AGO, Gynecologic Oncology, and ARO, 2005a). Several authors have demonstrated the accuracy of sonographic and stereotactic high-speed biopsy (Sittek et al., 2005b), which depends on the expertise of the examiner, the specialization of the center, and the amount of tissue gained (Liberman et al., 1994). Using sonographic and stereotactic high-speed biopsy, a sensitivity of 92-98% and a specifity up to 100% might be achieved (Fehr et al., 2002; Ikeda et al., 2002; Jackman et al., 1999; Jackman and Marzoni, Jr., 1997; Latosinsky et al., 2000; Lee et al., 1999; Liberman et al., 1997; Liberman, 2000; Liberman et al., 2000; Parker et al., 1991; Parker et al., 1993; Parker et al., 1994; Parker and Burbank, 1996; Schulz-Wendtland et al., 2003; Sittek et al., 2005b) as well as a positive predictive value (PPV) of up to 99,7% and a negative predictive value (NPV) of up to 97,3% (Buchberger et al., 2002) with a calculated false-negative rate for 14-G LCNB (large core needle biopsy) based on 3880 results of 0,4 % (Memarsadeghi et al., 2003) resp. between 1,1% (14-G LCNB) and 2,9% [14-G vacuum-assisted breast biopsy (V-ABB)] (Pfarl et al., 2002). Recommendations established by the “European Guidelines for Quality Assurance in Breast Cancer Screening and Diagnosis” (Perry et al., 2006) and the National S-3 Guideline on Breast Cancer (Kreienberg et al., 2004) state that more than 90% of patients subsequently proven to have breast cancer should have had a pre-operative fine-needle aspiration (FNA) or large core needle biopsy (LCNB) at the diagnosis of cancer and more than 70% of patients subsequently proven to have clinically occult breast cancer should have been assessed interventionally prior to open biopsy (Rutgers, 2001).

Two fields of biopsies can be distinguished: On the one hand **needle biopsy**, subdivided into fine needle **(FNB/FNA**), core needle (**LCNB**), and assisted breast biopsy (**ABB**)[11]. On the other hand **open biopsy,** subdivided into excisional (**EB**) and incisional biopsy (**IB**). While IB dissects only a part of the tissue in question, EB removes it entirely.

Until the end of the 1990s, a major problem in minimal invasive procedures used to be the extraction of an adequate tissue amount (Longo, 1998) to enable evaluation of histology, grade, invasiveness and to yield further molecular diagnostic information. Meanwhile, technical prerequisites allow extraction of sufficient tissue. Minimal invasive assessment is recommended for breast symptoms or lesions (LOE Ic/A+) and in the subsequent pre-therapeutic assessment of lesion extension (LOE Ic/A++), if clinical examination, mammography, and sonography (e.g. plus MRI) do not allow assessment of lesion extension (AGO, Gynecologic Oncology, and ARO, 2005c). Method of choice is the sonographically guided high speed large core needle biopsy [**14-gauge LCNB**] (Kreienberg et al., 2004; Schulz and Albert, 2003; Sittek et al., 2005b) which allows to dissect 15mg of tissue per biopsy (Nath et al., 1995; Sittek et al., 2002). Together with the histological work-up, it represents the most ap-

[11] *FNA* in the revised guidelines by the Tumorzentrum München (Sittek et al., 2005b) is recommended only in symptomtatic cystic tissue alteration. *LCNB* is referred to in German as ASB (automatisierte Stanzbiopsie) (Pfarl et al., 2002), and *ABB* in German is referred to as xAB (x-assistierte Stanzbiopsie) (Pfarl et al., 2002).

propriate technique for the detection of both invasive and noninvasive breast carcinomas (McIlhenny et al., 2002). Preoperative diagnostic work-up in mammographically suspicious lesions [BI-RADS 4][12] and in supposed DCIS/ADH and LCIS/ALH can be realized using **stereotactic core needle** and **vacuum biopsy** [LOE IIb/B++], extended by **wire guided excisional biopsy** [LOE IIb/B+] (AGO, Gynecologic Oncology, and ARO, 2005c).

For histological assessment in solid lesions, three to five biopsies and in microcalcifications five to ten biopsies are considered as optimum (Kreienberg et al., 2004; Schulz and Albert, 2003; Sittek et al., 2005b). Histologically, this allows to score the extracted sample from inadequate/unsatisfactory to malignant (EUSOMA, 2002; Perry and EUSOMA Working Party, 2001). If greater amounts of tumor tissue are required or the lesion in question shows microcalcifications or sonographically non-identifiable alterations, vacuum biopsy is the method of choice (Sittek et al., 2000; Sittek et al., 2002) enabling to dissect as much as 94mg of tissue (Nath et al., 1995). A comprehensive study on different biopsy systems regarding the breast parenchymal model is published by Sittek et al. (2002).

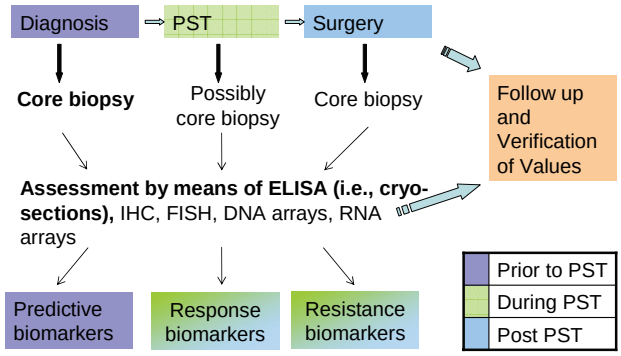


Figure 7 **Bioptical procedure prior, during, and post-PST**, modified from von Minckwitz (2002)

Using **14-G LCNB in the primary systemic setting (PST)** accuracy of diagnosis increases with the number of biopsies. Although stereotaxic **14-G LCNB** achieved a 99% diagnostic yield with five specimens, up to ten passes might be necessary (Liberman et al., 1994). Von Minckwitz (2002) on behalf of the AGO and McIlhenny (2002) defined highest accuracy to be achievable using LCNB before initiation of PST (figure 7) biopting a minimum of 3 biopsies from various locations within the primary tumor.

[12] *BI-RADS*: Breast Imaging Reporting and Data System, a quality assurance tool in breast evaluation (Pfarl, Helbich, and American College of Radiology, 2001).

2.9 Primary Systemic Therapy (PST)

PST is a valid option not only for advanced breast cancer stages, but also for all primary breast cancers (Kaufmann, von Minckwitz, and Rody, 2005). Synonymously it is known and described as neoadjuvant systemic chemotherapy (NST), preoperative systemic therapy or induction resp. downstaging therapy. However, primary systemic therapy (PST) is the term that has come into broad use (Kaufmann et al., 2006). PST takes into account the order of administration, the intended subsequent treatment, and the efficacy of the systemic intervention (Kaufmann et al., 2003). Referring to first post-diagnosis treatment (Kaufmann et al., 2003) prior to surgical intervention, PST aims at expanding local disease control and thereby improving the surgical options in order to determine the response to PST and to obtain complete histopathologic remission (pCR), thus improving long-term outcome (Bauerfeind et al., 2005; Kaufmann et al., 2006). Primary systemic therapy can be conducted using endocrine[13] or chemotherapeutic agents. It is generally administered in cycles with each period of treatment followed by a recovery period. The minimum quantity of cycles should number 4, with the total course of chemotherapy lasting at least 3 to 4 months (Kaufmann, von Minckwitz, and Rody, 2005). PST is not superior to possible other systemic therapy forms. It is as safe and effective as the same systemic postoperative treatment (Kaufmann et al., 2003). However, current data suggest PST having an advantage over adjuvant systemic therapy (AST) in some aspects (pCR) indicating good outcome. Prerequisites and state of the art recommendations for PST are extensively reviewed by Bauerfeind et al. (2005), Goldhirsch et al. (2005), Janni et al. (2005), and Kaufmann et al. (2005 and 2006).

Decade	Extended indications by decade for PST in breast cancer	Aims of PST	Alternative use
1970th	Non-operable breast cancer (locally advanced, i.e. ipsilateral supra/infraclavicular lymph nodes – N3) or inflammatory forms.	To enable operability for locally advanced tumors.	
1980th	Operable large tumor specimens (T greater than 5cm).	Improve breast conservation probability.	PST alternative to AST.
1990th	Operable small tumor specimens (T greater than 1-2cm).	Destroy/modify multicentric or multifocal tumor cells in breast carcinoma tissue (to diminish the ipsilateral recurrence rate).	
2000th and beyond	Mastectomy medically indicated patient however wants BCT.	Distinguish between chemoresistant and sensitive tumors.	PST alternative to mastectomy; prior to lumpectomy.
2000	Receptor-negative carcinoma.	Improved outcome for prognostically unfavorable receptor-negative subgroup (St. Gallen, Switzerland, 2005).	Core biopsy verified BC where AST is indicated by clinical and histological assessment of prognostic factors.

Table 6 **PST and development since the 1970s**, modified from Bauerfeind (2005) and Kaufmann (2003 and 2005)

Originally, **PST was introduced into clinical practice in the 1970s** based on the hypothesis of breast cancer being a systemic disease (table 6). In addition, supported by high tumor regression rates of up to 70%, malignant cells were believed to be more sensitive to PST than to AST. Accordingly, PST was favored as standard treatment for inoperable locally advanced or inflammatory breast cancer (Bonadonna, Hortobagyi, and Massimo-Gianni, 1997). Subsequently, in the **1980s**, application of PST increased the breast conserving surgery rate in patients with large operable tumors (Anderson et al., 1991; Bonadonna et al., 1990; Bonadonna et al., 1998; Calais et al., 1994; Chollet et al., 1997; Jacquillat et al., 1990; Smith et al., 1993; Smith et al., 1995). Similarly, in

[13] *Endocrine neoadjuvant systemic therapy* (prerequisite positive hormone receptor status): Tamoxifen or Aromatase inhibitors. *Endocrine adjuvant systemic therapy:* Premenopausal - GnRH-Analoga or similar ovar-suppressant; pre- and post-menopausal - Tamoxifen; post-menopausal – Tamoxifen and Aromatase inhibitors.

the **1990s**, eliminating disseminated micrometastases prior to development of multiple drug resistance came into focus. For the first time, PST was shown to allow correlation of primary tumor response to in vivo chemosensitivity (Kaufmann and Kubli, 1983). This facilitated **tailoring the most appropriate individualized therapy** (AGO, Gynecologic Oncology, and ARO, 2005b; AGO, Gynecologic Oncology, and ARO, 2005c). In vivo chemosensitivity testing is similar to resistance testing against anti-infectives; cells are incubated with different chemotherapeutical agents and the growth inhibition can be analyzed. However, PST not only offers an opportunity to obtain biological information correlated to tumor response. It also improves any local disease control by monitoring changes in the proliferation of breast cancer cells, which are associated with clinical response (Cleator, Parton, and Dowsett, 2002) and by observing the downstaging of the tumor mass (AGO, Gynecologic Oncology, and ARO, 2005b). This facilitates the decision-making for breast-conserving therapy [BCT] (Chen et al., 2005; Kaufmann, von Minckwitz, and Rody, 2005; Untch et al., 2002a; Veronesi et al., 1995) or operability in primarily inoperable tumors (AGO, Gynecologic Oncology, and ARO, 2005b; Fisher et al., 1998a; Gianni et al., 2002; Goldhirsch et al., 2005; Untch et al., 2002a). Assessing the response to PST after two to three cycles of chemotherapy, in several trials pCR has been linked to an independent prediction of improved DFS and OS (Aapro, 2001) while tumor progression predicts a poor prognosis (Anderson et al., 1991; Kuerer et al., 1999; van der Hage et al., 2001). Therefore, early response to PST is considered a predictor of pCR and may serve as a predictor for long-term outcome (Bauerfeind et al., 2005; von Minckwitz et al., 2005b). pCR is commonly considered a surrogate marker of complete eradication of distant micrometastatic residual disease, because of the favorable long-term outcome consistently seen in patients achieving pCR after PST (Bear et al., 2003).

Moreover, PST increases the proportion of patients, who are subsequently axillary N_0 (Kaufmann et al., 2003). With a low local recurrence rate in patients with complete clinical response and a high local recurrence rate in those failing, these results underline the hypothesis, that independently of the type of surgery poor response to PST predicts a poor prognosis and the high-risk of recurrence (Kaufmann et al., 2003). Furthermore, first data from prospective, randomized trials such as ECTO (European Cooperative Trial in Operable Breast Cancer), GeparDuo (Doxorubicin with Cyclophosphamid followed by Docetaxel), or AGO indicate a 3- to 4-fold higher rate of pCR in the subset of endocrine non-responsive (hormone receptor-negative) patients compared to endocrine responsive (hormone receptor-positive) patients. Thus, PST can change a formerly unfavorable prognostic marker into one that indicates a favorable prognosis, if pCR is achieved by PST (Bauerfeind et al., 2005; Goldhirsch et al., 2005; Kaufmann, von Minckwitz, and Rody, 2005).

	Level of Evidence and Grade		
Indications for PST	Oxford		AGO
	LOE	Grade	
Primary inoperable cancer	Ic	A	++
Inflammatory cancer	Ib	B	++
Operable cancer (minimum 2 cm)	I	B	+
Mastectomy medically indicated, patient however wishes BCT	I	B	++
Receptor-negative carcinoma	IIb	B	++
Indication for similar postoperative chemotherapy given	Ib	A	+*

Table 7 **Indication for PST with LOE and grade,** modified from AGO (2001)

*Study participation recommended (AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005b; Costa, 2001; Kaufmann et al., 2003; Untch et al., 2002a)

Based on current data of prospective clinical trials, recommendations for the use of preoperative systemic treatment depend upon potential endocrine responsiveness [table 8] (Kaufmann, von Minckwitz, and Rody, 2005). In summary, the "Gravenbruch Consensus 2001, AGO - State of the Art Meeting on Therapy of Primary Breast Cancer" gave preliminary prerequisites, updated in 2003 (AGO et al., 2003), 2005 (AGO, Gynecologic Oncology, and ARO, 2005b), and 2006 (Kaufmann et al., 2006) [tables 7 to 9]: An indication is given in primary inoperable cancer and inflammatory cancer. Optional indications are operable cancer (above or equal 2 cm) with the patient requesting downstaging to facilitate BCS or to enable a better cosmetic outcome (i.e., avoiding mastectomy), receptor-negative carcinoma, and a clear indication for a similar postoperative chemotherapy (AGO et al., 2001; AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005c; Costa, 2001; Janni et al., 2005; Kaufmann et al., 2003; Untch et al., 2002a; von Minckwitz et al., 2002). Regimes studied extensively in prospective randomized trials (table 9) are mostly anthracycline-containing and followed by taxanes (Bauerfeind et al., 2005; Kaufmann et al., 2003; Kaufmann et al., 2006; Kaufmann, von Minckwitz, and Rody, 2005). Combinations of chemotherapeutic drugs are more effective than a single drug, and increase the pCR rate. Administration strategies might consist of sequential, concurrent, and both sequential and concurrent delivery of agents as well as dose-dense approaches. Similarly to AST, the highest pCR rates have been described for anthracycline-containing regimes with mounting evidence that response rates are reflected by higher survival rates (Bauerfeind et al., 2005; Kaufmann et al., 2003). Suitable regimes comprise: Adriamycin/ Cyclophosphamid (AC) followed by Docetaxel; Docetaxel / Doxorubicin / Cyclophosphamid; Epirubicin / Paclitaxel / Cyclophosphamide / Methotrexate / Fluorouracil, and a dose-dense sequence of Epirubicin and Paclitaxel. A synopsis of clinical trials exploring the preoperative usage of taxanes in PST is published by Kaufmann et al. (2006). However, up to this point no strategy can be considered clearly superior in patients with operable breast cancer (Kaufmann et al., 2006). Therefore, chemotherapy for PST has not been standardized yet. As for endocrine preoperative therapy, in the subset of elderly post-menopausal endocrine-responsive patients, aromatase inhibitors yield a larger proportion of local response than Tamoxifen (Kaufmann, von Minckwitz, and Rody, 2005).

Tumor categorization	**PST form**
Endocrine unresponsive tumors	Chemotherapy
Endocrine responsive tumors	Chemotherapy and endocrine therapy
- Subgroup (elderly patients)	Endocrine therapy
Her-2 positive tumors (investigational)	Trastuzumab and chemotherapy +/- endocrine therapy

Table 8 **Recommendations for the use of preoperative (primary) systemic therapy,** modified from Kaufmann (2005)

	Level of Evidence and Grade		
PST - Therapy Protocols	Oxford		AGO
	LOE	Grade	
4 x AC (vs. 4x adjuvant AC)	Ib	A	+
FA (E) C (vs. adjuvant FA (E) C)	Ib	A	+
4 x AC → 4x D[14] (vs. ACx4)	Ib	A	+

Table 9 **Therapy protocols for PST with LOE and grade,** modified from AGO (2003 and 2005c)

The simultaneous application of chemotherapy and endocrine drugs should be viewed critically even in endocrine-responsive tumors (Kaufmann, von Minckwitz, and Rody, 2005).

If the regime applied fails or if the patient does not experience a response after 3 to 4 cycles, respectively, further response to alternative chemotherapy delivered as second line PST is less likely (Kaufmann et al., 2003). However, PST can reveal information about the tumor biology, which might be used both in tailoring a non-cross-

[14] GeparDuo (von Minckwitz et al., 2005b).

resistant second-line PST (Kaufmann et al., 2003) and an adjuvant treatment (Thomas et al., 2004) after definitive surgery. Yet, such an alternative approach comprises the necessity to monitor the patient closely in order to avoid inoperability due to uncontrolled tumor progression. Subsequent to PST, radiation therapy might be considered after having successfully achieved breast conserving therapy (Untch et al., 2002a). Surgery should be conducted as in primary breast cancer. The resection should be carried out within the non-infiltrated area (Costa, 2001). If the patient does not respond to PST, immediate surgery might be considered (Ikeda et al., 2002) with the possibility to adjust subsequent adjuvant chemotherapy (Thomas et al., 2004).

2.10 Diagnosis, Grading, Staging, and Response in Primary Systemic Therapy (PST)

Both **histological diagnosis** of cancer and accurate categorization of the corresponding tumor type are essential for developing a catalog of therapeutic measures and for choosing among different treatment options. Seeking to obtain sub-typing information in addition to histological diagnosis, biochemical and immunological tissue characterization has resulted in a high level of distinction between histologically similar tumors, which facilitates the selection of appropriate therapy guidelines (Slapak and Kufe, 1998).

Diagnosis of cancer used to be based on invasive tissue biopsy mainly, partly including less invasive forms such as FNA (Kuner et al., 2000). Currently, **patients considered for PST, are best diagnosed using core biopsy and histological examination**. Following mammography, these are considered to be the most suitable procedures for detecting either invasive and/or non-invasive breast carcinomas (Kaufmann et al., 2003). Beyond it, core needle biopsy helps to avoid over-treatment (Ikeda et al., 2002). At least three above or equal 14-gauge core needle biopsies from different locations within the primary tumor render for highest accuracy (McIlhenny et al., 2002) and help to categorize the tumor according to EUSOMA criteria (EUSOMA, 2002; Perry and EUSOMA Working Party, 2001). The extraction should provide sufficient amounts of tissue to carry out complex biological analyses at a later stage. Additionally, initial core biopsy will be the only source of tumor tissue for later tests in patients with pCR (Kaufmann et al., 2003).

The histological differentiation or **grading** correlates tightly with these four aspects: (1) Lymph node status, (2) receptor status, (3) relapse, and (4) mortality (Slapak and Kufe, 1998). **Staging** assists in determining prognostic information, selecting the most effective treatment plan as well as in measuring trends in cancer treatment and outcomes, enabling to minimize morbid complications (Slapak and Kufe, 1998). The extent of malignant disease is a key determinant in scheduling the most appropriate therapy regimen. It is developed by a combination of non-invasive and invasive diagnostic tests and procedures.

	Marker	Assessment	Intention
obligatory	Tumor grade	Core biopsy prior to PST	Histological tumor classification
	ER and PR receptors	Immunohistochemistry prior to PST	Possible effect on use of post-surgical endocrine therapy
Obligatory and/or optional	Number of involved axillary lymph nodes	Post-PST	Relevant prognostic level
		Prior to PST	Requested by the patient or important for radiotherapy decision making for mastectomy
Optional	Sentinel node biopsy - not yet finally decided upon		
	Additional factors/markers are optional, depending on subsequent use.		

Table 10 **Obligatory and Optional Marker assessment**, modified from Kaufmann (2003)

Two types are distinguished: (1) *Clinical staging* based on physical examination, radiographs, isotopic scans, computer tomography, and other imaging proce-

dures[15]. (2) *Pathologic staging* using information obtained during surgical procedure (Longo, 1998).

Due to the crucial role of the tumor stage, breast cancer *staging* is performed according to uniform criteria adapted by the UICC in terms of the TNM system originally developed by the American Joint Committee on Cancer (AJCC, 2002). Based on the above-mentioned clinical and histopathological assessment, the extent of disease is scored by considering the primary tumor size (T), the regional lymph nodes (N), and distant metastasis (M). The *stage* afterwards is categorized in different levels of T, N and M into 4 classes.

Prefixes are used to **stage patients** (Lebeau et al., 2005; Scharl, Costa, and Goehring, 2004). The **prefix "c" (cT, cN and cM)** stands for clinical evaluation in order to describe the tumor prior to therapy, while the **prefix "p"** describes postoperative histopathological findings **(pT, pN).** In addition to, the **prefix "y" (ypT, ypN)** is used to describe the tumor after or during PST [comprising chemo-, hormone- or radiation-therapy] (Feldman et al., 1986).

Based on the necessity to define an objective response to anticancer agents and to create a standardized approach of the recording of baseline data in cancer patients (Therasse et al., 2000), guidelines to assess **response in tumor treatment** were developed in the 1960s and 1970s. The WHO (1979) issued guidelines in the "WHO Handbook for Reporting Cancer Treatments". These were extended afterwards by Miller (1981), and further modified by the EORTC (2002), extending the WHO guidelines and taking into account criticism as outlined by Therasse et al. (2000). The re-revised WHO guidelines were then developed further into the so-called RECIST guidelines (Response Evaluation Criteria in Solid Tumors). The basic system with modification with respect to standardization and simplification was upheld (Therasse et al., 2000), using **four columns with respect to response, comprising CR** (complete response), **PR** (partial response), **NC** (no change or stable), **and PD** (progressive disease).

Neither the WHO nor the RECIST criteria were originally developed for PST. However, response after therapy has to be documented precisely. Therefore, as outlined in the NSABP-B18 trial, the basic system to assess response in PST is used (CR, PR, NC, PD) (Fisher et al., 1997). Feasible definitions, particularly adapted to report tumor response in PST, are outlined in table 11, the gold-standard in PST at present, however, is pCR (Kaufmann, von Minckwitz, and Rody, 2005).

Response in PST-treated patients:		
***C*linical definition**	cPR	Partial: reduction of tumor area lower or equal 50%
	cCR	Complete: no palpable mass detectable
***P*athologic definition**	-	Only focal invasive residuals in the removed breast tissue
	pCR inv	Only in situ tumor residuals in the removed breast tissue
	pCR	No invasive or in situ tumor cells in the removed breast tissue
	pCR breast + nodes	No malignant tumor cells in removed breast and lymph nodes
***I*maging definition**	iCR	No tumor visible by mammography and/or ultrasound and/or magnetic resonance imaging tomography

Table 11 Modified from Kaufmann (2003)

Response assessment after therapy aims to supply the surgeon with sufficient information to locate the tumor bed in case of complete tumor remission, and to estimate the initial tumor size in case of tumor shrinkage (Kaufmann et al., 2003). In accordance with a lately increasing rate of pCR in PST-patients, reliable response methods need further approval and development (Kaufmann et al., 2003; Kuroi et al., 2006). As for the role of tumor markers such as uPA and PAI-1, their utility with respect to prediction of response or resistance in PST

[15] Such as abdominal ultrasound of the liver, bone scan (PET), CAT scan.

needs to be investigated further.

Since **the thesis in hand** comprises data from patients treated with PST before any guidelines for the assessment of PST-treated patients were published, we **followed the suggestion to dichotomize two clinical subgroups comprising responding (CR/PR) and non-responding (NC/PD) patients** (table 12) as conducted by Pierga et al. (1997), Smith et al. (2002) and van Praagh et al. (2002).

Classification of *clinical response* and *progression* prior to recognition of PST guidelines

Responding to treatment	Failing to respond to treatment (**disease progression**)
1. complete (CR) and	3. stable (NC) and
2. partial (PR) response,	4. progressive (PD) disease

Table 12 Modified from Pierga (1997), Smith (2002), and van Praagh (2002)

3 Aim

Tumor specific factors such as uPA and PAI-1 may help to meet the clinical importance of early categorization of tumor characteristics and to develop individualized therapy concepts (Clark, 1996; Harbeck et al., 2002d; Harbeck et al., 2004b). In order to take into account the earlier and more efficient detection of breast tumors with an increase in incidence of smaller, and low-stage tumors (Cady, 1997; Harbeck and Thomssen, 2003; Hayes, 2005; Schmitt et al., 2002; Sweep et al., 2003; Thomssen and Janicke, 2000), one wanted to develop an applicable method to measure uPA and PAI-1 in small breast cancer tumor specimens (Schmitt et al., 2002).

1. **Methodically**, we developed a new micro determination procedure (micro ELISA) (Schmitt et al., 2002). We investigated the possibility to use cryostat sections as sample material representing the primary source of tumor material in order to determine uPA and PAI-1 biochemically by means of a standardized quality-approved and commercially available ELISA kit (ADI, American Diagnostica, Stamford, CT, USA).
2. In addition to the methodological aspects, we were interested to validate technical improvements **clinically**. To test the validity of uPA and PAI-1 in small amounts of tissue, uPA and PAI-1 levels were analyzed by ELISA in core biopsy (LCNB) specimens prior to PST (pre-PST) and in conventional biopsy specimens after PST (post-PST). In particular, we wanted to evaluate **(1)** the distribution of uPA and PAI-1 levels, **(2)** the change of uPA and PAI-1 levels under PST, **(3)** the correlation of uPA and PAI-1 between pre- and post-PST collectives, and **(4)** the correlation of uPA and PAI-1 in response to PST. Last but not least, we focused on **(5)** hormone receptor-status, menopausal status, HER-2/neu and Ki67 with respect to response, and whether **(6)** any of the applied chemotherapy regimens provided a practical benefit regarding the response to PST.

4 Material and Methods

4.1 Patient Collective

Retrospectively, we reviewed medical case records of female patients diagnosed with breast cancer between March 1990 and January 2001 and subsequently treated by PST at the Department of Obstetrics and Gynecology of the Technische Universität, München, Germany. **Forty-one** patients fulfilled the **inclusion criteria completion of primary systemic therapy (PST)** [figure 8] with **uPA and PAI-1 levels** obtained prior to PST via core-needle-biopsy and/or after PST in surgical specimens.

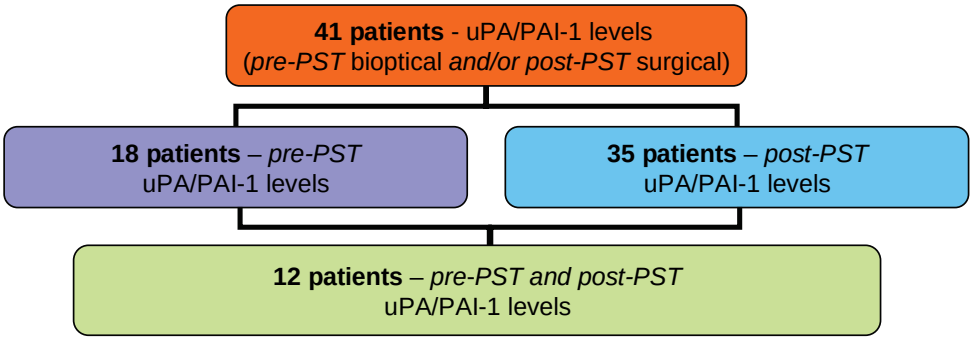


Figure 8 **Patient collective**

Data was primarily obtained from the Mamma CA (MaCA) Database in the Department of Obstetrics and Gynecology of the Technische Universität München, Germany, established by Prof Dr N. Harbeck and complemented with data from the Department of Pathology. The correctness of the data was reviewed by us again for this thesis in September 2006.

Subsequently, we defined three subgroups. Each collective and any corresponding reference or table are marked by a consistent color code throughout the thesis. The total collective **(n=41) [red]** split up into three subgroups as defined according to assessment of uPA and PAI-1:

1. uPA and PAI-1 levels **prior to PST** (pre-PST) measured in **core biopsy (n=18) [blue]**.
2. uPA and PAI-1 levels **after PST** (post-PST) measured in **surgical specimens (n=35) [light-blue]**.
3. uPA and PAI-1 levels **prior to PST** (pre-PST) measured in **core biopsy and after PST** (post-PST) measured in **surgical specimens (n=12) [green]**.

4.2 Tissue Sampling

Since our analysis was conducted prior to the revised guidelines of conducting three to five biopsies from various locations within the primary tumor using 14-G LCNB before initiation of PST (McIlhenny et al., 2002; Rutgers, 2001), only one biopsy was performed. Tumor assessment as conducted within our project is outlined in figure 9.

The **procedure of automated LCNB** comprises different systems with a variety of needle lengths and gauges**.** Basically, all systems use a 2-phase firing mechanism. Once discharged, an inner notched sheath presses forward. Following, a sharp outer cannula proceeds over the inner sheath, trapping a piece of tissue within the notch. If ultrasound guidance is used, the core-biopsy needle is advanced through the breast parenchyma to the edge of the lesion. Once the biopsy gun is discharged, a piece of the parenchyma is locked in the notch. In case of non-palpable lesion and necessary excisional biopsy, ultrasound might be used preoperatively to locate the

area in which the needle should be inserted. A hook wire can then be inserted percutaneously into the area of interest.

Most needle localizations are conducted by means of mammography with either a fenestrated compression or a stereotactic device. FNA and LCNB may both be executed either freehanded or aided by a biopsy guide. Both techniques must keep the needle midline and parallel with respect to the long axis of the ultrasound transducer. Using this approach, the entire corridor of the needle between the skin and the lesion is visible.

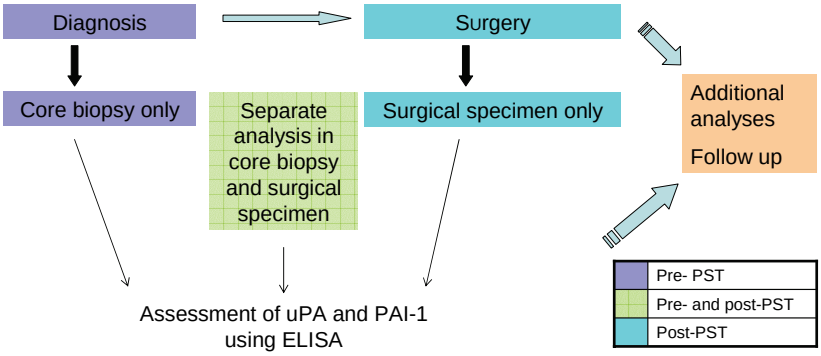


Figure 9 **Clinical tumor assessment of uPA and PAI-1 within our project**

While the transducer is immersed in glutaraldehyde solution, the patient is being prepared and draped under aseptic conditions. Subsequently, using ultrasound guidance, skin, underlying subcutaneous tissues and breast parenchyma are anesthetized within the limit of the lesion located. A small cut, easing the insertion of the LCNB, prepares the skin. The biopsy guide assists in directing the needle, which is penetrating from the breast parenchyma to the proper position, where the spring-loaded mechanism is discharged. Avoiding hematoma, the insertion area should become compressed manually for a short period of time after finish.

The specimen is forwarded for frozen section or put in formalin for histopathological examination. uPA and PAI-1 are analyzed as subsequently outlined using standardized laboratory determination procedure.

Procedure to obtain cryostat sections: To determine uPA and PAI-1 from cryosections of primary breast cancer, numerous 90µm thick cryostat sections are cut using a slicer and afterwards are extracted by the micro-method (figure 10). Sixteen adjacent sections are cut, pooled in sixteen different vials, extracted and then subjected to uPA, PAI-1, and protein analysis as illustrated by the respective standard curves (figure 11). Detailed tissue preparation after surgery and subsequent analysis using ELISA is outlined subsequently.

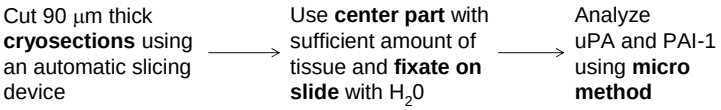


Figure 10 **Methodical assessment of uPA and PAI-1 using cryostat sections**

4.3 uPA and PAI-1 Determination

Using detergent-extracted (Triton X-100) breast cancer tissues and an ELISA technique (Janicke et al., 1993; Janicke, Schmitt, and Graeff, 1991), respectively, using so-called archived cytosol fractions (e.g., frozen tumor specimens) instead of detergent extracts (Foekens et al., 1992; Grondahl-Hansen et al., 1993; Janicke et al., 1994a; Spyratos et al., 1992; Sumiyoshi et al., 1992), uPA (Janicke et al., 1989) and PAI-1 (Duffy et al., 1990) were shown being of prognostic relevance in previous studies.

The assessment of uPA and PAI-1 - as used for this thesis - has been meticulously outlined by Prof Dr M. Schmitt as head of our clinical research group (Schmitt et al., 2006), supplementing our earlier publication (Schmitt et al., 2002). Our first publication on the technical considerations on how to assess uPA and PAI-1 using the micro-method, was based on internal laboratory protocols developed in this dissertation under his supervision (Schmitt et al., 2002). Subsequently, we give a brief summary on how to assess uPA and PAI-1 both using the micro- and the macro-method.

Prerequisites for a successful analysis include fresh-frozen breast cancer tissue [*micro-method*: fresh or thawed breast cancer tissue specimen(s) or five to ten 90µm thick cryosections cut from frozen breast cancer tissue specimen(s) or two to three core biopsies (fresh or thawed); *macro-method*: 100-300mg deep-frozen breast cancer tissue specimen], a disintegration device to pulverize the tissue in frozen state [*micro-method:* Potter-Elvehjem Tissue Homogenizer (Bellco Glass Incorporated; Vineland, USA); *macro-method*: Micro Dismembrator II (B. Braun AG, Melsungen, Germany, now represented by Sartorius AG, 37075 Göttingen, Germany)], detergent-(Triton X-100) containing Tris-buffered saline (Sigma-Aldrich Chemie GmbH, Munich, Germany) to extract uPA and PAI-1 from the pulverized breast cancer tissue, an ultracentrifuge to separate the detergent fraction from cellular debris, uPA and PAI-1 ELISA kits by ADI (American Diagnostica, Stamford, CT, USA; Kit #894 and #821), protein determination reagents, and a 96-well spectrophotometer (ELISA reader) to assess uPA, PAI-1, and total protein in the detergent extract. The BCA microtiter plate format is used as protein assay to determine the protein content (Kit #23225, Pierce Biotechnology, Rockford, IL, USA) with a Polystyrene 96-wells microtiter plate by Nunc (Wiesbaden, Germany). Bovine serum albumin A-7030 by Sigma-Aldrich (Munich, Germany) is used to prepare TBS-1 % BSA solution, which is used as a control sample/sample-dilution-buffer. Matching 1 ml cryovials are available from Nunc (Wiesbaden, Germany). The use of different ELISA kits from other companies is possible, however, only ADI kits have been quality controlled (Benraad et al., 1996). All materials used are commercially available.

Tables 13, 14, and 15 outline systematic and standardized approach to uPA and PAI-1 determination as approved by the EORTC and summarize information as conducted in our laboratory (Schmitt et al., 2002 and 2006). The general assessment comprises analysis in samples, control samples and standards. The entire procedure takes 3 days at the maximum. Several solutions - with a defined storage life - are necessary in order to assess the tumor tissue and extract the cytosol fraction, (tables 13 and 14). The temperature has to be kept between 0°C and 4°C.

	Preparation of solutions				
	TBS stock solution	**TBS working solution**	**10%Triton X-100 / TBS working solution**	**BCA working solution / BCA buffer**	**Sample-dilution-buffer**
SL[16]	4 weeks	1 week	4 weeks	1 week	1 week
Principle	2.42g (0.2mol/l) Tris in 75 ml aqua (5min at 4°C)	10ml TBS stock solution	1000mg Triton X-100	49.5ml TBS working solution	1000mg Serum Albumin bovine
		add	add	add	add
	Adjust ph to 8.5 with 37-40% HCl	90ml aqua	9ml TBS working solution	500µl 0.1% Triton X-100 and 0.05% Tween 20	100ml PBS-0.1% Triton X-100
	Add 7.305g (1.25 mol/l) NaCl, adjust with aqua to 100ml	Adjust ph to 8.5, store at 4°C	Adjust to 37°C		
	⇩	⇩	⇩	⇩	⇩
Yields	TBS stock solution	TBS working solution	10%Triton X-100 / TBS working solution	BCA working solution / BCA Buffer	1% BSA solution

Table 13 Modified from internal laboratory protocols of our Clinical Research Unit and Schmitt (2002 and 2006)

	Preparation of uPA and PAI-1 kit solutions						
	uPA standard solution	**PAI-1 standard solution**	**uPA resp. PAI-1 detection antibody solution**	**uPA resp. PAI-1 streptavidin-HRP conjugate solution**	**Wash-buffer**	**Enzyme reaction stop solution**	**Substrate solution**
SL[17]	Prepare per ELISA Kit					Use per ELISA Kit	
Principle	Add 1ml of H_2O dist. to each of the 1.0, 2.5, 5.0, 7.5, and 10.0ng uPA resp. PAI-1 standard vials provided with the kit. Agitate gently for 3min, do not shake.		Add 5.5ml of H_2O dist. to the biotinylated PAI-1 detection antibody - containing vial provided with the kit. Agitate gently for 3min, do not shake.	Add 20ml H_2O dist. to uPA and PAI-1 enzyme diluent vial provided with the kit. Take 12ml and mix with 12µl streptavidin-HRP conjugate provided with the kit.	1ml Triton X-100 add 1000ml PBS	0.5N H_2SO_4	Premixed perborate / 3,3'5,5'-tetramethyl-benzidine (TMB) solution is provided with the kit.
	⇩	⇩	⇩	⇩	⇩	⇩	⇩
Yields	uPA standard solution	PAI-1 standard solution	uPA and PAI-1 detection antibody solution	uPA and PAI-1 streptavidin-HRP conjugate solution	PBS-0,1% Triton X-100	Enzyme reaction stop solution	Substrate solution

Table 14 Modified from internal laboratory protocol of our Clinical Research Unit, American Diagnostica (ADI 2002a and b), and Schmitt (2002 and 2006)

[16] *SL*: Storage life at 4°C

	Step 1 / Day 1	Step 2 / Day 2	Step 3 / Day 3
Stepwise / Daily schedule for tissue preparation	Use the **micro-method** (cf. chapter 5.1) for fresh or thawed breast cancer tissue specimen(s) or five to ten 90µm thick cryosections cut from frozen breast cancer tissue specimen(s) or two to three core biopsies (fresh or thawed) Use the **macro-method** (cf. chapter 4.4) for deep-frozen breast cancer tissue specimen When the over night rotation/shaking (**macro-method**) respectively 2h rotation (**micro-method**) of the tube is finished proceed to **Step 2 / Day 2.**	Switch on ultra blender and set vacuum to 200. Turn off when vacuum at 200. Get rotor from freezer and place on cool-working-table. Remove tube from shaker and transfer content to plastic vial, fill rotor and close tightly. Place in centrifuge. Turn on vacuum, set to program 2 and press enter (1h/4°C/25000RPM). Finally, press start. Inscribe into the centrifuge book. Turn off vacuum; remove rotor and place on cool-working-table. (Leave centrifuge open for 1 h to cool out, to avoid condensation.) Recover clear supernatant and filtrate through gaze into Nunc-tube (=cytosol) [Nunc, Wiesbaden, Germany]. Discard lipid layer. Keep debris for another analysis. Aliquotate cytosol in red-lit tube and put in liquid nitrogen until use. Defrost one aliquot cytosol per patient and dilute for determination of **BCA**/**uPA**/**PAI-1**. Freeze all cytosol-aliquots in nitrogen tank. Pipette cytosol-dilution into ELISA-plates according to scheme. **uPA**/**PAI-1**: Add 100µl of standard, control, and sample to microtiter plate well. Cover plate with lid and incubate over night at 4 °C in a humid chamber. **BCA:** Pipette 50µl of standard curve resp. cytosol-dilution with 200µl color-solution (=20ml solution A with 400µl Solution B) ⇨ solution AB. Add 200µl reagent AB to each well of a 96-wells microtiter plate. Add 50µl of standard (0-400µg BSA/ml), control sample, or test sample to each well. If necessary, dilute samples with TBS, pH 8.5, plus 0.1% Triton X-100 and 0.05% Tween 20. Perform measurements in duplicate. Cover plate with lid and incubate over night at room temperature. Dilution for: **BCA**: 1:20= 20µl cytosol with 380µl BCA working solution / BCA buffer; 1:40= 150µl 1:20-dilution with 150µl BCA working solution / BCA buffer **uPA**/**PAI-1**: 1:20= 30µl cytosol with 570µl Sample-dilution-buffer; 1:40= 20µl cytosol with 780µl Sample-buffer **Proceed to Step 3 / Day 3**	**uPA**/**PAI-1**: Perform as instructed in the ELISA kit by ADI (American Diagnostica, Stamford, CT, USA). Perform measurements in duplicate. In the morning wash each plate 4 times with wash buffer and add 100µl antibodies (AB) in each well, cover plate with lid, incubate 1 h at room temperature. Wash each plate 4 times. Add 12µl of streptavidin-horseradish peroxidase conjugate to 12ml of enzyme conjugate diluent (provided with the kit). Add 100µl this conjugate-solution in each well. Cover plate with lid, incubate 1h at room temperature Wash each plate 4 times and add 100µl of wash solution in each well. Cover plate with lid and place it in the dark. Incubate 20min at room temperature. A blue color will develop. Stop the horseradish peroxidase reaction by adding 50µl of 0.5N H_2SO_4. The blue solution color will turn yellow. Read absorbances within 30min on a microtiter plate reader at a wavelength of 450nm. Deduct the background average of the blanks from the standards and sample readings. Construct standard curve by plotting the mean absorbance value calculated for each uPA/PAI-1 standard versus the corresponding uPA/PAI-1 concentration (figure 12). Calculate the uPA/PAI-1 concentrations in the test samples by use of this standard curve. Multiply result by dilution factor (i.e., if diluted 1:20, multiply by 20). **BCA:** After over-night incubation, a purple color will have developed. Measure absorbance at 540nm in a 96-wells microtiter plate reader. Deduct the background average of the blanks from the standards and sample readings. Construct standard curve by plotting the mean absorbance value calculated for each protein standard versus the corresponding protein concentration (figure 12). Calculate the protein concentrations in the test samples by use of this standard curve. Multiply result by dilution factor (e.g. if diluted 1:20, multiply by 20).

Table 15 Modified from Pierce Biotechnology (2002a) and Schmitt (2002 and 2006)

ELISA: ELISA kits by American Diagnostica (ADI, Stamford, CT, USA) [tables 16 and 17] are the most frequently used uPA and PAI-1 ELISAs according to literature (Schmitt et al., 2002). Moreover, as analyzed in the 1996 workshop of the EORTC-RBG (Benraad et al., 1996), these kits allowed reliable and reproducible data.

Applying the guidelines for measurement of uPA and PAI-1 as emphasized by the EORTC-RBG, the consequent use of an external standard replacing an "in-house" standard led to a significant reduction of the between laboratory coefficients of variation (CV): The consistent use of standard preparation #101094 lead to a decrease from 61% to 16% in case of uPA and from 42% to 19% in case of PAI-1 (Schmitt et al., 2002).

Independent of external or internal determination of uPA and PAI-1, basic measurement principles follow the identical steps (tables 13 to 17).

Standard curves for uPA and PAI-1 ELISA are outlined in figure 11, demonstrating the sensitivity range and performance of 96-wells microtiter plate ELISAs for uPA and PAI-1 (Schmitt et al., 2002). Information on the BCA microtiter plate, used to determine a standard curve of absorbance for measurement of uPA and PAI-1 content as ng analyte per mg of tumor tissue protein, is summarized in table 18 and can be obtained from Pierce Biotechnology (Rockford, IL, USA). Tables 16 and 17 summarize ELISA kits uPA #894 and PAI #821 by ADI (American Diagnostica, Stamford, CT, USA).

ADI uPA ELISA #894, enzyme-linked immunoassay for the quantitative determination of human urokinase-type plasminogen activator in breast cancer tissue extracts, plasma, and cell supernatants. Lower detection limit is 10 pg uPA/ml of sample. Single chain uPA and HMW-uPA forms of urokinase-type-plasminogen activator are all recognized by the assay, as is receptor bound uPA and uPA in complex with PAI-1 and PAI-2.	
Antibody-Enzyme Detection complex	
Capture antibody:	Murine monoclonal AB against uPA
Marking antibody:	Biotinylated AB recognizes bound uPA molecules
Streptavidin-conjugated horseradish peroxidase (HRP)	
Addition of tetramethylbenzidine (TMB), reaction with HRP	→ blue colored solution
Sensitivity increment by addition of sulfuric acid stop solution	→ yellow color
Quantification of uPA level by measuring absorbance of solution at 450nm	→ comparison with standard curve
Breast tissue extracts: Benign levels range from 0.02 to 1.22ng uPA/mg protein (median= 0.23)	Malignant primary tumor levels range from 0.13 to 15.17ng uPA/mg protein (median= 1.57)
Interpretation: uPA above 2.97ng/mg protein (detergent extracts) uPA above 1.15ng/mg protein (routinely prepared cytosol) high-risk of relapse and reduced life expectancy	

Table 16 Modified from American Diagnostica (ADI, 2002b)

ADI PAI-1 ELISA #821, enzyme-linked immunoassay for the determination of human PAI-1 in tissue extracts and cell culture supernatants. The essay detects latent (inactive) and active forms of PAI-1 and PAI-1 complexes and is insensitive to PAI-2.	
Antibody-Enzyme Detection complex	
Capture antibody:	Murine monoclonal anti-human PAI-1 AB
Marking antibody:	Biotinylated AB recognizes bound PAI-1 molecules
Streptavidin-conjugated horseradish peroxidase (HRP)	
Addition of tetramethylbenzidine (TMB), reaction with HRP	→ blue colored solution
Sensitivity increment by addition of a sulfuric acid stop solution	→ yellow color
Quantification of uPA level by measuring absorbance of solution at 450nm	→ comparison with standard curve
Breast tissue extracts: Benign levels range from 0.00 to 1.20ng PAI-1/mg protein (median= 0.00)	Malignant primary tumor levels range from 0.00 to 27.07ng uPA/mg protein (median= 1.02)
Interpretation: PAI-1 above 14ng/mg protein (detergent extracts) PAI-1 maintains level when measured from routinely prepared tumor cytosol (Foekens et al., 1994a; (Janicke et al., 1994a): high-risk of relapse and reduced life expectancy (Harbeck et al., 1999a)	

Table 17 Modified from American Diagnostica (ADI, 2002a)

The usage of the ADI ELISA kits combined with the microtiter applicable (Stoscheck, 1990) BCA protein assay (Smith) from Pierce Biotechnology (Rockford, IL, USA), has proven reliable in a prospective long term evaluation on the clinical relevance of uPA and PAI-1 for breast cancer prognosis in the Department of Obstetrics and Gynecology at the Technische Universität München, Germany (Schmitt et al., 2002) [figures 4 and 5].

Establishing standard curves

Standard curves give the amount of protein in the test tube. In protein assays, quantity measurement is performed using light absorbency as standard procedure. An unknown quantity can be estimated by preparing samples with known amounts of protein and comparing the differing absorbency rates. Table 18 outlines the basic steps to create a standard curve. Once all the tubes are prepared, the color reagent is added and the assay can be executed. Application by a pipette system allows rapid and accurate dispensing of the reagent. Since instability of either the color reagent or of the assaying conditions may occur, both establishment of the standard curve and the testing of the unknown concentration should be conducted at the same time. Absorbency can be read after approximately five minutes; the standard curve as illustrated in figure 11 can be prepared afterwards and the concentration of uPA or PAI, respectively, can be calculated.

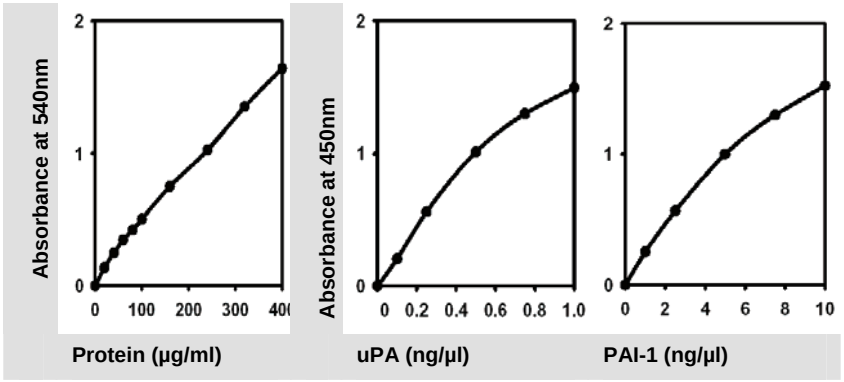


Figure 11 **Standard curves Protein, uPA and PAI-1,** modified from Schmitt et al. (2002)

	Prerequisite		
	• 2mg/ml IgG (Immunoglobulin G) and a quantity of buffer with unknown protein		
Preparation	**Reference**	**Preparation of 10 standard**	**Unknowns**
	• 100µl of buffer	• 10µl of 2mg/ml IgG plus 90 microliters of buffer; • 20µl of 2mg/ml IgG plus 80 microliters of buffer; • 30µl of 2mg/ml IgG plus 70 microliters of buffer; • … • up to 100µl of 2mg/ml IgG with no added buffer	• Unknowns containing known amounts of undiluted sample, each brought to a final volume of 100µl with buffer
Performance	• Add of 5ml color reagent to each tube of 100µl reference buffer, protein standard, or unknown • Mix content by vortexing tubes gently • Absorbance read ~ 3 to 5 minutes later by using a spectrophotometer		
	→ creation of standard curve		

Table 18 **"Bradford Method": Preparation of a standard curve for uPA and PAI-1 determination,** modified from internal laboratory protocol

4.4 Macro Method and Tissue Disintegration

Larger pieces of tumor tissue **(above 300mg, macro method)** should be processed as snap-frozen tumor tissue blocks and stored in liquid nitrogen until use (Janicke et al., 1994a) as outlined in (table 20), following recommendations of the EORTC-RBG and conducted in above mentioned quality approved laboratories.

Common macro-methods for disintegration of the primary tumor tissue and preparation of the tumor tissue extract are outlined in table 19 starting from initial fresh (or frozen) tumor tissue blocks (Schmitt et al., 2002). Tissue disintegration is performed by homogenizers (cell disrupters) such as centrifuges, vibrators, mortars, or other milling devices to fine-grind, disperse, and emulsify fresh tissue or biological samples to obtain an active tissue mush containing intracellular structural elements. For the macro method, we used a Micro-Dismembrator II (#853162/4), consisting of a ball mill with sample flask (pulverization device) made of Teflon® plus a grinding steel ball by B. Braun AG (Melsungen, Germany now represented by Sartorius AG, Göttingen, Germany).

Method	Procedure	T in °C	Tissue needed
Downs homogenizer	Squeezes fresh tissue	4	<100mg
UltraTurrax Blender (IKA)	Minces fresh tissue	4	>100mg
Pulverization	Pulverizes frozen tissue block, using:	-190	>100mg
-Dismembrator	Teflon® capsule steel balls		
-Mortar	Pistil		
-Weight	Heavy weight		
Sonication	Releases selected proteins	4	<100mg

Table 19 **Tissue disintegration methods**, modified from Schmitt (2002)

Addition of TBS (pH 8.5) to tissue powder with subsequent centrifugation brings about the so-called cytosol fraction. A large fraction of uPA and the largest part of PAI-1 is contained within this fraction. Addition of 1 % of the nonionic detergent Triton X-100 by Sigma-Aldrich (Munich, Germany) frees additional, membrane-bound uPA and uPA from intracellular stores, while additional PAI-1 is not released by this technique (Janicke et al., 1994a; Schmitt et al., 2002).

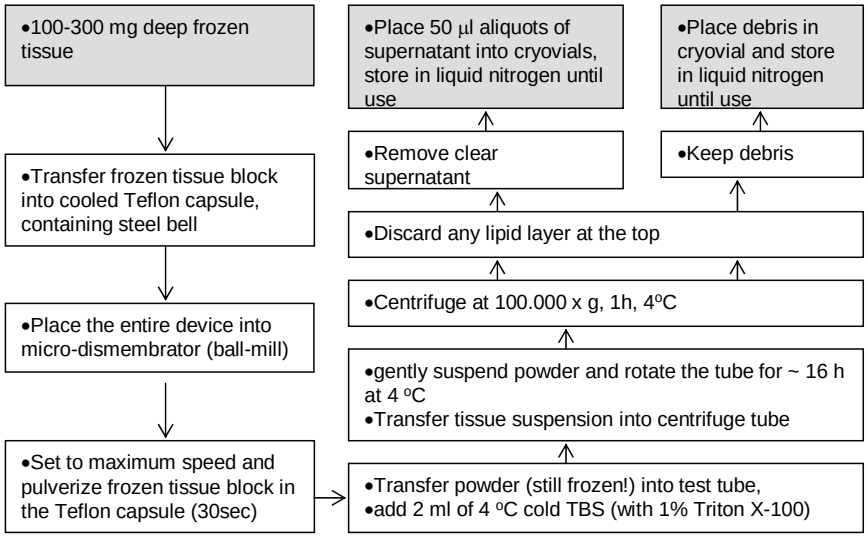


Table 20 **Macro method (above 300mg)**, modified from Schmitt (2002 and 2006)

4.5 Statistics

Several of the questions investigated here required comparison of distributions based on sampling from our groups. To test for statistically different means between two groups, for example to compare the means of uPA or PAI-1 within responding and non-responding patients, the **independent samples test (t-test)** was performed. If the underlying distributions are assumed to be normal, then under the null hypothesis the test statistic has a Student's t distribution[17] (Pospeschill, 2004).

Since departures from a normal distribution due to skewness of the measurements or outliers are possible, **robust statistical tests** were also carried out. These tests work by considering ranks instead of original measurements and require the data to have an ordinal structure[18] (Crichton, 2000a; Crichton, 2000b; Pospeschill, 2004). In this way, uPA and PAI-1 levels with respect to the applied chemotherapy-regime and with respect to dichotomized traditional prognostic factors were analyzed by the **Mann-Whitney U Test**, also known as the **Wilcoxon rank sum test**, a non-parametric test used to test for differences between the medians of two independent groups. To carry out the test, the groups are first combined, and the observations are then ranked (e.g., 1 for the smallest, 2 for the second smallest and so on). The test statistic is computed based on summing up the ranks for each group.

To test for an association between paired samples without making any assumptions about the frequency distribution of the variables, **Spearman's** Rank Correlation (**Rho**) **statistic** was used. It is a non-parametric measure of correlation, which may be used even if the data do not comply with a bivariate normal distribution, as required for the Pearson correlation. Here, Spearman's rho was used to estimate a rank-based measure of association between needle bioptical pre-chemo (pre-PST) and post-chemo (post-PST) conventional bioptical uPA or PAI-1 levels. Spearman's rank correlation works by transforming each variable according to rank and then performing a linear regression. The coefficient of determination (r^2) is calculated for both columns of ranks. The significance is then tested in the same way as the r^2 for a regression or correlation (Pospeschill, 2004).

Linear regression estimates the coefficients of the linear equation, involving one or more independent variables that best predict the value of the dependent variable (Lange and Bender, 2001; Urban and Mayerl, 2006). Here, we tested for an association between uPA and PAI-1 within the **(n=12)** pre- and post-PST setting using the uPA and PAI-1 ratios (table 31). The ratio of PAI-1 was defined as the dependent variable.

The ability of a test to discriminate non-responding from responding cases was evaluated using **Receiver Operating Characteristic (ROC) curve analysis [ROC AUC (area under the curve) statistic]**, solving the question of how accurate a classifier, e.g. PAI-1 is in identifying non-responding cases. In a ROC curve, the true positive rate (sensitivity) is plotted as a function of the false positive rate (100 - specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. The best possible prediction would yield a graph depicting a point in the upper left corner of the ROC space, i.e., 100% sensitivity (all true positives are found) and 100% specificity (no false positives are found). A completely random predictor would give a straight line at an angle of 45 degrees deviating from the horizontal origi-

[17] *Student's t-distribution:* Probability distribution that arises in the problem of estimating the mean of a normally distributed population when the sample size is small and when the population standard deviation is unknown and has to be estimated from the data.

[18] *Ordinal data*: Categories with a natural order (i.e., high, medium, low). Variables are either string-variables (alphanumeric) or numeric levels, representing different categories (i.e., 1= low, 2= medium, 3= high).

nating in the bottom left corner and ending in top right corner (the so-called 'line of no-discrimination')[19]. Results below this no-discrimination line would suggest a detector that gave wrong results consistently, and could therefore be used to make a detector that gave useful results by inverting its decisions (Zweig, 1993; Zweig and Campbell, 1993).

Kaplan-Meier survival analysis was used to analyze OS and DFS. It is most suitable to estimate model systems accurately presenting time to event in presence of censored cases. Censored cases - graphically depicted in the survival charts - are cases in which the event (i.e., death of patients or drop outs) is not monitored in the study (e.g., surviving patients). The Kaplan-Meier model estimates the conditional probability of an event occurring in the next time period assuming that no event has yet occurred (Ziegler, Lange, and Bender, 2002).

The **chi square (χ2) test** was used for comparison and correlation of additional factors with the response to therapy. It is a non-parametric test that compares observed frequencies in a contingency table with the expected frequencies (Pospeschill, 2004) and is appropriate if the test statistic has a chi-square distribution[20] under the null hypothesis.

P-values lower than 0.05 were considered significant in all tests. Statistical analysis was performed using SPSS 14.0.0 software (SPSS Inc., Chicago IL, USA). Technical assistance was provided by the Institute for Medical Statistics and Epidemiology (IMSE) of the Technische Universität, München, Germany (Head Prof A. Neiß) and by Dr R. Kates.

[19] As the threshold is raised, equal numbers of true and false positives would be let in.

[20] The chi-square distribution is a special case of the gamma distribution, a continuous probability distribution on the set of real numbers.

5 Results

5.1 Methodical Part – Micro ELISA

Early, more efficient detection of small breast tumors set a new task for clinicians: Tumors tend to be smaller at first time diagnosis and much of the increase in incidence of invasive breast cancer is associated with low-stage tumors. This emphasizes the necessity to develop or, ideally, to adapt a reliable and established method. Therefore, we developed and validated a miniaturized ELISA to meet the requirements for determination of uPA and PAI-1 in smaller pieces of breast cancer tissue. These small tumor specimens include fine needle aspirates, core biopsies, and cryostat sections.

The basic determination protocol has been published by Schmitt et al. (2002; 2006) and Thomssen (2003b). The principle of the micro ELISA and the quality assurance protocol are outlined in tables 21 and 42.

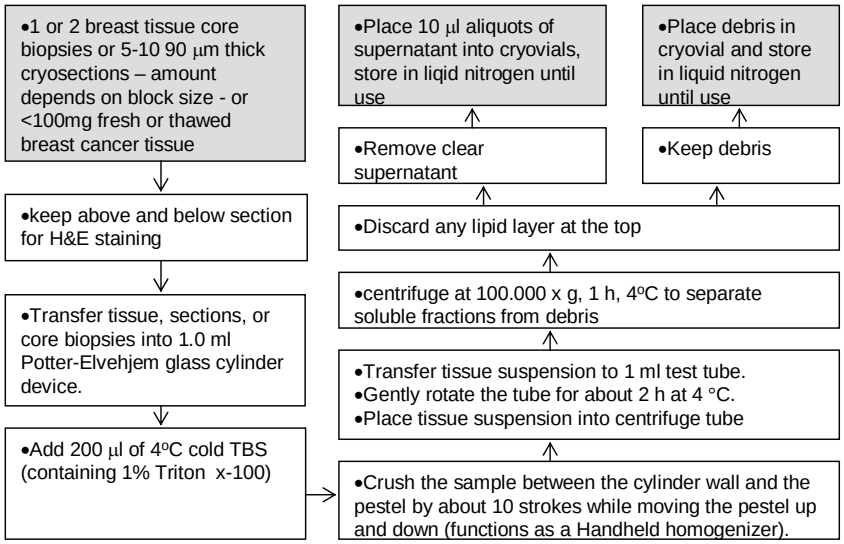


Table 21 **Micro method (90µm cryostat sections cut and extracted by micro method)**, modified from Schmitt (2002; 2006)

To determine uPA, PAI-1, and protein in primary breast cancer cryostat sections, consecutive 90µm thick cryostat sections of increasing numbers up to 16 were cut from one frozen breast cancer tissue block, pooled in 16 different vials, and subjected to uPA, PAI-1, and protein determination (figure 11) by the previously described micro method (tables 13 to 15) using a disintegration device to pulverize the tissue in frozen state [Potter-Elvehjem Tissue Homogenizer] (Bellco Glass Incorporated; Vineland, USA) and Triton X-100 (Sigma-Aldrich Chemie, Munich, Germany) to extract additional membrane and intercellular bound uPA (Duggan et al., 1995; Janicke et al., 1994a; Romain et al., 1995; Schmitt et al., 2002).

The standard curves (figure 11) illustrate the sensitivity range and performance of 96-wells microtiter plate ELISAs for uPA and PAI-1, measured by ELISA test kits for uPA and PAI-1 by ADI (American Diagnostica, Stamford, CT, USA). Following the protein determination by the Pierce method using the BCA test kit by Pierce Biotechnology (Rockford, IL, USA) [microtiter plate format], the protein content was verified by the Bradford method (figure 11 and table 18). Figures 12 to 16 describe the resulting content of protein, uPA, and PAI-1 as a function of a rising number of cryostat sections extracted (1 to 16 per vial).

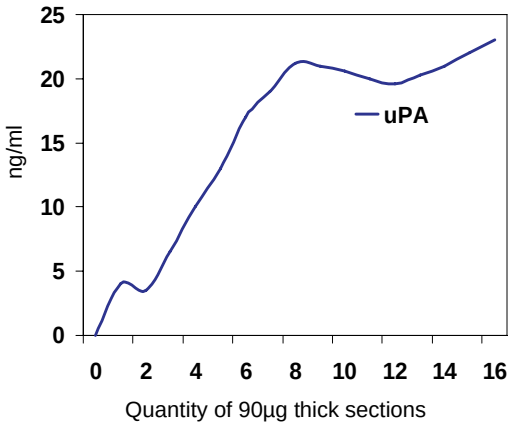


Figure 12 **Content of uPA per increasing number of cryostat sections (1 to 16)**, modified from Schmitt (2002)

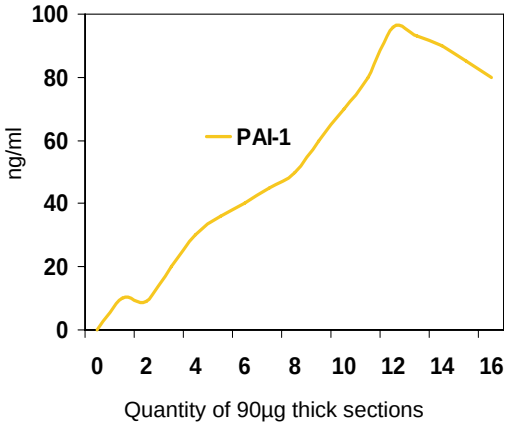


Figure 13 **Content of PAI-1 per increasing number of cryostat sections (1 to 16),** modified from Schmitt (2002)

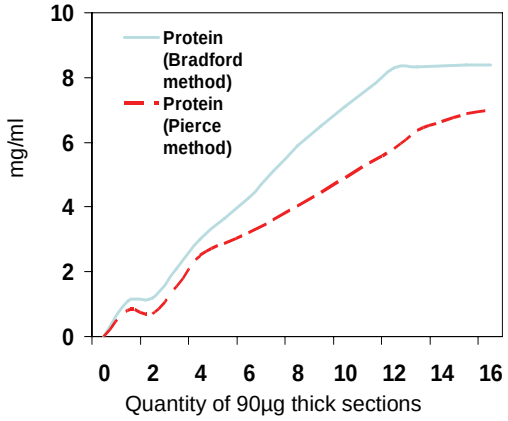


Figure 14 **Content of protein per increasing number of cryostat sections (1 to 16),** modified from Schmitt (2002)

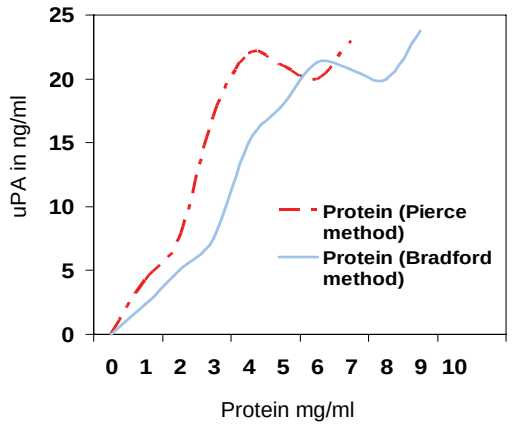


Figure 15 **Content of uPA in increasing number of cryostat sections/protein content determined**, modified from Schmitt (2002)

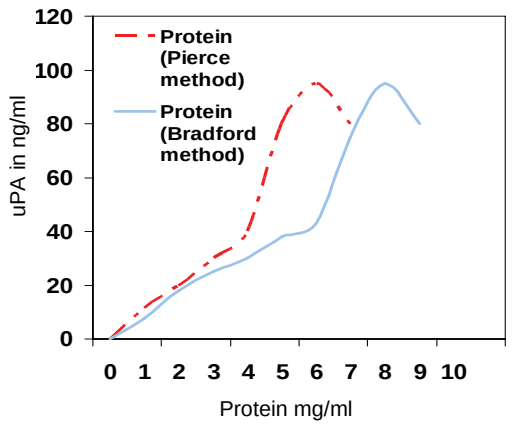


Figure 16 **Content of PAI-1 in increasing number of cryostat sections/protein content determined**, modified from Schmitt (2002)

Using an adapted microform of standard **ELISA**, our group evaluated a new **micro method's** practicability for clinical routine testing (Schmitt et al., 2002). Cryostat sections were used as an example for measurement on small amount of tumor tissue and to semi-precisely calculate the amount of tumor tissue needed. Afterwards, we tested for protein, uPA, and PAI-1 content.

Five to ten 90µm thick cryostat sections are sufficient to reach a plateau-point of protein content in the resulting tumor tissue extract (figures 14) as it is shown for uPA and PAI-1 content (figures 12 and 13).

Figures 15 and 16 give the content of uPA and PAI-1 antigen determined in an increasing number of cryostat sections as a function of the protein content determined. About 3ng uPA per mg protein and about 10 ng PAI-1 per mg of protein were determined, assessed from tumor specimen making use of above mentioned and validated uPA ELISA kit # 894 and PAI-1 ELISA kit #821 by ADI (American Diagnostica, Stamford, CT, USA). A guide how to uPA and PAI-1 were extracted from tumor tissue is given in table 21. A detailed description is available from Prof Schmitt (Schmitt et al., 2006).

Following the EORTC-RBG demands of reproducible standards (EORTC, 2002) and laboratory approval in regard to general determination of uPA and PAI-1, as stated above, we report that five to ten of 90µm thick cryosections of one frozen breast cancer tissue block, respectively one to two breast tissue core biopsies, are sufficient to consistently evaluate uPA and PAI-1 in the resulting tumor tissue extract.

Results have been published in the "Journal of the Clinical Ligand Society" together with Prof Dr M. Schmitt and Prof Dr N. Harbeck, Klinikum Rechts der Isar, Technische Universität, München, Germany [www.ingentaconnect.com/content/clas/jcla] (Schmitt et al., 2002), respectively in a complemented version again by Prof Dr M. Schmitt and Prof Dr N. Harbeck in "Methods in Molecular Medicine" (Schmitt et al., 2006).

5.2 Clinical Data

In completion to the methodological part, we were interested in transferring our findings into the clinical setting. Therefore, we **clinically** conducted a retrospective analysis on pre-PST needle-biopsies and post-PST surgical specimens.

Distribution of uPA and PAI-1 Levels

The total collective comprised **(n=41)** patients who were treated by PST and had uPA and PAI-1 levels assessed either using **pre-PST needle-biopsies** or **post-PST surgical specimens**. The collective was split into three subgroups (figure 17): A **(n=18)** pre-PST collective, a **(n=35)** post-PST collective and within the **(n=18)** and **(n=35)** collective a **(n=12)** pre- and post-PST collective. The mean age at diagnosis was 46.8 years. Unless otherwise mentioned, all levels are expressed in ng per mg tumor tissue extract protein.

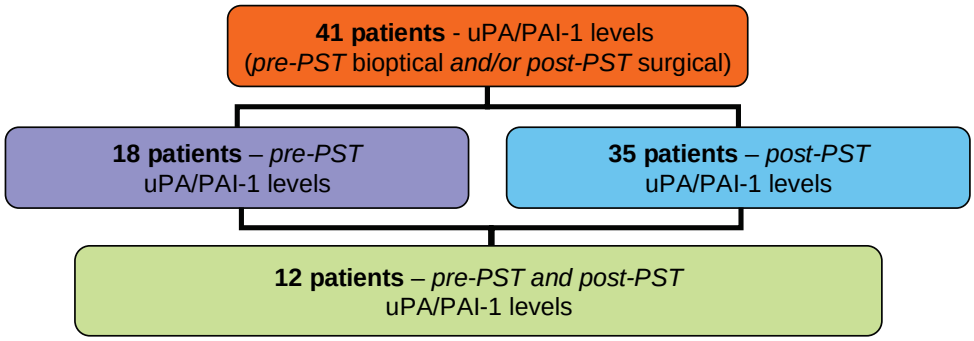


Figure 17 **Patient collective**

		N	Mean	Median	Spread	Minimum	Maximum
pre-PST	uPA	18	2.30	6.99	10.37	0.11	10.48
	PAI-1		9.01	0.94	52.69	1.11	53.80
post-PST	uPA	35	1.63	1.04	8.20	0.06	8.26
	PAI-1		10.11	5.56	51.89	0.85	52.74
pre- and post-PST	uPA pre-PST	12	2.20	0.94	10.37	0.11	10.48
	uPA post-PST		0.94	0.64	2.00	0.06	2.04
	uPA ratio		2.18	0.54	13.84	0.08	13.92
	PAI-1 pre-PST		10.63	7.76	52.58	1.22	53.80
	PAI-1 post-PST		9.03	4.32	51.82	0.92	52.74
	PAI-1 ratio		2.07	0.77	13.17	0.02	13.19

Table 22 **uPA and PAI-1 distribution (in ng/mg tumor tissue extract protein)**

In the **(n=41)** total **PST collective**, **pre-PST needle-biopsy** uPA levels ranged from 0.11 to 10.48 and PAI-1 levels from 1.11 to 53.80ng per mg tumor tissue extract protein. **Post-PST surgical specimen** uPA levels ranged from 0.06 to 8.26 and PAI-1 levels from 0.85 to 52.74ng per mg tumor tissue extract protein.

In the **(n=18)** **pre-PST collective** with uPA and PAI-1 levels determined in **core biopsies** uPA levels ranged from 0.11 to 10.48 (mean 2.30, median 6.99, spread 10.37) and PAI-1 levels from 1.11 to 53.80 (mean 9.01, median 0.94, spread 52.69) ng per mg tumor tissue extract protein. Pre-PST PAI-1 levels are more spread out with a negative skew, while pre-PST uPA levels are less spread out with a positive skew with uPA mostly lower than PAI-1. The mean (ng/mg) of pre-PST uPA (2.30) is lower than that of pre-PST PAI-1 (9.01) [tables 22, 23;

figures 18 to 20].

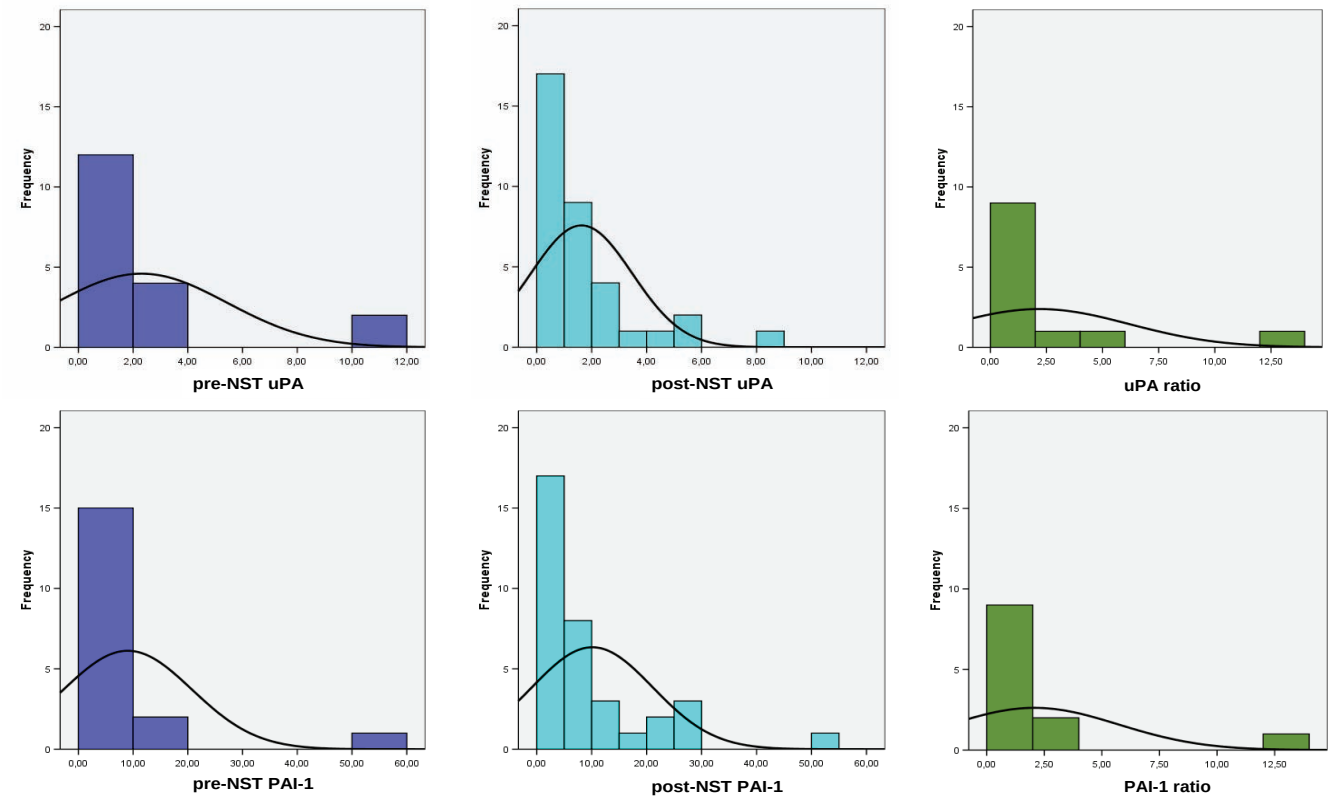
In the **(n=35)** **post-PST collective** with uPA and PAI-1 levels determined in **surgical specimens** uPA levels ranged from 0.06 to 8.26 (mean 1.63, median 1.04, spread 8.20) and PAI-1 levels from 0.85 to 52.74 (mean 10.11, median 5.56, spread 51.89) ng per mg tumor tissue extract protein. Post-PST PAI-1 levels are more spread out with a positive skew while post-PST uPA levels are less spread out with a minimal positive skew again with uPA mostly lower than PAI-1. The mean (ng/mg) of post-PST uPA (1.63) is lower than that of post-PST PAI-1 (10.11) [tables 22, 23; figures 18 to 20].

In the **(n=12)** **pre- and post-PST collective pre-PST** uPA levels ranged from 0.11 to 10.48 (mean 2.20, median 0.94, spread 10.37) and **pre-PST** PAI-1 levels from 1.22 to 53.80 (mean 10.63, median 7.76, spread 52.58) ng per mg tumor tissue extract protein. **Post-PST** uPA levels ranged from 0.06 to 2.04 (mean 0.94, median 0.64, spread 2.00) and **post-PST** PAI-1 levels from 0.92 to 52.74 (mean 9.03, median 4.32, spread 51.82) ng per mg tumor tissue extract protein. Post-PST levels for **uPA** are lower and less spread out with a more positive skew compared to pre-PST levels. **PAI-1** levels in the post-PST setting are also less spread with a positive skew compared to the pre-PST negative skew. The means (ng/mg) of both pre-PST PAI-1 (10.63) and post-PST PAI-1 (9.03) are higher than those of both pre-PST uPA (2.20) and post-PST uPA (0.94) [tables 22, 23; figures 21, 22].

The **(n=12)** **uPA and PAI-1 ratios** express post-PST levels divided by pre-PST levels (i.e., post-PST uPA/pre-PST uPA vs. post-PST PAI-1/pre-PST PAI-1 levels). They were used to additionally assess the change of uPA and PAI-1 levels during PST. uPA ratio levels ranged from 0.08 to 13.92 (mean 2.18, median 0.54, spread 13.84) and PAI-1 levels from 0.02 to 13.19 (mean 2.07, median 0.77, spread 13.17) ng per mg tumor tissue extract protein (tables 22, 23; figures 20).

pre-PST N=18			post-PST N=35						pre- and post-PST N=12						
uPA	PAI-1	*Pat ID*	uPA	PAI-1	*Pat ID*	uPA	PAI-1	*Pat ID*	uPA pre-PST	uPA post-PST	PAI-1 pre-PST	PAI-1 post-PST	uPA ratio	PAI-1 ratio	*Pat ID*
0.25	2.2	*1*	5.17	28.87	*19*	0.49	3.21	*9*	0.72	0.57	6.46	3.78	0.79	0.59	*6*
1.79	9.69	*2*	3.26	3.28	*20*	0.62	4.69	*10*	2.26	0.66	13.55	5.56	0.29	0.41	*7*
0.67	5.28	*3*	0.31	28.8	*21*	1.48	7.92	*12*	0.43	0.34	4.42	3.10	0.79	0.70	*8*
10.19	8.89	*4*	1.74	8.69	*22*	0.27	0.92	*14*	3.07	0.49	9.05	3.21	0.16	0.35	*9*
0.35	1.11	*5*	0.46	2.78	*23*	1.92	3.94	*15*	3.51	0.62	10.16	4.69	0.18	0.46	*10*
0.72	6.46	*6*	0.21	2.96	*24*	1.4	8	*16*	1.16	1.48	9.21	7.92	1.28	0.86	*12*
2.26	13.55	*7*	0.43	4.27	*25*	2.04	11.72	*17*	0.28	0.06	4	52.74	0.21	13.19	*13*
0.43	4.42	*8*	0.37	3.56	*26*	1.52	2.74	*18*	3.4	0.27	53.8	0.92	0.08	0.02	*14*
3.07	9.05	*9*	0.26	1.88	*27*	2.56	22.18	*34*	0.69	1.92	3.01	3.94	2.78	1.31	*15*
3.51	10.16	*10*	0.34	3.86	*28*	1.3	14.1	*35*	10.48	1.4	9.62	8	0.13	0.83	*16*
1.76	7.53	*11*	1.82	15.91	*29*	8.26	8.62	*36*	0.36	2.04	3.08	11.72	5.67	3.81	*17*
1.16	9.21	*12*	2.11	7.82	*30*	1.39	22.25	*37*	0.11	1.52	1.22	2.74	13.82	2.25	*18*
0.28	4	*13*	0.7	1.97	*31*	4.86	27.53	*38*							
3.4	53.8	*14*	5.82	9.19	*32*	0.2	9.76	*39*							
0.69	3.01	*15*	0.7	0.85	*33*	1.04	2.1	*40*							
10.48	9.62	*16*	0.57	3.78	*6*	2.37	14.38	*41*							
0.36	3.08	*17*	0.66	5.56	*7*	0.06	52.74	*13*							
0.11	1.22	*18*	0.34	3.10	*8*										

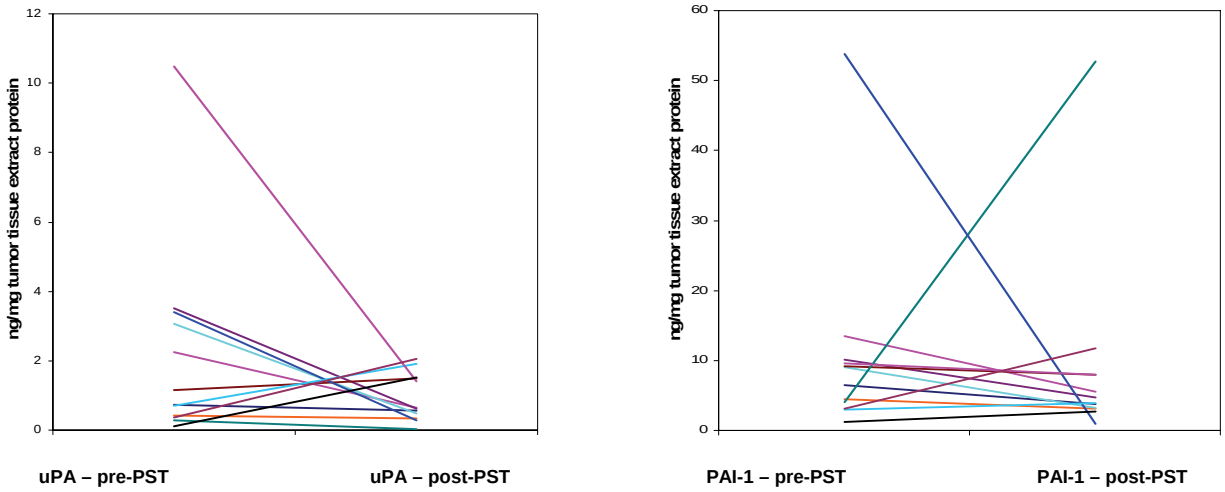
Table 23 **uPA and PAI-1 levels per patient in the corresponding collectives** (in ng/mg tumor tissue extract protein)**; patient number**: please note, that patients in the table overlap partly only (cf. figure 17). **uPA and PAI-1 ratios**: post-PST uPA divided by pre-PST uPA vs. post-PST PAI-1 divided by pre-PST PAI-1 levels



Figures 18, 19, and 20 **Distribution of uPA and PAI-1 levels in the (n=18), (n=35), and (n=12) collectives**

Correlation of uPA and PAI-1 in the pre-PST and post-PST Setting

Measured differences between uPA and PAI-1 were rather consistent throughout the **(n=18)**, **(n=35)** and **(n=12)** collectives with a **diminishment in spread, mean and median of uPA and PAI-1** levels between **pre- and post-PST levels** and a significant **correlation of pre-PST uPA and PAI-1 (n=18)**, resp. **post-PST uPA and PAI-1** levels **(n=35)**. To test for a correlation between the paired samples uPA and PAI-1 without making any assumptions about the frequency distribution of the variables, **Spearman's Rho** test was used. However, we were not able to find any significant correlation between the **(n=12) pre-PST and post-PST** uPA resp. PAI-1 levels (tables 24, 25; figures 21, 22).



Figures 21 and 22 **uPA (left) and PAI-1 (right) levels pre- and post-PST (n=12) [in ng/mg tumor tissue extract protein]**

Correlation of uPA and PAI-1 within the Pre-PST and Post-PST Setting

Spearman's Rho test was again used to test for a correlation between the paired samples uPA and PAI-1 without making any assumptions about the frequency distribution of the variables. uPA and PAI-1 significantly correlate within the **(n=18) pre-PST** setting with r=0.86 (p=0.00) and within the **(n=35) post-PST** setting with r=0.39 (p=0.03). In addition, the ratios of uPA and PAI-1 (post-PST levels divided by pre-PST levels) in the **(n=12) pre-PST and post-PST setting** correlated significantly with r=0.66 (p=0.02) [tables 24, 25].

Unexpected, correlation of uPA and PAI-1 in the pre-PST setting was stronger than in the post-PST setting. We therefore continued with separate analysis of responders and non-responders, referring to previously portrayed clinical dichotomized categorization [responding patients - CR (complete response) or PR (partial response) and non-responding patients - NC (no change) or PD (progressive disease)] (Pierga et al., 1997; Smith et al., 2002; van Praagh et al., 2002).

	Spearman's rho	uPA pre-PST	uPA post-PST	PAI-1 pre-PST	PAI-1 post-PST
uPA pre-PST	Correlation Coefficient	1.00	-0.18	0.86(**)	-0.04
	Sig. (2-tailed)		0.59	0.00	0.89
	N=	18	12	18	12
uPA post-PST	Correlation Coefficient	-0.18	1.00	-0.39	0.37(*)
	Sig. (2-tailed)	0.59		0.19	0.03
	N=	12	35	12	35
PAI-1 pre-PST	Correlation Coefficient	0.86(**)	-0.39	1.00	-0.05
	Sig. (2-tailed)	0.00	0.19		0.88
	N=	18	12	18	12
PAI-1 post-PST	Correlation Coefficient	-0.04	0.37(*)	-0.05	1.00
	Sig. (2-tailed)	0.89	0.03	0.88	
	N=	12	35	12	35

Table 24 **Correlation of uPA and PAI-1** [21] and (*) resp. (**)

	Spearman's rho	uPA ratio	PAI-1 ratio
uPA ratio	Correlation Coefficient	1.00	0.66(*)
	Sig. (2-tailed)		0.02
	N=	12	12
PAI-1 ratio	Correlation Coefficient	0.66(*)	1.00
	Sig. (2-tailed)	0.02	
	N=	12	12

Table 25 **Correlation of uPA and PAI-1 ratios** [22] and (*) resp. (**)

[21] uPA and PAI-1 ratios: post-PST uPA/pre-PST uPA vs. post-PST PAI-1/pre-PST PAI-1 levels. (*) Correlation is significant at the 0.05 level (2-tailed); (**) Correlation is significant at the 0.01 level (2-tailed).

uPA and PAI-1 According to Response

Discriminating the collectives separately according to response (table 26) in the **(n=18) pre-PST collective,** 15 patients had responded to therapy, 1 did not (progressive disease, PD). Consequently, no measurable difference between responders and non-responders could be established. 2 patients were not documented clinically before surgery and thus were excluded. uPA levels ranged from 0.11 to 10.48 (mean 2.05, median 1.16, spread 10.37) and PAI-1 levels from 1.11 to 53.80 (mean 9.81, median 7.53, spread 52.69) ng per mg tumor tissue extract protein.

Concerning the **(n=35) post-PST collective,** 22 patients responded to therapy, 9 did not (3 had progressive disease [PD], 6 had no change [NC]). 4 patients were not documented clinically. Patients not having responded to therapy (PD/NC, n=9) express higher, more spread out PAI-1 levels with a positive skew (mean 19.30, median 14.38, spread 50.64) compared to those responding (CR/PR, n=22) and expressing decreased less spread out PAI-1 levels (mean 5.03, median 3.82, spread 15.06). As for uPA, patients not having responded to therapy (PD/NC, n=9) express higher, more spread out uPA levels with a positive skew (mean 2.45, median 1.39, spread 8.20) compared to those responding (CR/PR, n=22) expressing decreased less spread out uPA levels (mean 1.12, median 0.64, spread 5.61).

In the **(n=12) pre-PST and post-PST collective,** 11 patients responded to therapy, 1 did not (progressive disease, PD). In **the pre-PST setting uPA within the responding tumors** ranged from 0.11 to 10.48 (mean 2.38, median 1.16, spread 10.37), **PAI-1** ranged from 1.22 to 53.80 (mean 11.42, median 9.21, spread 52.58). In the **post-PST setting, uPA within the responding tumors** ranged from 0.27 to 2.04 (mean 1.03, median 0.66, spread 1.77). **PAI-1** ranged from 0.92 to 11.72 (mean 5.05, median 3.94, spread 10.80). Considering the total process, spread, mean, and median diminish from pre- to post-PST regarding both uPA and PAI-1.

	pre-PST uPA and PAI-1 levels N=18		post-PST uPA and PAI-1 levels N=35		pre-PST uPA and PAI-1 levels N=12		post-PST uPA and PAI-1 levels N=12	
	responding	**not-responding**	**responding**	**not-responding**	**responding**	**not-responding**	**responding**	**not-responding**
	CR/PR	PD/NC	CR/PR	PD/NC	CR/PR	PD/NC	CR/PR	PD/NC
uPA	N=15	N=1	N=22	N=9	N=11	N=1	N=11	N=1
-Spread	10.37		5.61	8.20	10.37		1.77	
-Mean	2.05		1.12	2.45	2.38		1.03	
-Median	1.16		0.64	1.39	1.16		0.66	
-Minimum	0.11		0.21	0.06	0.11		0.27	
-Maximum	10.48		5.82	8.26	10.48		2.04	
PAI-1	N=15	N=1	N=22	N=9	N=11	N=1	N=11	N=1
-Spread	52.69		15.06	50.64	52.58		10.80	
-Mean	9.81		5.03	19.30	11.23		5.05	
-Median	7.53		3.82	14.38	9.21		3.94	
-Minimum	1.11		0.85	2.10	1.22		0.92	
-Maximum	53.80		15.91	52.74	53.80		11.72	

Table 26 **uPA and PAI-1 values (in ng/mg protein) versus response to PST in the (n=18), (n=35), and (n=12) collective**[22]

[22] Note, please, that some information was not completely documented in patient records. Therefore, numbers may defer from the collective size. The one non-responding patient in the (n=12) and (n=18) pre-PST collective had progressive disease (PD) with uPA-level of 0.28 and PAI-1 level of 4 ng/mg protein.

To test for statistically different means between uPA and PAI-1 within responding and non-responding patients, the **independent samples test (t-test)** was used provided that the underlying distributions can be assumed to be normal (table 27). PAI-1 in the **(n=35) post-PST** setting and the **(n=12)** PAI-1 **ratio** were the only factors to discriminate between responders and non-responders (p=0.00). uPA in the **(n=35) post-PST** setting in contrast was barely non-significant (p=0.06). Since data was not normally distributed (figures 18 to 20), we proceeded with the **Mann-Whitney test** (table 28) to analyze for differences between the medians.

t-test for Equality of Means	Equal variances		t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	assumed	not-assumed						Lower	Upper
uPA pre-PST N=18	X		0.66	14	0.52	1.77	2.69	-3.99	7.52
		X				1.77			
uPA post-PST N=35	X		-1.95	29	0.06	-1.33	0.68	-2.73	0.06
		X	-1.46	9.49	0.18	-1.33	0.91	-3.38	0.71
PAI-1 pre-PST N=18	X		0.44	14	0.66	5.81	13.11	-22.31	33.94
		X				5.81			
PAI-1 post-PST N=35	X		-4.29	29	0.00	-14.27	3.32	-21.06	-7.47
		X	-2.85	8.41	0.02	-14.27	4.99	-25.69	-2.83
uPA ratio N=12	X		0.49	10	0.63	2.15	4.34	-7.52	11.82
		X				2.15			
PAI-1 ratio N=12	X		-10.67	10	0.00	-12.14	1.14	-14.67	-9.60
		X				-12.14			

Table 27 Independent samples test (t-test) of uPA or PAI-1 levels vs. response to PST

	Dichotomized Response	N=	Mean Rank	Sum of Ranks	Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)	Exact Sig. [2*(1-tailed Sig.)]
uPA pre-PST N=18	responding	15	8.93	134.00					
	not-responding	1	2.00	2.00	1.00	2.00	-1.41	0.16	0.25(a)
	N=	16							
uPA post-PST N=35	responding	22	14.73	324.00					
	not-responding	9	19.11	172.00	71.00	324.00	-1.22	0.22	0.24(a)
	N=	31							
PAI-1 pre-PST N=18	responding	15	8.73	131.00					
	not-responding	1	5.00	5.00	4.00	5.00	-0.76	0.45	0.63(a)
	N=	16							
PAI-1 post-PST N=35	responding	22	12.64	278.00					
	not-responding	9	24.22	218.00	25.00	278.00	-3.22	0.00	0.00(a)
	N=	31							
uPA ratio N=12	responding	11	6.64	73.00					
	not-responding	1	5.00	5.00	4.00	5.00	-0.44	0.67	0.83(a)
	N=	12							
PAI-1 ratio N=12	responding	11	6.00	66.00					
	not-responding	1	12.00	12.00	0.00	66.00	-1.59	0.11	0.17(a)
	N=	12							

Table 28 Mann-Whitney test and Mean Rank (in ng/mg protein) of uPA or PAI-1 levels vs. response to PST [23] and (a)

[23] Note, please, that some information was not completely documented in patient records. Therefore, numbers may defer from the collective size. [(a)] Not corrected for ties.

PAI-1 in the **(n=35)** **post-PST** setting remained a strong factor to discriminate between responders and non-responders (p=0.00), whereas concerning the **PAI-1 ratio** **(n=12)** no significance could be detected. Interestingly, the mean ranks for **PAI-1** in the **(n=35)** **post-PST** setting were quite different (responding 12.64ng/mg vs. non-responding 24.22ng/mg, difference 11.58ng/mg), whereas for **uPA** with 4.38ng/mg they were in close range (responding 14.73ng/mg vs. non-responding 19.11ng/mg, difference 4.38ng/mg).

As one would expect, values in the **(n=35)** **post-PST** setting were quite scattered (figure 23) and using the Pearson test, no significant correlation could be established. In contrast, applying the **Spearman's Rho** test on the subset of responding patients to test for an association between uPA and PAI-1 again, correlation of uPA and PAI-1 in the (n=22) responding patients of the **(n=35)** **post-PST** setting increased to r=0.61 (p=0.003), while in the (n=15) responding patients of the **(n=18)** **pre-PST** setting the correlation remained rather stable at r=0.89 (p=0.00) [table 29].

The same effect was seen in the ratios of uPA and PAI-1 in the **(n=12)** **pre- and post-PST** setting. Their significant correlation increased to r=0.84 (p=0.001) [table 30].

	Spearman's rho	uPA pre-PST	uPA post-PST	PAI-1 pre-PST	PAI-1 post-PST
uPA pre-PST	Correlation Coefficient	1.00	-0.45	0.89(**)	0.16
	Sig. (2-tailed)		0.17	0.00	0.65
	N=	15	11	15	11
uPA post-PST	Correlation Coefficient	-0.45	1.00	-0.55	0.61(**)
	Sig. (2-tailed)	0.17		0.08	0.003
	N=	11	22	11	22
PAI-1 pre-PST	Correlation Coefficient	0.89(**)	-0.55	1.00	0.08
	Sig. (2-tailed)	0.00	0.08		0.81
	N=	15	11	15	11
PAI-1 post-PST	Correlation Coefficient	0.16	0.61(**)	0.08	1.00
	Sig. (2-tailed)	0.65	0.003	0.81	
	N=	11	22	11	22

Table 29 **Correlation of uPA and PAI-1 according to dichotomized response to PST** [24] and (*) resp. (**)

	Spearman's rho	uPA ratio	PAI-1 ratio
uPA ratio	Correlation Coefficient	1.00	0.84(**)
	Sig. (2-tailed)		0.001
	N=	11	11
PAI-1 ratio	Correlation Coefficient	0.84(**)	1.00
	Sig. (2-tailed)	0.001	
	N=	11	11

Table 30 **Correlation of uPA and PAI-1 ratios according to dichotomized response to PST** [25] and (*) resp. (**)

[24] uPA and PAI-1 ratios: post-PST uPA/pre-PST uPA vs. post-PST PAI-1/pre-PST PAI-1 levels. (*) Correlation is significant at the 0.05 level (2-tailed); (**) Correlation is significant at the 0.01 level (2-tailed).

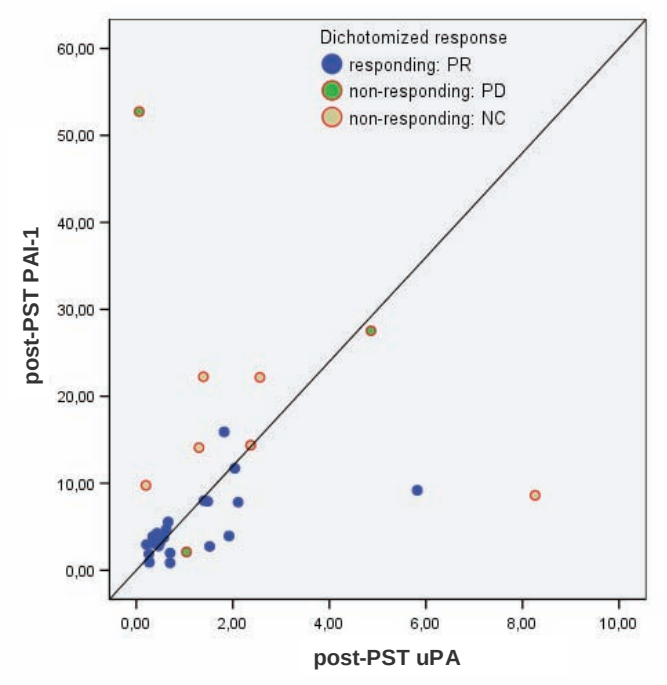


Figure 23 **Scatter plot on the distribution of uPA and PAI-1 in the (n=35) post-PST setting**

Furthermore, we applied a **linear regression analysis** to test for an association between uPA and PAI-1 within the **(n=12) pre- and post-PST** setting using the uPA and PAI-1 ratios (table 31). The unstandardized coefficient resembles the slope of the reference line in the scatter plot shown in figure 24. Including responders only, the unstandardized coefficient is 1/4 (0.25). We further conducted one linear regression analysis, excluding the unexpected high uPA (13.82ng/mg) resp. PAI-1 (13.19ng/mg) ratios. The latter resulted in an unstandardized coefficient of 2/3 (0.65). However, statistically there is no rationale excluding these high values.

	N=	Unandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
uPA ratio (without non-responding patient)	11	0.25	0.06	0.78	3.99	0.00	0.11	0.392
uPA ratio (without high uPA and PAI-1 ratios)	10	0.65	0.05	0.97	12.18	0.00	0.53	0.77

Table 31 **Association between uPA and PAI-1 within the (n=12) pre- and post-PST setting using the uPA and PAI-1 ratios**[25]

[25] uPA and PAI-1 ratios: post-PST uPA/pre-PST uPA vs. post-PST PAI-1/pre-PST PAI-1 levels. Dependent variable: PAI-1 ratio; linear regression through the origin.

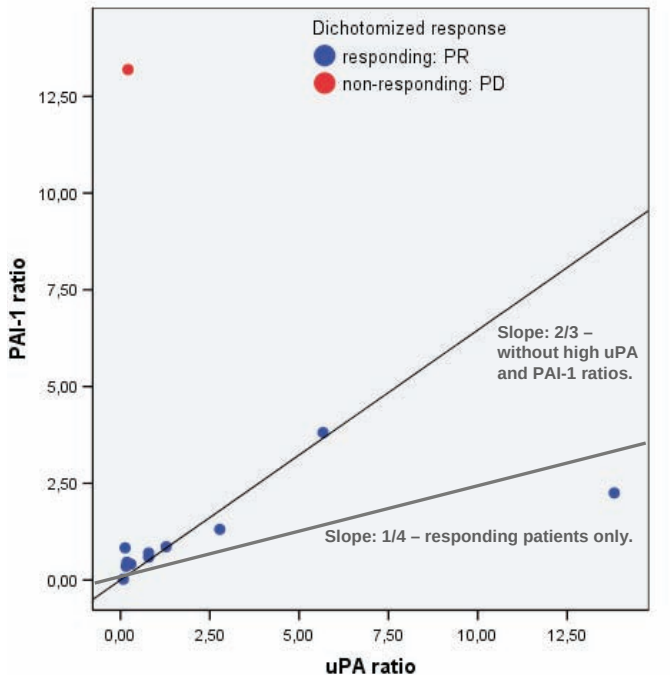


Figure 24 **Scatter plot on the distribution of uPA and PAI-1 in the (n=12) pre- and post-PST setting using the uPA and PAI-1 ratios**

In the **scatter plot** depicting the **(n=12) pre- and post-PST** setting (figure 24), primarily, the only non-responding patient with a very high **PAI-1 ratio** of 13.19ng/mg comes into focus, whereas the responding patients (except for one with an uPA ratio of 13.82ng/mg) appear to scatter slightly above (slope 1/4) resp. around the reference line (slope 2/3). Secondly, the slope of the reference line is < 1/1, which is surprising only at the first instant. Including our additional findings of **PAI-1** in the **(n=35) post-PST** setting as being a strong factor to discriminate between responders and non-responders, this substantiates the theory of a possible discriminative function of PAI-1 as discussed in chapter 6.2.

Contrary to uPA, **PAI-1** in the **(n=35) post-PST** setting discriminates between responding and non-responding patients (tables 27; 28). Moreover, patients having responded to PST appear to have lower PAI-1 levels (figures 23 and 24). We further used a **ROC** diagram to calculate the probability of non-response to PST with respect to PAI-1 levels (table 32; figure 25). ROC is suitable to evaluate the ability of a test to discriminate non-responding from responding cases. Ultimate sensitivity (89%) and specificity (82%) of PAI-1 with respect to response to PST were seen at PAI-1 8.31ng/mg, leaving the area under the curve at 0.87 (p=0.00). This is further discussed in chapter 6.2.

Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity	Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity	Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity
0.89	1.00	0.95	3.82	0.89	0.50	10.74	0.67	0.09
1.40	1.00	0.91	3.90	0.89	0.45	12.91	0.67	0.05
1.93	1.00	0.86	4.11	0.89	0.41	14.24	0.56	0.05
2.04	1.00	0.82	4.48	0.89	0.36	15.15	0.44	0.05
2.42	0.89	0.82	5.13	0.89	0.32	19.05	0.44	0.00
2.76	0.89	0.77	6.69	0.89	0.27	22.22	0.33	0.00
2.87	0.89	0.73	7.87	0.89	0.23	24.89	0.22	0.00
3.03	0.89	0.68	7.96	0.89	0.18	40.14	0.11	0.00
3.16	0.89	0.64	8.31	0.89	0.14	53.74	0.00	0.00
3.39	0.89	0.59	8.91	0.78	0.14			
3.67	0.89	0.55	9.48	0.78	0.09			

Table 32 Coordinates of the receiver-operating curve in the (n=35) post-PST setting[26]

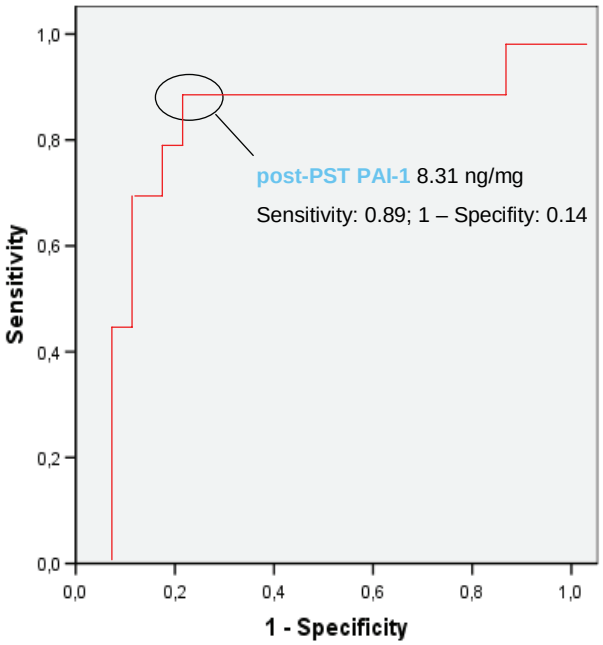


Figure 25 Probability of non-response to PST with respect to PAI-1 levels in the (n=35) post-PST setting using a receiver-operating curve, the area under the curve is 0.87 (p=0.00)

[26] The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values. Response was assessed in n=31 patients (cf. table 26).

Survival Analysis

We proceeded with a Kaplan-Meier survival analysis and focused on the cumulated overall survival of the patients categorized according to dichotomized response to PST in the **(n=41) total collective**. However, we found no statistically significant results (Log Rank p=0.53) [table 33, figure 26].

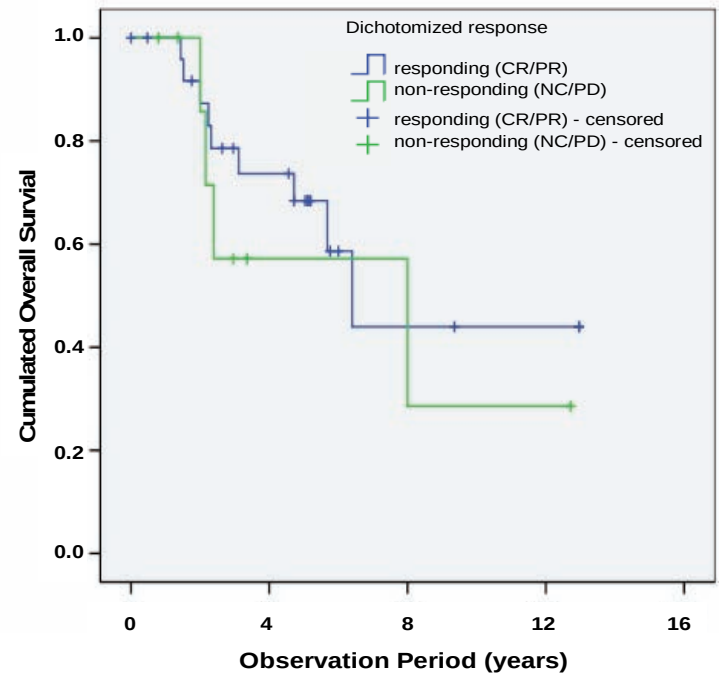


Figure 26 **Cumulated overall survival of all patients, (n=41) total collective (p=0.53)**

	Mean(a)				Median			
			95% Confidence Interval				95% Confidence Interval	
Dichotomized response	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
responding (CR/PR)	100.04	15.74	69.19	130.89	80.00	9.68	61.03	98.97
non-responding (NC/PD)	85.71	22.53	41.56	129.87	100.00	54.54	0.00	206.91
Overall	95.98	12.94	70.62	121.34	80.00	18.67	43.42	116.58

Table 33 **Means and medians for survival time (in months), (n=41) total collective** [27]

Response to PST with Respect to the PST Chemotherapy Regimen

The **chi square (χ2)** test was used to compare observed frequencies in a contingency table with the expected frequencies using the sample distribution. Dichotomized response to therapy was compared to PST chemotherapy, categorized according to anthracycline (tables 34 and 35). Within the **(n=41) total collective,** 30 patients received an anthracycline-containing regime. 5 patients received a different scheme. 6 patients did not have any clinical response documented and thus were excluded. No advantage could be portrait for any of the applied regimes. It is noteworthy, however, that patients with an anthracycline-containing regime have an 8-fold higher rate of responders than non-responders (24 responding vs. 3 non-responding).

[27] Estimation is limited to the largest survival time if it is censored.

		Chemotherapy regime					
Dichotomized response		Triple M	FEC	EC	EPI/Taxol	EPI	N=
responding	CR	0	0	1	0	0	1
	PR	2	0	13	9	1	25
non-responding	NC	2	0	2	2	0	6
	PD	1	1	1	0	0	3
N=		5	1	17	11	1	35

Table 34 **Chemotherapy regime according to dichotomized response, (n=41) total collective**

		Anthracycline		
Dichotomized response		+	-	N=
responding	CR	1	0	1
	PR	23	2	25
non-responding	NC	2	4	6
	PD	1	2	3
N=		30	5	35

Table 35 **Anthracycline containing regime vs. dichotomized response, (n=41) total collective**

Patient Characteristics and Additional Analysis

Menopausal status was assessed in the **(n=18) pre-PST** collective in n=12 (65%) patients (table 36). Of these n=3 (17%) were pre-menopausal, n=8 (43%) were post-menopausal, and n=1 (5%) was peri-menopausal. In the (n=35) post-PST collective n=34 (97%) had menopausal status assessed. Of these n=17 (49%) were pre-menopausal, n=15 (42%) were post-menopausal, and n=2 (6%) were peri-menopausal (table 36). Using the **chi-square (χ2)** test to compare the **menopausal status and the response to PST,** no significant correlation could be established.

The distribution of the hormone receptor status **within the sub collectives is outlined in table 36. Within the (n=18) pre-PST** collective n=15 (93%) patients were responding to therapy, of these n=10 (71%) had negative and n=3 (21%) displayed a positive **progesterone-receptor** status. N=1 (6%) patient did not respond to therapy. Of the n=15 (93%) responding patients, n=6 (38%) had negative and n=7 (44%) had a positive **estrogen-receptor** status. Of the n=16 patients who had **response documented**, n=15 (83%) were responding to therapy. Of these, n=5 (28%) showed PR and ER negative and n=10 (56%) had a PR and/or ER positive-receptor status. N=1 (6%) patient did not respond to therapy. Within the (n=35) **post-PST** collective, n=22 (63%) patients were responding to therapy, with n=10 (28%) negative and n=6 (17%) positive **progesterone-receptor** status. N=9 (25%) patient did not respond to therapy. Of the n=22 (63%) responding patients, n=8 (22%) had negative and n=9 (26%) had positive **estrogen-receptor** status. Of the n=31 patients who had **response assessed,** n=22 (63%) were responding to therapy. Of these, n=4 (11%) had PR and ER negative and n=18 (51%) showed a PR and/or ER positive-receptor status. N=9 (26%) patients did not respond to therapy.

Her2/neu was assessed in n=7 patients. Of the n=7 patients, n=6 were responding patients (n=4 either 2+ or 3+ and n=2 0 to 1+) and n=1 patient was non-responding (table 38). 0 to 1+ is normal and the result is HER2-negative. 2+ means that a moderate amount of the HER2 protein is present at the cell membrane. 3+ means over expression and the result is HER2-positive.

			N=18 pre-PST		N=35 post-PST	
Prognostic factor			**N**	**%**	**N**	**%**
Menopausal status	Pre		3	17	17	49
	Post		8	43	15	42
	Peri		1	5	2	6
	not documented		6	35	1	3
Steroid Hormone Receptor Status	PR and ER negative		5	28	8	23
	positive (one or both > 0) including not documented		13	72	27	77
	not documented		2	11	6	17
Grade cG = pre-PST ypG = post-PST	1/2		3	17	10	29
	3/4		12	66	23	66
	not documented		3	17	2	5
Lymph node status cN = pre-PST ypN = post-PST	Negativ		4	22	10	29
	Positiv		10	56	22	64
	not documented		4	22	3	9
Tumor size cN = pre-PST ypN = post-PST	T1 ≤ 2cm		-	-	11	31
	T2 > 2cm		15	84	22	63
			CR/PR	**NC/PD**	**CR/PR**	**NC/PD**
Steroid Hormone Receptor Status vs. Clinical Response	PR and ER negative		5	-	28	4	2	17
	PR and/or ER positive including not documented		10	1	62	18	7	72
Progesterone Receptor	Negative		10	1	61	10	4	40
	Positive		3	-	16	6	4	29
Estrogen Receptor	Negative		7	-	39	8	2	29
	Positive		6	1	39	9	6	43
Clinical Response vs. Therapy Scheme	Anthracycline	yes	15	1	89	20	6	74
		no	-	-	-	2	3	14

Table 36 **Patient characteristics for the (n=18) pre-PST and (n=35) post-PST collective** [28]

An **independent samples test** (t-test) was used to discriminate for an association of **Her2/neu, progesterone receptor, and estrogen receptor levels** with response to PST (table 37). No statistical correlation was obtained. And, since **Her2/neu** was only assessed in n=7 patients, we refrained from further evaluation (table 38).

[28] Please note that some information was not completely documented in patient records and therefore numbers may defer from the collective size.

t-test for Equality of Means	Equal variances assumed		t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
	yes	no						Lower	Upper
Her2/neu	X			3		-20.00	0.00	-20.00	-20.00
		X				-20.00			
Progesterone receptors (exact value)	X		3.30	0.08	-1.10	26	0.281	-1.98	1.80
		X			-0.94	9.89	0.368	-1.98	2.09
Estrogen receptors (exact value)	X		0.27	0.61	-0.71	27	0.487	-1.44	2.03
		X			-0.72	13.41	0.481	-1.44	1.98

Table 37 **Independent samples test** (t-test) of **Her2/neu, progesterone receptor, and estrogen receptor levels vs. response to PST**

	Response	
Her2/neu	**CR/PR**	**NC/PD**
2+/3+	4	-
0/1+	2	1

Table 38 **Dichotomized Her2/neu and distribution according to response (n=7)**

Proliferation marker Ki67 was assessed in n=25 patients (tables 39 and 40). Of these, n=1 had a proliferation rate of 0%, n=3 were not documented with respect to response. Of the n=22 patients documented with respect to response, n=16 had responded (n=4 above 30% and n=12 lower or equal 30% proliferation rate). N=6 did not respond with n=3 above 30% and n=3 lower or equal 30% proliferation rate. Using the **Mann-Whitney test** to detect, whether Ki67 discriminated between responders and non-responders, no significant results were found (p=0.33). Ki67 did not significantly decrease or increase during application of PST, with 64 per cent of patients lower or equal 30% proliferation rate in the n=11 responding **pre-PST (n=18)** patients and 76 per cent of patients lower or equal 30% proliferation rate in the n=13 responding **post-PST** (n=35) patients.

Ki67 (proliferation rate in %)	N=	Frequency (in %)
0	1	2.4
5	5	12.2
10	1	2.4
20	1	2.4
25	5	12.2
30	3	7.3
35	1	2.4
40	4	9.8
50	3	7.3
70	1	2.4
N=	25	61.0
Not documented	16	39.0

Table 40 **Ki67 distribution (frequency in %)**

	CR/PR	NC/PD	Ki67 (proliferation rate in %)
total collective N=41	4	3	**>30**
	12	3	**≤30**
pre-PST N=18	4	1	**>30**
	7	-	**≤30**
post-PST N=35	3	3	**>30**
	10	3	**≤30**
pre- and post-PST N=12	3	1	**>30**
	5	-	**≤30**

Table 39 **Dichotomized Ki67 (in % proliferation rate) according to response in the collectives**[29]

[29] Please note that some information was not completely documented in patient records and therefore numbers may defer from the collective size.

6 Discussion

6.1 Methodical Part – Micro ELISA

A central question concerning the discovery and identification of single protein biomarkers encompasses the validation and development of appropriate assays. Novel technical approaches call for expensive equipment, well-trained scientists and production of specific protein reagents. This is often complicated by a wide range of methods and the presentation of data in a variety of formats, resulting in drawbacks regarding significant and reproducible results. Thus, it may take time to determine whether presence or absence of a given protein is specific for a given disease, and whether the assay is specific for a given protein. Therefore, it is sensible to implement **an easily, widely available** and **validated method** allowing cost-cutting also. Hence, we postulated that it is feasible not only to use ELISA, but also to miniaturize it in order to measure uPA and PAI-1 in increasingly smaller amounts of breast cancer tissue.

Consequently, we developed and validated a new micro determination procedure (micro ELISA) using an adapted microform of standard ELISA [American Diagnostica, Stamford, CT, USA] (Schmitt et al., 2002). Cryostat sections were used as an example for measurement on small amount of tumor tissue and to calculate the amount of tumor tissue needed semi-precisely. Afterwards, we tested for protein, uPA, and PAI-1 content: Five to ten of 90µm thick cryosections of one frozen breast cancer tissue block, respectively one to two breast tissue core biopsies, are sufficient to consistently evaluate uPA and PAI-1 in the resulting tumor tissue extract using micro ELISA (Schmitt et al., 2002; Schmitt et al., 2006).

ELISA

To this point, no reports in scientific literature have been published contradicting the prognostic impact of uPA and PAI-1 levels measured by ELISA in breast cancer. This underlines the strong biological role of uPA and PAI-1 in tumor spread and metastasis (Harbeck et al., 2002d; Schmitt et al., 2002). Still, a limiting factor with respect to evaluation of biomarkers in tissues, blood, or bodily fluids are the different assay formats and approaches concerning sample collection, storage, processing, cut-off levels, or scoring systems. These diminish the quality with respect to comparability of marker levels (Schrohl et al., 2003). This was first recognized by Blankenstein (1995) who emphasized the necessity to define a standardized approach for assessment of tumor prognostic factors. Consequently, a workshop in Nijmegen (1996), Netherlands, under the patronage of the EORTC-RBG and the BIOMED-1 consortium on “Clinical Relevance of Proteases in Tumor Invasion and Metastasis” (Benraad et al., 1996) was held: Focusing on the immunological potential of various standards, detection limits, parallelism, and within as well as between assay variations of six different ELISA kits used in clinical studies, high correlations for any two of the four uPA ELISA kits finally applied were obtained. Additionally, commercially available uPA and PAI-1 ELISAs were compared with laboratory specific “in-house” ELISAs and the results proved to be comparable and satisfactory (Benraad et al., 1996; Sweep et al., 1998). The main outcome of the workshop was the recommendation for every multicentric study to use one type of ELISA in all laboratories as well as stable reference material (tumor tissue extract). Applying the guidelines for measurement of uPA and PAI-1 as emphasized by the EORTC-RBG, the consistent use of an external standard led to a significant reduction of the between laboratory CV (Schmitt et al., 2002). Guaranteeing comparability of uPA/PAI-1 levels and corresponding cut-off levels specified by using ADI ELISA kits (American Diagnostica, Stamford, CT, USA), the Institute for Ex-

perimental Endocrinology of the University Nijmegen together with the EORTC-RBG developed calculation tables in order to simplify the comparison of different results (Benraad et al., 1996; Thomssen, 2003a). An external uPA and PAI-1 control (reference) preparation can be obtained from Prof Dr F. Sweep, University of Nijmegen, Netherlands (f.sweep@ace.umcn.nl).

Additional techniques next to biochemical ELISA technique are immunohistochemistry (IHC) and immuncytochemistry (ICC), both parts of **immunochemistry** (Sweep et al., 2003)**,** as well as **activity assays** (Duffy et al., 1988), and quantitative real time **polymerase chain reaction** [PCR] (Bader et al., 2005)..

IHC refers to the process of localizing proteins in cells of a tissue section, while exploiting the principle of antigens binding to their respective antibodies in the tissue tested. Visualization is enabled by tagging the specific antibody with color producing tags. Typical examples include horseradish peroxidase or alkaline phosphatase. The ideal chemical produces the color required using different redox dyes. **ICC** refers to immunocytochemistry, or the staining of cell suspensions on slides, or cytospins. **Activity assays** are based on colorimetric detection also. Originally, activity assays were used to report on uPA with respect to clinical relevance (Duffy et al., 1988), today they are used rarely due to the exposition to methodological variations. Especially tissue collection and sample processing may cause discrepancies. Moreover, the enzyme activity is subject to enzyme inhibitors as well as to other proteases released during the extraction procedures. **PCR** exponentially amplifies a fragment or a sequence of interest of nucleic acid by enzymatic replication. For instance, in this respect, analysis of uPA and PAI-1 mRNA by quantitative real time PCR or array based nucleic acid detection platforms could be an alternative using less tissue (Bader et al., 2005).

Table 41 outlines advantages and disadvantages of both ELISA and IHC, irrespective to components of the plasminogen activation system. PCR is not discussed further.

With respect to components of **the plasminogen activation system** and in addition to characteristics outlined in table 41, IHC and ELISA both display certain advantages and disadvantages as summarized by Ferrier (1999), Janicke (1990), and Sweep (2003). Furthermore, the relation between ELISA and IHC was reported ambiguous: Though a higher IHC score category was constantly associated with an increased median ELISA value of uPA, an overlap of ELISA values from different IHC scoring classes was seen (Ferrier et al., 1999). However, irrespective of subsequently outlined disadvantages of IHC, the comparison of IHC and ELISA to measure components of the plasminogen activation system in various human tumor tissues showed comparable results (Christensen et al., 1996; Ferrier et al., 1999; Janicke et al., 1990; Kobayashi, Fujishiro, and Terao, 1994; Pappot et al., 1997; Sier et al., 1991).

The advantages of **ELISA to test for components of the plasminogen activation system** comprise viable results from multicentre studies, standardized measurements, and an established clinical value (Ferrier et al., 1999; Harbeck et al., 2002d; Schmitt et al., 2007; Sweep et al., 1998; Sweep et al., 2003). Additionally, amount of tissue extract needed per each measurement is very small [table 43] (Schmitt et al., 2006; Schmitt et al., 2007). **Disadvantages of ELISA** include, that the application of different kits by different laboratories results in differing cut-off levels, since differing antibodies manifest in a defined specifity and affinity for the various forms of uPA [e.g., pro-uPA, high molecular weight (HMW) –uPA, low molecular weight (LMW) –uPA], the aminoterminal fragment (ATF), and complexes with inhibitors (PAI-1 and PAI-2). Up to this point, clinical validation for N_0 patients has only been conducted for ADI ELISA kits (Look et al., 2002; Sweep et al., 2003).

Advantages of **IHC to test for components of the plasminogen activation system** comprise, that both frozen

sections and routinely processed paraffin-embedded tissue can be used (Ferrier et al., 1998; Ferrier et al., 1999). **Disadvantages of IHC** include, that the reliable scoring of uPA and PAI-1 is difficult, since the use of IHC is hampered by the expression of factors in tumor tissue and surrounding stroma (Harbeck et al., 2004b). Beyond it, as outlined in table 41, concentration of the end product is questionable (Boenisch et al., 2003), and, to date no standard operating procedure (SOP) on the use of IHC on uPA and PAI-1 has been published.

	ELISA
Advantages:	Parallel analysis on a large number of specimens (Ferrier et al., 1999). ELISA is a competitive method, thus more sensitive than IHC. Even with very low amounts of antigen over a large section, the stained signal will still be recognized using ELISA (Boenisch et al., 2003; Goldsby et al., 2003). Hence, ELISA allows to assess very small amounts of tissue (Boenisch et al., 2003; Santella, 2006). Discrimination of proteins is possible even with very high analyte concentrations (Ferrier et al., 1999). Results are more easily reproducible (Ferrier et al., 1999; von Minckwitz et al., 2002), since objective quantification of analyte levels by measuring a quantitative endpoint against a defined standard is used (Ferrier et al., 1999; Harbeck et al., 2002d; Schmitt et al., 2007; Sweep et al., 1998; Sweep et al., 2003). Concentration of the end product has to exceed the solubility constant in order to reveal antigens on a section immunoenzymatically. If the concentration is below, the product diffuses and will bind non-specifically to the entire section (e.g., on proteins). This is similar for both ELISA and IHC. However, in ELISA binding will take place on the tube wall and thus the micro-plate reader will still recognize the signal (Boenisch et al., 2003; Goldsby et al., 2003).
Disadvantages:	Possible cross-reaction of antibodies results in errors in quantitation and possible influences due to sensitivity and/or specificity of the antibody (Boenisch et al., 2003; Santella, 2006). Vulnerability of the antigen/antibody interaction to variations of sample matrix, pH, temperature, and tumor tissue extraction buffers (Ferrier et al., 1999; Sweep et al., 2003). Not possible on paraffin sections, restricted to cryostat sections (von Minckwitz et al., 2002) [table 42]. However, cryostat sections give much better antigen preservation than paraffin sections [allows retrospective studies using archival material] (Boenisch et al., 2003).
	IHC
Advantages:	Widely available determination technique (Boenisch et al., 2003; Santella, 2006). Insight into tissue heterogeneity and distribution of an antigen over the different cell types [the clinical relevance of expression of an antigen by a particular cell type can be studied] (Ferrier et al., 1999). Can be performed on both frozen sections and routinely processed paraffin-embedded tissue [allows retrospective studies using archival material] (Ferrier et al., 1999; Santella, 2006). When used with cryostat sections, immunochemist can select a differing optimal fixative for each antigen, all from the same block (Boenisch et al., 2003).
Disadvantages:	Possible cross-reaction of antibodies results in errors in quantitation and possible influences due to sensitivity and/or specificity of the antibody (Boenisch et al., 2003; Santella, 2006). Not as sensitive as the competitive ELISA, IHC precision depends on the tissue treatment (Boenisch et al., 2003; Santella, 2006). Not possible to use IHC above the antigen level causing maximum staining (Ferrier et al., 1999). Hampered by expression of factors in tumor tissue and surrounding stroma, thus making reliable scoring difficult (Harbeck et al., 2004b). At best semi-quantitative information (Ferrier et al., 1999). Commonly used on paraffin sections (Ferrier et al., 1999), however, cryostat sections give much better antigen preservation than paraffin sections (Boenisch et al., 2003). Subjective assessment, thus control experiments are needed to reveal both the specificity of cell staining as well as the reliability of results (Boenisch et al., 2003; Santella, 2006). Concentration of the end product has to exceed the solubility constant in order to reveal antigens on a section immunoenzymatically. If the concentration is below, the product diffuses and binds to the entire section non-specifically [e.g., on proteins] (Boenisch et al., 2003; Goldsby et al., 2003). In case of allocation of low amounts of antigen over a large section, the signal may microscopically not become visible in IHC (solubility constant), while using ELISA it may still be detectable (Boenisch et al., 2003; Goldsby et al., 2003).

Table 41 Characteristics of IHC and ELISA

Regarding the choice of preservation, cryostat sections allow much better antigen preservation than paraffin sections (Boenisch et al., 2003). And, availability of an ultra-deep frozen specimen collection may even facilitate future retrospective assessment of markers using ELISA (Blankenstein, 1995). Sledge (2001) further reported that the storage of stained paraffin sections in hospitals is less frequent nowadays and many hospitals tend to dispose of unused sections. In general, the usage of paraffin sections will most likely be restricted to clinically relevant markers and a basic prognostic factor panel.

Even though IHC and ELISA showed comparable results with respect to uPA and PAI-1 determination (Christensen et al., 1996; Ferrier et al., 1999; Janicke et al., 1990; Kobayashi, Fujishiro, and Terao, 1994; Pappot et al., 1997; Sier et al., 1991), the **results** up to this point **promote** the **use of ELISA**. ELISA has extensively been proven viable in multicentre studies and standardized measurements are of established clinical value, allowing objective quantification of analyte levels by measuring a quantitative endpoint against a defined standard (Ferrier et al., 1999; Funke et al., 2005; Harbeck et al., 2002d; Schmitt et al., 2007; Sweep et al., 1998; Sweep et al., 2003). The determination of uPA and PAI using ELISA is now part of a standard operating procedure (SOP) as subsequently outlined (Schmitt et al., 2006; Schmitt et al., 2007). Nevertheless, for a national or international assessment of uPA and PAI-1 and depending on the amount of tissue and the method of fixation available, IHC would be ideal, possibly complemented by specific and standardized antibodies and automated scoring systems. Further research might render that ultimately both IHC and ELISA may be used complementarily.

Tissue Disintegration

The field of tumor-associated biomarkers has expanded rapidly recently and assay results for the same marker can be quite heterogeneous (Schmitt et al., 2007). As demanded by the EORTC-RBG and the BIOMED-1 in Nijmegen in 1996, this outlines the necessity to establish simplified, but standardized, and reproducible guidelines on how to disintegrate the tumor tissue in its frozen state for assessment of tumor tissue-associated biomarkers. Schmitt et al. (2007) put effort in establishing an easy to follow SOP under the patronage of the Patho-Biology Group of the EORTC. Using disintegration techniques portrayed by Schmitt et al., it is possible to yield reproducible results and a still frozen tissue powder containing not only tumor biomarkers, but also RNA isolated from the pulverized tissue, and genomic DNA from the resulting tissue cell debris sediment. And, it is possible to obtain usable biomaterial after tissue disruption without subsequent (bio-) chemical treatment directly. This is especially important for the exact determination of uPA, since usage of acidic buffer favors the activity of cysteine–type proteases cathepsin B and L. Within the pH range of pH 3 and 5, the enzymatically inactive proform of the urokinase-type plasminogen activator (pro-uPA) is activated by the proteolytic action of cathepsins B and L resulting in higher values of uPA at low pH [cf. figure 2 in chapter 2.5] (Goretzki et al., 1992; Kobayashi et al., 1991). Again, all materials used are commercially available. It is even possible to upgrade commonly available “older” systems in use such as the Micro-Dismembrator II (B. Braun AG, Melsungen, Germany, now represented by Sartorius AG, 37075 Göttingen, Germany) to be operated with containers for the latest Micro-Dismembrator S (Sartorius AG, 37075 Göttingen, Germany) with a conversion kit (#BBI-8531986; Sartorius AG, 37075 Göttingen, Germany).

SOP - Standard Operating Procedure

As previously outlined, the utilization of either assay should be allowed only after having passed an approved and strict SOP (Ferrier et al., 1999). Meeting the demands by Goldhirsch (2005) and Hayes (2005) to facilitate detection using micro ELISA or IHC and to further validate the prognostic utility of micro ELISA results (Ferrier et al., 1999; Schmitt et al., 2002; Schmitt et al., 2006; Schrohl et al., 2003; Sweep et al., 2003), such a SOP was published recently by Schmitt et al. (2005; 2006) recently. Detailed information can be obtained from Prof Dr M. Schmitt, Klinikum Rechts der Isar, Germany (manfred.schmitt@lrz.tu-muenchen.de) and Prof Dr N. Harbeck, Klinikum Rechts der Isar, Germany (nadia.harbeck@lrz.tu-muenchen.de), or from the EORTC (http://www.eortc.be/), alternatively. A brief guideline on how to proceed with tumor tissue gained out of direct reach of a quality-assured laboratory is given in table 42.

Ideally, samples should at best be forwarded to a "Quality Assurance Protocol" participating laboratory (initiated by the Experimental Endocrinology University of Nijmegen, the Universitäts-Frauenklinik Hamburg, the EORTC Receptor and Biomarker Study Group and the BIOMED-2 program)	
1.	Place fresh tumor tissue on ice for cry diagnostics and transport to pathologist
2.	After pathological examination excise representative tumor tissue (approximately 200 to 500mg)
3.	Transfer tissue block into cryogenic tube(s) 5011 (Nalgene Europe Ltd, Neerijse, Belgium)
4.	Snap-freeze in liquid nitrogen (for quality assurance, additional histological slice should be assessed for independent verification).
5.	Transfer cryogenic tube into liquid nitrogen tank storing device for storage at deep temperature
6.	Transport to a participating laboratory [30] using, e.g. special liquid nitrogen transport vessels. A detailed guide how to transport frozen biological material is available from Glode and Gillum (2006)

Table 42 **Guideline to quality assured external uPA and PAI-1 assessment,** modified from Sweep (1998) and Schmitt (2005)

Micro ELISA

The **micro tissue extraction method** is **easy, convenient,** and **fast.** The necessary **technical requirements** are already **available in all laboratories** focusing on **standardized measurement of uPA** and **PAI-1.** The method utilizes routinely available cryostat sections but may be adapted for core biopsy specimens. Consequently, this method will smoothen the way for both swift and sensitive routine determination of uPA and PAI-1, while permitting even the assessment of increasingly smaller tumor samples as provided by different means of biopsy (Sweep et al., 2003).

At present, due to on the extensively proven clinical value (Benraad et al., 1996; Janicke et al., 1993; Janicke et al., 1994a), the unanimous interpretation (Ferrier et al., 1999), and the weaving in of the ELISA into current SOPs (Schmitt et al., 2007), ELISA is employed regularly to assess uPA and PAI-1. Further on, the inspiring results of the German randomized therapy trial in N_0 breast cancer patients (Janicke et al., 1994b; Janicke et al., 2001) and the questionable IHC staining for uPA and PAI-1 antigen in formalin-fixed breast cancer specimens (Janicke, Schmitt, and Graeff, 1995) promote the adoption of the newly developed micro ELISA technique.

[30] [cf. Table 42] Participating laboratories for quality assured external uPA and PAI-1 assessment: e.g., Prof Dr Henner-Graeff, Prof Dr Manfred Schmitt, Prof Dr Nadia Harbeck; Frauenklinik und Poliklinik der Technischen Universität München; Klinikum rechts der Isar; Ismaningerstr. 22; 81675 München, Germany; or: Prof Dr Fritz Janicke, Prof Dr Klaus Pantel, Prof Dr Christoph Thomssen, Frau Antje Andreas MTA; Onkologisches Forschungslabor; Frauenklinik und Poliklinik der Universität Hamburg; Universitäts-Krankenhaus Eppendorf; Martinistr. 52; 20246 Hamburg, Germany.

Summarizing clinically relevant results on uPA and PAI-1 relating to breast cancer prognosis and the response to therapy, the majority of results have been obtained by ELISA (Harbeck et al., 2002b; Janicke et al., 2001; Schmitt et al., 1997a) for both N_0 and N+ breast cancer patients (Bouchet et al., 1999; Duffy et al., 1988; Ferno et al., 1996; Foekens et al., 1994; Grondahl-Hansen et al., 1993; Harbeck et al., 1999b; Harbeck et al., 1999a; Janicke et al., 1994a). ELISAs are quality assured, reliable and robust enough for clinical routine use, and even allow application as a micro method (Harbeck et al., 2002d; Schmitt et al., 2002; Sweep et al., 1998; Sweep et al., 2003). Protein extracts can be prepared from 100µg of tumor-tissue (corresponding to about 1µg protein extract) only, or from five to ten 90µm thick cryostat sections, respectively [micro method] (Schmitt et al., 2002; Schmitt et al., 2006). This allows the application to a broad range of specimens such as core needle biopsies or cryostat sections (Harbeck et al., 2002d; Schmitt et al., 2002; Schmitt et al., 2006). Methodologically, ELISA surpasses any other method. No satisfying data has been published yet, making use of standardized IHC or other techniques regarding the measurement of uPA and PAI-1 in breast cancer tissue (Funke et al., 2005). This emphasizes that the tissue heterogeneity and the expression of uPA and PAI-1 in different cell types is best accounted for by ELISA. However, further studies to position ELISA and IHC within the context of clinical use are recommended; IHC and ELISA may be used complementary to a certain degree, depending on the amount of tissue and the method of fixation available (Ferrier et al., 1999), and whether a SOP will be developed.

Prerequisites for and performance of the micro method and advantages vs. disadvantages
Needs frozen tissue block
Small tissue sample (e.g., five to ten 90µm thick cryostat sections)
Yields about 200µl of tissue extract
Little amount of tissue extract per each measurement:
- Protein Bradford or Pierce Method (Pierce Biotechnology, 2002a): 1-5µl
- uPA ELISA [e.g. ADI #894 (American Diagnostica, Stamford, CT, USA)]: 1-5µl
- PAI-1 ELISA [e.g. ADI #821 (American Diagnostica, Stamford, CT, U-SA)]: 1-5µl
Equipment: Downs homogenizer, ultracentrifuge, ELISA plate, ELISA reader
Advantages: easy, convenient, fast
Disadvantage: not possible from paraffin sections

Table 43 Modified after Schmitt (2002; 2006)

6.2 Clinical Data

In order to transfer our findings on the micro ELISA from the methodological part into the clinical setting, we analyzed uPA and PAI-1 levels in small amounts of tumor tissue obtained by large-core needle biopsy (LCNB) specimens prior to primary systemic therapy (pre-PST, micro-method) as well as in conventional biopsy specimens after primary systemic therapy (post-PST, macro-method).

Corresponding to previous studies on uPA and PAI-1 both in core biopsies and surgical specimens, we postulated the following: Assuming that uPA and PAI-1 have a predictive value in breast cancer as proven in the adjuvant setting (Harbeck and Thomssen, 2003), we supposed that such a predictive value could be portrayed in the PST setting as well. Specifically, we expected uPA and PAI-1 to discriminate between responders and non-responders prior to and after completion of therapy. However, it was not our aim to prove any prognostic impact as this has already been proven extensively (Harbeck et al., 1998a; Harbeck et al., 1999a; Harbeck et al., 2000; Harbeck et al., 2001b; Harbeck et al., 2001a; Harbeck et al., 2002d; Harbeck et al., 2002b; Harbeck et al., 2002c; Harbeck et al., 2004a; Harbeck, Kates, and Schmitt M, 2002; Harbeck and Thomssen, 2003; Janicke et al., 2001; Look et al., 2002). Since response to PST has been reported the most important surrogate marker of PST success and predictor of long-term outcome (Kaufmann, von Minckwitz, and Rody, 2005; von Minckwitz et al.,

2005b), we were interested in a possible survival difference. However, due to the retrospective setting, the assessment of the actual response was conducted primarily clinically (van Praagh et al., 2002; WHO, 1979) and not standardized histopathologically as recommended at the present (von Minckwitz et al., 2005b). Accordingly, the response to PST was dichotomized following Pierga et al. (1997), Smith et al. (2002), and Van Cure (2002) by differentiating between (clinical) responders vs. non-responders. Corresponding to previous studies, we further expected uPA and PAI-1 to correlate with each other (Bouchet et al., 1994; Foekens et al., 2000; Fox et al., 2001; Grebenchtchikov et al., 2005; Grondahl-Hansen et al., 1993; Grondahl-Hansen et al., 1995; Janicke et al., 1994b; Janicke et al., 1993; Reilly et al., 1992). Moreover, we anticipated to find a correlation of uPA and PAI-1 between pre-PST core-bioptical and post-PST surgical specimens (Abraha et al., 2003; Pierga et al., 1997). More important, we analyzed for a possible improvement in response to PST, while applying anthracycline-based multiple-combination schemes (AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005c; Costa, 2001; Howell and Wardley, 2005; Kaufmann et al., 2003; Levine et al., 2005; Untch et al., 2002b; von Minckwitz et al., 2005b). Finally, in addition to the evidence-based predictive factors hormone receptor-status, menopausal status, and HER-2/neu-status in breast cancer (Funke et al., 2005), we analyzed cell proliferation (Ki67).

Circumstances Influencing Results in our Analyses

In addition to the results discussed below, the following circumstances are comprehensive for all analyses: **First**, tumor characteristics might have been different from previous studies and patients might have presented at larger tumor stages. Beyond it, Gershtein and Kushlinskii (2001) reported, that uPA and PAI-1 are distributed differently in different stage cancer specimens with an early stage increase paralleling the tumor expansion, while both parameters decrease with further tumor growth. **Second**, within the **(n=18)** pre-PST setting, uPA and PAI-1 were assessed using core needle biopsies with subsequently outlined possible consequences. Furthermore, previous core needle biopsies together with the administered chemotherapy might have had an implication on levels in the **(n=35)** post-PST setting (Kuroi et al., 2006). **Third**, levels in the **(n=35)** post-PST setting might depend on and reflect response to PST. Over and above, chemotherapy alters both neoplastic and non-neoplastic tissues (Kuroi et al., 2006). **Fourth**, as outlined by Noel (1997), tumor cells rely on more than the proteolytic system for efficient proteolysis, and there might be an unidentified independent factor influencing uPA and PAI-1 levels (Harbeck et al., 2000). **Fifth**, our total collective is rather small and comprised **(n=41)** patients only. What's more, the three subgroups of **(n=18)** pre-PST and **(n=35)** post-PST patients, respectively **(n=12)** pre- and post-PST patients were even smaller. **Sixth**, only very few studies have focused on distribution, change, and correlation of uPA and PAI-1 in pre-PST compared to post-PST settings (Kuner et al., 2000; Pierga et al., 1997).

Distribution of uPA and PAI-1 Levels

Unanticipated, uPA and PAI-1 levels within any of our collectives were not normally distributed (figures 18, 19 and 20). Reasons comprise previously outlined causes.

Comparing the **(n=18)** pre-PST median of PAI-1 to levels previously reported in a pre-PST setting (Pierga et al., 1997), there appears to be only a slight difference with a lower mean and spread in our collective (table 44). Reasons again comprise previously outlined ones.

Comparing the **(n=18)** pre-PST median of uPA and PAI-1 to levels from a surgical "pre"-AST setting (Schmitt et

al., 2002), a higher mean and spread are noticeable in the AST setting (table 44). One should have expected the opposite with higher levels of uPA and PAI-1 in the PST setting, because PST was mainly applied to patients with larger tumor masses at the time our patient population was selected. One additional reason might be that the assessment of uPA and PAI-1 in the AST setting was conducted from surgical specimens.

As expected and subsequently discussed, levels of uPA and PAI-1 in the already treated **(n=35)** post-PST setting are lower compared to levels in the not yet treated "pre"-AST setting (table 44) (Schmitt et al., 2002).

However, we refrained from statistical comparison of uPA and PAI-1 levels from our setting to others, since our setting was fairly small and a statistically valid analysis will have to take into consideration amongst other factors the TNM-classification of tumors. A broader study using a PST setting might yield more comparable results in order to resolve these questions.

		N	Mean	Median	Spread	Minimum	Maximum
pre-PST	**uPA**	**18**	2.30	6.99	10.37	0.11	10.48
	PAI-1		9.01	0.94	52.69	1.11	53.80
pre-PST (Pierga. 1997)	PAI-1	58	12	7.6	67.5	0.5	68
post-PST	**uPA**	**35**	1.63	1.04	8.20	0.06	8.26
	PAI-1		10.11	5.56	51.89	0.85	52.74
AST setting (Schmidt. 2002)	uPA	764	3.1	2.06	65.96	0.04	66.0
	PAI-1		14.7	9.1	247.12	0.06	247.2

Table 44 **uPA and PAI-1 levels in the (n=18) pre-PST and (n=35) post-PST setting, in an AST setting [before AST]** (Schmitt et al., 2002), **and PAI-1 in a PST setting [prior to PST]** (Pierga et al., 1997)

Correlation of uPA and PAI-1 in the Pre-PST and Post-PST Setting

Since uPA and PAI-1 levels were not normally distributed, we used **Spearman's Rho statistic** to test for an association of uPA and PAI-1 between the pre-PST and the post-PST setting (tables 24, 25). Although uPA and PAI-1 did not remain at constant levels and **diminished in spread, mean, and median** in the **(n=12) post-PST** setting compared to the **(n=12) pre-PST** setting, there is no statistically significant correlation between the **(n=12) pre-PST and post-PST** uPA or PAI-1 levels (table 24; figures 21, 22). Results are distinct to findings from Pierga et al. (1997). Additional explanations supplementing previous causes include: **First**, using core biopsy, there remains the risk of not having biopsied within the area of interest or just having touched the margins as subsequently outlined. **Second**, unlike histological tumor parameters, which were reported to remain mostly unchanged under therapy (Faneyte et al., 2003), uPA and PAI-1 are sensitive and alterable tumor-biological factors. Thus, biopsy or therapy might have altered uPA and PAI-1 levels. **Third,** only very few needle biopsies were obtained which Pierga et al. (1997) considered as a possible weakness in their own study as well.

Correlation of uPA and PAI-1 within the Pre-PST and Post-PST Setting

Since uPA and PAI-1 levels were not normally distributed, we again used **Spearman's Rho statistic** to test for an association of uPA and PAI-1 within the pre- and the post-PST setting (tables 24, 25). As expected, uPA and PAI-1 significantly correlated within the **(n=18)** pre-PST setting, the **(n=35)** post-PST setting, and the **(n=12)** pre-PST and post-PST setting. Such a correlation between uPA and PAI-1 is consistent with findings from several

authors (Bouchet et al., 1994; Foekens et al., 2000; Fox et al., 2001; Grebenchtchikov et al., 2005; Grondahl-Hansen et al., 1993; Grondahl-Hansen et al., 1995; Janicke et al., 1994b; Janicke et al., 1993; Reilly et al., 1992).

Complementally, we initially expected to find a higher correlation of uPA and PAI-1 within the **(n=35)** post-PST surgical specimens due to merely technical considerations: **First**, the greater amount of tumor tissue available might be more representative regarding the total tumor. **Second**, each tumor consists of both malignant and normal tissue, together with a tumor heterogeneity. Thus, the relatively "blind" core biopsy might yield protein outside the area of interest or might be "contaminated" with non-cancerous tissue (Kuner et al., 2000). **Third**, therapy and response to therapy might alter uPA and PAI-1 levels (Kuner et al., 2000).

Surprisingly, the correlation of uPA and PAI-1 in the **(n=18)** pre-PST setting (r=0.86; p=0.00) was stronger than in the **(n=35)** post-PST setting (r=0.39; p=0.03). Partly, findings might be explicable **first** by a substantial histological remission rate (pCR) after PST (Bear et al., 2003; San Antonio Breast Cancer Symposium, 2005). The known patterns of biomarkers such as uPA and PAI-1 may undergo changes during the cause of aggressive chemotherapy (Kuner et al., 2000) either due to the therapy itself or caused by yet unknown factors. **Second**, as subsequently outlined, any biopsy might eventually alter uPA and PAI-1 levels (Kramer, Schaefer, and Reinartz, 1995; Romer et al., 1991; Scully, 1991). Yet, such an alteration would be expected to occur in the neighborhood of the biopsy. **Third**, as previously discussed, tumor characteristics might have been different and patients had different tumor stages, which have been reported to influence uPA and PAI-1 levels (Gershtein and Kushlinskii, 2001).

Response Assessment

Coherent with the WHO proposal (Miller and Hoogstraten, 1981; WHO, 1979) for response assessment and previous studies in the primary systemic setting, the basic system to assess response in PST was used [CR, PR, NC, PD] (Fisher et al., 1997) and two clinical subgroups comprising responding [CR (complete response) or PR (partial response)] and non-responding patients [NC (no change) or PD (progressive disease)] were dichotomized (Pierga et al., 1997; Smith et al., 2002; van Praagh et al., 2002) [cf. chapter 2.10].

Viewed critically, although physical examination is the best noninvasive predictor of the real size of locally advanced primary breast cancer, the combination of physical examination with standardized methods such as mammography or sonography significantly improves the accuracy of noninvasive assessment of tumor dimensions (Billgren, 2002; Herrada et al., 1997). Beyond that, the assessment of the response as set by the WHO (Miller and Hoogstraten, 1981; WHO, 1979) was critically reflected by Therasse et al. (2000, 2005). And following Billgren (2002), compared to WHO criteria, the degree of response of breast cancer to primary chemotherapy, depicted by mammography and ultrasound, is less marked than the degree of response seen at clinical examination, thus suggesting response rates being too high, if the tumor is assessed clinically only compared to mammographically using UICC criteria. Further studies should use the gold-standard in PST, the pathologic definition pCR (Kaufmann, von Minckwitz, and Rody, 2005). However, reliable response methods need further approval and development with respect to PST (Kaufmann et al., 2003), and assessment of pCR needs to be standardized (Kuroi et al., 2006).

uPA and PAI-1 According to Response

To analyze uPA and PAI-1 with respect to dichotomized response [responding patients - CR (complete response) or PR (partial response) and non-responding patients - NC (no change) or PD (progressive disease)], we used an **independent samples test** [t-test] (table 27) and the **Mann-Whitney test** (table 28). PAI-1 in the **(n=35)** post-PST setting was the only factor discriminating between responders and non-responders (p=0.00). In contrast, uPA was barely non-significant (p=0.06). Additionally, the mean ranks for PAI-1 in the post-PST setting (table 28) differed substantially (responding 12.64ng/mg vs. non-responding 24.22ng/mg, difference 11.58ng/mg), whereas for uPA, values were closer together (responding 14.73ng/mg vs. non-responding 19.11ng/mg, difference 4.38ng/mg). Applying the **Spearman's Rho** test in the subset of responding patients, the correlation of uPA and PAI-1 in the (n=22) post-PST responding patients **(n=35)** increased to r=0.61 (p=0.003), and in the ratios of uPA and PAI-1 in the **(n=12)** pre-PST and post-PST setting to r=0.84 (p=0.001). In contrast, in the (n=15) responding pre-PST patients **(n=18)**, the correlation remained rather stable at r=0.89 (p=0.00) [tables 29, 30]. The **linear regression analysis** (figure 24) to test for an association between uPA and PAI-1 within the **(n=12)** pre- and post-PST setting, using the uPA and PAI-1 ratios, has a slope of < 1/1. This can be interpreted as responding patients after therapy to have lower levels of PAI-1 than non-responding patients. A slope of 1/1 would be found, if the ratios of uPA and PAI-1 remained stable, i.e., if there was no change between pre- and post-PST levels (e.g., caused by a short interval between biopsy and surgery), or if uPA and PAI-1 levels were independent of PST, therapy, or response, or if there was a constant substantial contamination, e.g. with "normal" tissue. A slope of > 1/1 could be expected, if PAI-1 was constantly higher than uPA, e.g., if there was an increased angiogenesis resp. tissue healing as a reaction to core biopsy (Kramer, Schaefer, and Reinartz, 1995), or if PAI-1 was always higher after PST than uPA. Supporting this interpretation, even though levels in the **(n=35)** post-PST setting were quite scattered, responding patients appear to have lower PAI-1 levels than non-responding ones in the post-PST tissue.

In addition to the **Mann-Whitney test** results (table 28) that PAI-1 in the **(n=35)** post-PST setting is suitable to discriminate between responders and non-responders (p=0.00), we used **ROC AUC statistics**. Thereby, we evaluated the ability of the test to discriminate non-responding from responding cases, solving the question of how accurate a classifier PAI-1 is in identifying non-responding cases (table 32, figure 25). Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. The best possible prediction with ultimate sensitivity (89%) and specificity (82%) of PAI-1 with respect to response to PST was seen at a post-PST PAI-1 level of 8.31ng/mg (p=0.00). Thus, **PAI-1 in the (n=35) post-PST setting appears to be able to discriminate between responders and non-responders as subsequently discussed**. Thus, **PAI-1 in the (n=35) post-PST setting appears to be able to discriminate between responders and non-responders as subsequently discussed**. However, due to the rather small sample size, we refrained from defining an optimized cut-off point.

uPA and PAI-1 as Prospective and Predictive Tumor Markers in Breast Cancer

Since breast cancer is a heterogeneous disease, general treatment recommendations are not sufficient. Instead, an assessment of the risk of recurrence should be used to avoid over-treatment. However, risk-assessment is only possible, if the tumor specimen can be categorized meticulously. A precise categorization is facilitated, e.g., by using tumor prognostic and predictive markers (Thomssen and Janicke, 2000). Previously outlined (cf. chap-

ter 2.4), evaluation guidelines for clinical routine markers in breast cancer were adapted (Clark, 1992; Clark, 1994; Graeff, Janicke, and Schmitt, 1991; Harbeck et al., 2000; Harbeck et al., 2001b; Harbeck, 2001; Hayes et al., 1996; McGuire, 1991), and prerequisites for **prospective and predictive tumor markers** were outlined: A **predictive marker** projects response or resistance to a specific therapy, while a **prognostic marker** correlates with the patients´ outcome (i.e., DFS or OS) and predicts relapse or progression independently of future treatment effects. Summarizing, both prospective and predictive tumor markers have to support therapeutic decision making (ASCO, 1996; Cianfrocca and Goldstein, 2004; Clark, 1992; Graeff, Janicke, and Schmitt, 1991; Harbeck et al., 2001b; Harbeck, 2001; Harbeck et al., 2002a; Hayes et al., 1996; Hayes, 2006; Hayes, Isaacs, and Stearns, 2001; Kaufmann and Scharl, 2000; McGuire, 1991; Ravdin, 1998; Schrohl et al., 2003).

Unlike inconsistent data on several supposedly clinically relevant factors as reviewed by Hayes (2005) and Thomssen and Janicke (2000), uPA and PAI-1 have a meaningful prognostic and predictive value (Harbeck et al., 1999a; Harbeck et al., 2002d; Harbeck et al., 2002b; Harbeck et al., 2004b; Harbeck, Kates, and Schmitt M, 2002; Harbeck and Thomssen, 2003; Janicke et al., 2001). Furthermore, they are approved according to the highest level of evidence LOE I, A + [cf. chapters 2.6] (AGO, Gynecologic Oncology, and ARO, 2005c; Brunnert et al., 2001; Janicke et al., 2001; von Minckwitz et al., 2002). Since the LOE I validation of uPA and PAI-1 was published shortly after publication of the 2003 St. Gallen (Switzerland) consensus recommendations (Goldhirsch et al., 2003; NIH, 2000), these results were not included into the breast cancer management guidelines in 2003. However, the measurement of uPA and PAI-1 is recommended by the AGO guidelines (AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005c) and within the “Tumorzentrum München: Manual Mammakarzinome” (Funke et al., 2005). In the 2005 St. Gallen (Switzerland) consensus recommendations, uPA and PAI-1 were extensively discussed (Goldhirsch et al., 2005). It was agreed upon, that high levels (as measured in tissue extracts using ELISA) indicate a poor prognosis in N_0 patients with an increased risk of disease recurrence (Harbeck et al., 2002d; Harbeck et al., 2004a; Harbeck, Kates, and Schmitt M, 2002). In contrast, patients with low uPA/PAI-1-levels show a particularly good prognosis and therefore, may be spared the burden of adjuvant chemotherapy; respectively no evidence was found for a subgroup defined by uPA and PAI-1, which does not respond to adjuvant endocrine therapy (Harbeck et al., 2004b; Harbeck and EORTC RBG, 2005). However, research on recommendations for therapy schemes with respect to high and low uPA and PAI-1 levels is continued (Harbeck and Thomssen, 2003): The NNBC-3 trial in N_0 breast cancer [AGO, EORTC-RBG] (Harbeck et al., 2002d; NNBC-3 Europe Studie, 2006; Paepke et al., 2006) and the now finished ADEBAR trial in patients with 4 or more involved axillary lymph nodes (estimate, 2005).

Promising results on the use of uPA and PAI-1 in the AST setting suggest focusing on determination with respect to new therapeutical settings such as PST. Analogous to the AST setting, **subgroup selection in the PST setting is of great interest**, enabling to further individualize systemic therapy (Harbeck et al., 2004b; Kaufmann et al., 2006; Kaufmann, von Minckwitz, and Rody, 2005; Shannon and Smith, 2003). Although Therasse et al. (2000) ruled out the use of **tumor markers to assess response,** the prognostic and predictive value of uPA and PAI-1 has been reported by several authors (Cufer, Vrhovec, and Borstnar, 2002; Harbeck et al., 1999a; Harbeck et al., 2002b; Harbeck et al., 2004b; Harbeck, Kates, and Schmitt M, 2002; Harbeck and Thomssen, 2003; Janicke et al., 2001). Additional findings in the adjuvant setting imply that **high levels of uPA and/or PAI-1 do reflect an aggressive phenotype**, which may be overcome or suppressed by early systemic therapy, but may be too advanced for response to palliative therapy at a later stage (Harbeck et al., 2001c; Harbeck et al., 2004b). Our data suggest for the first time that **PAI-1 does not only have predictive power in the adjuvant (or pre-**

PST) setting, but post-PST PAI-1 changes also reflect response to PST.

Pierga et al. (1997) reported, that pre-PST PAI-1 and response did not correlate. Yet, he admitted that their results were preliminary due to the lack of subsequent core-biopsies during the course of disease. However, if **post-PST PAI-1** as demonstrated in our analysis is capable of discriminating between responders and non-responders after completion of, e.g. 6 cycles of PST, it might be interesting to study, whether such a discriminative function is already present at an earlier moment. **Clinical consequences** of such an earlier discriminative potential of PAI-1 could be that decisions about treatment can take place at the earliest after two to three cycles of PST. Our findings are coherent with a study from Cufer (2002) demonstrating, that PAI-1 levels could be used as a biological marker to identify patients with a higher risk of local relapse already at the time of primary treatment. Beyond, such earlier discriminative ability has been reported for cCR as well (Beresford, Stott, and Makris, 2007; Muller et al., 2007). Complementary, consecutive core biopsies could be used to monitor the change of the tumor as conducted in the GeparTrio and GeparQuattro Trials (German Breast Group (GBG), 2007; von Minckwitz et al., 2005a).

However, whether PAI-1 levels might enable to adapt therapy at an earlier time will have to be validated in a broader study as, e.g. scheduled by Dittmer in 2008 (Dittmer, 2006).

Tissue Sampling and uPA and PAI-1 Assessment

The advantages in patient care regarding percutaneous breast biopsy have been well documented in literature. Based on the available evidence, results using core biopsy are clinically acceptable and core biopsy is now recommended before definite surgery [cf. chapter 2.8] (AGO, Gynecologic Oncology, and ARO, 2005c; Krainick et al., 2002; McIlhenny et al., 2002; Memarsadeghi et al., 2003; Pfarl et al., 2002; von Minckwitz et al., 2002).

Since our analysis was conducted retrospectively, biopsy was performed prior to the initiation of PST solely instead of gaining tumor specimens prior, during and post-PST as proposed by von Minckwitz (2002). A fact diminishing the quality in our study as well as a similar study on pre-PST PAI-1 by Pierga (1997).

Reasons to **support minimal invasive techniques** are various: An early confirmation of exact diagnosis will facilitate to plan the proximate steps more carefully, thus promoting individualized therapy concepts and avoiding unnecessary therapy (Harris et al., 2003; Sittek et al., 2005a). It is less invasive and painful than excisional biopsy, does not deform the breast, causes minimal or no scarring on subsequent mammograms, and can be performed quickly (Meloni et al., 2002). Moreover, it facilitates timely patient management (Harris et al., 2003; Liberman, 2000). The complication-rate is between 0,2% (Buchberger et al., 2002) and 1.2% (Jackman et al., 1999; Pfarl et al., 2002) with a frequency of hematoma or infection each less than one in 1000 patients (Parker et al., 1994). Women, who have undergone percutaneous biopsy, receive fewer surgeries (Liberman, 2000) and have a lower cost of diagnosis (Groenewoud et al., 2004; Liberman et al., 1998; Liberman, 2000; Sabel and Staren, 1997). Additionally, chemosensitivity of vital tumor tissue can be assessed, since once the tumor tissue has been treated chemotherapeutically by PST an assessment of biological factors is often less viable (Kuner et al., 2000).

Using a 14-gauge assisted ultra-sound-guided high speed needle together with biochemical ELISAs by American Diagnostica (Stamford, CT, USA) and a protein-assay by Pierce Biotechnology (Rockford, IL, USA), the **assessment of the tumor-biological factors uPA and PAI-1 from minimal invasive biopsy** has also been sug-

gested for clinical practice (Funke et al., 2005; Kuner et al., 2000; Schmitt et al., 2006; Sittek et al., 2005b; Tumorzentrum Muenchen, 2005). An additional comparison of uPA and PAI-1 values by using needle biopsy vs. excisional biopsy is scheduled by Dittmer in 2008 [University of Halle, Germany] (Dittmer, 2006).

Reasons to **question the general applicability as well as the results of needle bioptical procedure** comprise the following: **First**, there exists the possibility of a delay in diagnosing breast cancer (Liberman, 2000). **Second**, if a non-standardized method is used or a less experienced examiner conducts the examination, comparability is cut down and the quality of the results might be diminished. Furthermore, a minimum of 3 sonographically guided LCNBs from various locations within the primary tumor is necessary (von Minckwitz et al., 2002), but in order to achieve a high diagnostic accuracy (McIlhenny et al., 2002) even five or more passes may be required (Schulz-Wendtland et al., 2003). **Third,** using sonographical guidance, the lesion must be sonographically evident (Liberman, 2000).

In addition, several points need to be considered before **uPA and PAI-1 assessment using minimal invasive biopsy material**: **First**, any biopsy might alter subsequent uPA and PAI-1 levels in the surgical specimen, since in tissue healing, components of the plasminogen activation system facilitate localized proteolysis of the ECM at the leading edge of migrating keratinocytes (Kramer, Schaefer, and Reinartz, 1995; Scully, 1991) and in the wound area, expression of uPA and PAI-1 by keratinocytes increases significantly following regeneration of penetrating incisional wounds (Grondahl-Hansen et al., 1988; Huang et al., 2002; Jones, Cohen, and Chambers, 2002; Romer et al., 1991). Still, questions remain how fast any alteration occurs, how long-lasting the effects are, and how many biopsies are needed to modify levels especially in conjunction with elevated levels due to cancerogenic activity. **Second**, a smaller amount of tissue assessed might still yield less protein (Schmitt et al., 2002; Schmitt et al., 2006). **Third**, a rather “blindly” performed biopsy in comparison to a standard biopsy under visual control might increase the risk of biopting at the periphery or outside the area of interest resulting in modified levels (Kuner et al., 2000). **Fourth**, a tumor is an inhomogeneous area, implying the difficulty to obtain a representative sample especially if prognostic or predictive factors are of interest (Harris et al., 2003). **Fifth**, even if uPA and PAI-1 should correlate, this could still occur at the margin of the tumor region, possibly resulting in diminished overall levels. **Sixth**, elevated levels of uPA and PAI-1 may even be found in normal breast cancer tissue (Costantini et al., 1996), although, as previously outlined (cf. chapter 2.6) results have proven clearly that the biological role of uPA and PAI-1 is strongly associated with malignancy. **Seventh**, depending on the stage of disease at diagnosis, values for uPA and PAI-1 may vary (Gershtein and Kushlinskii, 2001).

Summarizing, there is no reason not to conduct core biopsy and to measure uPA and PAI-1 levels. Findings from our analysis both validate, that core biopsy is a viable alternative to assess uPA and PAI-1 prior to therapy (Krainick et al., 2002) and that the results obtained are reliable.

Survival Analyses

The survival analyses (table 33; figure 26) conducted with respect to response to therapy did not yield any statistically significant results. Partly, reasons may be explained by: **First,** the rather small collective. **Second,** patients were treated by different drugs, dosages, and schedules. **Third**, by the clinically crude assessment of response and the retrospective setting of our analysis: Some patients with no palpable residual disease may have been categorized complete clinical responders, while radiographically and histologically these patients might have had residual disease. This fact may diminish the quality of our study as well as of a similar study on response and

survival in a PST setting by Gajdos et al. (2002). **Fourth**, in addition to the crude assessment of clinical tumor response and the retrospective setting, the TNM status was not completely documented in patient records. However, clinical tumor response was reported to be significantly related to initial tumor size in several studies (Fisher et al., 1997; Gajdos et al., 2002; Kuerer et al., 1999). Since survival analyses in our study were not adapted to tumor size, they might have been biased. As previously outlined, future studies should use the pathologic definition pCR which is the gold-standard in PST at present (Kaufmann, von Minckwitz, and Rody, 2005) [cf. chapter 2.10].

Systemic Breast Cancer Therapy

Choosing from several treatment options, breast cancer can be treated systemically applying either primary systemic therapy (PST) after histological diagnosis, but prior to surgery, or AST subsequent to surgery. Both PST and AST can be conducted using endocrine or chemotherapeutic agents. Prerequisite and state of the art recommendations for both as well as other therapeutic options are extensively reviewed by (Bauerfeind et al., 2005; Goldhirsch et al., 2005; Janni et al., 2005; Kahlert et al., 2005).

Recommendations for **adjuvant chemotherapeutic systemic therapy** are based on studies made in the late 1980s. During this decade, the NCI extended adjuvant treatment recommendations for N+ to N_0 patients, based on patient mortality of 30% after 10 years follow-up and on the supposedly systemic disease during diagnosis. Moreover, traditional prognostic factors were considered reliable enough to allow risk assessment (Kaufmann and Scharl, 2000). However, preference of **adjuvant chemotherapy was reflected critically** by a number of investigational groups (Hayes, 2000). In January 2005, the St. Gallen (Switzerland) expert consensus meeting finally agreed on modified guidelines and recommendations for adjuvant breast cancer therapy (Janni et al., 2005), taking into account new evidence (Goldhirsch et al., 2005): The first consideration is endocrine responsiveness, further differentiated by menopausal status. Thereafter, patients are sub-grouped into low-, intermediate- and high-risk categories. Current recommendations advise chemotherapy for endocrine non-responsive disease; endocrine therapy as the primary therapy for endocrine responsive disease, adding chemotherapy for some intermediate- and all high-risk patients in this category; and both chemotherapy and endocrine therapy for all patients in the uncertain endocrine response category except those in the low-risk group (Goldhirsch et al., 2005). Still, the debate continues, which chemotherapy regimen is to be recommended: Complementally to the CMF regime in the low-risk setting (Bonadonna et al., 1995), evidence has accumulated that anthracycline-based multiple-combination schemes [e.g., the Canadian FEC120 or French FEC100 (Bonneterre) Protocol] provide a significantly better survival (Howell and Wardley, 2005; Levine et al., 2005). To day, Taxanes are considered standard for N+ patients (AGO, Gynecologic Oncology, and ARO, 2005b).

Comparing previously described **PST to AST** (cf. chapter 2.9), the **main clinical questions to date** are, if PST reduces the mastectomy rate and if response translates into improved disease-free and overall survival (Kaufmann, von Minckwitz, and Rody, 2005). Therefore, several PST protocols (e.g. PREPARE, TECHNO and NOAH) have been completed under the patronage of the AGO (www.ago-online.org) as well as Gepar-protocols (GBG, www.germanbreastgroup.de). They are focusing on the possibility to increase the rate of BCT, local and loco-regional response, to correlate clinical apparative and histological remission, as well as to improve DFS and OS (Bauerfeind et al., 2005).

At present, the following factors **promote the use of PST in breast cancer**: **First**, DFS and OS are equivalent in

patients treated by the same adjuvant or primary systemic regimen. To this point, PST is considered as safe and effective as the same systemic postoperative treatment, when patients are treated by identical drug combinations (AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005b). However, current data from the NSABP B-18 trial followed by the B-27 trial, and the European Cooperative Trial in Operative Breast Cancer (ECTO) suggest a possible additional benefit of PST (Eiermann et al., 2003; Gianni et al., 2002; San Antonio Breast Cancer Symposium, 2005) based on improved pCR (Bear et al., 2003) and the subsequent improvements in DFS and recurrence free survival (RFS). pCR was shown to be the most important surrogate marker of PST success, demonstrating a correlation between pCR and improved DFS and OS (Kaufmann, von Minckwitz, and Rody, 2005). This is coherent with findings from several authors (Bear et al., 2003; Buzdar et al., 2005; Fisher et al., 1998b; Kaufmann et al., 2003; Kuroi et al., 2006; Untch et al., 2002b; von Minckwitz et al., 2005a). Clinically, PST has the advantage of improving the surgical options for BCT (Dixon, Anderson, and Miller, 2002; Goldhirsch et al., 2003; Hutcheon, Heys, and Sarkar, 2003; Ikeda et al., 2002; Pierga et al., 2000; Valero et al., 2002). In addition, patients with a sufficient clinical down-staging allowing BCT have the best long-term outcome, while those still requiring mastectomy are at a higher risk of relapse and of developing contra-lateral cancers (Cance et al., 2002). Beyond, PST might be more effective due to a pre-surgically still intact vessel system of the original tumor, elimination of occult metastases (Wallwiener, 2001), and a smaller micrometastatic disease volume (Hortobagyi et al., 1983). **Second**, response to PST is a predictor of long-term outcome. Although validation is outstanding, that increased pCR rates with more active regimens improve OS, pCR has been associated with improved DFS and OS (von Minckwitz et al., 2005b). Response to PST is a predictor of long-term outcome and gives prognostic information after a short-term interval in contrast to adjuvant trials, which do not show their results before a 5- to 10-year follow-up (Kaufmann, von Minckwitz, and Rody, 2005). However, to date the lymph node status after PST still remains the most important prognostic marker (Bonadonna et al., 1990; Kaufmann et al., 2006; Kuerer et al., 1999). **Third**, the study of cancer biology and cancer as a biological model is facilitated (Ikeda et al., 2002). Assessing both “real-time” chemosensitivity to the applied agents in vivo (Bauerfeind et al., 2005) and responsiveness to systemic therapy (Colleoni, 2003; Colleoni et al., 2001) diminishes the risk of developing resistance in a rapidly dividing cell-population (Wallwiener, 2001). As a result, ineffective therapy or any change to the worse allows immediate adoption of the applied medication (Valero et al., 2002). **Fourth**, individualization of the therapy regime is becoming more important (Kaufmann et al., 2006). Predictive and prognostic clinical and pathological factors are a promising approach regarding this matter (Bauerfeind et al., 2005). This again might increase the chance of pCR for a broader collective (Kaufmann et al., 2003). **Fifth**, patient management after completion of PST is improved. In case of PST failure, a non-cross-resistant second-line PST (Kaufmann et al., 2003) or AST (Thomas et al., 2004) can be chosen. And, early scheduling of adjuvant radiation after surgery is expedited (Bauerfeind et al., 2005). **Sixth**, endocrine-non-responsive tumors more often show pCR than endocrine-responsive tumors (Bauerfeind et al., 2005; Goldhirsch et al., 2005; Kaufmann, von Minckwitz, and Rody, 2005). **Seventh**, sentinel node-biopsy after PST might be a reasonable approach in experienced hands and has to be considered as a criterion, whether the patient has to undergo axillary surgery (Pockaj and Gray, 2004; San Antonio Breast Cancer Symposium, 2002). **Eight**, sequential minimal invasive biopsies (e.g., LCNB) together with previously validated methods (e.g. micro ELISA) allow monitoring the course of disease prior, during and after PST more precisely (Mamounas and Fisher, 2001). **Additionally**, minimal invasive surgery will help to identify tissue left of the primary tumor after PST and in the future may help to decide whether surgery is necessary (Mamounas, 2002; Mamounas and Fisher, 2001). **Finally**, a visible tumor reduction increases patient compliance and together with the lowered probability of mastectomy, this minimizes the psychological distress (Bauerfeind et

al., 2005; Kaufmann, von Minckwitz, and Rody, 2005).

Disadvantages of PST comprise the potential tumor stage modification, while treatment of patients with progressive disease (PD) might be delayed (Ikeda et al., 2002). Furthermore, residual intraductal components may be overlooked after breast conserving surgery, which may be avoidable by assessment of the shrinkage pattern using MRI (Ikeda et al., 2002). In certain cases, over-treatment has been reported (Ikeda et al., 2002). Despite potential advantages, no statistically significant difference in disease free, distant disease-free, or overall survival in patients receiving the same adjuvant or primary systemic regimen has been observed (Fisher et al., 1998b; Goldhirsch et al., 2003).

Unresolved problems of PST: First, the search for the optimal drug combination and sequence as well as of the duration of application is still a challenge for further clinical trials. And, what will be the best treatment after PST (Kaufmann, von Minckwitz, and Rody, 2005)? Data from the NSABP B-27, Aberdeen and GeparTrio studies suggest that 'non-cross-resistant' adjuvant treatment may not be effective (Hanrahan, Hennessy, and Valero, 2005). Hence, which therapy-regime is suitable to proceed with, if patients lack response or remission after PST (Hanrahan, Hennessy, and Valero, 2005; Janni et al., 2005; Kaufmann et al., 2006; Thomas et al., 2004; von Minckwitz et al., 2005a)? Results for primary systemic endocrine (PSE) therapy are promising, too (Shannon and Smith, 2003). Therefore, simultaneous use of chemotherapy and endocrine therapy has to be reflected carefully (Kaufmann, von Minckwitz, and Rody, 2005). **Second**, how can response to PST be defined and measured? The use of PST regimes may provide a pathologically complete response (van Praagh et al., 2002), which correlates with prolonged periods of remission (Valero et al., 2002). However, the term complete response with respect to PST still has to be defined more thoroughly: Definitions of pCR used by various authors are inconsistent and methods of assessment are not standardized (Kaufmann et al., 2006; Kuroi et al., 2006; Untch et al., 2002a). In this regard, one of the most critical problems is the optimal imaging method for monitoring tumor response (Kaufmann et al., 2003; Kaufmann, von Minckwitz, and Rody, 2005). Further on, it might be necessary to assess response differently in the preoperative endocrine therapy and chemotherapy setting (Dixon, Anderson, and Miller, 2002). **Third**, as for sentinel node-biopsy: Is it precise enough to replace the standard conventional axillary lymphonodectomy after PST (Pockaj and Gray, 2004)? When should it be conducted [pre-PST vs. post-PST] (Kaufmann, von Minckwitz, and Rody, 2005)? **Fourth**, following Solomayer et al. (2003) who found tumor cell dissemination not to being positively influenced by PST, this has to be tested in further trials. **Finally**, PST provides molecular tests with follow up results within short-term intervals. Thus, DNA micro array technology could give further insights into the biology of breast cancer, enabling patient sub-grouping in the context of PST (Kaufmann, von Minckwitz, and Rody, 2005).

Response to PST with Respect to the PST Chemotherapy Regimen

Most modern PST regimes are anthracycline-containing (cf. chapter 2.9) and sequential approaches are promising (AGO et al., 2003; AGO, Gynecologic Oncology, and ARO, 2005c; Bauerfeind et al., 2005; Costa, 2001; Howell and Wardley, 2005; Kaufmann et al., 2003; Kaufmann et al., 2006; Kaufmann, von Minckwitz, and Rody, 2005; Levine et al., 2005; Untch et al., 2002b; von Minckwitz et al., 2005b). Therefore, we used a **chi square (χ2)** test to test for an advantage of any of the applied regimes with emphasis on anthracycline-containing regimes (tables 34 and 35). However, no regime was found to be clearly superior to the others, which is coherent with findings from Kaufmann et al. (2006). Though it is noteworthy, that patients with an anthracycline-containing

regime hold an 8-fold higher rate of responders than non-responders (24 responding vs. 3 non-responding). Reasons again might be **first** the small collective, **second** the variety of regimes used in our analysis, **third** the clinical assessment of response, and **fourth,** that the ideal regime is outstanding and was none of the ones applied.

As previously discussed, PAI-1 is not predictive for response in the pre-PST setting (Pierga et al., 1997) while **post-PST PAI-1**, as demonstrated in our analysis, is capable of discriminating between responders and non-responders after completion of PST. Should **post-PST PAI-1** be validated as a "**surrogate marker of response**" in further studies, it may be used to test for chemosensitivity at an early stage. Furthermore, these patients could then be offered an individualized primary systemic therapy regime as outlined by Cufer et al (2002).

Patient Characteristics and Additional Analyses

Using **chi-square (χ2)** test to compare the menopausal status to response to PST, no significant correlation could be established. It could have been expected that consistent with reports in literature pre-menopausal patients had a higher rate of responders (Funke et al., 2005). An **independent samples test** [t-test] (table 37) was used to analyze for an association of Her2/neu, progesterone, and estrogen levels with response to PST. However, we did not find any statistically significant correlation. As for **Her2/neu**, it could have been expected, that over-expression independently predicted response to primary systemic chemotherapy as reported by Penault-Llorca et al. (2003) and known from adjuvant anthracycline-based chemotherapy (Paik et al., 2000). Since the **hormone receptor-status** is known to predict response to therapy and patients with negative hormone receptor status are reported to show better response to PST than patients with positive hormone receptor status (Funke et al., 2005; Gianni et al., 2002; Tumorzentrum Muenchen, 2005; Untch et al., 2002b; Untch et al., 2002a), we expected to find similar results.

Using the **Mann-Whitney test** to detect, whether proliferation marker Ki67 discriminated between responders and non-responders, no significant results were found (p=0.33). Ki67 proliferation rate did not significantly decrease or increase during application of PST, with 64 per cent of patients lower or equal 30% proliferation rate in the n=11 responding pre-PST **(n=18)** patients and 76 per cent of patients lower or equal 30% proliferation rate in the n=13 responding post-PST **(n=35)** patients (tables 39 and 40). These findings are coherent with Burcombe et al. (2006) that neither pre-treatment nor post-chemotherapy median Ki67 proliferation rate differed significantly between clinical responders and non-responders over a defined course of time. Moreover, Billgren et al. (1999) demonstrated, that although a decrease of more than 25% within the proliferating fraction occurred, and PST predicted a reduced risk of disease recurrence, there was no correlation with local objective response. Overall, the prognostic significance of pre-treatment Ki67 index in breast tumors seems to vary as outlined by Burcombe et al. (2006).

Lack of conclusive data within our analysis with respect to menopausal, Her2/neu, and hormone-receptor status may be explained by our small collective. Further prospective clinical trials on primary systemic chemotherapy with parallel biological marker studies on histological tissue taken at various stages before, during, and after primary systemic chemotherapy will promote the search for clinically useful predictive biomarkers as well as bringing about more comparable results (Abraha et al., 2003; Burcombe et al., 2006; Jones and Smith, 2006; Pierga et al., 1997).

6.3 Synopsis

In summary, our main results of the methodical part on mirco-ELISA and the clinical part on uPA and PAI-1 determination using core biopsies, and on uPA and PAI-1 levels during primary systemic therapy were:

1. Our newly developed **micro determination procedure (micro ELISA**) for uPA and PAI-1 measurement is reliable for clinical routine testing.
2. **PAI-1 after** completion of **primary systemic therapy** may have a **possible discriminative function** between responders and non-responders, supporting that post-PST PAI-1 may function as a "**surrogate marker of response" to PST**.
3. **Core biopsy** can be considered a viable **alternative to assess uPA and PAI-1** prior to therapy.

Although Therasse et al. (2000) ruled out the use of **tumor markers to assess response** and Pierga et al. (1997) did not find a correlation between pre-PST PAI-1 and response, our findings support the hypothesis of a **possible discriminative function of post-PST PAI-1** between responders and non-responders after the completion of primary systemic therapy. PAI-1 may function as a "**surrogate marker of response" to PST**. Thus, in addition to the predictive power of PAI-1 in the adjuvant (or pre-PST) setting (cf. chapter 2.6), PAI-1 might have predictive power in the post-PST setting or even during application of PST.

However, given the limitations of our study, we are not able to differentiate whether the change of PAI-1 levels are due to the applied PST-regime or due to the response to PST. Since our collective is rather small and there is one not responding patient only, we refrain from making a statement about patients who have not responded to therapy. Still, a key role of PAI-1 in tumor aggressiveness is supported by several studies (cf. chapter 2.5). Additionally, PAI-1 is reported to play an important role in the vascular remodeling in human cancer (Fox et al., 2001). And, as reviewed by Harbeck et al. (1999b), PAI-1 is a strong prognostic factor after long-term follow-up both for primary breast cancer and following first relapse.

uPA and PAI-1 correlate within the **pre-PST core-biopsy and the post-PST surgical specimens**, which is consistent with findings from other groups (Bouchet et al., 1994; Foekens et al., 2000; Fox et al., 2001; Grebenchtchikov et al., 2005; Grondahl-Hansen et al., 1993; Grondahl-Hansen et al., 1995; Janicke et al., 1994b; Janicke et al., 1993; Reilly et al., 1992). This correlation is now substantiated by our findings. Thus, core biopsy can be considered as a viable alternative to assess uPA and PAI-1 prior to therapy as reported by other authors (AGO, Gynecologic Oncology, and ARO, 2005c; Krainick et al., 2002; Kuner et al., 2000; McIlhenny et al., 2002; Pierga et al., 1997; Schmitt et al., 2006; Sittek et al., 2005b; von Minckwitz et al., 2002).

On the whole, the validity of uPA and PAI-1 determination using core biopsy as well as the previously reported discriminative function of PAI-1 with respect to response after primary systemic therapy will have to be confirmed in a larger setting. Carefully taking into consideration ethical aspects, it might be applicable to conduct sets of subsequent core-biopsies prior to, during, and after PST to monitor the change of uPA and PAI-1 levels as discussed by several authors (Abraha et al., 2003; German Breast Group (GBG) and Minckwitz von, 2007; Jones and Smith, 2006; Pierga et al., 1997; von Minckwitz et al., 2005a). Afterwards, core biopsy uPA and PAI-1 levels could be compared to levels measured in surgical specimens. In addition, our **micro ELISA** could be integrated, using five to ten of 90µm thick cryosections of a frozen breast cancer tissue block, respectively one to two breast tissue core biopsies (Schmitt et al., 2002; Schmitt et al., 2006).

Such a study might bring about a suitable pre-PST uPA and/or PAI-1 cut-off, predicting response to PST. Outlined by Jones and Smith (2006), the primary systemic approach allows the tumor to be used as a measure of treatment response in vivo and together with information on the use of clinical, pathological, and molecular endpoints, these can be used as surrogate markers to predict the long-term outcome in the adjuvant setting. If not as predictor to long-term outcome, as outlined by Cufer et al. (2002), post-PAI-1 may still be used as a marker for chemosensitivity which might allow to optimize the subsequent therapy accordingly. This is consistent with findings by Harbeck et al. (2002c, 2004b) which emphasize the necessity to define the aggressive phenotype of the individual cancer at an early stage.

Further questions are, **(1)** how nodal involvement could be integrated into the therapy concept. In the 2005 St. Gallen consensus recommendations on adjuvant therapy (Goldhirsch et al., 2005) it was agreed on, that in N_0 patients high levels of uPA and PAI-1 indicate a poor prognosis with an increased risk of disease recurrence (Harbeck et al., 2002d; Harbeck, Kates, and Schmitt M, 2002) while patients with low uPA/PAI-1-levels show a good prognosis and therefore may be spared adjuvant chemotherapy (Harbeck et al., 2004b; Harbeck and EORTC RBG, 2005). As for the PST setting, patients with extensive nodal involvement after primary systemic chemotherapy have been reported to have a poor outcome (Pierga et al., 2000). **(2)** In which manner should a second-line treatment be integrated (Harbeck, Kates, and Schmitt M, 2002)? And **(3)**, why was there only a predictive function for PAI-1 and not for uPA? This contrasts to findings by Harbeck, Kates, and Schmitt (2002), that the clinical relevance of the two tumor-invasion factors uPA and PAI-1 is greatest, when used in combination.

Resolving these questions might further substantiate the role of uPA and PAI-1 in the subset of primary systemic treatment.

6.4 Evaluating our Results by Comparison with Other Studies

We are aware of the small collective we present here. Hence, we decided to interpret our results as “pilot” results, encouraging further studies with the goal to confirm our results in larger collectives. Although statistical analysis and patient selection are solid, our results must be interpreted with caution due to the retrospective setting, where clinical response was based on crude physical examination only. Unlike clinical prospective trials, in our study patient recruitment and subsequent analysis depended on several factors and were strongly influenced by data availability, patient charts, and laboratory determination procedures at the time. Besides, unlike clinical drug trials, any retrospective design of protocols and in- or exclusion of data with respect to the prognostic power of a given biological marker is certainly somewhat less rigid. The tissue sampling by needle biopsy (LCNB) should at best have been conducted prospectively. Thereby, assessment suiting the LOE (cf. chapter 4) could have been accounted for more precisely. However, since the quality of patient care in our clinic has always followed Good Clinical Practice [ICH-GCP] (EMEA, 2002) and the current guidelines (AGO, Gynecologic Oncology, and ARO, 2005c) [cf. chapter 4], we do consider our retrospective results to be of high enough validity to propose further prospective studies. As outlined by Harbeck et al. (2004b), although prospective randomized studies are considered the gold standard for evaluating therapy response, such studies are often not feasible or unethical to perform. Hence, as shown in our analysis for PAI-1 in a PST setting, retrospective analysis may yield valuable information, especially since uPA and PAI-1 had not been used for therapy decision-making in that setting. We are aware of possible criticism relating to the clinical part, caused by the determination of uPA and PAI-1 before the guidelines of EORTC-RBG were set. However, uPA and PAI-1 levels were determined by our clinical re-

search group laboratory, which followed the EORTC “Quality Assurance Protocol” for quite some time. Moreover, both Prof Dr N. Harbeck and Prof Dr M. Schmitt are members of the EORTC-RBG panel and have substantially influenced establishment of quality assured laboratory determination. Furthermore, using previously illustrated assay formats by American Diagnostica (Stamford, CT, USA) and Pierce (Rockford, IL, USA), uPA and PAI-1 levels already have been determined in our clinic since 1987, also for a prospective study in primary breast cancer (Janicke et al., 1990; Janicke et al., 1994a).

6.5 Conclusion, Prospects, and Perspectives

The promising and convincing experimental and clinical data demonstrating the essential role of uPA and PAI-1 in tumor cell invasion and metastasis has made the uPA/PAI-1 system an important target for cancer therapy (Muehlenweg et al., 2001; Rosenberg, 2000; Schmitt et al., 1997a; Schmitt et al., 2000). **uPA and PAI-1 ELISAs** have been submitted for Food and Drug Administration (FDA) approval by the manufacturer (ADI, 2002a; ADI, 2002b; Janicke et al., 2001). So far, the FDA has approved the PAI-1 ELISA Kit No. 822 (FDA, 2003). Meanwhile, on basis of the already established uPA and PAI-1 **ELISAs**, ADI developed a new Communauté Européenne (**CE)-labeled**[31] kit including uPA and PAI-1 ELISAs, which is meant for use in **clinical routine testing** (ADI, 2005). In addition to the **Chemo N_0** trial and the promising results of the first two interim analysis (Harbeck et al., 2001c; Janicke et al., 2001), the **NNBC-3** Europe follow-up trial is currently recruiting concentrating on the optimal chemotherapy for the subgroup of N_0 breast cancer patients with elevated uPA/PAI-1 levels (Harbeck et al., 2002d; NNBC-3 Europe Studie, 2006; Paepke et al., 2006) as well as the **ADEBAR** trial for the subgroup of N+ patients (estimate, 2005). With **PST** being an established alternative to adjuvant therapy (Bauerfeind et al., 2005; Janni et al., 2005; Kaufmann et al., 2003; Kaufmann, von Minckwitz, and Rody, 2005), the in vivo measurement of chemosensitivity will help testing new chemotherapeutical compounds and tailoring more individualized therapy regimes (Cleator, Parton, and Dowsett, 2002; Faneyte et al., 2003; Ikeda et al., 2002; Kaufmann and Kubli, 1983; Shannon and Smith, 2003; Wallwiener, 2001). **Minimal invasive tissue biopsy** is a feasible and established method (AGO, Gynecologic Oncology, and ARO, 2005c; McIlhenny et al., 2002; Rutgers, 2001; Sittek et al., 2002). When used with PST, conservation of tissue prior to, during, and after PST will possibly allow later identification of predictive factors associated with an increased response to therapy (von Minckwitz et al., 2002). **uPA and PAI-1** as well as other biological surrogate markers of response are important tools aiding clinicians in solving crucial questions such as early diagnosis, estimation of patient prognosis, prediction of therapy response, and individualizing systemic therapy regimens (Shannon and Smith, 2003). At the same time, **novel therapy concepts** such as different synthetic uPA inhibitor classes (Steinmetzer, 2003) have shown promising results in pre-clinical testing and are undergoing early phase clinical testing such as the uPA inhibitor WX-UK1 (Ertongur et al., 2004; Harbeck et al., 2002d; Muehlenweg et al., 2001; WILEX, 2002; WILEX, 2006).

Micro ELISA at present enables to use small amounts of tumor tissue such as core biopsies (Schmitt et al., 2002; Schmitt et al., 2006). **Future ELISA** - particularly adapted to quantify uPA/PAI-1 interactions only - will offer the option to envisage the clinical relevance of such complexes further. Besides, in the near future **more sensitive techniques** including, e.g. fluorescence (antibody chips) and plasma resonance techniques, might extend the field of (pre-)clinical testing to quantify biomarker levels more precisely (Sweep et al., 2003).

31 *Communauté Européenne*: By affixing the mandatory CE marking, the manufacturer, or its representative, or the importer assures that the item meets all the essential requirements of all applicable EU directives with respect to essential health and safety requirements.

Elevated tumor tissue levels of uPA and PAI-1 correlate with tumor aggressiveness and poor patient outcome not only in breast cancer, but also in other malignancies (cf. chapter 2.6). However, clinical consequences originating from measurement of uPA and PAI-1 are so far limited to breast cancer (Schmitt et al., 2000). Approaches such as non-invasive uPA and PAI-1 assessment in **nipple fluids** (Qin et al., 2003) and in **blood** (Hamer et al., 2001), principally not requiring special extraction methods, suggest utility of uPA and PAI-1 in a widened field. Still, the determination of uPA and PAI-1 in the blood of breast cancer patients yields no prognostic relevance at present (Grebenchtchikov et al., 2005).

Finally, data from several **prospective therapy trials** as summarized by Harbeck (2004b) and the availability of approved strict **standard operating procedures** on the usage of ELISA (Schmitt et al., 2007) both will aid in increasing the practical value of uPA and PAI-1 (Goldhirsch et al., 2005), by validating their clinical utility further (Schrohl et al., 2003), in defining the most promising chemotherapeutic regimens for high-risk patients according to their high uPA/PAI-I levels, and in determining which chemotherapy-regimes may be best suited in combination with novel therapeutics targeting the uPA system. Additionally, markers must continuously prove useful in improving patient outcome, quality of life, and in lowering costs of care. Described by several authors, different procedures (sample-collection, -storage, -processing) and different assay formats may yield different results, which diminish the crucial role of reproducibility and of quality control. Hence, meeting **protocols of quality assurance and standardization** such as the **EORTC preliminaries** (www.eortc.be) or previously described "**Evaluation guidelines for possible new markers**" or **Standard Operating Procedures** (Graeff, Janicke, and Schmitt, 1991; Schmitt et al., 2007) are indispensable and will create the highest quality with respect to quality development, management, and analysis.

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Publications

In accordance with the regulations issued by the deanship of the Technische Universität, München, Germany, part of the thesis in hand has been published in the “Journal of Clinical Ligand Assay” (www.ingentaconnect.com/content/clas/jcla) “The urokinase protease system as a target for breast cancer prognosis and therapy: Technical considerations” under the patronage of Prof Dr Schmitt and Prof Dr Harbeck (Schmitt et al., 2002). The author of the thesis in hand is co-author to the publication.

A second publication is scheduled for “Breast Cancer” (www.karger.com/brc_guidelines).

Index of Figures and Tables

List of Materials

Materials used and corresponding companies

Product	Company
96 Well Optical Bottom Plates	Nunc GmbH & Co.KG; Wiesbaden, Germany
Bicinchoninic acid protein assay kit (BCA)	Pierce Biotechnology, P.O. Box 117, Rockford, IL, USA
Cryobank vials and Bank-It™ - Tube System	Nunc GmbH & Co.KG, Wiesbaden, Germany
Cryogenic Vials (#5011 1.2ml)	Nalgene Europe Ltd, Neerijse, Belgium
ELISA kit uPA # 894	American Diagnostica Incorporated, Stamford, CT, USA
ELISA kit PAI-1 #821	American Diagnostica Incorporated, Stamford, CT, USA
Lowry assay (Modified)	Pierce Biotechnology, P.O. Box 117, Rockford, IL, USA
Micro-Dismembrator II (#853162/4), Micro Dismembrator II conversion kit (#BBI-8531986)	B. Braun AG, Melsungen, Germany, now represented by Sartorius AG, Göttingen, Germany
Micro Dismembrator S (#BBI-8531609)	Sartorius AG, Göttingen, Germany
Cryobank vials and Bank-It™ - Tube System	Nunc GmbH & Co.KG; Wiesbaden, Germany
Potter-Elvehjem Tissue Homogenizer	Bellco Glass Incorporated, Vineland, NJ, USA
Sigma Albumin from bovine serum BSA A7030	Sigma-Aldrich Chemie GmbH, Munich, Germany
SPSS software packets for windows, 14.0.0	SPSS Inc., Chicago, IL, USA
Triton X-100	Sigma-Aldrich Chemie GmbH, Munich, Germany

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Curriculum Vitae

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Kongressbeiträge und Veröffentlichungen

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