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Analysis of Urokinase-type Plasminogen Activator (uPA), Plasminogen Activator Inhibitor Type-1 (PAI-1), and Urokinase Plasminogen Activator Receptor (uPAR) Protein Expression by Immunohistochemistry in Triple-Negative Breast Cancer (TNBC)

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1 Introduction

1.1 Breast cancer - a heterogeneous disease

Breast cancer affects millions of women worldwide and is by far the most common cancer in women. One in four women diagnosed with cancer has breast cancer, and in 2018, 2.1 million females were newly diagnosed with breast cancer. In the same year, breast cancer was the most common cause of cancer death in women and was responsible for 626,700 deaths a year *(Bray et al. 2018)*. In Germany, breast cancer comprises 30.5% of malignant diseases in women. One of eight German women is diagnosed with breast cancer during her lifetime. In 2014, 17,670 women died of breast cancer in Germany *(Kaatsch et al. 2017)*. Risk factors for breast cancer are, amongst others, early menarche, late menopause, childlessness, genetic disposition (breast cancer 1 and 2 gene (BRCA1/2) mutation), obesity, physical inactivity, recent use of oral contraceptives, menopausal hormone therapy, alcohol abuse, and smoking *(Kaatsch et al. 2017, Bray et al. 2018)*. Since around 1990, the mortality rate in developed countries has been stable or has decreased *(Torre et al. 2015)*. Because of both early detection and improved therapeutic strategies, the five-year survival rate in Germany is 88% *(Kaatsch et al. 2017, MunichCancerRegistry 2018)*.

Breast cancer is a complex and heterogeneous disease. Tumors strongly differ in their clinicopathological characteristics, response to therapy, and patient outcome. Multiple molecular subtypes of breast cancer have been distinguished via gene expression profiling: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, basal-like breast, normal breast-like, and claudin-low cancer *(Perou et al. 2000, Sorlie et al. 2001, Sorlie et al. 2003, Herschkowitz et al. 2007, Parker et al. 2009, Sotiriou and Pusztai 2009, Prat and Perou 2011)*. Surrogate definitions of molecular subclasses are obtained by immunohistochemical measurement of the estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67. Moreover, multigene expression assays provide additional information for the classification of tumors. Resulting clinicopathologic surrogate definitions are luminal A-like, luminal B-like (HER2-), luminal B-like (HER2+), HER2-positive (non-luminal), and triple-negative, as depicted in **Table 1** *(Goldhirsch et al. 2013)*. Patients with luminal A breast cancer have the best prognosis. In contrast, luminal B breast

cancer is associated with a poorer prognosis. Patients with HER2 overexpressing or basal-like tumors have the poorest survival *(Sorlie et al. 2001, Voduc et al. 2010, Prat and Perou 2011)*.

Intrinsic subtype	Clinicopathologic surrogate definition	%
Luminal A	**'Luminal A-like'** ER-positive PR-positive HER2-negative Ki-67 low[a] Recurrence risk low[b]	37.5
Luminal B	**'Luminal B-like (HER2 negative)'** ER-positive HER2-negative and Ki-67 high[a] or PR-negative or low or Recurrence risk high[b]	43.2
	'Luminal B-like (HER2 positive)' ER-positive HER2 overexpressed	8.0
HER2 overexpression	**'HER2 positive (non-luminal)'** ER-negative PR-negative HER2 overexpressed	3.1
'Basal-like'	**'Triple-negative'** ER-negative PR-negative HER2-negative	8.2

[a]The definition of an optimal cut point of Ki-67 levels is difficult. Ki-67 scores can be interpreted as follows: Ki-67-low: <10%, Ki-67-high: >20%-29% *(Coates et al. 2015)*.
[b] Recurrence risk-'high' or 'low' based on multigene expression assay (if available).

Table 1. Surrogate definitions of intrinsic subtypes of breast cancer *(Goldhirsch et al. 2013, MunichTumorCenter 2017)*.
ER: estrogen receptor, HER2: human epidermal growth factor receptor 2, PR: progesterone receptor.

1.2 Prognostic and predictive factors in breast cancer

The key to improved cancer treatment is personalized medicine, or rather, precision medicine *(Dowsett and Dunbier 2008, Duffy and Crown 2008, Harbeck et al. 2010)*. In the past, every patient with breast cancer received similar treatment. However, as mentioned above, breast cancer displays immense heterogeneity. The different molecular subtypes of breast cancer are characterized by distinct clinicopathological behavior and therapeutic responses *(Perou et al. 2000, Sorlie et al. 2001, Herschkowitz et al. 2007, Parker et al. 2009, Sotiriou and Pusztai 2009, Prat and Perou 2011)*. Undifferentiated treatment approaches in breast cancer patients can lead to undertreatment or overtreatment *(Dowsett and Dunbier 2008)*. Therefore, individual assessment of every tumor is essential for well-founded decision making concerning the therapeutic strategy *(Dowsett and Dunbier 2008, Duffy and Crown 2008, Harbeck et al. 2010, Harbeck et al. 2014)*. Over the last several years, cancer researchers focused on finding new factors that enabled the determination of prognosis and prediction of the response to therapy and provided new targets for personalized treatment options *(Dowsett and Dunbier 2008, Duffy and Crown 2008, Harbeck et al. 2010, Schmitt et al. 2010, Crown et al. 2012)*. Established parameters to evaluate prognosis and predict the response to therapy are tumor size, nodal status, lymphovascular invasion, distant metastasis, histological subtype, grading, and age. Besides the TNM-staging system and histological classification, determination of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) statuses are performed in routine clinical practice *(Senkus et al. 2015, Curigliano et al. 2017, MunichTumorCenter 2017, AGOBreastCommittee 2018, AWMF-S3-Leitlinie 2018)*. Expression of the estrogen and/or PR is associated with better survival and indicates therapy with tamoxifen, a selective ER modulator. High expression of the hormone receptor (HR) is associated with a poor response to chemotherapy. Conversely, the negativity of the HR is predictive for a good response to neoadjuvant chemotherapy *(MunichTumorCenter 2017, AGOBreastCommittee 2018)*. Amplification of HER2 is associated with a poor survival and indicates anti-HER2 therapy. In addition, HER2-positive breast cancer responds better to neoadjuvant chemotherapy *(MunichTumorCenter 2017, AGOBreastCommittee 2018)*.

Since 2007, the guidelines of both the American Society for Clinical Oncology (ASCO) and the German AGO Breast Committee (Arbeitsgemeinschaft Gynäkologische Onkologie) recommend two other forecast cancer biomarkers, urokinase plasminogen activator (uPA) and plasminogen activator inhibitor type-1

(PAI-1) *(Harris et al. 2016, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. Both uPA and PAI-1 are prognostic and predictive biomarkers that have reached the highest level of evidence, LOE-1. LOE-1 is attained by fulfilling the criteria of systematic reviews of randomized controlled trials *(Schmitt et al. 2010, AGOBreastCommittee 2018)*. High levels of uPA and PAI-1 are associated with a poor prognosis *(Schmitt et al. 2008, Schmitt et al. 2010, Duffy et al. 2014)*. It has been found that patients with high expression of uPA and PAI-1 benefit more from adjuvant cytotoxic chemotherapy than do patients with low levels of uPA and PAI-1 *(Harbeck et al. 2002a, Harbeck et al. 2002b, Manders et al. 2004, Borstnar et al. 2010)*. Based on the results of the Chemo-N0 trial, both ASCO and the German AGO Breast Committee guidelines recommend determination of uPA and PAI-1 in breast cancer patients with a borderline indication for adjuvant cytotoxic chemotherapy *(Harbeck et al. 2013, Harris et al. 2016, AGOBreastCommittee 2018)*. Analysis of uPA and PAI-1 status is advised in node-negative, HR-positive, HER2-negative, G2 breast cancer *(MunichTumorCenter 2017)*. Use of uPA and PAI-1 are currently not recommended to guide the decision on chemotherapy in triple-negative breast cancer (TNBC) *(Harris et al. 2016)*. The clinical relevance of uPA and PAI-1 are described in detail in chapter 1.4.2.1.

Another prognostic and predictive biomarker in breast cancer is Ki-67, which is associated with cell proliferation *(Denkert et al. 2013, Coates et al. 2015)*. However, there is controversy regarding the interpretation of Ki-67 levels because no optimal cut point has been determined for Ki-67 scores. Currently, the level of Ki-67 is interpreted in routine clinical tests as follows: Ki-67 low: <10%, Ki-67 high: >20%-29%. Medium Ki-67 levels are not recommended to guide the decision on chemotherapy *(Coates et al. 2015, Curigliano et al. 2017, MunichTumorCenter 2017, AWMF-S3-Leitlinie 2018)*. Besides the difficulty of defining an optimal Ki-67 cut point, the measurement of Ki-67 lacks reproducibility *(Harris et al. 2016, AWMF-S3-Leitlinie 2018)*. Because of technical problems with determining the level of Ki-67, ASCO recommends against the widespread clinical use of Ki-67 for decision making in adjuvant chemotherapy *(Harris et al. 2016)*. In contrast, guidelines of the German AGO Breast Committee and the AWMF (Association of the Scientific Medical Societies in Germany) advise the determination of Ki-67 as both a prognostic and predictive parameter in breast cancer *(AGOBreastCommittee 2018, AWMF-S3-Leitlinie 2018)*. The determination of Ki-67 is used to differentiate between luminal A and luminal B-like breast cancer *(Coates et al. 2015, MunichTumorCenter 2017)*. Moreover, Ki-67 is a strong predictor of a pathologic complete response (pCR). High levels of Ki-67 are associated with an increased response to neoadjuvant chemotherapy of

HR-positive disease *(Denkert et al. 2013, Nitz et al. 2014, Sonnenblick et al. 2015, Gluz et al. 2016)*. In contrast to ER-/PR-positive breast cancer, Ki-67 is not recommended as a predictive factor in TNBC to guide the decision on neoadjuvant chemotherapy *(AWMF-S3-Leitlinie 2018)*. In addition to its predictive value, Ki-67 is also a prognostic parameter. High Ki-67 expression is associated with a poor prognosis, particularly after neoadjuvant chemotherapy. The prognostic effect of Ki-67 is proven in patients with breast cancers of all subtypes and the subgroup of HR-positive patients, but not in patients with triple-negative disease *(de Azambuja et al. 2007, Denkert et al. 2013, Coates et al. 2015, Petrelli et al. 2015)*. Even though Ki-67 is significantly correlated with pCR, it has no prognostic effect in HR-negative breast cancer. Patients with HR-negative disease and high Ki-67 levels have a rather better outcome because of the high response rate to chemotherapy *(Denkert et al. 2013)*.

Commercially available molecular multigene tests, such as Oncotype DX®, MammaPrint®, Endopredict®, and Prosigna® are also in clinical use and provide enhanced prognostic information *(Duffy et al. 2014, Harbeck et al. 2014, Gyorffy et al. 2015)*. All four named tests have reached LOE-1 *(Harbeck et al. 2014, AGOBreastCommittee 2018)*. The MINDACT study analyzed the use of MammaPrint® in patients with early stage breast cancer and a high clinical risk of recurrence. It was found that MammaPrint® can define a patient group who is at low genomic risk for recurrence and, therefore, these patients can be spared from cytotoxic chemotherapy *(Cardoso et al. 2016)*. The West German Study Group (WSG) PlanB trial evaluated clinically high-risk pN0-1 early breast cancer patients with HR-positive, HER2-negative tumors, and a low Oncotype DX® Recurrence Score®. These patients were treated with adjuvant endocrine therapy alone, and their five-year disease-free survival without adjuvant chemotherapy was excellent (>94%). Given these findings, the use of Oncotype DX® can help to select lymph node-positive patients (pN0-1) who do not require cytotoxic chemotherapy, despite their high clinical risk of recurrence *(Nitz et al. 2017)*. Currently, the use of molecular multigene tests in routine clinical practice is only recommended for patients with HR-positive, HER2-negative breast cancer *(MunichTumorCenter 2017, AGOBreastCommittee 2018, Sestak et al. 2018)*. All four tests (Oncotype DX®, MammaPrint®, Endopredict®, Prosigna®) provide significant prognostic information in node-negative breast cancer patients to determine whether chemotherapy is indicated *(MunichTumorCenter 2017, Sestak et al. 2018)*. Patients with limited node-positive (pN0-1) disease also benefit from molecular multigene tests *(AGOBreastCommittee 2018, Sestak et al. 2018)*. However, it is essential to

combine clinical and genomic information to select these lymph node-positive patients who do not require chemotherapy *(Sestak et al. 2018)*. Similar to Ki-67, uPA, and PAI-1, the use of molecular multigene tests is presently not recommended to guide the decision on chemotherapy in TNBC patients *(MunichTumorCenter 2017)*.

The presence of tumor-infiltrating lymphocytes (TILs) at diagnosis and tumor response at the time of surgery are two parameters that are especially valuable in TNBC. A pathologic complete response (pCR) is defined as the absence of all cancer cells in the breast, angioinvasion, and lymph node metastasis after treatment with neoadjuvant cytotoxic chemotherapy. Achievement of a pCR is associated with a favorable outcome. This applies in particular to triple-negative and HER2-positive breast cancer *(von Minckwitz et al. 2012, Amoroso et al. 2015, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. Another prognostic and predictive marker in breast cancer is the presence of TILs at diagnosis. Lymphocyte-predominant breast cancer (LPBC) shows a score of over 50% TILs. An increased TILs score can predict the response to neoadjuvant cytotoxic chemotherapy. A prognostic impact has been found solely in the triple-negative and HER2-positive breast cancer subtypes: high TIL concentration is associated with better survival *(Salgado et al. 2015, MunichTumorCenter 2017, AGOBreastCommittee 2018, Denkert et al. 2018)*. However, at present, TILs should not be taken into account for the therapeutic strategy outside of investigational studies *(MunichTumorCenter 2017, AWMF-S3-Leitlinie 2018)*.

Many other new prognostic and predictive parameters in breast cancer are currently being evaluated in ongoing studies *(MunichTumorCenter 2017)*.

1.3 Triple-negative breast cancer (TNBC) - a challenging breast cancer subtype

About 10%-15% of all breast cancers are triple-negative tumors *(Bauer et al. 2007, Dent et al. 2007, Rakha et al. 2007)*. Triple-negative breast cancer (TNBC) is characterized by the lack of estrogen receptor (ER < 1%) and progesterone receptor (PR < 1%) expression as well as the lack of human epidermal growth factor 2 (HER2) amplification. Overexpression of HER2 will be diagnosed, if there is intense immunohistochemical staining of over 10% of breast cancer cells (3+ staining pattern). In borderline cases (2+ staining pattern), additional analysis via in situ hybridization (ISH) is required *(AGOBreastCommittee 2018)*. The diagnosis of TNBC is technically easy compared with related intrinsic subtypes, such as basal-like breast cancer (BLBC) or claudin-low breast cancer, and therefore, is established in routine clinical practice. For the precise identification of intrinsic subtypes of breast cancer, the use of gene expression profiling is necessary. The different subtypes are defined by specific gene expression profiles. For example, amongst others, expression of basal cytokeratins (CK5/6) and epidermal growth factor receptor (EGFR) characterizes BLBC *(Perou et al. 2000, Nielsen et al. 2004, Prat and Perou 2011, Valentin et al. 2012)*. In contrast, claudin-low tumors are characterized by several properties including the low expression of claudins (3, 4, 7), occludin, and E-cadherin *(Herschkowitz et al. 2007)*. Since the exact diagnosis of intrinsic subtypes of breast cancer is a very complicated matter, a surrogate subtype classification system has been established in clinical practice based on conventional clinicopathological factors, such as ER, PR, HER2, or Ki-67. Consequently, triple-negative tumors are roughly assigned to the intrinsic subtype BLBC. However, not all basal-like tumors display triple-negativity. Only about 60%-80% of BLBCs are attributed to the triple-negative subgroup *(Bertucci et al. 2008, Foulkes et al. 2010, Prat and Perou 2011)*. Furthermore, not all TNBCs have a basal-like phenotype. Only about 50%-80% of triple-negative tumors are basal-like *(Foulkes et al. 2010, Lehmann et al. 2011, Perou 2011, Prat and Perou 2011, Kalimutho et al. 2015)*. TNBC is a highly diverse group of tumors. It can be subdivided by gene expression profiling into several subtypes including two basal-like, an immunomodulatory, a mesenchymal, a mesenchymal stem-like, and a luminal androgen receptor subtype *(Lehmann et al. 2011)*.

Despite the heterogeneity of TNBC, its triple-negativity is significantly correlated with several characteristics. TNBC is associated with younger age at diagnosis (<40 years), African-American and Hispanic ethnicity, and breast cancer 1/2 gene (BRCA1/2) mutations (**Figure 1**) *(Lakhani 2002, Carey et al. 2006, Bauer et*

al. 2007, Azim et al. 2012, Boyle 2012, Couch et al. 2015). About 10%-20% of patients with TNBC have mutations in the BRCA1 and BRCA2 genes *(Atchley et al. 2008, Chacón and Costanzo 2010, Couch et al. 2015)*. About 60%-90% of BRCA1-related tumors display a triple-negative phenotype *(Haffty et al. 2006, Chacón and Costanzo 2010, Foulkes et al. 2010)*. Consequently, it is suggested to determine the BRCA1/2 status of all TNBC patients, and in particular, in patients who are younger than 60 years *(Couch et al. 2015, Collignon et al. 2016, Curigliano et al. 2017, AGOBreastCommittee 2018)*.

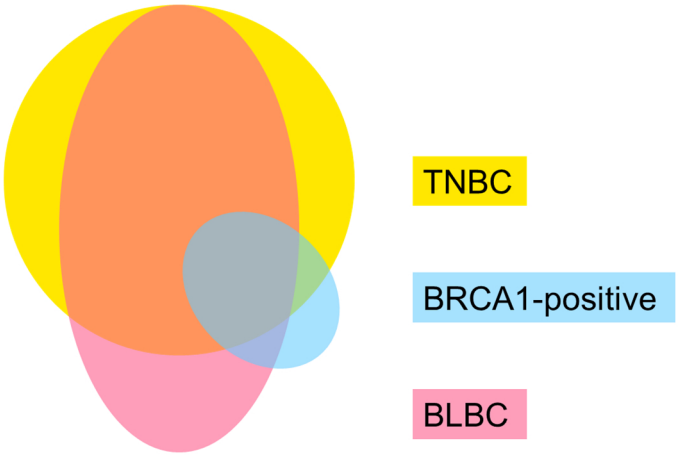

Figure 1. Schematic illustration of the overlap between TNBC, BLBC, and BRCA1-related tumors *(Pal et al. 2011)*.
BLBC: basal-like breast cancer, BRCA1: breast cancer 1 gene, TNBC: triple-negative breast cancer.

TNBCs show more aggressive clinical behavior compared with luminal tumors and patients with TNBC have a worse prognosis *(Haffty et al. 2006, Foulkes et al. 2010, Boyle 2012)*. At the time of diagnosis, the cancer is often advanced, and TNBC commonly displays a larger tumor size and shows a higher rate of lymph node positivity *(Criscitiello et al. 2012)*. However, there is no correlation between the tumor size and lymph node status of TNBC patients as 55% of women with small tumors (≤1 cm) present already with positive lymph nodes *(Dent et al. 2007, de Ruijter et al. 2011)*. The tumor aggressively metastasizes, preferably to the lung and the brain *(Liedtke et al. 2008, Dent et al. 2009)*. Metastatic TNBC rapidly progresses compared with other breast cancer subtypes. The triple-negative subgroup is associated with short survival after the diagnosis of distant metastasis *(Lobbezoo et al. 2013)*. Histological aspects can partially explain the characteristic behavior of TNBC. Most of these tumors are invasive ductal carcinomas of histological grade 3, showing high mitotic and proliferative indices, and necrotic areas *(Dent et al. 2007, Reis-Filho*

and Tutt 2008, Gluz et al. 2009). Consequently, Ki-67 expression is often elevated in TNBC *(de Ruijter et al. 2011)*. Relapse-free survival is shorter compared with non-triple-negative tumors. The risk of recurrence is increased, especially within 3 years after diagnosis and the 5-year mortality rate is higher. Interestingly, the difference in the risk of both recurrence and death diminishes after 5 years of therapy *(Sorlie et al. 2003, Dent et al. 2007, Rakha et al. 2007, Tischkowitz et al. 2007, Liedtke et al. 2008)*.

Locoregional treatment of TNBC involves surgery and radiation therapy and is similar to the treatment of other breast cancer subtypes. The combination of breast conservative surgery and adjuvant radiation therapy is the standard treatment. Since about 10%-20% of TNBC patients have a mutation in the BRCA1 gene, determination of BRCA status before surgery is recommended. Patients with the BRCA1 mutation should be counseled regarding the possible surgical option of risk-reducing mastectomy *(Senkus et al. 2015, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. Besides surgery and radiation therapy, presently, the only established systemic treatment option for TNBC patients is cytotoxic chemotherapy *(Coates et al. 2015, Senkus et al. 2015, Curigliano et al. 2017, AGOBreastCommittee 2018, AWMF-S3-Leitlinie 2018, Hwang et al. 2019)*. Use of agents, such as anthracyclines, taxanes, or platinum-based antineoplastics, is an effective treatment option for TNBC. Neoadjuvant chemotherapy should be the preferred therapeutic approach in TNBC patients *(Hanf et al. 2015, Curigliano et al. 2017)*. International and national guidelines, published by the National Comprehensive Cancer Network (NCCN), the European Society for Medical Oncology (ESMO), and the German AGO Breast Committee, and the St. Gallen International Expert Consensus Conference recommend an anthracycline-/taxane-based regimen for non-BRCA-associated TNBC *(Senkus et al. 2015, Curigliano et al. 2017, MunichTumorCenter 2017, AGOBreastCommittee 2018, Hwang et al. 2019)*. There is a controversy about whether carboplatin should be administered additionally. The addition of platinum compounds increases the rate of pCR but is associated with higher toxicity *(Senkus et al. 2015, Bartsch and Bergen 2017, Curigliano et al. 2017, MunichTumorCenter 2017, AWMF-S3-Leitlinie 2018, Loibl et al. 2018)*. The Munich Tumor Center (Tumorzentrum München), the German AGO Breast Committee, and ASCO (American Society of Clinical Oncology) recommend the addition of carboplatin in TNBC patients *(Bartsch and Bergen 2017, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. In contrast, both the ESMO and members of the St. Gallen International Expert Consensus Conference recommend against routine use of carboplatin in unselected TNBC patients *(Senkus et al. 2015, Curigliano et al.*

2017). Nevertheless, the ESMO 2018 guideline states that carboplatin is an important treatment option for advanced TNBC *(Cardoso et al. 2018)*. Contrary to unselected TNBC patients, there is clear agreement that BRCA1/2 mutation carriers benefit of treatment with the addition of carboplatin. Therefore, guidelines advise the use of platinum-based chemotherapy to treat BRCA-related tumors *(Senkus et al. 2015, Curigliano et al. 2017, MunichTumorCenter 2017, AGOBreastCommittee 2018)*.

Chemotherapy is very beneficial in TNBC patients. It even improves the outcome of TNBC patients to a greater extent compared with patients with ER-positive tumors. However, only a few patients have a pathologic complete response (pCR) and, therefore, an excellent outcome *(Foulkes et al. 2010, von Minckwitz et al. 2011, Cortazar et al. 2014)*. Most TNBC patients still have residual tumor after neoadjuvant chemotherapy and, thus, a relatively poor prognosis. Compared with patients with non-triple-negative tumors, the clinical outcome after chemotherapy remains worse *(Carey et al. 2007, Liedtke et al. 2008, De Laurentiisa et al. 2010, Foulkes et al. 2010, Ismail-Khan and Bui 2010, MunichTumorCenter 2017)*.

Currently, there is no established targeted therapy option available to treat TNBC *(Hwang et al. 2019)*. Some new agents are under investigation in TNBC, e.g., antiangiogenic agents targeting vascular endothelial growth factor (VEGF), poly(adenosine diphosphate (ADP)-ribose) polymerase (PARP) inhibitors, and immune checkpoint modulators *(Miller et al. 2007, Santana-Davila and Perez 2010, Carey et al. 2012, Baselga et al. 2013, O'Shaughnessy et al. 2014, Hein et al. 2015)*.

Bevacizumab, a monoclonal anti-VEGF antibody, is approved for therapy of metastatic breast cancer by the European Medicines Agency (EMEA) *(Marme and Schneeweiss 2015)*. The German AGO Breast Committee and the AWMF (Association of the Scientific Medical Societies in Germany) recommend adding bevacizumab to first-line cytotoxic therapy (paclitaxel or capecitabine) in metastatic TNBC *(AGOBreastCommittee 2018, AWMF-S3-Leitlinie 2018)*. However, the efficacy of treatment with bevacizumab is controversial *(Hwang et al. 2019)*. Several trials showed conflicting results evaluating the therapeutic benefit of bevacizumab in TNBC patients. The latest study, the randomized phase III BEATRICE trial, evaluating adjuvant bevacizumab-containing therapy has shown no improvement in long-term outcomes for early TNBC *(Bell et al. 2017, Curigliano et al. 2017)*. Therefore, bevacizumab is currently not approved to treat breast cancer by the FDA (Food and Drug Administration) *(Hwang et al. 2019)*.

PARP inhibitors (olaparib, veliparib) block the activity of the enzyme poly(ADP-ribose) polymerase , which is involved in the repair of DNA

(deoxyribonucleic acid) damage. Presently, the use of olaparib is recommended in BRCA mutation carriers with TNBC as part of a study *(Bartsch and Bergen 2017, MunichTumorCenter 2017, AGOBreastCommittee 2018, Hwang et al. 2019)*. The phase III OlympiAD trial demonstrated that olaparib monotherapy provides a significant benefit over standard chemotherapy amongst HER2-negative metastatic breast cancer patients with a BRCA mutation *(Robson et al. 2017)*. In contrast to olaparib, the use of veliparib did not significantly improve the outcome in patients with BRCA-associated TNBC *(Curigliano et al. 2017, Han et al. 2018)*. The BrighTNess study, evaluating the addition of veliparib to chemotherapy, also showed no additional benefit in TNBC patients *(Bartsch and Bergen 2017, Loibl et al. 2018)*.

Other new therapeutic approaches in TNBC are immune checkpoint modulators, such as nivolumab, pembrolizumab, and atezolizumab. Nivolumab and pembrolizumab are monoclonal antibodies that bind to the receptor PD-1 (programmed cell death protein 1) on lymphocytes and thereby allow the immune system to destroy cancer cells. Atezolizumab is also a monoclonal antibody, but, contrary to nivolumab and pembrolizumab, it is directed against PD-L1 (programmed death ligand 1). Still, the effect of atezolizumab is similar to nivolumab and pembrolizumab, since it reverses T-cell suppression. Early phase clinical trials showed promising results investigating immune checkpoint modulators in metastatic TNBC *(van Rooijen et al. 2015, Adams et al. 2018, Rinnerthaler et al. 2018, Schmid et al. 2018)*. Schmid et al. evaluated atezolizumab and nab-paclitaxel (albumin-bound paclitaxel) in metastatic TNBC. Treatment of TNBC patients with a combination of atezolizumab and nab-paclitaxel prolonged progression-free survival significantly *(Schmid et al. 2018)*.

There are multiple other experimental treatment approaches for TNBC. Guidelines recommend participation in clinical studies for patients with TNBC *(MunichTumorCenter 2017, AGOBreastCommittee 2018)*.

1.4 Urokinase plasminogen activator receptor (uPAR) interactome

1.4.1 Characteristics of the uPAR interactome

The plasminogen activation system consists of three major components: serine proteases (uPA, tPA), inhibitors of these serine proteases (PAI-1, PAI-2), and the specific receptor of uPA (uPAR/CD87) *(Mengele et al. 2010)*. Both uPA (urokinase-type plasminogen activator) and tPA (tissue-type plasminogen activator) are serine proteases activating plasminogen to plasmin. While tPA is primarily involved in intravascular fibrinolysis, uPA plays a role in cell migration, cell signaling, cell adherence and motility, degradation of the extracellular matrix, and cancer cell dissemination and metastasis *(Andreasen et al. 1997, Schmitt et al. 2003)*. Both PAI-1 and PAI-2 (plasminogen activator inhibitor type-1 and type-2) are not limited to their inhibitory function on uPA; PAI-1 is an independent regulating factor in cell adhesion, and migration and PAI-2 is involved in cell death by inhibiting apoptosis *(Stefansson and Lawrence 1996, Kjøller et al. 1997, Medcalf and Stasinopoulos 2005)*. The cell surface receptor of uPA, uPAR (urokinase plasminogen activator receptor), is associated with multiple other ligands, expanding the functions of the uPAR system *(Eden et al. 2011)*.

1.4.1.1 Characteristics of uPAR

Urokinase plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol (GPI)-anchored cell surface receptor comprising three domains, D1-D3. Having no intracellular domain, uPAR laterally interacts with several ligands, such as integrins, receptor tyrosine kinases, and G protein-coupled receptors (**Figure 2**) *(Reuning et al. 2003a, Smith and Marshall 2010, Eden et al. 2011)*. Currently, 42 proteins have been shown to interact directly with the uPAR, 33 lateral ligands, and 9 soluble partners. These soluble ligands comprise urokinase-type plasminogen activator (uPA), its inhibitor plasminogen activator inhibitor type-1 (PAI-1), and vitronectin (VN), a cofactor of PAI-1 *(Eden et al. 2011)*.

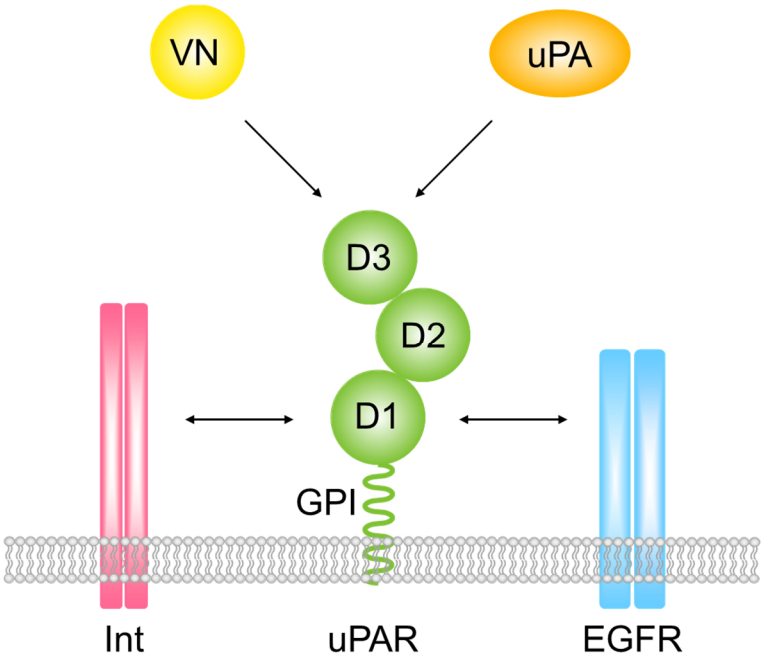

Figure 2. Structure of uPAR, consisting of a glycosylphosphatidylinositol (GPI)-anchor and three other domains (D1-D3). Lateral interaction of uPAR is necessary since it lacks an intracellular domain. Transmembrane proteins laterally interact with uPAR, for example, integrins and epidermal growth factor. There are several soluble ligands of uPAR, such as uPA and VN *(Mengele et al. 2010)*.
D1-D3: domain 1-3, EGFR: epidermal growth factor receptor, GPI: glycosylphosphatidylinositol, Int: integrin, uPA: urokinase-type plasminogen activator, uPAR: urokinase plasminogen activator receptor, VN: vitronectin.

uPAR holds a strategic position in multiple metabolic processes. The uPAR interactome is involved in blood coagulation, tissue remodeling, chemotaxis, and cell proliferation. It regulates fibrinolysis, cell growth, angiogenesis, and apoptosis. Furthermore, the uPAR system influences cell adherence, cell migration, and cell dissemination. In malignant tissues, it affects tumor cell invasion, tumor cell dissemination, and metastasis. Several processes increase the concentration of components of the uPAR interactome, e.g., inflammation, infection, malignancy, tissue injury, and hypoxia *(Blasi and Carmeliet 2002, Reuning et al. 2003b, Ulisse et al. 2009, Blasi and Sidenius 2010, Mengele et al. 2010, Eden et al. 2011)*.

1.4.1.2 Characteristics of uPA

The serine protease urokinase-type plasminogen activator (uPA) is the main ligand of the urokinase plasminogen activator receptor (uPAR). Pro-uPA, the enzymatically inactive precursor form of uPA, can also bind to uPAR. Several proteases, e.g., plasmin, can activate pro-uPA to high molecular weight (HMW)-uPA. HMW-uPA consists of two chains; chains A and B. Chain A contains the receptor-reactive growth factor domain (GFD), which allows binding to the uPAR. Furthermore, a kringle domain and an internal domain are located on the A-chain. Chain B contains the catalytic center. HMW-uPA can lose its capacity of binding to uPAR, e.g., by cleavage by plasmin. The resulting low molecular weight (LMW)-uPA lacks the amino-terminal fragment (ATF), consisting of the GFD and the kringle domain. Both HMW-uPA and LMW-uPA are catalytically active forms and can convert plasminogen into plasmin. The primary function of plasmin is fibrinolysis, i.e., the cleavage of fibrin, but plasmin also degrades other constituents of the extracellular matrix. **Figure 3** depicts the interplay of these important molecules within the plasminogen activation system *(Blasi and Carmeliet 2002, Ulisse et al. 2009, Mengele et al. 2010, Eden et al. 2011)*.

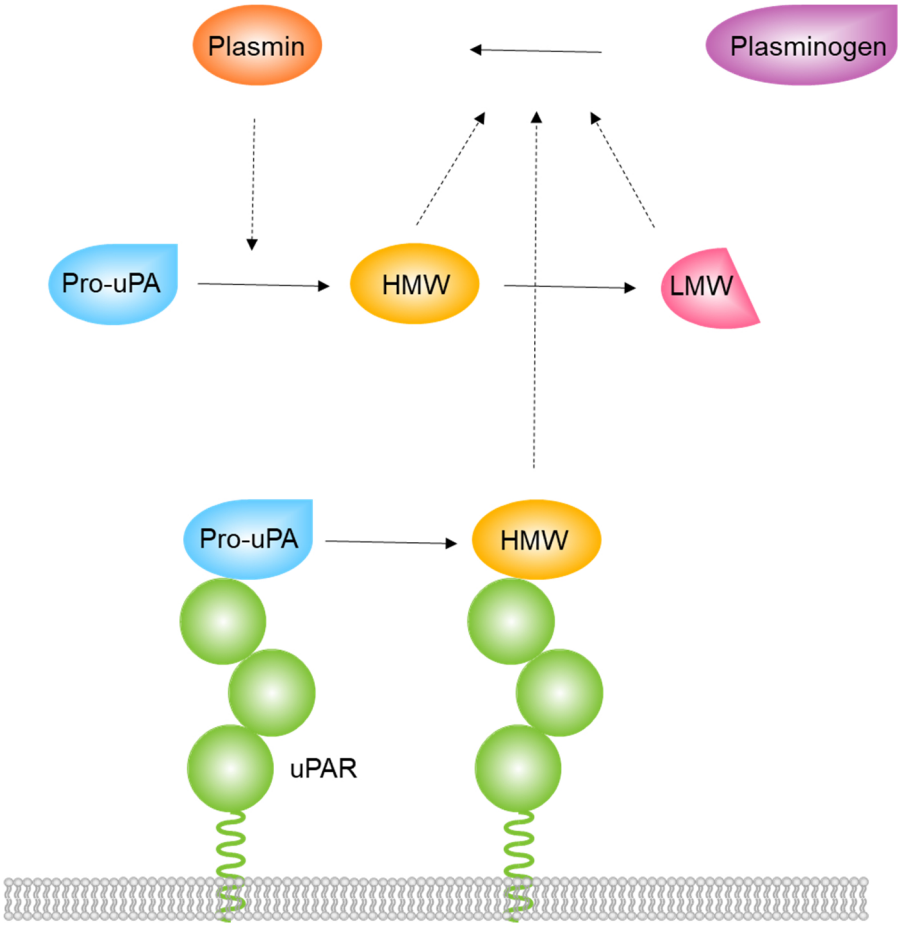

Figure 3. Interplay of uPA within the plasminogen activation system. The main ligand of uPAR is uPA. Pro-uPA, the enzymatically inactive precursor form of uPA, is activated by binding to uPAR or interacting with plasmin. The active forms of uPA, HMW-uPA, and LMW-uPA are both able to convert plasminogen into plasmin. In contrast to HMW-uPA, LMW-uPA lacks the amino-terminal fragment and, therefore, loses the capacity of binding to uPAR *(Mengele et al. 2010)*.
HMW-uPA: high molecular weight urokinase-type plasminogen activator, LMW-uPA: low molecular weight urokinase-type plasminogen activator, uPA: urokinase-type plasminogen activator, uPAR: urokinase plasminogen activator receptor.

1.4.1.3 Characteristics of PAI-1

Urokinase-type plasminogen activator (uPA)-dependent proteolysis is modulated by several plasminogen activator inhibitors; the main inhibitor is plasminogen activator inhibitor type-1 (PAI-1) (**Figure 4**). PAI-1 interacts with both uPA and vitronectin (VN). The binding of PAI-1 to high molecular weight urokinase-type plasminogen activator (HMW-uPA) inhibits the enzymatic activity of uPA. Besides its inhibitory function, PAI-1 is involved in multiple cellular processes. The interaction of PAI-1 with the uPA-uPAR (urokinase plasminogen activator receptor) complex results in both the internalization of the uPA-uPAR-PAI-1 triad via endocytosis and disruption of cell-signaling pathways of uPAR. Integrins, which are bound to uPAR, are simultaneously internalized, and both are recycled and returned to the cell surface. Internalization of uPA and PAI-1 induce their degradation. The active conformation of PAI-1 is quite unstable with a half-life of approximately 1-3 h, but binding to VN approximately doubles the half-life of PAI-1. Interaction with VN not only stabilizes PAI-1 but also expands its functions. VN is a glycoprotein and a component of the extracellular matrix. It interacts with PAI-1, uPAR, and multiple integrins *(Blasi and Carmeliet 2002, Ulisse et al. 2009, Mengele et al. 2010, Eden et al. 2011)*.

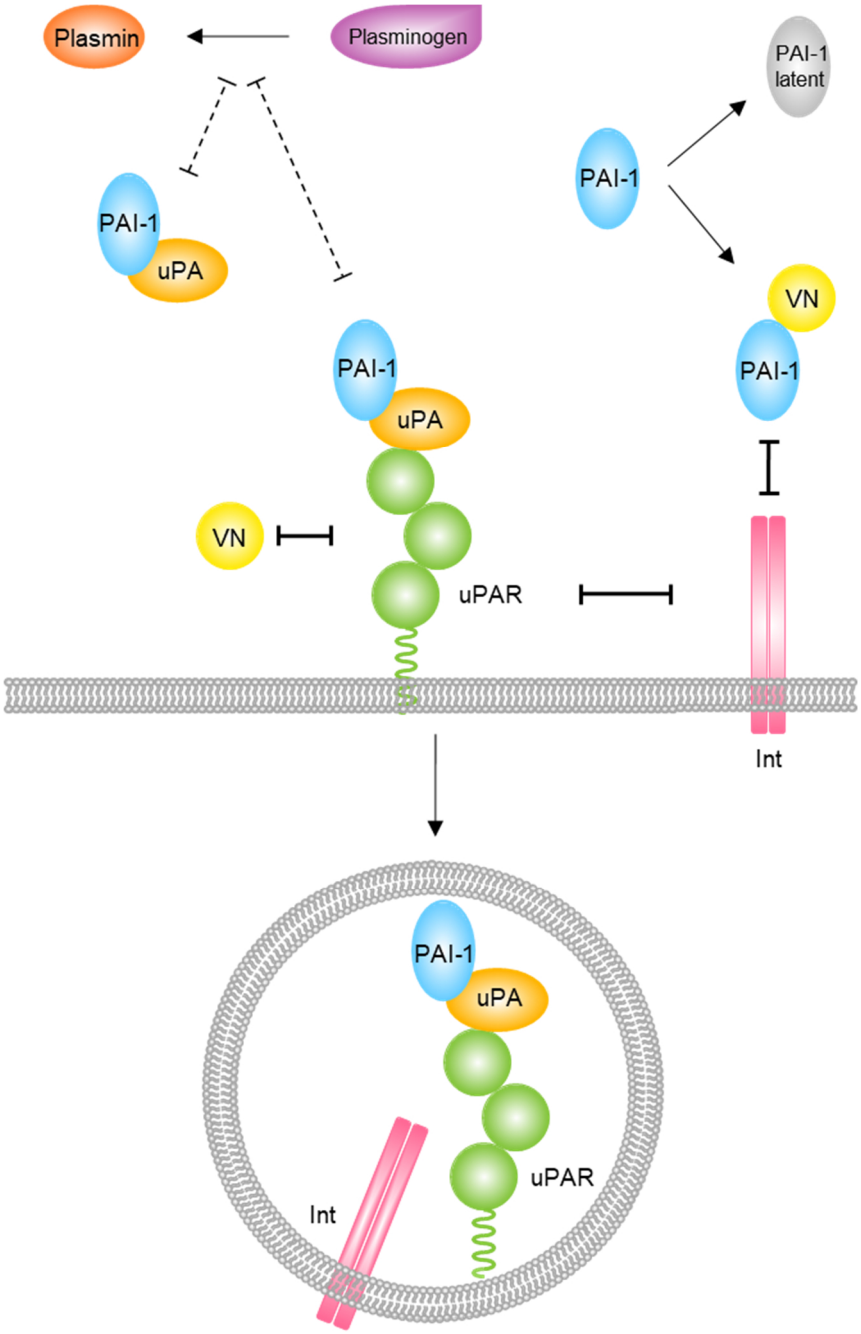

Figure 4. Interplay of PAI-1 within the uPAR interactome. The main modulator of uPA-dependent proteolysis is PAI-1. Binding of PAI-1 to HMW-uPA inhibits the enzymatic activity of uPA. Interaction of PAI-1 with the uPA-uPAR complex results in internalization of the uPA-uPAR-PAI-1 triad via endocytosis and disruption of cell-signaling pathways of uPAR. PAI-1 can bind to VN, which approximately doubles the half-life of PAI-1. The PAI-1-VN complex inhibits integrin mediated cell signaling *(Mengele et al. 2010)*.
Int: integrin, PAI-1: plasminogen activator inhibitor type-1, uPA: urokinase-type plasminogen activator, uPAR: urokinase plasminogen activator receptor, VN: vitronectin.

1.4.2 Clinical relevance of uPA, PAI-1, and uPAR

1.4.2.1 Clinical relevance of uPA and PAI-1

Expression of uPA (urokinase plasminogen activator) and PAI-1 (plasminogen activator inhibitor 1) is elevated in most solid cancers, such as malignant tumors of the ovary, esophagus, stomach, colorectum, kidney, lung, liver, or breast *(Schmitt et al. 2000)*. Overexpression of uPA and PAI-1 in malignant tissue is correlated with tumor aggressiveness, shorter disease-free survival, and worse outcome *(Duffy et al. 1988, Janicke et al. 1989, Jänicke et al. 1990, Jänicke et al. 1991, Harbeck et al. 2007)*. The uPA/PAI-1 system is associated with tumor cell proliferation, invasion, dissemination, and metastasis. Among others, it is involved in fibrinolysis and both degradation and remodeling of the extracellular matrix *(Jänicke et al. 1991, Mengele et al. 2010, Duffy et al. 2014)*.

The biomarkers, uPA and PAI-1, have been studied intensively in breast cancer in the past. **Figure 5** provides a summary of important steps to raise clinical awareness about both uPA and PAI-1 *(Schmitt et al. 2011)*. Both biomarkers have proven to be of independent prognostic and predictive value in breast cancer and consequently have reached the level of evidence 1 *(Duffy et al. 2014)*. High levels of uPA or PAI-1 are significantly associated with a poor prognosis *(Look et al. 2002, Schmitt et al. 2008, Schmitt et al. 2010, Duffy et al. 2014)*. The prospective clinical therapy trial Chemo-N0 demonstrated that both uPA and PAI-1 are valid biomarkers for classifying risk groups in node-negative breast cancer patients. Node-negative breast cancer patients with low levels of uPA and PAI-1 have a low risk of disease recurrence (<10%). These patients can be spared from adjuvant systemic chemotherapy. Patients with high uPA and PAI-1 expression have a high risk of metastasis and, therefore, benefit from adjuvant systemic chemotherapy. The risk of recurrence in high-risk patients is reduced by 40% because of treatment with chemotherapy *(Jänicke et al. 2001, Annecke et al. 2008, Schmidt et al. 2009, Kantelhardt et al. 2011, Harbeck et al. 2013, Jacobs et al. 2013, MunichTumorCenter 2017)*. Based on the results of the Chemo-N0 trial, since 2007, guidelines of the American Society for Clinical Oncology (ASCO) and the German AGO Breast Committee (Arbeitsgemeinschaft Gynäkologische Onkologie) recommend the measurement of both uPA and PAI-1 in breast cancer patients with a borderline indication for adjuvant cytotoxic chemotherapy *(Harris et al. 2016, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. Analysis of uPA and PAI-1

status is advised in node-negative, hormone receptor (HR)-positive, HER2-negative, G2 breast cancer *(MunichTumorCenter 2017)*.

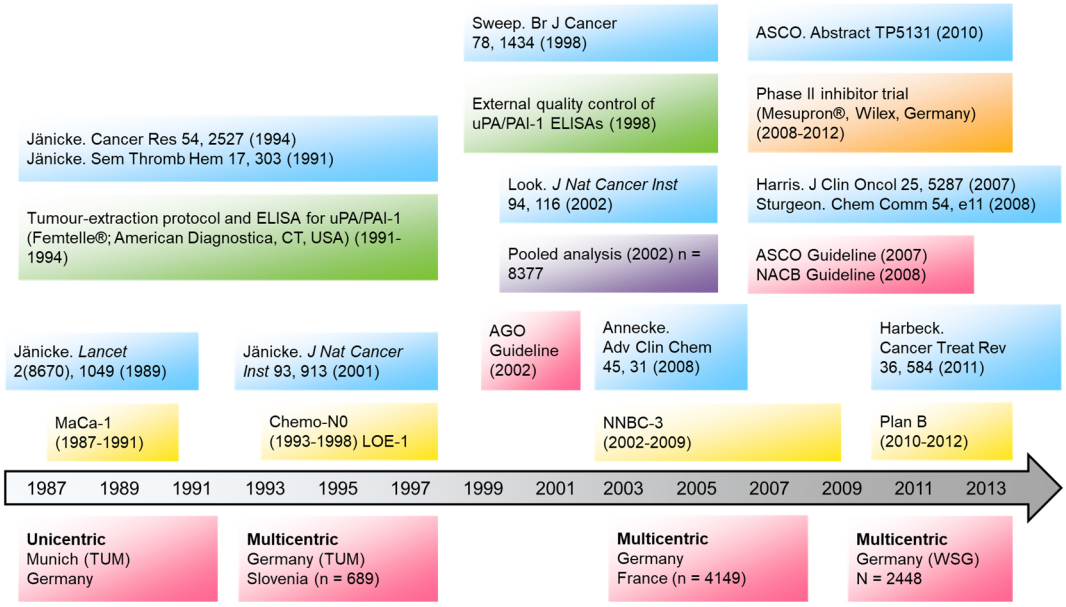

Figure 5. Important steps in assessing the clinical relevance of uPA and PAI-1 *(Janicke et al. 1989, Jänicke et al. 1991, Jänicke et al. 1994, Sweep et al. 1998, Jänicke et al. 2001, Look et al. 2002, Harris et al. 2007, Annecke et al. 2008, Sturgeon et al. 2008, Harbeck et al. 2010, Kantelhardt et al. 2011, Schmitt et al. 2011, Harbeck et al. 2013, Nitz et al. 2017)*. AGO: Arbeitsgemeinschaft Gynäkologische Onkologie, ASCO: American Society of Clinical Oncology, PAI-1: plasminogen activator inhibitor type-1, TUM: Technical University of Munich, uPA: urokinase-type plasminogen activator, WSG: West German Study Group.

Both uPA and PAI-1 are used in routine clinical practice, especially in Germany. FEMTELLE® is a commercial ELISA (enzyme-linked immunosorbent assay) test to determine the concentration of uPA/PAI-1 in fresh-frozen breast cancer tissue. Guidelines recommend 100-300 mg tissue for measuring uPA/PAI-1 *(Sturgeon et al. 2008, Kantelhardt et al. 2011, IQWiG 2014)*. Thomssen et al. showed that FEMTELLE® is also a reliable test in very small tumor tissue samples (10-30 mg), obtained by preoperative needle biopsy *(Thomssen et al. 2009)*. Accordingly, the company LOXO GmbH, which sells FEMTELLE®, recommends using at least 50 mg tissue for the determination of uPA and PAI-1 levels by ELISA *(LOXOGmbH 2019)*.

Besides their value as prognostic and predictive biomarkers, both uPA and PAI-1 are possible targets enabling the targeted treatment of cancer. The synthetic small molecule WX-UK1 is capable of inhibiting the catalytic activity of uPA. Various studies demonstrated the anti-metastatic and anti-proliferative activity of the 3-amidinophenylalanine-based WX-UK1 in vitro and in vivo *(Stürzebecher et al. 1999, Ertongur et al. 2004, Setyono-Han et al. 2005)*. WX-671 (upamostat), a prodrug of the active drug WX-UK1, can be administered orally and has so far been investigated in phase II clinical trials *(Schmitt et al. 2010)*. A double-blind, randomized phase II study compared combination treatment with upamostat, and capecitabine with capecitabine monotherapy in HER2-negative metastatic breast cancer. Goldstein et al. observed that upamostat significantly improved progression-free survival (PFS) in patients who had received prior adjuvant chemotherapy *(Goldstein et al. 2013)*. Another phase II clinical trial evaluated the combination of upamostat and gemcitabine versus gemcitabine alone in non-resectable, locally advanced pancreatic cancer. The treatment with upamostat improved patient survival; however, the difference between patient groups was not significant *(Heinemann et al. 2013)*. Both studies observed that upamostat was safe and well tolerated *(Goldstein et al. 2013, Heinemann et al. 2013, Duffy et al. 2014)*.

1.4.2.2 Clinical relevance of uPAR

In contrast to urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1), urokinase plasminogen activator receptor (uPAR) is not an established breast cancer biomarker in routine clinical tests. However, the expression of uPAR has been investigated in multiple studies and is elevated in breast carcinoma *(Needham et al. 1987, Jankun et al. 1993, Bianchi et al. 1994, Costantini et al. 1996, Fisher et al. 2000, Kotzsch et al. 2010)*. High levels of uPAR are found not only in breast cancer tissue but also are expressed in ovarian, colorectal, hepatocellular, gastric, esophageal, pancreatic, and bladder cancer tissue *(de Bock and Wang 2004)*. Especially at invasive fronts of the tumor, the expression of uPAR was found to be greater, which suggests that uPAR plays an important role in malignant cell invasion *(Carriero et al. 1994, Fisher et al. 2000)*. Expression of uPAR was also found to be elevated in stromal tissue of the tumor *(de Bock and Wang 2004, Kotzsch et al. 2010)*. Multiple studies analyzed the association between the expression level of uPAR and survival. The increased level of uPAR in breast

cancer tissue is associated with poor survival *(Carriero et al. 1994, Duggan et al. 1995, Grøndahl-Hansen et al. 1995, Costantini et al. 1996, Dublin et al. 2000, Guyton et al. 2000, Giannopoulou et al. 2007, Kotzsch et al. 2010)*. However, compared with uPA and PAI-1, the prognostic impact of uPAR in breast cancers seems to be less powerful *(de Bock and Wang 2004)*.

1.4.3 Determination of uPA, PAI-1, and uPAR by immunohistochemistry

In this study, urokinase plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1), and urokinase plasminogen activator receptor (uPAR) are analyzed by immunohistochemistry (IHC), which is a reliable method for determination of uPA, PAI-1, and uPAR levels *(Jänicke et al. 1990, Reilly et al. 1992, Schmitt et al. 2008, Kotzsch et al. 2010, Mengele et al. 2010, Eljuga et al. 2011, Lang et al. 2013)*. The enzyme-linked immunosorbent assay (ELISA) is an alternative method for uPA, PAI-1, and uPAR measurement allowing a quantitative determination. In contrast to IHC, ELISA requires fresh or frozen tissue, and ideally, a rather large amount of material (about 100-300 mg) *(Sturgeon et al. 2008, Kantelhardt et al. 2011, IQWiG 2014)*. IHC-based analysis can also use formalin-fixed, paraffin-embedded material. Besides, IHC requires only small amounts of tissue. Another advantage over ELISA is that IHC provides additional information on tumor morphology. Several previous studies compared IHC and ELISA for the measurement of uPA and PAI-1. Staining intensity of uPA significantly correlates with uPA values gained by ELISA; the same applies to PAI-1 *(Jänicke et al. 1990, Reilly et al. 1992, Lang et al. 2013)*. In contrast to uPA and PAI-1, there are no direct comparisons available between IHC and ELISA for evaluating uPAR levels. However, both methods were frequently used in previous studies and proved to enable reliable determination of uPAR *(Hildenbrand et al. 2009, Kotzsch et al. 2010)*.

2 Aim

Prognostic and predictive factors are essential for more personalized cancer treatment. However, most established biomarkers are only recommended in non-triple-negative breast cancer. For example, the AWMF (Association of the Scientific Medical Societies in Germany) guideline advises against the use of Ki-67 as a predictive factor in triple-negative breast cancer (TNBC) *(AWMF-S3-Leitlinie 2018)*. Furthermore, the prognostic impact of Ki-67 is limited on hormone receptor-positive disease *(Denkert et al. 2013)*. Similar to Ki-67, the use of molecular multigene tests, such as Oncotype DX® or MammaPrint®, is currently not recommended to guide the decision on systemic treatment in TNBC. Guidelines of the German AGO (Arbeitsgemneinschaft Gynäkologische Onkologie) Breast Committee and the Munich Tumor Center advise limiting the use of multigene assays on estrogen receptor (ER)-positive, human epidermal growth factor 2 (HER2)-negative breast cancer *(MunichTumorCenter 2017, AGOBreastCommittee 2018)*. The prognostic and predictive biomarkers, urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1), are also not established in TNBC. ASCO (American Society of Clinical Oncology) advises against the measurement of uPA and PAI-1 in TNBC. Because of the lack of data, clinicians should not use uPA and PAI-1 to guide their decision on chemotherapy in the patient group with triple-negative disease *(Harris et al. 2016)*. Therefore, studies evaluating the prognostic and predictive impact of uPA and PAI-1 in TNBC are necessary. Several previous studies observed an inverse correlation between uPA/PAI-1 expression and ER/progesterone receptor (PR) expression, irrespective of HER2 status *(Jänicke et al. 1990, Jänicke et al. 1991, Bouchet et al. 1999, Look et al. 2002)*. The same applies to the receptor of uPA and PAI-1, the urokinase plasminogen activator receptor (uPAR). Both ER- or PR-negative breast cancer tissue express higher levels of uPAR *(Grøndahl-Hansen et al. 1995, Bouchet et al. 1999, de Witte et al. 2001)*. Since TNBC lacks both ER and PR expression *(AGOBreastCommittee 2018)*, analysis of uPA, PAI-1, and uPAR in TNBC is very interesting. Expression of uPA, PAI-1, and uPAR is expected to be elevated in TNBC, compared with other breast cancer subtypes. The overexpression of uPA, PAI-1, or uPAR in TNBC could be potential new targets of personalized therapy for TNBC patients. So far, the only established systemic treatment option for TNBC is cytotoxic chemotherapy *(Coates et al. 2015, Senkus et al. 2015, Curigliano et al. 2017,*

AGOBreastCommittee 2018). Patients with the triple-negative disease do not benefit from personalized therapy options, such as trastuzumab, a monoclonal antibody interfering with HER2, or tamoxifen, a selective ER modulator *(Joensuu and Gligorov 2012)*.

Therefore, the aim of this thesis is to analyze uPA, PAI-1, and uPAR in TNBC. Immunohistochemistry is used to demonstrate the histologic localization of uPA, PAI-1, and uPAR in TNBC tissue, and to determine the level of uPA, PAI-1, and uPAR. The data collected is correlated with follow-up data to study the clinical relevance of uPA, PAI-1, and uPAR in TNBC patients.

3 Materials and methods

3.1 Patient collective

The Klinik und Poliklinik für Frauenheilkunde des Klinikums rechts der Isar at the Technical University Munich routinely collects tissue of primary breast cancer patients. All patients included in the present study gave their written informed consent. The tissue derives from standard breast cancer surgery, e.g., mastectomy and breast conservative surgery. The tumor bank is linked with clinicopathological data, e.g., tumor stage, therapy received, and follow-up data. Based on this extensive bio- and data bank, a patient collective was built consisting of 251 patients with triple-negative breast cancer (TNBC), who have been treated in Klinikum rechts der Isar between 1988 and 2013. The Institute of Pathology at the Technical University Munich provided formalin-fixed, paraffin-embedded breast cancer tissue of these patients with TNBC, and constructed nine tissue microarrays (TMAs) containing core biopsies of all 251 paraffin blocks. Furthermore, the receptor status of every tumor was determined again to confirm the triple-negativity.

3.2 Materials

Table 2. Alphabetical list of reagents and equipment used for immunohistochemical staining.

Material	Company
Antibody diluent	ZUC025-500, Zytomed Systems, Berlin, Germany
Antibodies	
Polyclonal antibody #399R directed against urokinase-type plasminogen activator receptor, rabbit, anti-human	Sekisui Diagnostics (formerly American Diagnostica), Pfungstadt, Germany

Monoclonal antibody #3689 directed against urokinase-type plasminogen activator (B-chain), mouse, anti-human Monoclonal antibody #3471 directed against urokinase-type plasminogen activator (amino-terminal fragment), mouse, anti-human Monoclonal antibody #3785 directed against plasminogen activator inhibitor type-1, mouse, anti-human Monoclonal antibody #3786 directed against plasminogen activator inhibitor type-1, mouse, anti-human Monoclonal antibody IID7 directed against urokinase-type plasminogen activator receptor, mouse, anti-human.	In-house, provided by the Frauenklinik, Technical University of Munich, Munich
Citric acid monohydrate	C1909-500G, Sigma-Aldrich®, St. Louis, Missouri
Citric buffer	2,1g Citric acid monohydrate, 1l aqua dist., adjust ad a pH 6,0 adding sodium hydroxide solution
Coverslips	R. Langenbrinck, Teningen, Germany
DAB substrate kit high contrast	DAB5000plus, Zytomed Systems, Berlin, Germany
Electronic balance	BP 310 S, Sartorius, Göttingen, Germany
Ethanol 96%/70%	In-house, provided by the Institute of Pathology, Technical University of Munich, Munich
Heating plate	Rommelsbacher, Dinkelsbühl, Germany

Hematoxylin	Mayer's haemalaun, A0884,1000, AppliChem, Darmstadt, Germany
HRP-kit broad spectrum, ZytoChem Plus, biotinylated secondary antibody polyvalent (2)	HRP125, Zytomed Systems, Berlin, Germany
HRP-kit broad spectrum, ZytoChem Plus, streptavidin-HRP-conjugate (3)	HRP125, Zytomed Systems, Berlin, Germany
HRP one-step polymer, ZytoChem Plus, anti-mouse/-rabbit/-rat	ZUC053-100, Zytomed Systems, Berlin, Germany
Humidity chamber	
HCl (2 mol/L)	4328, Carl Roth, Karlsruhe, Germany
H_2O_2 (3%)	Aqua dist. + hydrogen peroxide 30%, K44176710305, 1.07210.0250, Emsure®, Merck, Darmstadt, Germany
Isopropanol	In-house, provided by the Institute of Pathology, Technical University of Munich, Munich
Light microscope	Axioskop, Carl Zeiss, Jena, Germany
Magnetic stirrer, RCT basic	IKA Labortechnik, Staufen, Germany
Microtome	Microm HM 335E, Microm GmbH, Walldorf, Germany
Microscope slides	SuperFrost Plus, # 03-0060, R. Langenbrinck, Teningen, Germany
Mounting medium	Pertex®, Medite GmbH, Burgdorf, Germany
Nanozoomer	Nanozoomer 2.0 HAT slide scanner, Hamamatsu Photonics, Herrsching am Ammersee, Germany
Paraffin	In-house, provided by the Institute of Pathology, Technical University of Munich, Munich
pH-meter	Schott, Mainz, Germany
Pipettes	Eppendorf
Pipette tips	Eppendorf

Pressure cooker	Ankoch-Automatik, WMF, Geislingen/ Steige, Germany
Scanning software NDP.view2	NanoZoomer virtual microscopy, Hamamatsu Photonics, Herrsching am Ammersee, Germany
NaCl	1.06404, Merck, Darmstadt, Germany
NaOH (2 mol/L)	HC383947,1.09136.1000, TitriPUR®, Merck, Darmstadt, Germany
Tris-buffered saline (TBS) application solution	tenfold dilution of TBS-stock in aqua dist.
TBS-stock	60,5 g Trisma®Base, 700 ml aqua dist., HCl (2 N), 90 g NaCl, adjust ad a pH 7.6 adding HCl (2N), storing at RT
Trizma Base (Tris[hydroxymethyl]aminomethane)	T-1503, Sigma®, St. Louis, Missouri
Vortex shaker	MS1 minishaker, Roth, Karlsruhe, Germany
Xylene	In-house, provided by the Institute of Pathology, Technical University of Munich, Munich

Table 2. Reagents and equipment used for immunohistochemical staining. DAB: 3,3‘-diaminobenzidine tetrahydrochloride, H_2O_2: hydrogen peroxide, HCl: hydrogen chloride, HRP: horseradish peroxidase, NaCl: sodium chloride, NaOH: sodium hydroxide, RT: room temperature, TBS: tris-buffered saline.

3.3 Tissue fixation and processing

The first step in the routine of tissue fixation and processing was that the surgical specimens were immediately transported to the Institute of Pathology at the Technical University Munich. The untreated tissue was systematically examined by a pathologist followed by at least eight hours of fixation in formalin (3.7%). Dehydration and paraffinization were performed using the protocol provided in **Table 3**. The formalin-fixed, paraffin-embedded tissue was cut using a microtome. The resulting serial sections (3 µm) were transferred onto slides and dried at room temperature overnight.

#	Step of procedure
1	Dehydration in ascending graded row of alcohols: 70% ethanol (1 h) 96% ethanol (45 min) 96% ethanol (1 h) 96% isopropanol (45 min) 96% isopropanol (2 x 1 h) Xylene (2 x 1 h)
2	Bathing in liquid paraffin (60 °C): 2 x 15 min 1 x 30 min 1 x 60 min
3	Embedding in paraffin
4	Cooling

Table 3. SOP for rehydration and paraffinization of the untreated surgical specimen.
SOP: standard operating procedures.

3.4 Construction of tissue microarray

Application of tissue microarrays (TMA) is an efficient and rapid method for immunohistochemical staining. Small sections of different tumors are arranged on one standard glass slide that allows the efficient handling of resources and rapid staining of many tumor samples *(Wan et al. 1987)*. Only small cylinders are punched out causing minimal destruction of the original tissue blocks *(Kononen et al. 1998)*. This technique reduces the consumption of both valuable antibodies and tissue material. For example, a single antibody preparation stains multiple tissue samples on one slide *(Wan et al. 1987)*.

The Institute of Pathology at the Technical University Munich produced nine TMAs comprising the material of all 251 TNBC cases of our patient collective (**Figure 6**). Standard histologic sections of formalin-fixed, paraffin-embedded tissue, selected by a pathologist, were punched by using a hollow needle (diameter 1.25 mm) Up to 40 tissue cores were relocated to corresponding holes in an acceptor block *(Rimm et al. 2015)*. To guarantee consistent and comparable results, it is necessary to punch at least two cores from each tumor when using a 1

mm core *(Badve et al. 2013)*. The TMA was completed by adding several control tissues (placenta, lymph node, and kidney). The new paraffin block was cut into 3 µm sections. The preparation of an exact map enabled the correct identification of all inserted tissue samples *(Badve et al. 2013)*.

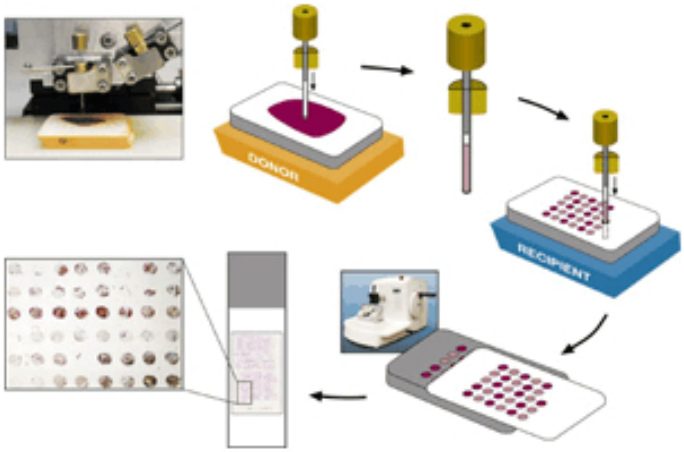

Figure 6. Construction of tissue microarrays. Standard histologic sections of formalin-fixed, paraffin-embedded tissue are punched by using a hollow needle (diameter 1.25 mm). Up to 40 tissue cores can be relocated to corresponding holes in an acceptor block. The new paraffin block usually is cut into 3 µm sections *(Rimm et al. 2015)*.

3.5 Immunohistochemical staining procedure

Immunohistochemistry allows the determination of protein expression in tissue. This technique is based on the affinity of antibodies to an antigen. The binding of antibodies to their specific antigen is visualized via complex detection systems. Standard operating procedures, which were established and used in this study, are depicted in the Results chapter.

3.5.1 Pretreatment

Several steps are necessary before antibody application on tissue slides can be successful.

3.5.1.1 Deparaffinization and rehydration

Only complete removal of wax from the tissue enables sufficient infiltration of aqueous reagents. Dewaxing paraffin-covered tissue slides is done by heating overnight at 53 °C, or for 20 min at 58 °C followed by incubation with xylene and 100% ethanol. For rehydration, slides are passed through a row of descending graded alcohol solutions (96%, 70%) *(Badve et al. 2013)*.

3.5.1.2 Antigen retrieval

Fixation with formalin leads to the modification of tissue structure. Changes in antigen conformation, such as the development of cross-links, prevent antibodies from binding to its specific epitope. The loss of its immunoreactivity can often be corrected by heat retrieval or enzymatic treatments of the tissue *(Badve et al. 2013)*. Heat-induced epitope retrieval (HIER) is performed by using a pressure cooker, water bath, autoclave, or microwave oven. There are several options of buffers to use for heat retrieval. Citrate buffer (pH 6.0) is commonly used in a multitude of laboratories. Another method of antigen demasking is proteolytic pretreatment that is achieved by the application of enzymes like proteinase k, pepsin, or trypsin. This limited proteolytic step can restore the original conformation of formalin-fixed tissue *(Badve et al. 2013)*.

3.5.1.3 Blocking

The presence of endogenous enzymes in tissue can be the cause of background staining, especially if endogenous enzymes are identical to those used for visualization of the antibody reaction. For example, horseradish peroxidase and alkaline phosphatase are commonly used in immunohistochemistry. Both enzymes physiologically exist in many tissues. False-positive staining, caused by endogenous enzymes, is reduced via application of enzyme blockers. In the case of horseradish peroxidase, a treatment option is to treat with 3% hydrogen peroxide, which inhibits its endogenous activity *(Badve et al. 2013)*.

3.5.2 Antibody incubation

After completion of the pretreatment procedure, diluted antibodies are applied to the tissue slide for a defined period. Pure antibody diluent is applied to the negative control tissue slide. Incubation of the antibody is performed in a humidity chamber to preserve dehydration of tissue slides.

Antibodies are classified into different groups. Polyclonal antibodies are a heterogeneous mix of antibodies and have a binding affinity for several epitopes of an antigen. Monoclonal antibodies are specifically directed against a single epitope. A primary antibody binds directly to the biomarker of interest, whereas a secondary antibody recognizes the constant part (Fc) of the primary antibody, linking the primary antibody to an enzyme complex *(Badve et al. 2013)*.

3.5.3 Visualization of the antigen-antibody-reaction

Multiple techniques allow the visualization of the primary reaction between the biomarker of interest and the primary antibody, such as the labeled streptavidin-biotin (LSAB) method and the polymer method. The LSAB method and the polymer method are both indirect visualization systems using a secondary antibody to detect the reaction of the primary antibody. The advantage of indirect staining is its increased sensitivity that allows the visualization of the smallest amounts of tissue-bound primary antibody *(Badve et al. 2013)*. An optimization process of repeated staining with changed parameters reveals optimal pretreatment, antibody dilution, and incubation time.

3.5.3.1 Labeled streptavidin-biotin (LSAB) method

The labeled streptavidin-biotin (LSAB) method is based on the strong affinity of streptavidin to biotin. First, the primary antibody binds to the tissue antigen. As a next step, a biotinylated secondary antibody is linked to the primary antibody. Furthermore, a streptavidin-peroxidase conjugate is added. Because of the strong affinity of streptavidin to biotin, the streptavidin-peroxidase complex binds to the biotinylated secondary antibody (**Figure 7**). In the next step, the peroxidase

enzyme molecules convert the added colorless 3,3'-diaminobenzidine tetrahydrochloride (DAB) to a brown product. Therefore, brown staining of the tissue indicates the target antigen. This method results in the linkage of one primary antibody to multiple peroxidase molecules. The resultant high enzyme-to-antibody ratio greatly improves sensitivity compared with direct staining methods.

Despite its advantages, the LSAB method has several limitations. One challenge is the nonspecific binding of streptavidin to other tissue components. However, compared with the Avidin-Biotin Complex (ABC) method, nonspecific tissue binding is significantly decreased using streptavidin instead of avidin. Another problem is the existence of endogenous biotin in tissues. Although both fixation and paraffinization significantly reduce endogenous biotin, background staining is observed occasionally. Blocking methods can further reduce endogenous biotin *(Badve et al. 2013)*.

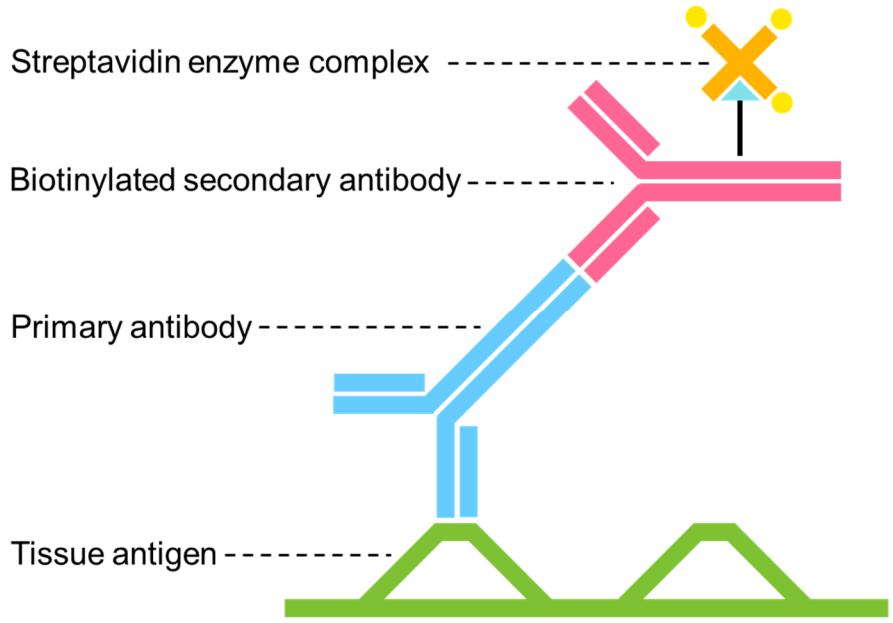

Figure 7. Labeled streptavidin-biotin method. This technique is based on the strong affinity of streptavidin to biotin. First, the primary antibody binds to the tissue antigen. The second step links a biotinylated secondary antibody to the primary antibody. Furthermore, the streptavidin-peroxidase complex is added and binds to the biotinylated secondary antibody. This method results in the linkage of one primary antibody to multiple peroxidase molecules. The enzyme peroxidase converts added colorless DAB to a brown substrate and, therefore, enables the target antigen to be determined by brown staining of the tissue *(Badve et al. 2013)*.

3.5.3.2 Polymer method

The polymer method is an advanced visualization system avoiding limitations of the streptavidin-biotin method, such as nonspecific binding of streptavidin or the existence of endogenous biotin. A long dextran polymer backbone is labeled with about 10 secondary antibodies and up to 70 horseradish peroxidase enzymes. This big construct binds to the primary antibody, which is directed against the tissue antigen to study. This technique results again in the linkage of one primary antibody to many peroxidase molecules. Similar to the LSAB method, added DAB is transformed by the enzymes into a brown product and therefore allows determining the target antigen by brown staining of the tissue (**Figure 8**). Binding of two different types of secondary antibodies (anti-mouse and anti-rabbit) to one dextran backbone facilitates universal detection of any primary mouse or rabbit antibody. Compared with the LSAB method, sensitivity is even a little higher.

A limitation of the polymer method can be the large molecule size of the polymer complex. Binding to some epitopes is possibly be hindered because of steric hindrance caused by the relatively large polymer backbone *(Badve et al. 2013)*.

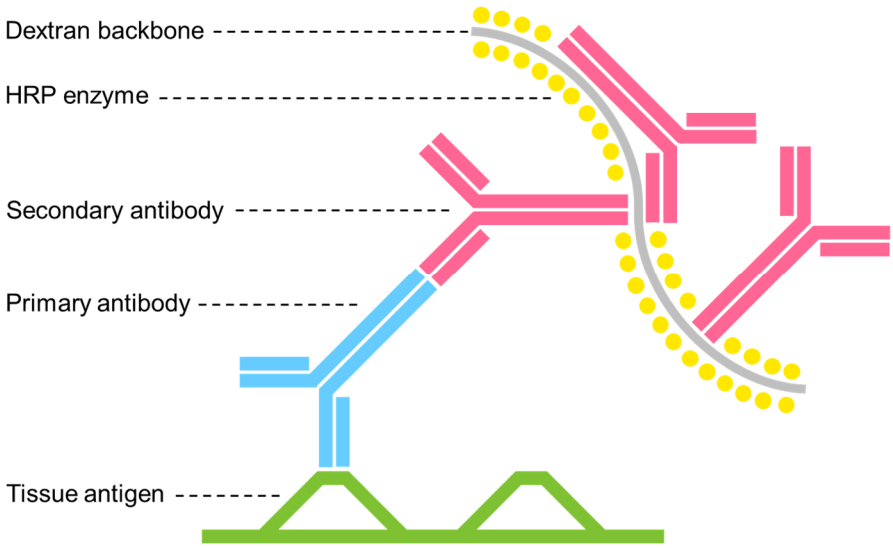

Figure 8. The polymer method. First, the primary antibody binds to the tissue antigen. The next step binds a long dextran polymer backbone, which is labeled with about 10 secondary antibodies and up to 70 enzymes, to the primary antibody. This technique results in linkage of one primary antibody to many peroxidase molecules. The enzyme peroxidase converts added colorless DAB to a brown substrate and, therefore, allows determining the target antigen by brown staining of the tissue *(Badve et al. 2013)*.
HRP: horseradish peroxidase.

3.5.4 Counterstaining

The tissues slides are counterstained by hematoxylin, which is commonly used in immunohistochemistry. The blue chromogen colors the nucleus allowing for improved assessment of the tissue.

3.6 Scanning of the stained tissue slides for digitalization

All stained tissue slides were scanned for digitalization using the Hamamatsu Nanozoomer 2.0. HAT slide scanner, Hamamatsu Photonics.

3.7 Histologic evaluation of immunohistochemical staining

Histologic evaluation of immunohistochemical staining was performed by the Institute of Pathology at the Technical University Munich. Prof. Dr. Michaela Aubele evaluated the stained tissue categorizing intensity groups. The staining of uPA and PAI-1 was classified into three groups (score 0-2). Tissue that showed no or weak staining intensity was assigned score 0. Score 1 was attributed to moderately stained breast cancer specimens. Intense staining of the tissue was assigned score 2. In contrast to uPA and PAI-1, uPAR was classified into 4 groups (score 0-3) because of its robust expression patterns: no or very weak staining intensity (score 0), moderately low staining intensity (score 1), moderately high staining intensity (score 2), and strong staining intensity (score 3).

3.8 Statistical analysis

Statistical analysis was performed using the software package SPSS statistics (IBM). To compute the statistical significance, two-tailed tests were used. A *p*-value of < 0.05 was considered statistically significant. Associations between clinicopathologic characteristics of TNBC patients and uPA/PAI-1/uPAR intensity scores of their tumors were analyzed using Pearson's Chi-squared test.

Histopathological and clinical characteristics, such as grading, tumor stage, distant metastasis, lymph node status, and menopausal status were analyzed. The Kaplan-Meier analysis was used to estimate the survival function. Three types of survival rates were differentiated: event-free survival (EFS), overall survival (OS), and disease-free survival (DFS). EFS described the time after primary treatment that the patient remained free of any events. Examples of an event were disease recurrence or death. OS was defined as the period between the time of primary surgery and the time of death; the cause of death was not specified. DFS was defined as the period between the time of eliminated disease and the time of relapse. The comparison of different Kaplan-Meier curves was made by the log-rank test (Mantel-Cox test).

4 Results

4.1 Optimization of immunohistochemical staining procedures for assessment of uPA, PAI-1, and uPAR protein expression in breast cancer tissue

The staining procedure was tested repeatedly to optimize staining results. The identification of optimal parameters, such as pretreatment, primary antibody concentration, the incubation time of the first antibody, incubation temperature, and visualization system is necessary to obtain high-quality staining. For optimization of the staining method, usage of first cuts of tissue microarrays (TMAs) is recommended *(Badve et al. 2013)*. The stained tissue was analyzed in cooperation with Prof. Dr. Michaela Aubele (Institute of Pathology at the Technical University Munich, Helmholtz Center Munich). Overall, five primary antibodies were tested, two monoclonal antibodies (mAb) directed against uPA (mAb #3689, mAb #3471), one monoclonal and one polyclonal antibody (pAb) directed against uPAR (mAb IID7, pAb #399R), and one monoclonal antibody directed against PAI-1 (mAb #3786). During the optimization process, two antibodies were ruled out because of their staining patterns: mAb #3471 directed against uPA and pAb #399R directed against uPAR. **Table 4-6** present resulting parameters gained in the optimization process that evaluated every step of the staining procedure. Different methods were compared with repeated testing of the staining procedure to identify optimal parameters.

	mAb #3689 (directed against uPA)
Antigen	uPA, chain B
Company	Sekisui Diagnostics (formerly American Diagnostica)
Clonality	Monoclonal
Host	Mouse
Isotype	IgG
Visualization	LSAB method
Dilution	1:2,100
Incubation time	2 h (RT)
Pressure cooking	No

Table 4. Characteristics of mAb #3698 (directed against uPA) used for visualization of uPA *(AD/SD 2015)*.
Ig: immunoglobulin, LSAB: labeled streptavidin-biotin, mAb: monoclonal antibody, uPA: urokinase-type plasminogen activator, RT: room temperature.

	mAb #3786 (directed against PAI-1)
Antigen	PAI-1
Company	Sekisui Diagnostics (formerly American Diagnostica)
Clonality	Monoclonal
Host	Mouse
Isotype	IgG
Visualization	LSAB method
Dilution	1:400
Incubation time	2 h (RT)
Pressure cooking	No

Table 5. Characteristics of the mAb #3786 (directed against PAI-1) used for visualization of PAI-1 *(AD/SD 2015)*.
Ig: immunoglobulin, LSAB: labeled streptavidin-biotin, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, RT: room temperature.

	mAb IID7 (directed against uPAR)
Antigen	uPAR
Company	In-house, provided by the Frauenklinik, Technical University of Munich, Munich
Clonality	Monoclonal
Host	Mouse
Isotype	IgG
Visualization	Polymer method
Dilution	1:600
Incubation time	Overnight (4 °C)
Pressure cooking	Yes

Table 6. Characteristics of the mAb IID7 (directed against uPAR) used for visualization of uPAR *(AD/SD 2015)*.
Ig: immunoglobulin, mAb: monoclonal antibody, uPAR: urokinase-type plasminogen activator receptor.

4.1.1 Standard operating procedures (SOPs) for immunohistochemical staining of uPA, PAI-1, and uPAR

SOPs containing detailed instructions to allow reproducibility of the complicated procedure were established. **Table 7 and 8** depict SOPs for the optimized staining procedure using the LSAB (uPA/PAI-1) and the polymer (uPAR) method for visualization. These SOPs are the result of repeated testing of the staining procedure.

#	Steps of procedure
1	Deparaffinization and rehydration in a descending graded row of alcohols: 2 x 10 min xylene 2 x 5 min 100% isopropanol 1 x 5 min 96% ethanol 1 x 5 min 70% ethanol
2	Washing in Tris-buffered saline (TBS) for 5 min, one intervening buffer change
3	Blocking of endogenous peroxidase activity by 3% hydrogen peroxide and incubation for 20 min at RT
4	Washing in normal tap water for 2 min
5	Washing in TBS for 5 min, one intervening buffer change
6	Application of one of the diluted primary antibodies, 120 µl per slide: - mAb #3689 (directed against uPA): dilution: 1:2,100 incubation for 2 h at RT - mAb #3786 (directed against PAI-1): dilution: 1:400 incubation for 2 h at RT
7	Washing in TBS for 5 min, one intervening buffer change
8	Application of the biotinylated secondary antibody and incubation for 30 min at RT, 120 µl per slide
9	Washing in TBS for 5 min, one intervening buffer change
10	Application of the streptavidin-HRP-conjugate and incubation for 30 min at RT, 120 µl per slide

11	Washing in TBS for 5 min, one intervening buffer change
12	Application of the chromogen DAB (dilution: 1:50) and incubation for 8 min at RT, 120 µl per slide
13	Washing in TBS for 5 min, one intervening buffer change
14	Counterstaining with hematoxylin for 50 sec
15	Blue-dyeing: washing under flowing normal tap water for 5 min
16	Transfer into distilled water
17	Dehydration in ascending row of graded alcohol each 3 min 1 x 3 min 70% ethanol 1 x 3 min 96% ethanol 2 x 3 min 100% isopropanol 2 x 3 min xylene
18	Sealing of slides with permanent mounting medium

Table 7. SOP for immunohistochemical staining of uPA/PAI-1 using the LSAB method for visualization of the primary antibody reaction.
DAB: 3,3‘- diaminobenzidine tetrahydrochloride, HRP: horseradish peroxidase, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, RT: room temperature, SOP: standard operating procedure, TBS: tris-buffered saline, uPA: urokinase-type plasminogen activator.

#	Steps of procedure
1	Deparaffinization and rehydration in a descending graded row of alcohols: 2 x 10 min xylene 2 x 5 min 100% isopropanol 1 x 5 min 96% alcohol ethanol 1 x 5 min 70% ethanol
2	Washing in TBS for 5 min, one intervening buffer change
3	Blocking of endogenous peroxidase activity by 3% hydrogen peroxide and incubation for 20 min at RT
4	Washing in normal tap water for 2 min
5	Washing in TBS for 5 min, one intervening buffer change
6	Pressure cooking for 4 min in citrate buffer (pH 6.0) Cooling down by washing in flowing normal tap water Washing in TBS for 5 min, one intervening buffer change

7	Application of the diluted primary antibody (mAb IID7 (directed against uPAR)), 120 µl per slide, and overnight incubation at 4 °C
8	Washing in TBS for 5 min, one intervening buffer change
9	Application of the HRP polymer and incubation for 30 min at RT, 120 µl per slide
10	Washing in TBS for 5 min, one intervening buffer change
11	Application of the chromogen DAB (dilution: 1:50) and incubation for 8 min at RT, 120 µl per slide
12	Washing in TBS for 5 min, one intervening buffer change
13	Counterstaining with hematoxylin for 50 sec.
14	Blue-dyeing: washing under flowing normal tap water for 5 min
15	Transfer into distilled water
16	Dehydration in ascending row of graded alcohol each 3 min 1 x 3 min 70% ethanol 1 x 3 min 96% ethanol 2 x 3 min 100% isopropanol 2 x 3 min xylene
17	Sealing of slides with permanent mounting medium

Table 8. SOP for immunohistochemical staining of uPAR using the polymer method for visualization of the primary antibody reaction.
DAB: 3,3‘- diaminobenzidine tetrahydrochloride, HRP: horseradish peroxidase, mAb: monoclonal antibody, RT: room temperature, SOP: standard operating procedure, TBS: tris-buffered saline, uPAR: urokinase-type plasminogen activator receptor.

4.2 Scoring results of immunohistochemical staining of uPA, PAI-1, and uPAR in triple-negative breast cancer (TNBC)

Immunohistochemical staining of formalin-fixed, paraffin-embedded (FFPE) TNBC specimens were scored by Prof. Dr. Michaela Aubele (Institute of Pathology at the Technical University Munich, Helmholtz Center Munich) evaluating the staining intensity of the nuclei of tumor cells, cytoplasm of tumor cells, and tumor stroma.

4.2.1 Scoring results of immunohistochemical staining of uPA in TNBC

Immunohistochemical staining of urokinase-type plasminogen activator (uPA) in FFPE TNBC specimens was performed using the monoclonal antibody (mAb) #3689 directed against uPA. Stained tissue slides were categorized into three staining categories: weak staining intensity (score 0), moderate staining intensity (score 1), and strong staining intensity (score 2). When comparing all stained tumor slides using mAb #3689 directed against uPA, several similarities were observed. The nuclei of tumor cells showed no or weak staining, whereas the cytoplasm of tumor cells presented intense staining. The tumor stroma was slightly stained because of the non-cellular part of the tumor stroma. **Table 9** depicts the distribution of intensity scores in immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA.

Score	n	%
0	71	45
1	72	46
2	14	9
Σ	157	100

Table 9. Scoring of immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA.
0: weak staining intensity, 1: moderate staining intensity, 2: strong staining intensity, FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator.

Figure 9 compares differently scored examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA.

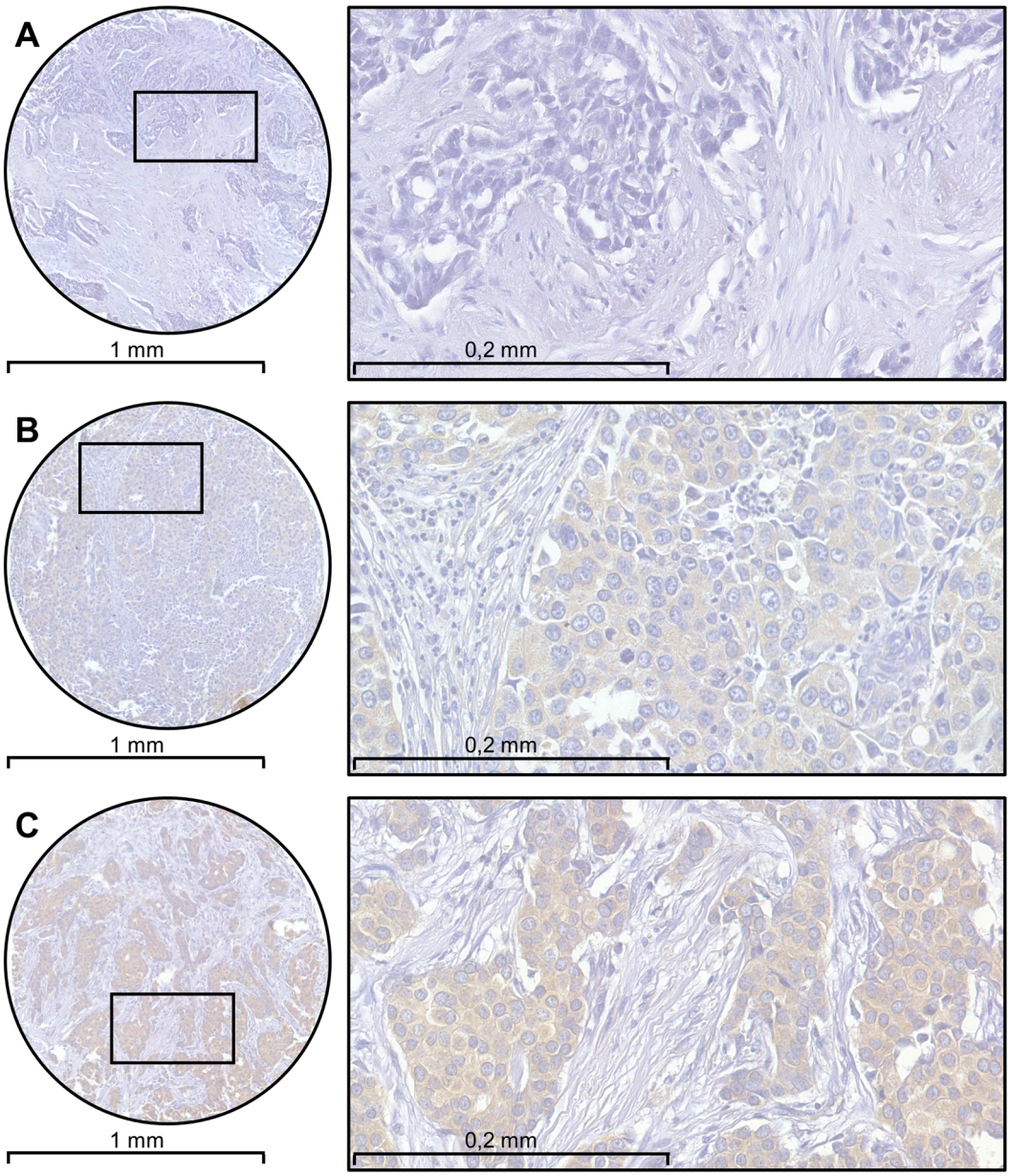

Figure 9. Comparison of differently scored immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA. **(A)** Intensity score 0. Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Intensity score 1. Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Intensity score 2. Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer.

Figure 10-12 depict examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA. Three different tissues of TNBC, that were assigned score 0 (no or weak staining intensity), are shown in **Figure 10**. **Figure 11** presents three tissue samples with score 1 (moderate

staining intensity). Score 2 (strong staining intensity) was attributed to the tissue examples in **Figure 12**.

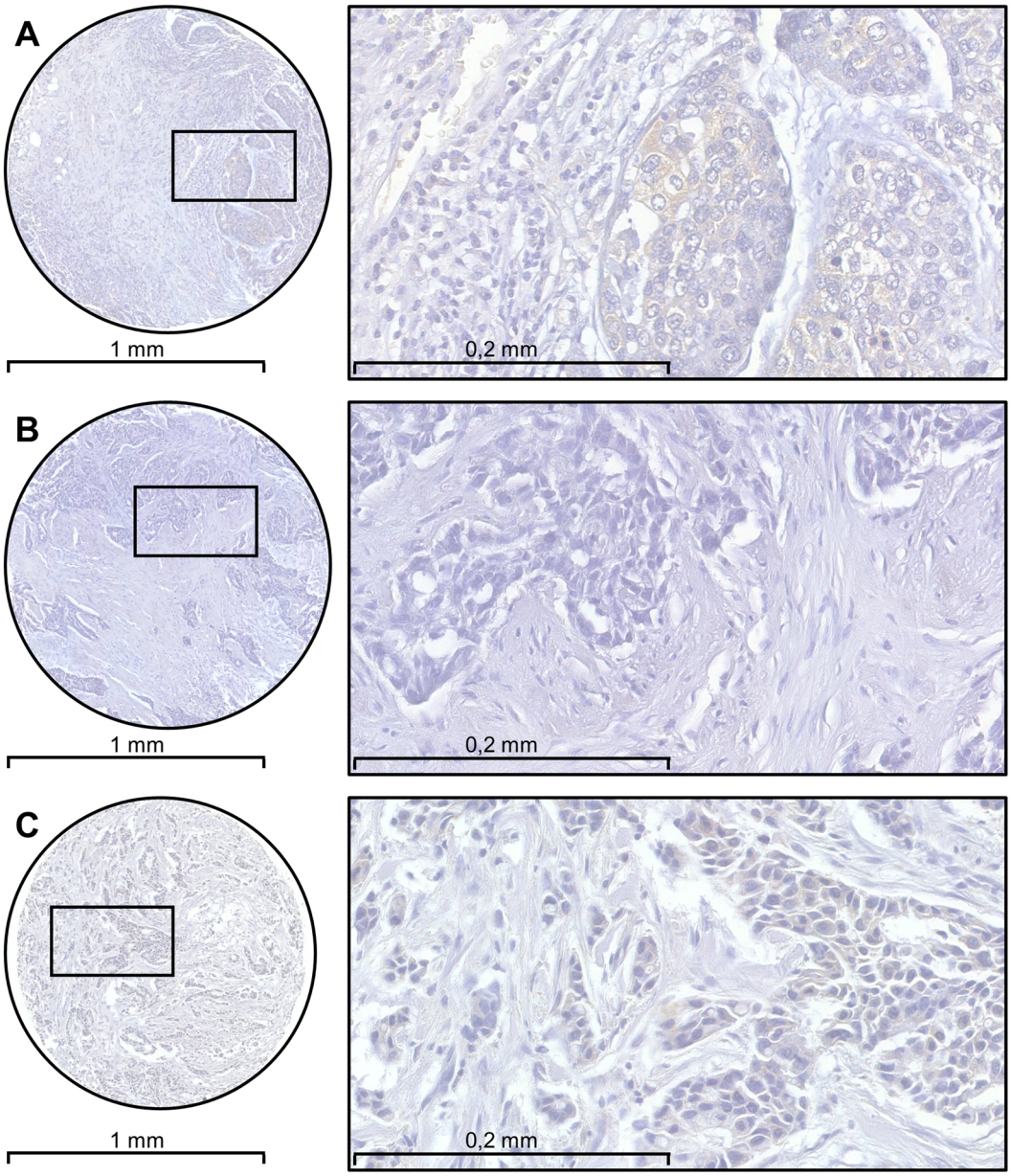

Figure 10. Intensity score 0, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer.

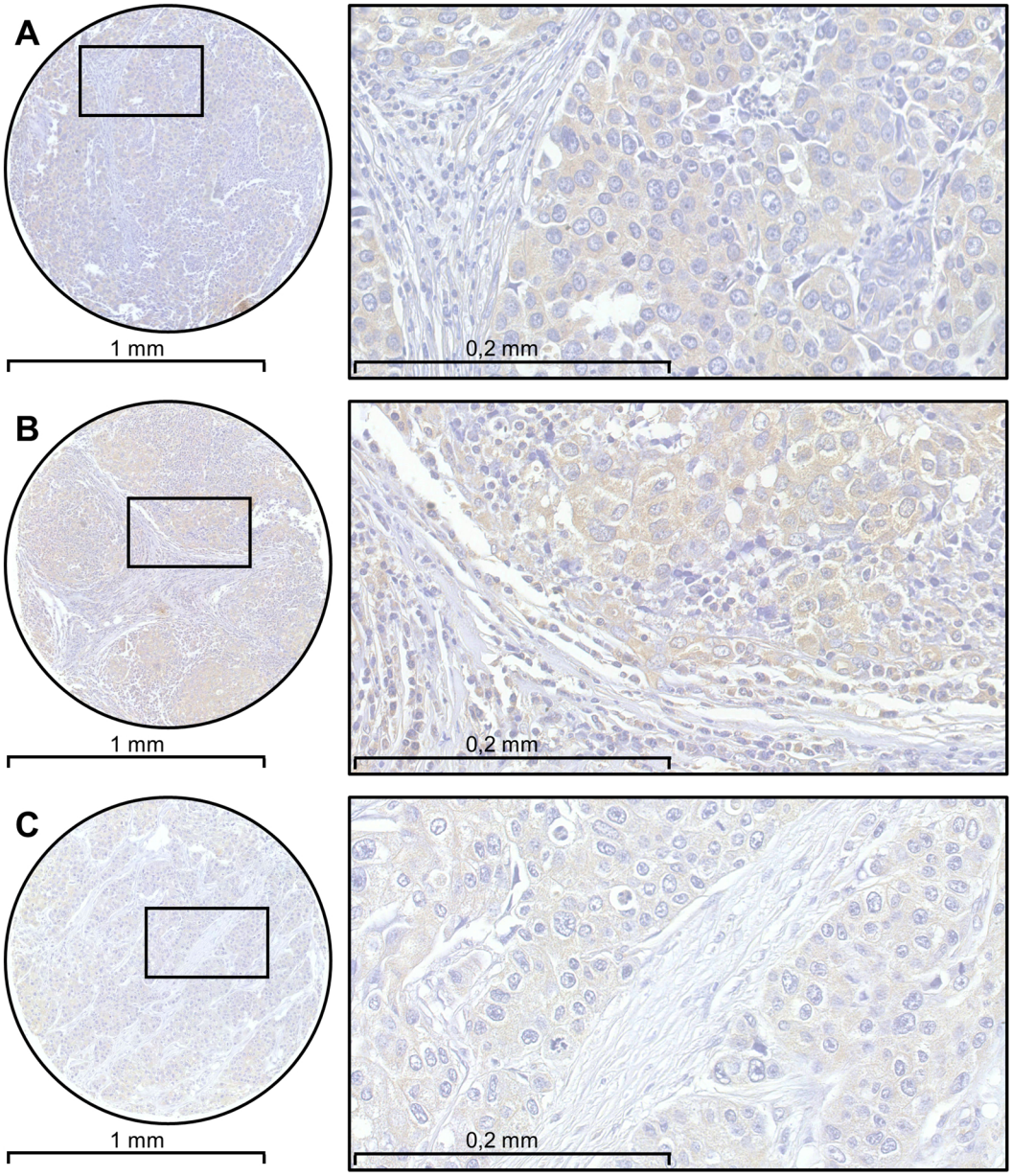

Figure 11. Intensity score 1, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive medullary carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer.

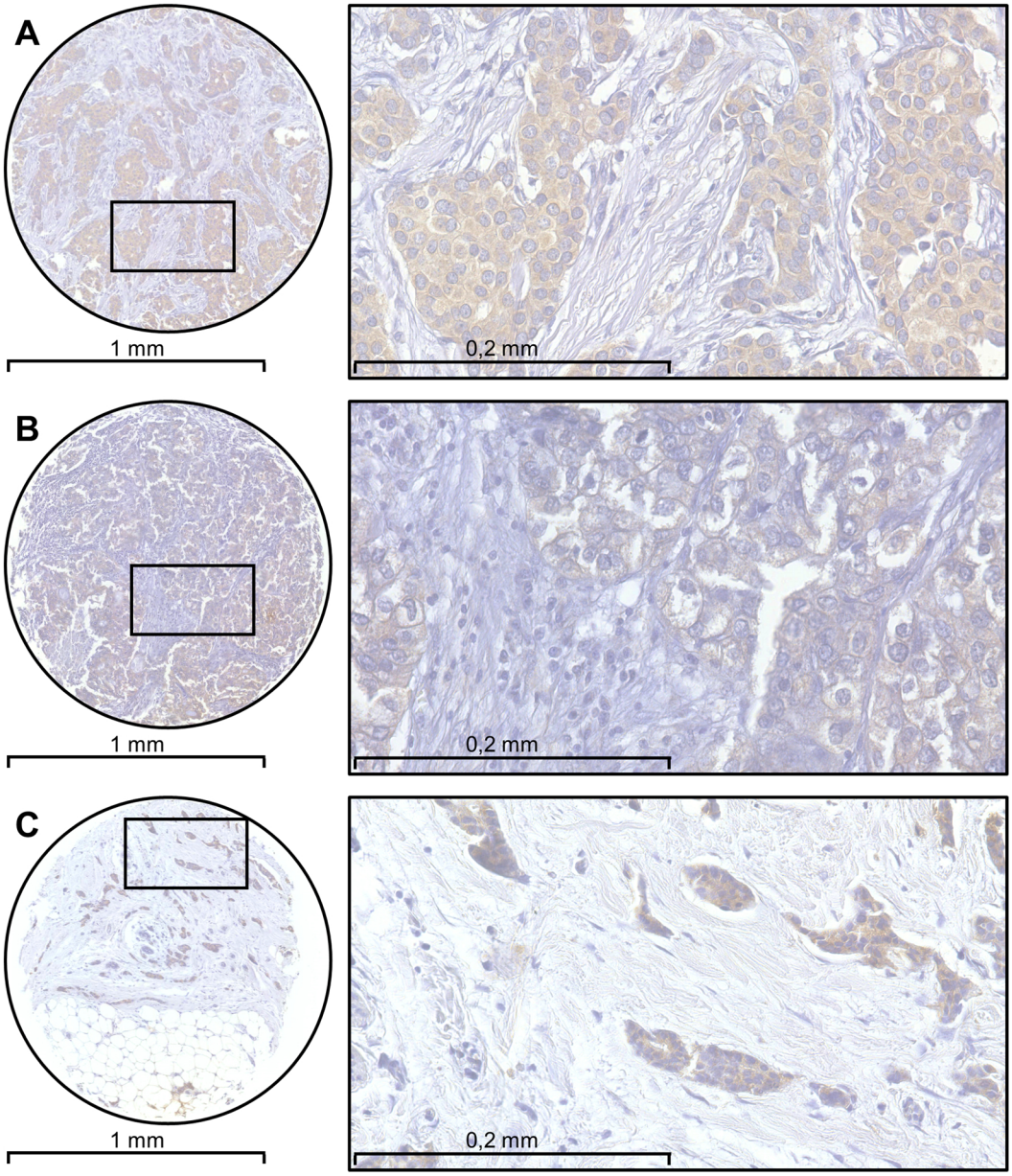

Figure 12. Intensity score 2, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3689 directed against uPA. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, apocrine subtype, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G2, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer.

4.2.2 Scoring results of immunohistochemical staining of PAI-1 in TNBC

Immunohistochemical staining of plasminogen activator inhibitor type-1 (PAI-1) in FFPE TNBC specimens was performed using the mAb #3786 directed against PAI-1. Similar to the IHC analysis of uPA expression, stained tissue slides were categorized into three staining categories. The staining patterns of tissue using the mAb #3786 directed against PAI-1 look very similar to those using the mAb #3689 directed against uPA. The nuclei of tumor cells also show no or weak staining, whereas their cytoplasm of tumor cells demonstrate strong staining. In contrast to the cellular part of the tumor stroma that showed no staining, the non-cellular part of the tumor stroma presents weak staining. **Table 10** shows the distribution of intensity scores of immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1.

Score	n	%
0	56	38
1	69	47
2	23	15
Σ	148	100

Table 10. Scoring of immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1. 0: weak staining intensity, 1: moderate staining intensity, 2: strong staining intensity, FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

Figure 13 compares differently scored immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1. **Figure 14-16** depict examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1. Three different tissues of TNBC, that were assigned score 0 (no or weak staining intensity), are shown in **Figure 14**. **Figure 15** presents three tissue samples with score 1 (moderate staining intensity). Score 2 (strong staining intensity) was attributed to tissue examples shown in **Figure 16**.

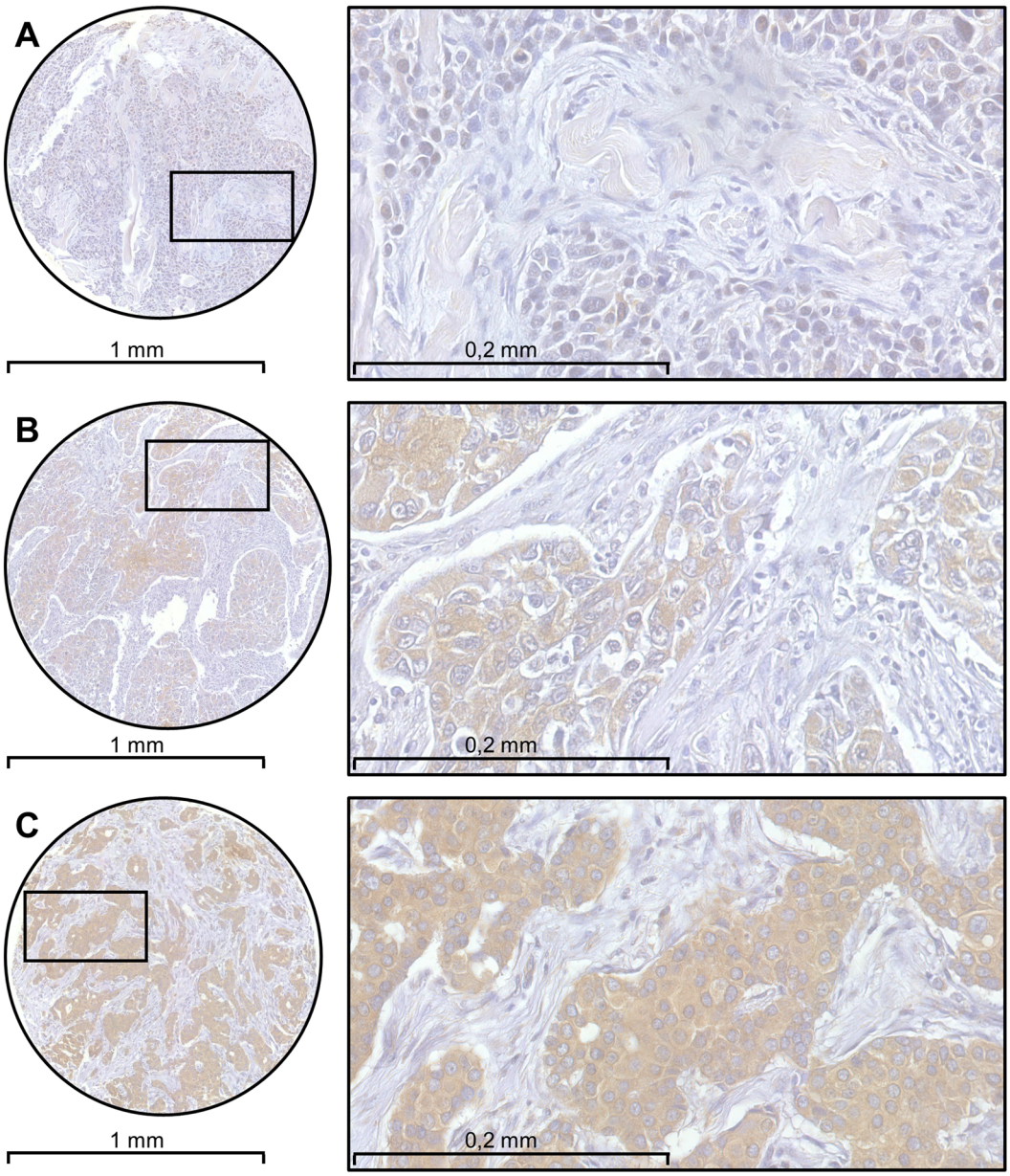

Figure 13. Comparison of differently scored immunohistochemical staining of TNBC specimens using the mAb #3786 directed against PAI-1. **(A)** Intensity score 0. Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Intensity score 1. Left: overview: G, atypical medullary carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Intensity score 2. Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

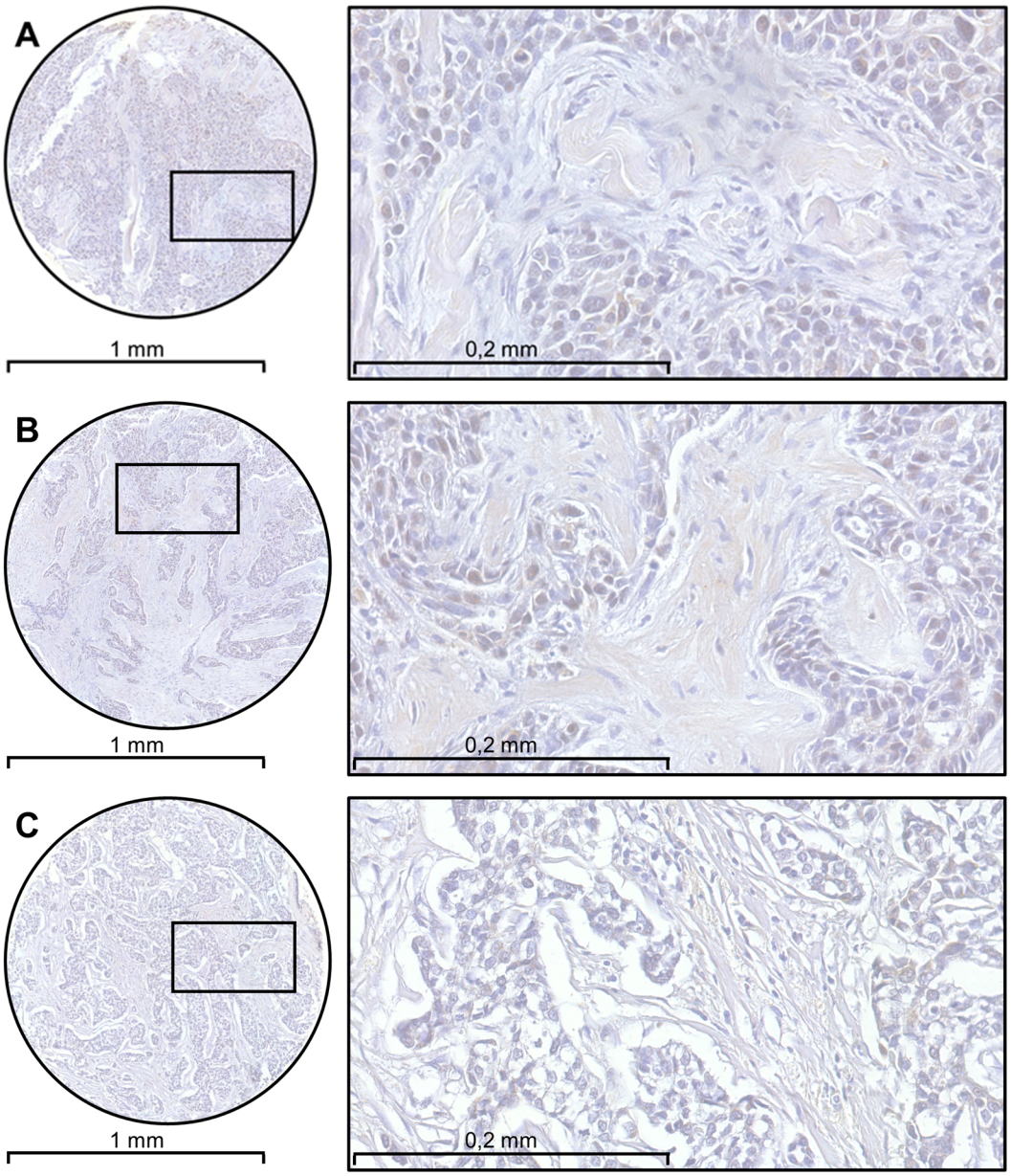

Figure 14. Intensity score 0, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G2, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

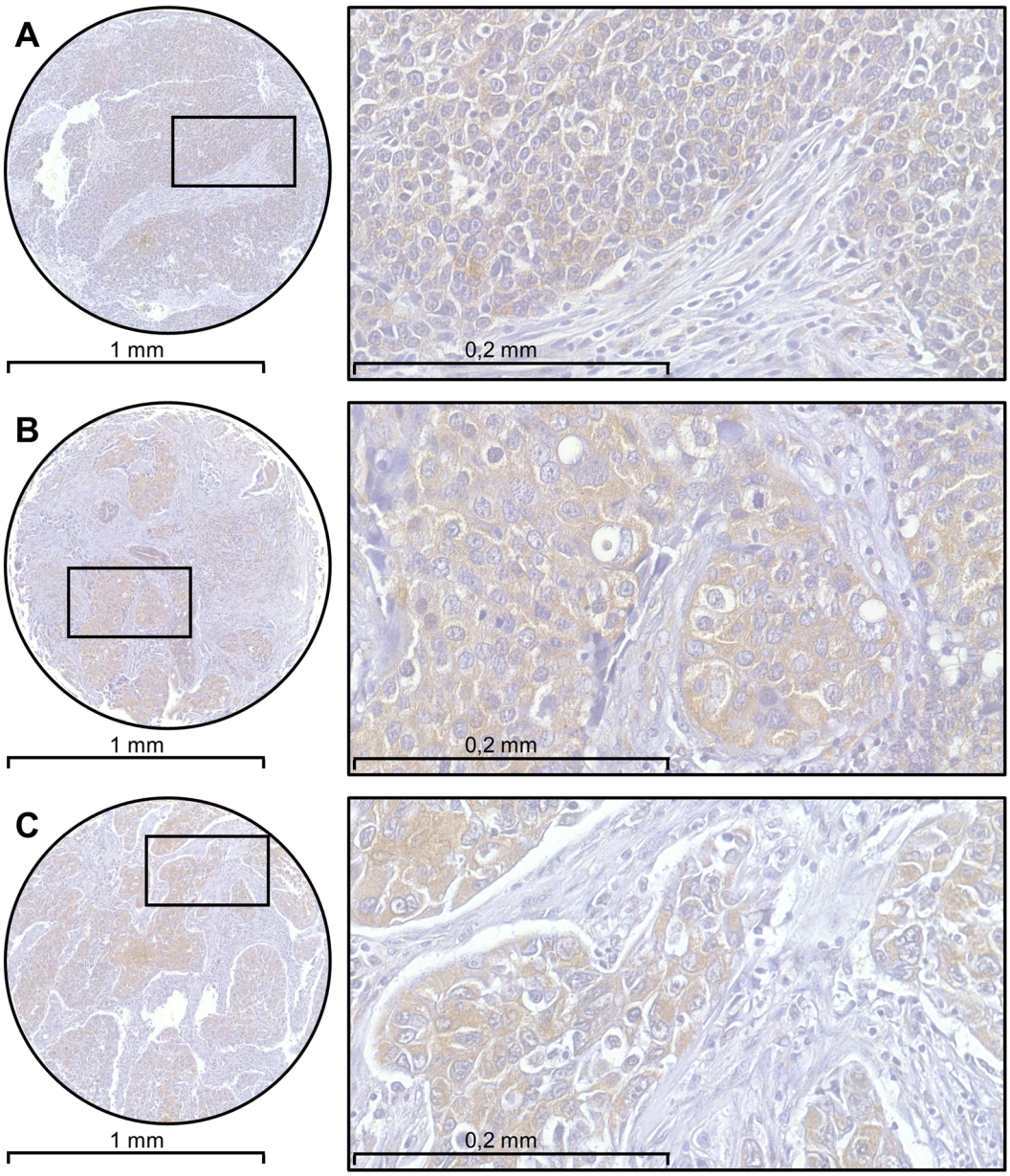

Figure 15. Intensity score 1, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G, atypical medullary carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

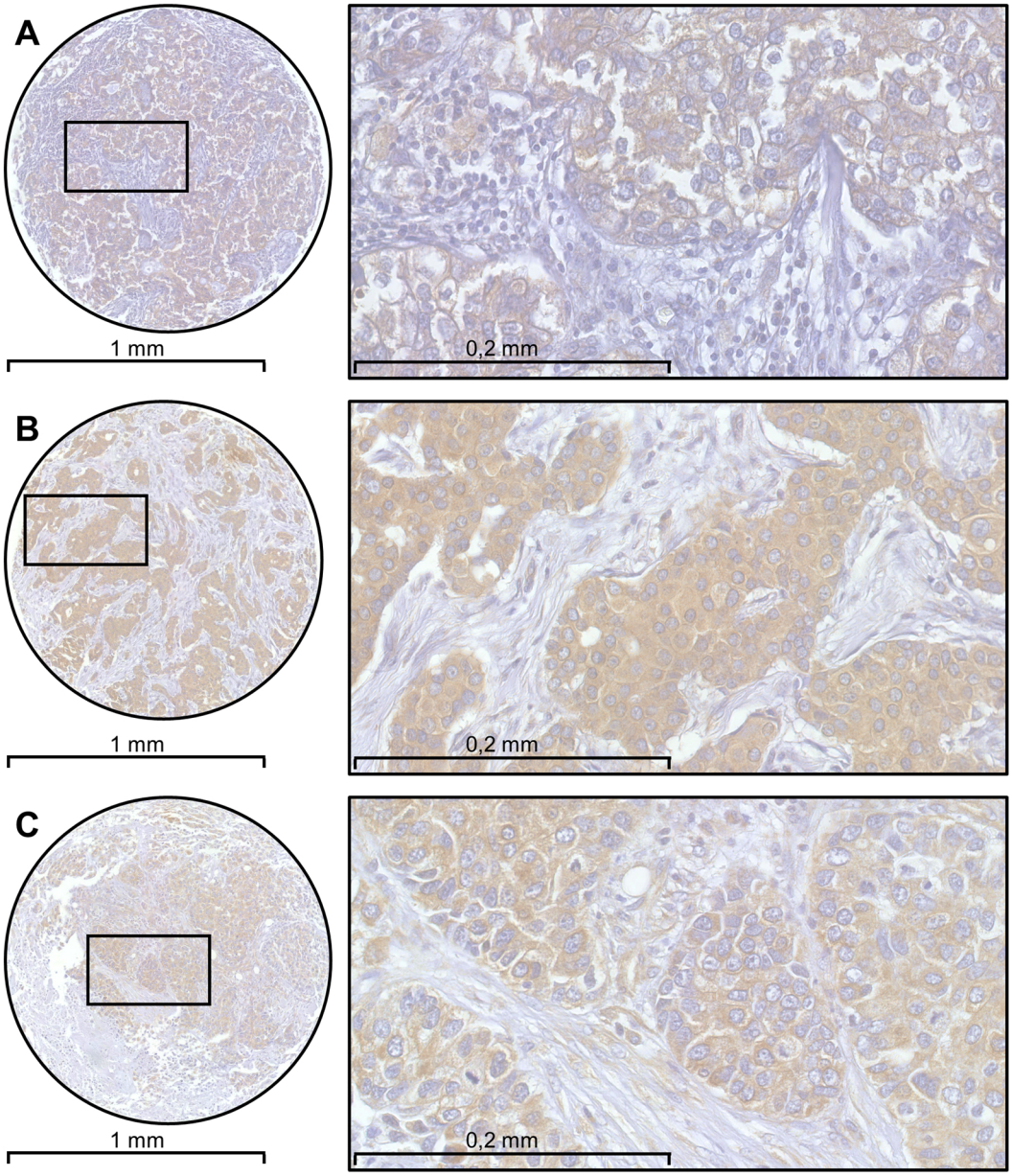

Figure 16. Intensity score 2, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb #3786 directed against PAI-1. **(A)** Left: overview: G3, invasive ductal carcinoma, apocrine subtype, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

4.2.3 Scoring results of immunohistochemical staining of uPAR in TNBC

Immunohistochemical staining of urokinase-type plasminogen activator receptor (uPAR) in FFPE TNBC specimens was performed using the mAb IID7 directed against uPAR. In contrast to the IHC analysis of uPA and PAI-1 expression, stained tissue slides were categorized into four staining categories: weak staining intensity (score 0), moderate low staining intensity (score 1), moderate high staining intensity (score 2), and strong staining intensity (score 3). Comparing all stained tumor slides using the mAb IID7 directed against uPAR, several similarities are observed. The nuclei of tumor cells show no or weak staining, whereas the cytoplasm of tumor cells present strong staining. The tumor stroma is slightly stained because of its non-cellular part. In some tumor tissues with score 3, membranous staining of tumor cells could be observed. **Table 11** lists the distribution of intensity scores in immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR.

Score	n	%
0	6	4
1	43	29
2	72	49
3	26	18
Σ	147	100

Table 11. Scoring of immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR.
0: weak staining intensity, 1: moderate low staining intensity, 2: moderate high staining intensity, 3: strong staining intensity, FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, uPAR: urokinase-type plasminogen activator receptor, TNBC: triple-negative breast cancer.

Figure 17 compares examples of differently scored immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. **Figure 18-21** depict examples of immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. Three different tissues of TNBC that were assigned score 0 (no or weak staining intensity) are shown in **Figure 18**. **Figure 19** presents three tissue samples with score 1 (moderate staining intensity). Score 2 (strong staining intensity) was attributed to tissue examples in **Figure 20**. **Figure 21** depicts TNBC specimens that showed very strong staining and were assigned score 3.

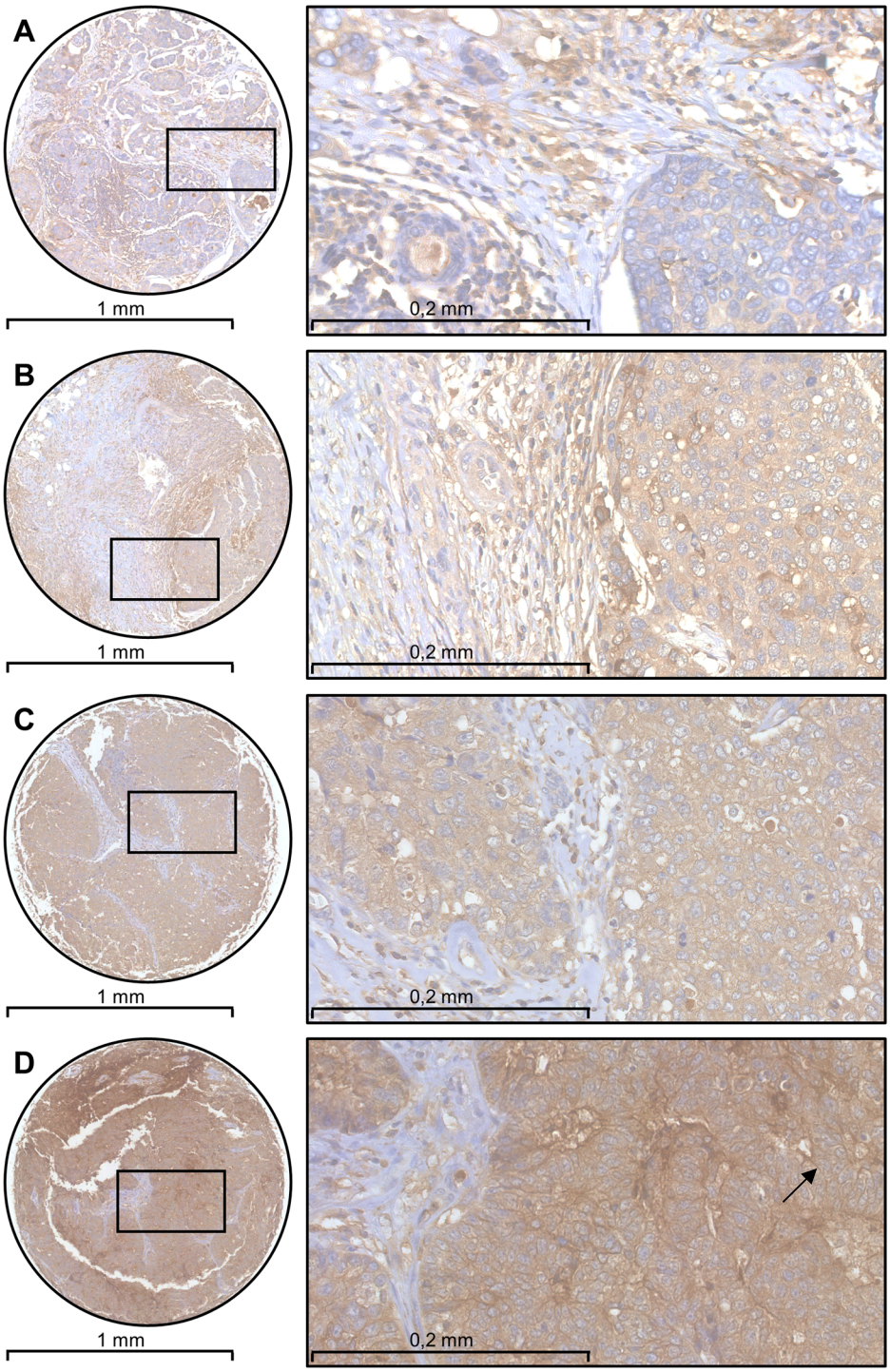

Figure 17. Comparison of differently scored immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(D)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Membranous staining of tumor cells (→) Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

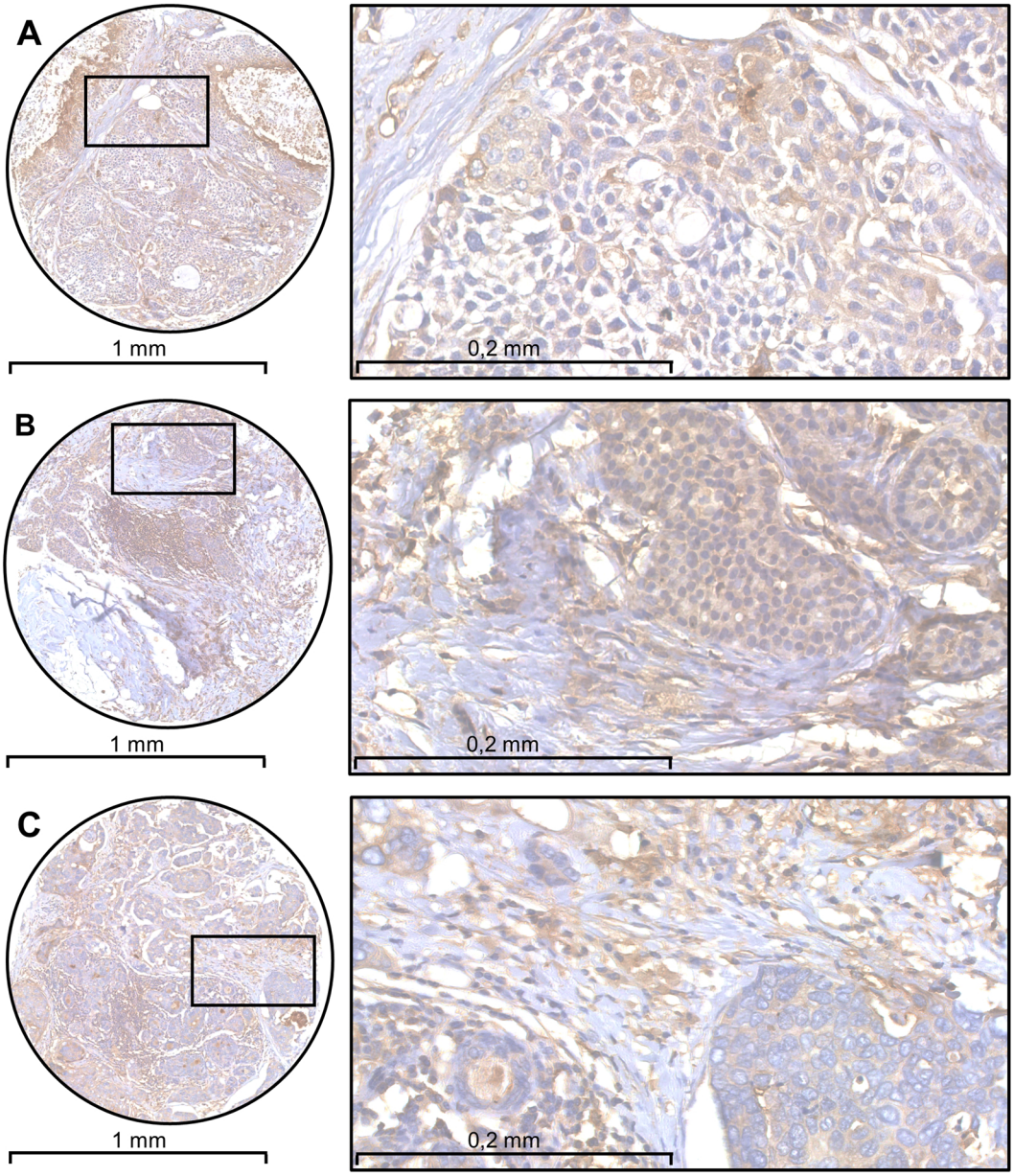

Figure 18. Intensity score 0, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive lobular carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

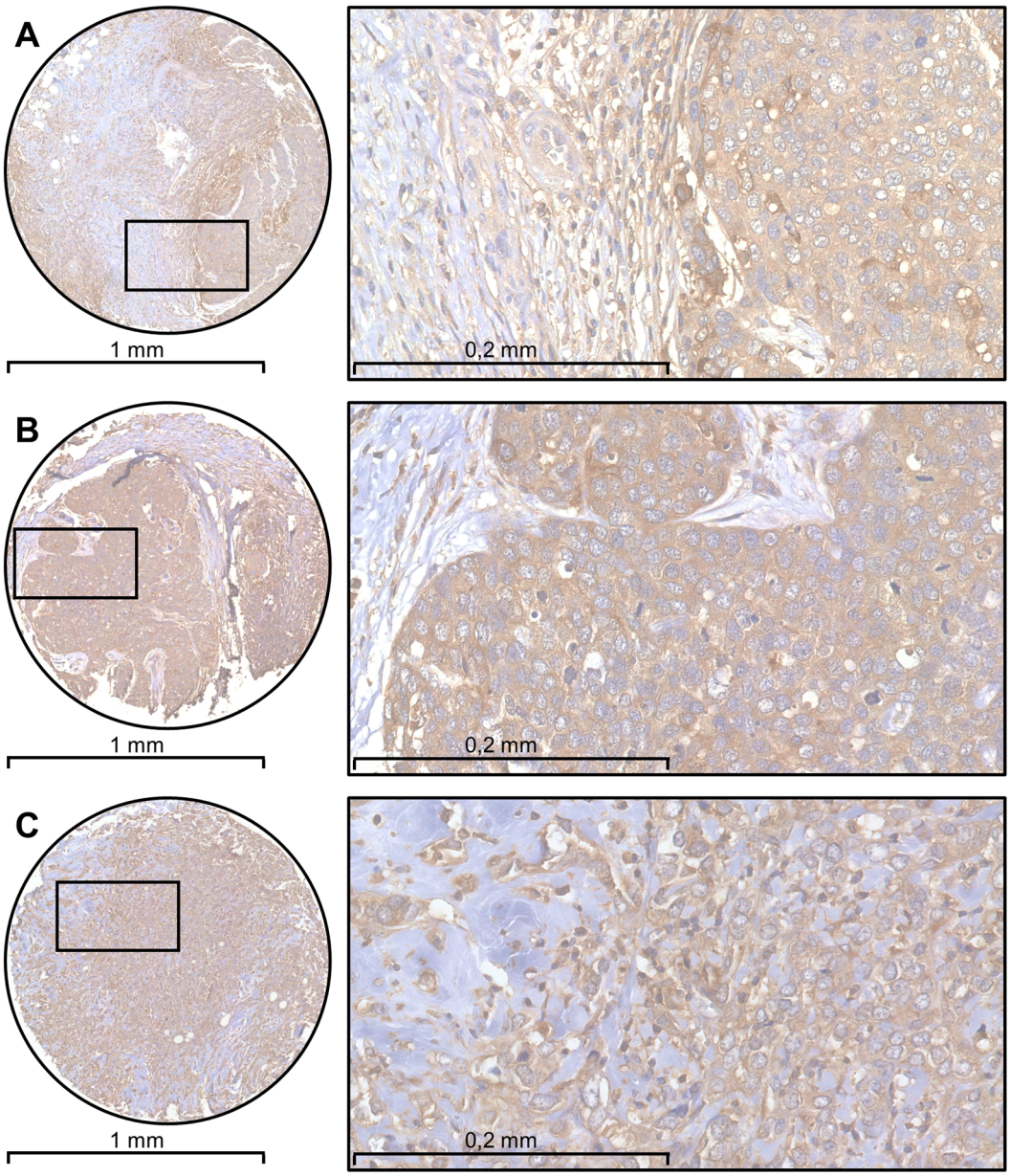

Figure 19. Intensity score 1, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

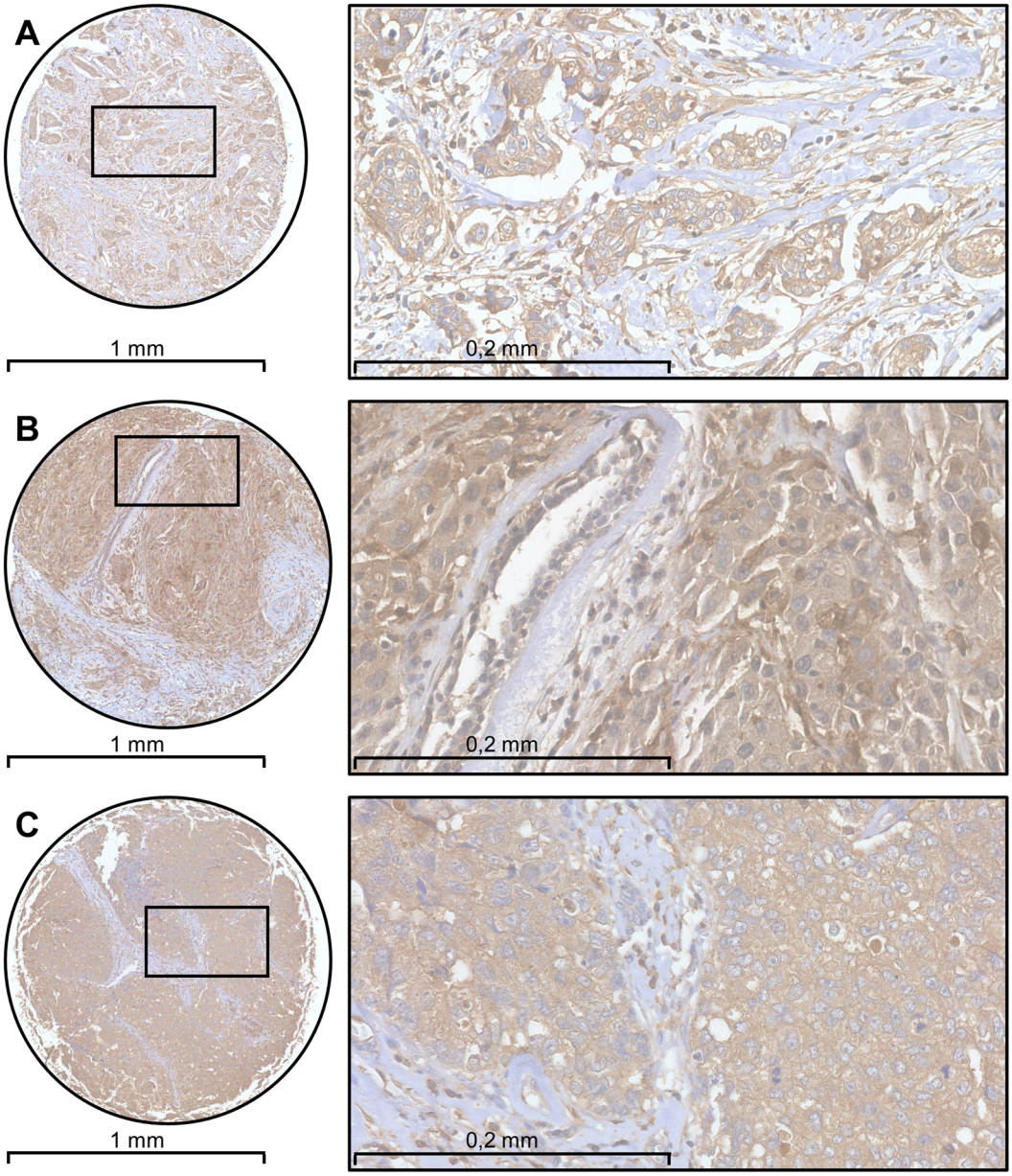

Figure 20. Intensity core 2, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

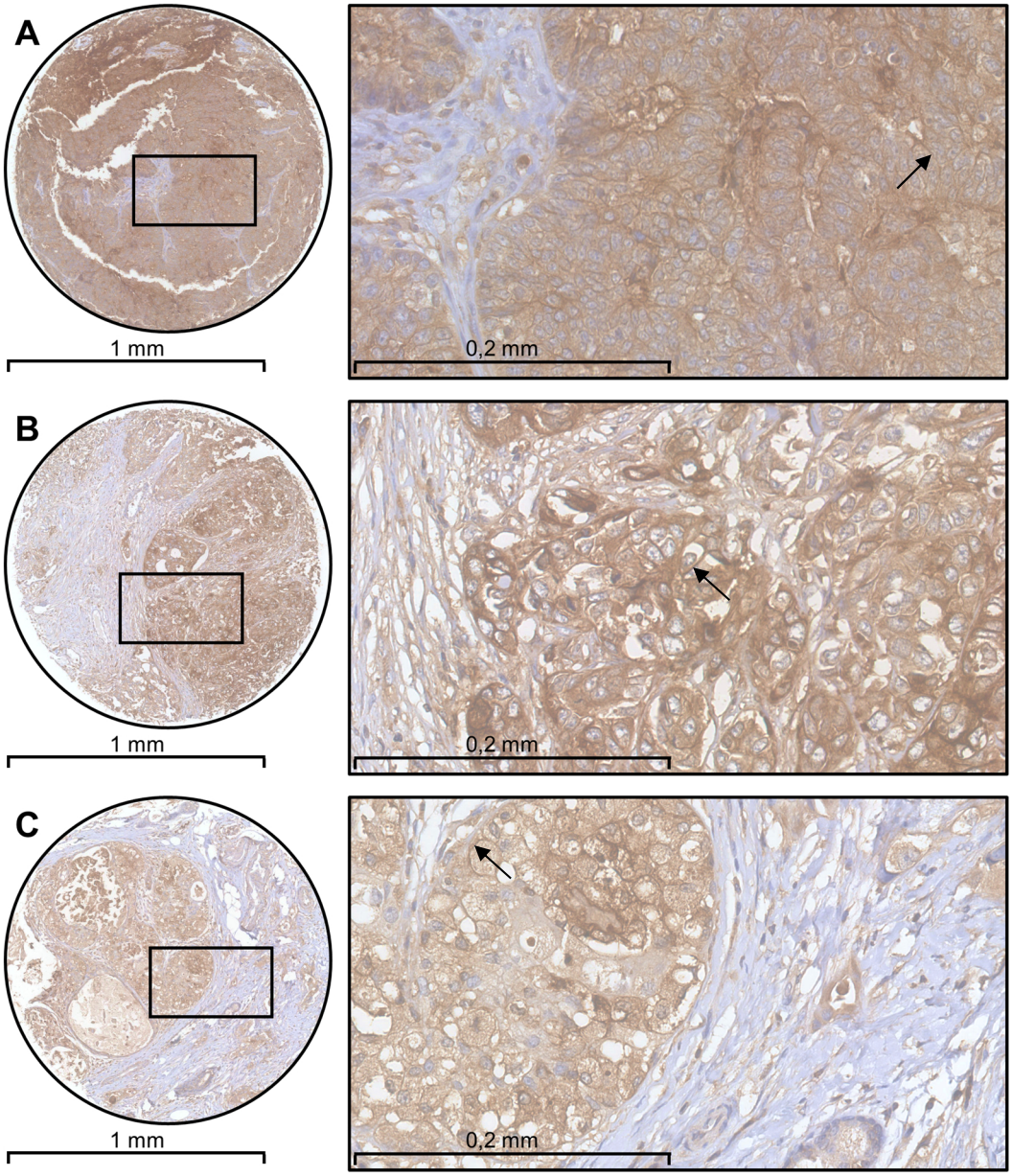

Figure 21. Intensity score 3, examples of immunohistochemical staining of FFPE TNBC specimens using the mAb IID7 directed against uPAR. **(A)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Membranous staining of tumor cells (→) **(B)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Membranous staining of tumor cells (→) **(C)** Left: overview: G3, invasive ductal carcinoma, 5x zoom; Right: detailed view of tumor cells and tumor stroma, 40x zoom. Membranous staining of tumor cells (→). Slides were scanned by Hamamatsu Nanozoomer 2.0 HAT slide scanner.
FFPE: formalin-fixed, paraffin-embedded, mAb: monoclonal antibody, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

4.3 Triple-negative breast cancer patient collective

The analyzed patient collective includes 172 triple-negative breast cancer (TNBC) patients. Initially, 251 cases of TNBC were analyzed by immunohistochemistry; 18 patients were excluded because of treatment with neoadjuvant chemotherapy. Exclusion of another 61 patients had several reasons, including, for example, incomplete follow-up data, missing triple-negativity upon retesting, or lack of material on the stained tissue microarray slide. The patient cohort shows typical characteristics corresponding to the TNBC phenotype **(Table 12)**. At the time of the first diagnosis, the median age is 58, ranging from 27 to 96 years and 24% of our patients are premenopausal. The majority of the cohort is classified into G3 (82%). A high proportion of patients is afflicted with invasive ductal carcinoma accounting for 78% of all patients. Other histological subtypes, such as medullary breast cancer (9%) and lobular breast cancer (5%) are present. In 32% of our patients, tumors are classified as tumor stage pT1 at the time of diagnosis, 50% are diagnosed with pT2 tumors, 7% with pT3, and 10% with pT4. **Figure 22** depicts the tumor size of 3 different patient groups: the left graph illustrates the histologic extent of the primary tumor of our TNBC patient cohort and, compared with that, the middle and right graphs chart the data of Bavarian breast cancer patients published by the Munich Cancer Registry *(MunichTumorCenter 2017)*. In the middle chart, the data of the primary tumor size of about 1,160 TNBC patients are presented. It is apparent that tumors in the presently analyzed TNBC patient cohort are slightly larger compared with the TNBC comparison group. The right graph depicts the extent of the primary tumor of about 13,020 Bavarian breast cancer patients with non-TNBC (luminal A-like, luminal B-like, HER2+ non-luminal). Comparison of these graphs demonstrates that triple-negative tumors are slightly larger at the time of the first diagnosis.

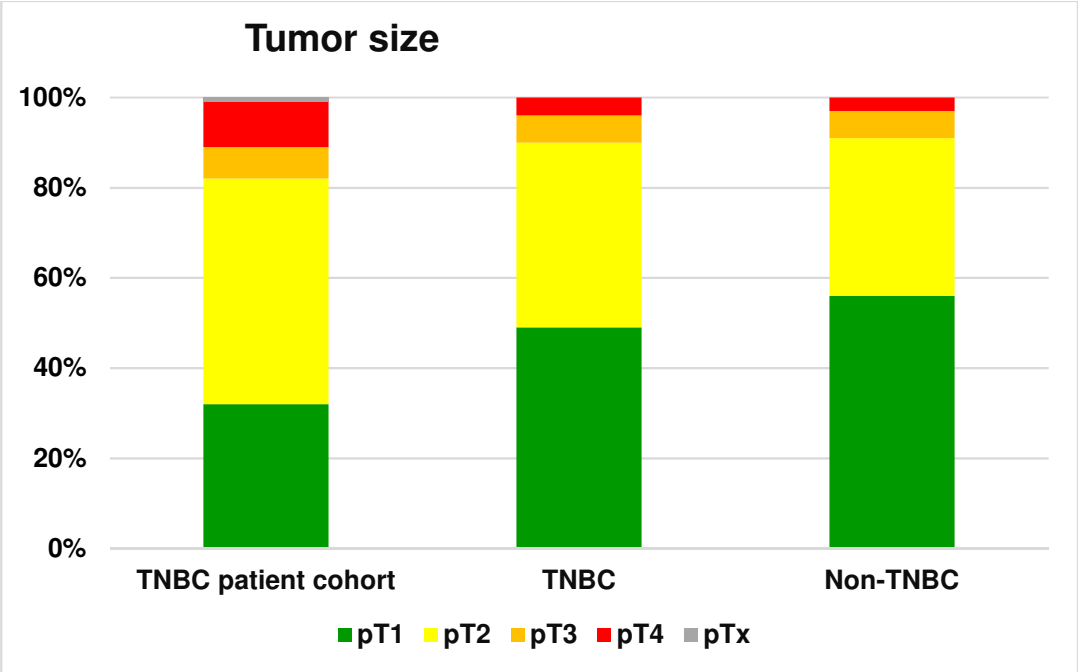

Figure 22. Tumor size of the triple-negative patient cohort (n = 172, left graph). Compared with this, the middle and right graphs depict data of Bavarian breast cancer patients published by the Munich Cancer. In the middle, the primary tumor sizes of about 1,160 TNBC patients are presented. It is apparent that tumors of our TNBC patients are slightly bigger compared with the TNBC comparison group. The right graph depicts the extent of the primary tumors of about 13,020 breast cancer patients with all other breast cancer subtypes (luminal A-like, luminal B-like, HER2+ non-luminal). Comparison of these graphs demonstrates that triple-negative tumors are slightly larger at the time of the first diagnosis. Left graph: pT1: 32%, pT2: 50%, pT3: 7%, pT4: 10%, pTx: 1%. Middle graph: pT1: 49%, pT2: 41%, pT3: 6%, pT4: 4%. Right graph: pT1: 56%, pT2: 35%, pT3: 6%, pT4: 3% *(MunichTumorCenter 2017)*.
pT1: histopathologic tumor size up to 2 cm, pT2: histopathologic tumor size >2-5 cm, pT3: histopathologic tumor size >5 cm, pT4: histopathologic invasion of thoracic wall or skin, pTx: no information on tumor size, TNBC: triple-negative breast cancer.

The left graph in **Figure 23** illustrates the histopathologic lymph node status of our TNBC patient cohort. Lymph node positivity was diagnosed in 43% of the patients at the time of diagnosis. 49% of the patient cohort showed no metastasis in the locoregional lymph nodes. In comparison to that, the middle and right graphs depict data of Bavarian breast cancer patients published by the Munich Cancer Registry *(MunichTumorCenter 2017)*. The middle graph depicts the lymph node status of about 1,160 TNBC patients. It is apparent that our TNBC patients demonstrate lymph node positivity more often compared with the TNBC comparison group. The right graph depicts the lymph node status of about 13,020 Bavarian breast cancer patients with the non-triple-negative disease. Comparing the middle and the right graph, it is notable, that a smaller proportion of patients demonstrate lymph node positivity in the triple-negative subgroup than in breast cancers of all subtypes. One might expect the opposite, given that previous studies

have shown that tumor size of TNBC is not correlated with lymph node status. For example, 55% of women with small triple-negative tumors (≤1 cm) present with positive lymph nodes *(Dent et al. 2007, de Ruijter et al. 2011)*.

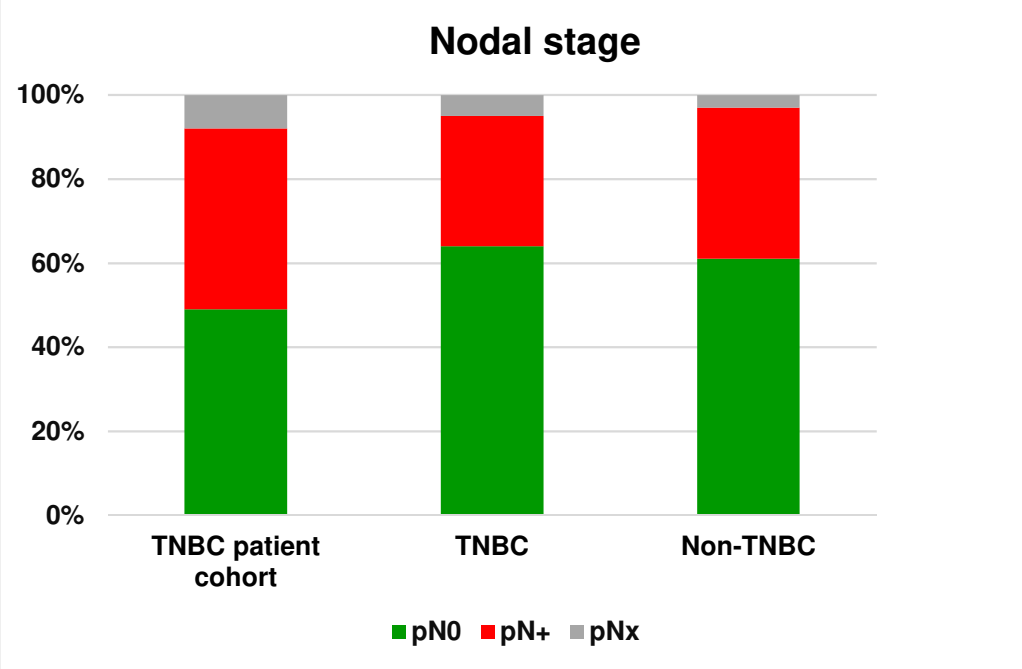

Figure 23. Nodal status of the triple-negative patient cohort (n = 172, left graph). In comparison to this, the middle and right graph chart data of Bavarian breast cancer patients published by Munich Cancer Registry. The middle graph depicts the lymph node status of about 1,160 TNBC patients. It is apparent that our TNBC patients demonstrate lymph node positivity more often compared with the TNBC comparison group. The right graph depicts the extent of the primary tumor of about 13,020 breast cancer patients with all other breast cancer subtypes (luminal A-like, luminal B-like, HER2+ non-luminal). Comparing the middle and the right graph, it is notable that a smaller proportion of TNBC patients demonstrate lymph node positivity. Left graph: pN0: 49%, pN+ 43%, pNx: 8%. Middle graph: pN0: 64%, pN+: 31%, pNx: 5%. Right graph: pN0: 61%, pN+: 36%, pNx: 3%. *(MunichTumorCenter 2017)*.
pN0: no regional lymph node metastasis present based on histopathological analysis, pN+: lymph node metastasis present based on histopathological analysis, pNx: no information on lymph node status, TNBC: triple-negative breast cancer.

The median observation period was 58 months. According to the aggressive clinical behavior of TNBC, a high incidence of metastasis, and both disease recurrence and progression was observed. 8% of patients suffered from the primary metastasized disease. A similar percentage was observed by the Munich Cancer Registry, which found 8% of TNBC patients had distant metastases that were detectable at the time of diagnosis. When compared with that, primary metastasis occurs in approximately 4% of patients with other breast cancer subtypes *(MunichTumorCenter 2017)*. Distant metastasis occurred in 27% of patients. In 39% of cases, cancer recurred or progressed, and 60 patients died

during the time of observation (35%). Standard breast cancer surgery was performed on almost every patient; 60% underwent breast conservative surgery, and 39% underwent a mastectomy. A large proportion of the patient cohort received adjuvant therapy, 62% were treated with anthracycline/cyclophosphamide-based chemotherapy, and 72% received radiation therapy. Interestingly, the use of endocrine therapy was documented; 17% of patients received endocrine treatment despite the triple-negativity of tumors. Furthermore, about 2% of TNBC patients were treated with trastuzumab. Current guidelines recommend against hormonal therapy or treatment with trastuzumab in TNBC because of its negative hormone receptor status and lack of HER2 overexpression *(Harris et al. 2016, MunichTumorCenter 2017, AGOBreastCommittee 2018, AWMF-S3-Leitlinie 2018)*.

Characteristics	**n**	**%**
Age at diagnosis (27-96 years)		
<= 50	51	30
> 50	118	68
Unknown	3	2
Median age	58	
Mean age	58	
Menopausal status		
Premenopausal	41	24
Perimenopausal	8	5
Postmenopausal	121	70
Unknown	2	1
Histological subtype		
Invasive ductal	134	78
Medullary	15	9
Lobular	9	5
Other subtypes	12	7
Unknown	2	1

Tumor stage		
pT1	55	32
pT2	86	50
pT3	12	7
pT4	17	10
pTx	2	1
Lymph node status		
pN0	84	49
pN+	74	43
pNx	14	8
Metastasis at time of diagnosis		
M0	149	86
M1	13	8
Mx	10	6
Histological grade		
G1	3	2
G2	23	13
G3	141	82
Gx	5	3
Treatment of primary tumor		
Breast conservative surgery	103	60
Mastectomy	68	39
Unknown	1	1
Adjuvant therapy		
Cytotoxic chemotherapy	106	62
Radiotherapy	123	72
Distant metastasis		
No	123	71
Yes	46	27
Unknown	3	2
Disease recurrence/progression		
No	98	57
Yes	67	39
Unknown	7	4

Case of death		
No	79	46
Yes	60	35
Unknown	33	19

Table 12. Clinicopathological characteristics of all patients.
G1: well-differentiated tumor (low grade), G2: moderately-differentiated tumor (intermediate grade), G3: poorly-differentiated tumor (high grade), Gx: no assessment of grading, M0: no distant metastasis present, M1: distant metastasis present, Mx: no information on status of distant metastasis, pN0: no regional lymph node metastasis present based on histopathological analysis, pN+: lymph node metastasis present based on histopathological analysis, pNx: no information on lymph node status, pT1: histopathologic tumor size up to 2 cm, pT2: histopathologic tumor size >2-5 cm, pT3: histopathologic tumor size >5 cm, pT4: histopathologic invasion of thoracic wall or skin, pTx: no information on tumor size.

4.4 Statistical analysis

4.4.1 Association between uPA/PAI-1/uPAR intensity scores and clinicopathologic characteristics of TNBC patients

Associations between clinicopathologic characteristics of triple-negative breast cancer (TNBC) patients and the urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor type-1 (PAI-1) and urokinase-type plasminogen activator receptor (uPAR) intensity scores of their tumors were analyzed using Pearson's Chi-squared tests. Menopausal status of TNBC patients is significantly associated with uPA intensity scores (p = 0.013). Pre- and perimenopausal status is correlated with low uPA expression. In contrast, postmenopausal status is correlated with high uPA expression. **(Table 17)**. No correlation was found when analyzing other clinicopathologic characteristics of TNBC patients, such as primary tumor size, lymph node status, distant metastasis, or histologic grading **(Table 13-17)**.

		Tumor size			Chi² test
		pT1/pT2	**pT3/pT4**	**Σ**	***p***
uPA	**low**	59	12	71	
	high	67	17	84	
	Σ	126	29	155	
					n.s.
PAI-1	**low**	46	10	56	
	high	74	17	91	
	Σ	120	27	147	
					n.s.
uPAR	**low**	41	7	48	
	high	78	19	97	
	Σ	119	26	145	
					n.s.

Table 13. Association between uPA, PAI-1, uPAR intensity scores, and primary tumor size of TNBC patients. This contingency table depicts the frequency distribution of uPA, PAI-1, and uPAR IHC intensity scores and primary tumor size. Pearson's Chi-squared test was used to analyze the association (asymptotic significance). Expression levels of uPA, PAI-1, and uPAR are statistically independent of primary tumor size.
IHC: immunohistochemistry, PAI-1: plasminogen activator inhibitor type-1, pT1: histopathologic tumor size up to 2 cm, pT2: histopathologic tumor size >2-5 cm, pT3: histopathologic tumor size >5 cm, pT4: histopathologic invasion of thoracic wall or skin, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator, uPAR: urokinase-type plasminogen activator receptor.

		Lymph node status			Chi² test
		pN0	**pN+**	**Σ**	***p***
uPA	**low**	36	29	65	
	high	37	41	78	
	Σ	73	70	143	
					n.s.
PAI-1	**low**	26	26	52	
	high	41	41	82	
	Σ	67	67	134	
					n.s.
uPAR	**low**	25	19	44	
	high	44	45	89	
	Σ	69	64	133	
					n.s.

Table 14. Association between uPA, PAI-1, uPAR intensity scores, and nodal status of TNBC patients. This contingency table depicts the frequency distribution of uPA, PAI-1, and uPAR IHC intensity scores and lymph node status. Pearson's Chi-squared test was used to analyze association (asymptotic significance). Expression levels of uPA, PAI-1, and uPAR are statistically independent of nodal status.
IHC: immunohistochemistry, pN0: no regional lymph node metastasis present based on histopathological analysis, pN+: lymph node metastasis present based on histopathological analysis, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator, uPAR: urokinase-type plasminogen activator receptor.

		Distant metastasis			Chi² test
		M0	**M+**	**Σ**	***p***
uPA	**low**	54	17	71	
	high	58	25	83	
	Σ	112	42	154	
					n.s.
PAI-1	**low**	43	13	56	
	high	64	25	89	
	Σ	107	38	145	
					n.s.
uPAR	**low**	38	9	47	
	high	70	27	97	
	Σ	108	36	144	
					n.s.

Table 15. Association between uPA, PAI-1, uPAR intensity scores, and distant metastasis status of TNBC patients. This contingency table depicts the frequency distribution of uPA, PAI-1, and uPAR IHC intensity scores, and distant metastasis status. Pearson's Chi-squared test was used to analyze the association (asymptotic significance). Expression levels of uPA, PAI-1, and uPAR are statistically independent of distant metastasis status.
IHC: immunohistochemistry, M0: no distant metastasis present, M1: distant metastasis present, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator, uPAR: urokinase-type plasminogen activator receptor.

		Histologic grading			Chi² test
		G1/G2	**G3**	**Σ**	***p***
uPA	**low**	13	58	71	
	high	11	70	81	
	Σ	24	128	152	
					n.s.
PAI-1	**low**	8	46	54	
	high	14	75	89	
	Σ	22	121	143	
					n.s.
uPAR	**low**	10	37	47	
	high	13	82	95	
	Σ	23	119	142	
					n.s.

Table 16. Association between uPA, PAI-1, uPAR intensity scores, and histologic grading of TNBC patients. This contingency table depicts the frequency distribution of uPA, PAI-1, and uPAR IHC intensity scores and histologic grading. Pearson's Chi-squared test was used to analyze the association (asymptotic significance). Expression levels of uPA, PAI-1, and uPAR are statistically independent of the clinicopathologic parameter of histologic grading.
G1: well-differentiated tumor (low grade), G2: moderately-differentiated tumor (intermediate grade), G3: poorly-differentiated tumor (high grade), IHC: immunohistochemistry, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator, uPAR: urokinase-type plasminogen activator receptor.

		Menopausal status			Chi² test
		premenopausal/ perimenopausal	**postmenopausal**	**Σ**	***p***
uPA	**low**	27	42	69	
	high	18	68	86	
	Σ	45	110	155	
					0.013
PAI-1	**low**	20	34	54	
	high	23	69	92	
	Σ	43	103	146	
					n.s.
uPAR	**low**	10	37	47	
	high	33	65	98	
	Σ	43	102	145	
					n.s.

Table 17. Association between uPA, PAI-1, uPAR intensity scores, and menopausal status of TNBC patients. This contingency table depicts the frequency distribution of uPA, PAI-1, and uPAR IHC intensity scores, and menopausal status. Pearson's Chi-squared test was used to analyze the association (asymptotic significance). There is a statistically significant association between IHC intensity scores of uPA and menopausal status. Expression levels of PAI-1 and uPAR are statistically independent of the clinicopathologic parameter of distant metastasis status.
IHC: immunohistochemistry, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator, uPAR: urokinase-type plasminogen activator receptor.

4.4.2 Impact of uPA, PAI-1, and uPAR expression levels analyzed by immunohistochemistry (IHC) on the probability of event-free survival (EFS), overall survival (OS), and disease-free survival (DFS) of TNBC patients

Kaplan-Meier analysis was used to estimate the impact of urokinase-type plasminogen activator (uPA)/plasminogen activator inhibitor type-1 (PAI-1)/urokinase plasminogen activator receptor (uPAR) expression levels in primary tumor tissues analyzed by immunohistochemistry (IHC) on the probability of event-free survival (EFS), disease-free survival (DFS), and overall-survival (OS) of triple-negative breast cancer (TNBC) patients.

4.4.2.1 Impact of uPA expression levels analyzed by IHC on the probability of EFS, OS, and DFS of TNBC patients

Two groups of TNBC patients were differentiated and characterized by urokinase-type plasminogen activator (uPA) expression of tumor tissue: patients with low levels of uPA (n = 71) and patients with high levels of uPA (n = 86). Initially, scoring of stained tissue resulted in three TNBC patient groups: score 0 (low intensity, n = 71), score 1 (medium intensity, n = 72), and score 2 (high intensity, n = 14). Patients with medium uPA intensity scores were combined with the high intensity score group. **Figure 24 A** shows the analysis of the whole TNBC patient cohort. Furthermore, different subcategories of TNBC patients were analyzed; patients with invasive ductal carcinoma **(Fig. 24, B)**, patients with high-grade TNBC **(Fig. 24, C)**, patients with lymph node-negative TNBC **(Fig. 25, A)**, and patients with lymph node-positive TNBC **(Fig. 25, B)**. Also, patients who received adjuvant chemotherapy **(Fig. 26, A)** and patients who did not receive adjuvant chemotherapy **(Fig. 26, B)** were evaluated. **Figure 24-26** show comparisons of generated Kaplan-Meier curves using the log-rank test (Mantel-Cox test).

TNBC patients with elevated uPA expression levels tend to show worse EFS, OS, and DFS than the group of patients with low uPA expression. This effect seems to be highest in high-grade tumors, invasive ductal carcinoma, lymph node-positive patients, and patients that have received adjuvant chemotherapy. However, statistical significance was not attained. Analyzing the subgroup of TNBC patients with high-grade tumors, elevated uPA expression levels display a trend toward significance ($p = 0.097$) in the case of DFS **(Fig. 24, C)**.

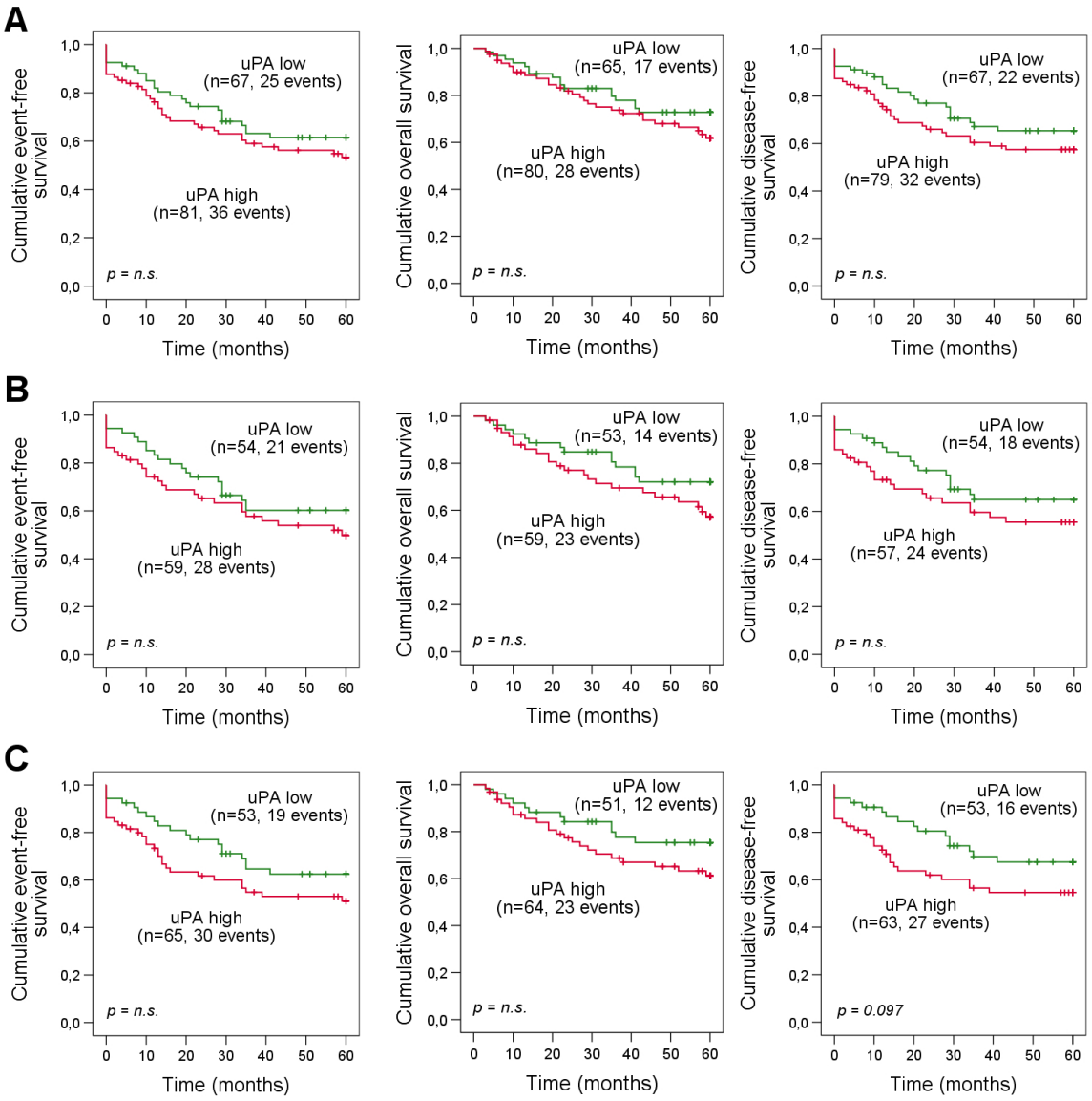

Figure 24. Probability of EFS, OS, and DFS of patients with TNBC as stratified by uPA expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) TNBC patients with elevated uPA expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPA expression; however, there is no statistically significant difference between these groups. **(B)** TNBC patients with invasive ductal carcinoma and elevated uPA expression levels tend to show worse EFS, OS, and DFS than the group of patients with low uPA expression; however, there is no statistically significant difference between these groups. **(C)** TNBC patients with high-grade tumors and elevated uPA expression levels display a trend toward significance (p = 0.097) in the case of DFS (right panel), but not EFS (left panel) and OS (middle panel). Analysis of TNBC patients with low-grade and intermediate-grade tumors is not depicted because of the small number of cases.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator.

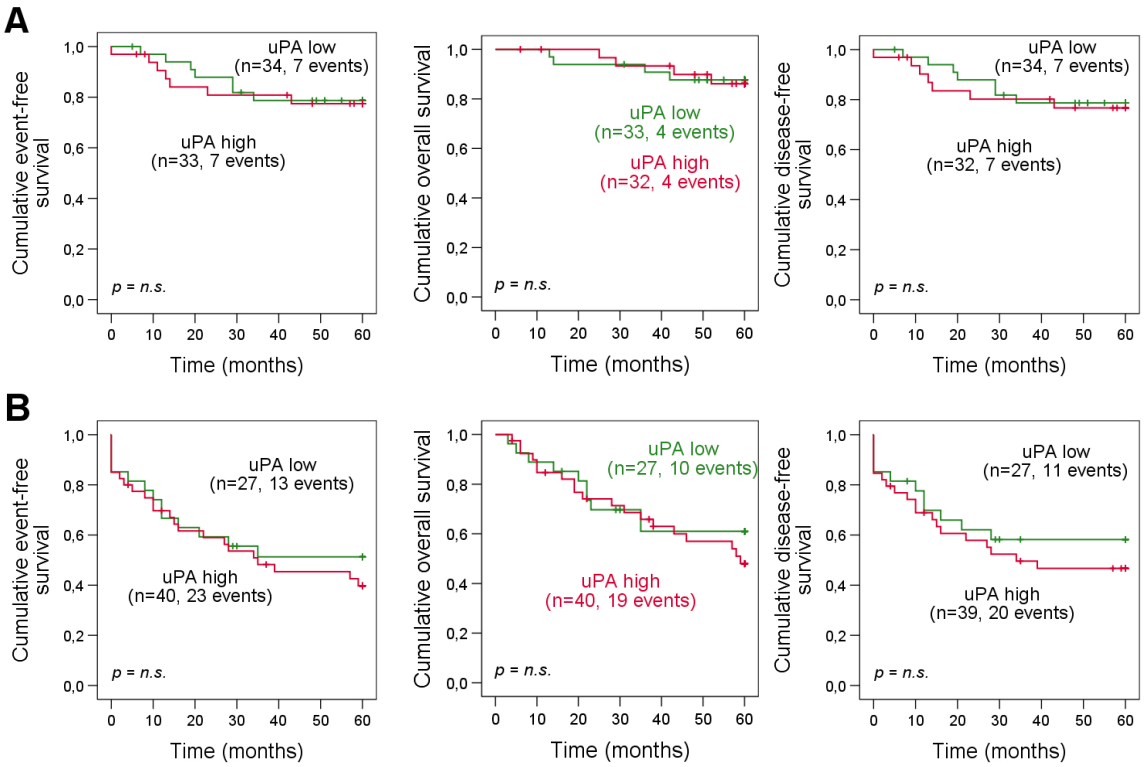

Figure 25. Probability of EFS, OS, and DFS of patients with TNBC as stratified by uPA expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) Lymph node-negative TNBC patients with elevated uPA expression levels show no significant difference in EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPA expression. **(B)** Lymph node-positive TNBC patients with elevated uPA expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPA expression; however, there is no statistically significant difference between these groups.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator.

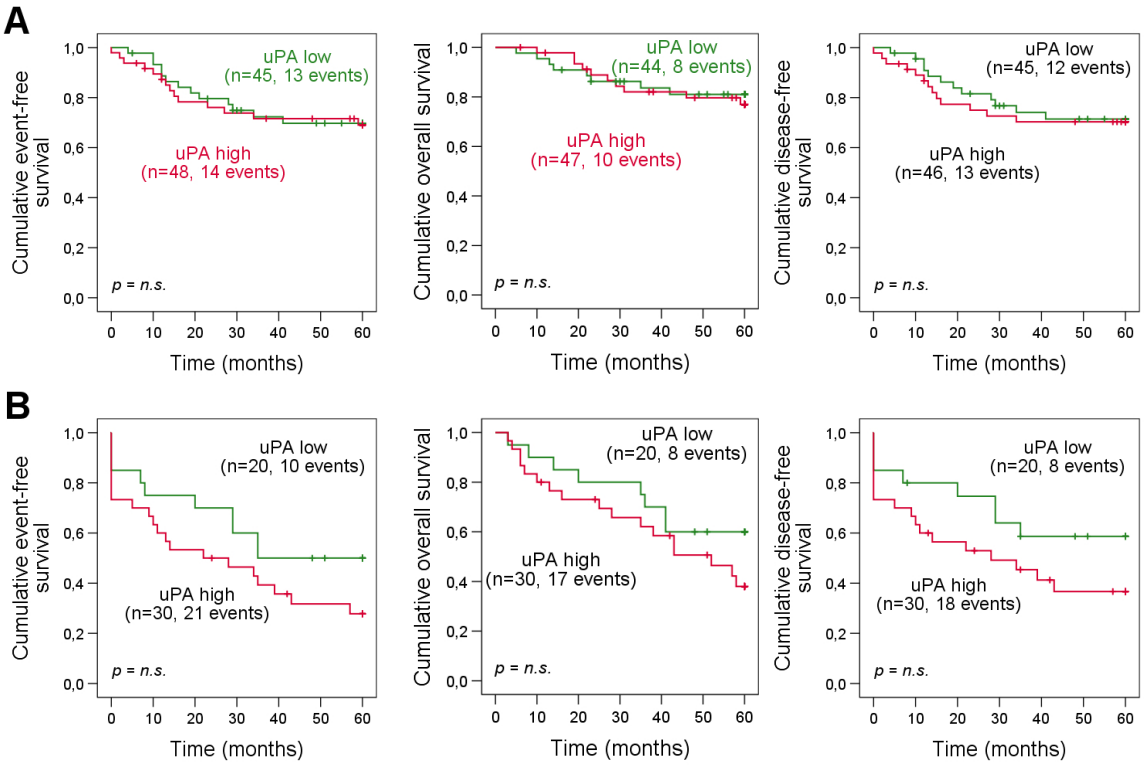

Figure 26. Probability of EFS, OS, and DFS of patients with TNBC as stratified by uPA expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) TNBC patients that have received adjuvant chemotherapy with elevated uPA expression levels show no significant difference in EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPA expression. **(B)** TNBC patients with high uPA expression levels, that have received no adjuvant chemotherapy, tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPA expression; however, there is no statistically significant difference between these groups.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, TNBC: triple-negative breast cancer, uPA: urokinase-type plasminogen activator.

4.4.2.2 Impact of PAI-1 expression levels analyzed by IHC on the probability of EFS, OS, and DFS of TNBC patients

Two groups of TNBC patients were differentiated and characterized by plasminogen activator inhibitor type-1 (PAI-1) expression of tumor tissue: patients with low levels of PAI-1 (n = 56) and patients with high levels of PAI-1 (n = 92). Initially, the scoring of stained tissue resulted in three TNBC patient groups: score 0 (low intensity, n = 56), score 1 (medium intensity, n = 69), and score 2 (high intensity, n = 23). Patients with medium PAI-1 intensity scores were assigned to the high intensity score group. **Figure 27, A** shows the analyzation process of the whole TNBC patient collective. Different subcategories of TNBC patients were analyzed; patients with invasive ductal carcinoma **(Fig. 27, B)**, patients with high-grade TNBC **(Fig. 27, C)**, patients with lymph node-negative TNBC **(Fig. 28, A)**, and patients with lymph node-positive TNBC **(Fig. 28, B)**. Patients who received adjuvant chemotherapy **(Fig. 29, A)** and patients who did not receive adjuvant chemotherapy **(Fig. 29, B)** were also evaluated. **Figure 27-29** show the comparison of generated Kaplan-Meier curves using the log-rank test (Mantel-Cox test).

An analysis of the whole collective indicated that TNBC patients with elevated PAI-1 expression levels tend to have worse EFS, OS, and DFS than patients with low PAI-1 expression levels. Statistical significance was attained by analyzing the three subgroups of TNBC; invasive ductal carcinoma, high-grade tumors, and patients who have received adjuvant chemotherapy. TNBC patients with invasive ductal carcinoma and elevated PAI-1 expression levels show significantly worse OS than the group of patients with low PAI-1 expression (p = 0.037) **(Fig. 28, B)**. Furthermore, TNBC patients with high-grade tumors and elevated PAI-1 expression levels show significantly worse OS than the group of patients with low PAI-1 expression (p = 0.033) **(Fig. 27, C)**. In cases of EFS, these patients display a trend toward significance (p = 0.077) **(Fig. 27, C)**. In addition, the patient subgroup that received adjuvant chemotherapy and with elevated PAI-1 expression levels show significantly worse OS than the group of patients with low PAI-1 expression (p = 0.044) **(Fig. 29, A)**.

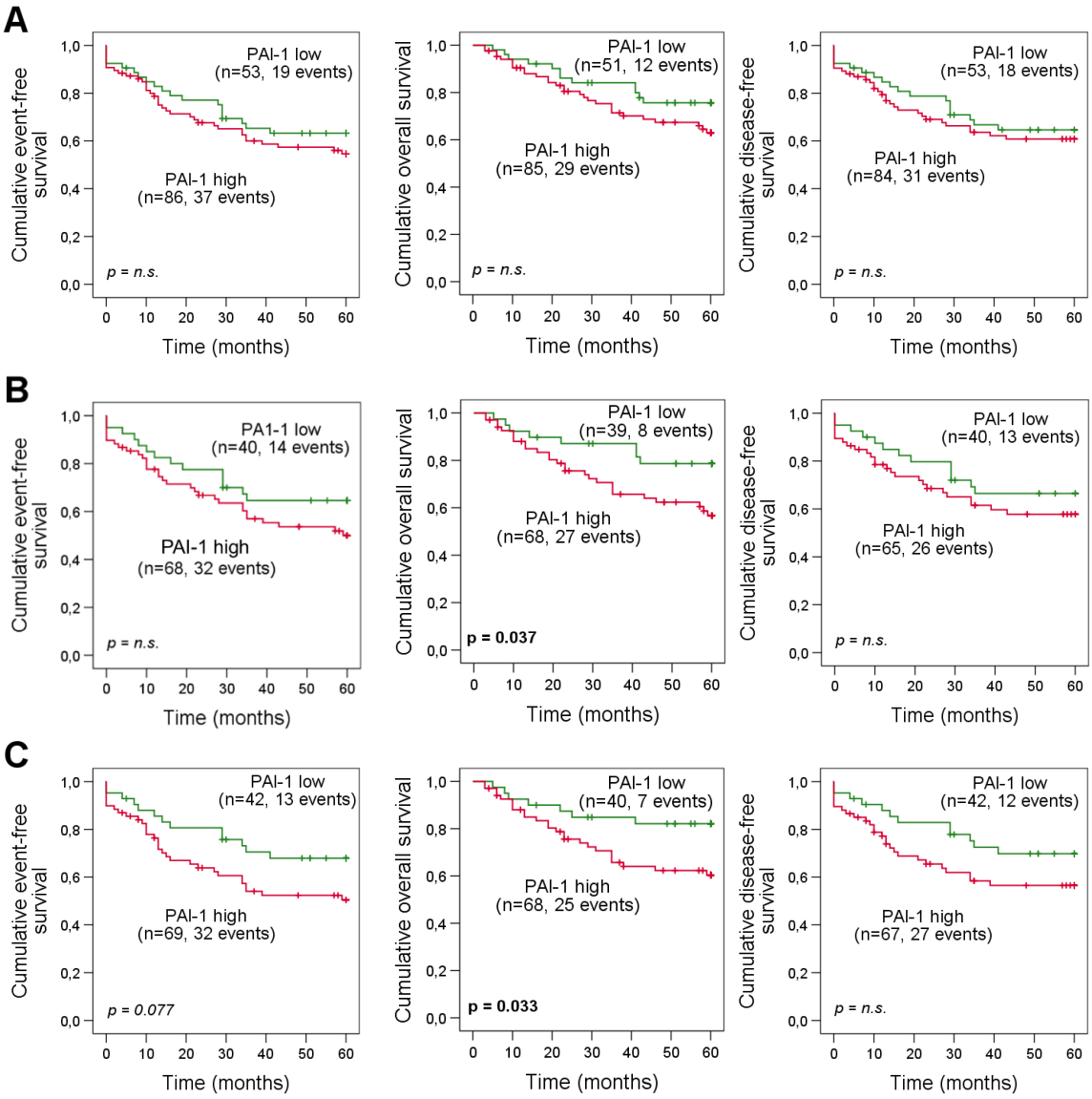

Figure 27. Probability of EFS, OS, and DFS of patients with TNBC as stratified by PAI-1 expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) TNBC patients with elevated PAI-1 expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low PAI-1 expression; however, there is no statistically significant difference between these groups. **(B)** TNBC patients with invasive ductal carcinoma and elevated PAI-1 expression levels show significantly worse OS than the group of patients with low PAI-1 expression (p = 0.037, middle panel). **(C)** TNBC patients with high-grade tumors and elevated PAI-1 expression levels show significantly worse OS than the group of patients with low PAI-1 expression (p = 0.033, middle panel). In the case of EFS (left panel) these patients display a trend toward significance (p = 0.077). Analysis of TNBC patients with low-grade and intermediate-grade tumors is not depicted because of the small number of cases.

DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

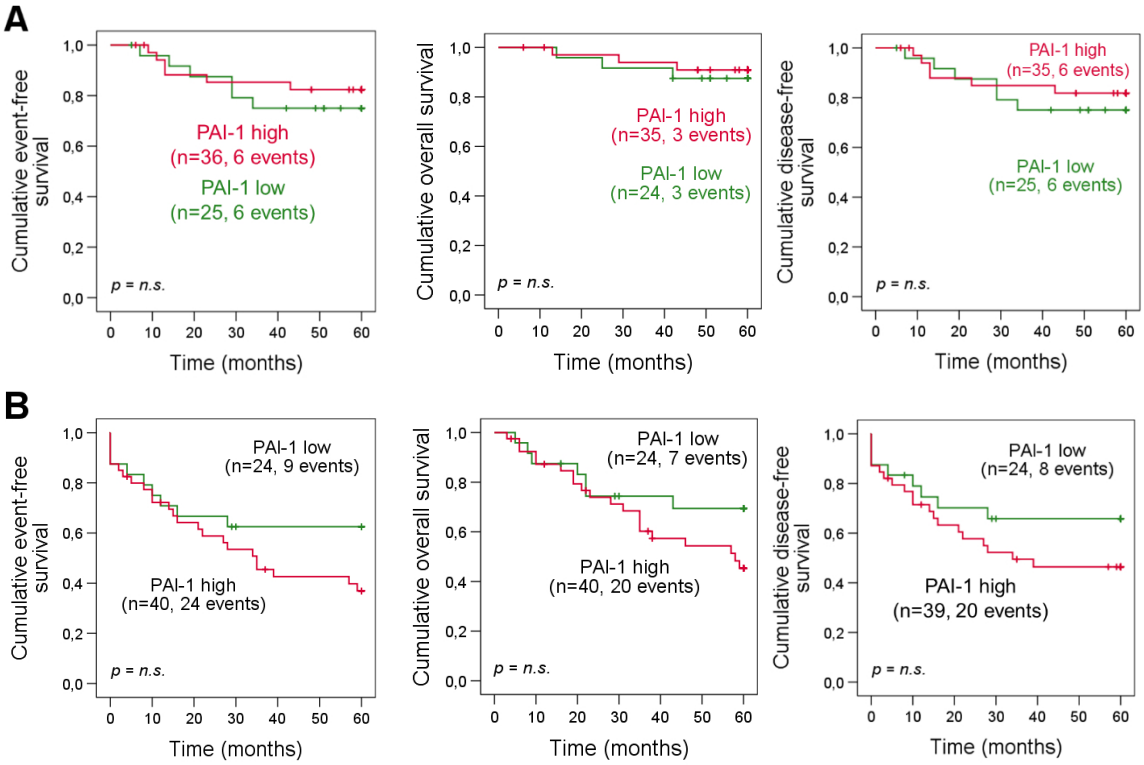

Figure 28. Probability of EFS, OS, and DFS of patients with TNBC as stratified by PAI-1 expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) Lymph node-negative TNBC patients with elevated PAI-1 expression levels show no significant difference in EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low PAI-1 expression. **(B)** Lymph node-positive TNBC patients with elevated PAI-1 expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low PAI-1 expression; however, there is no statistically significant difference between these groups.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

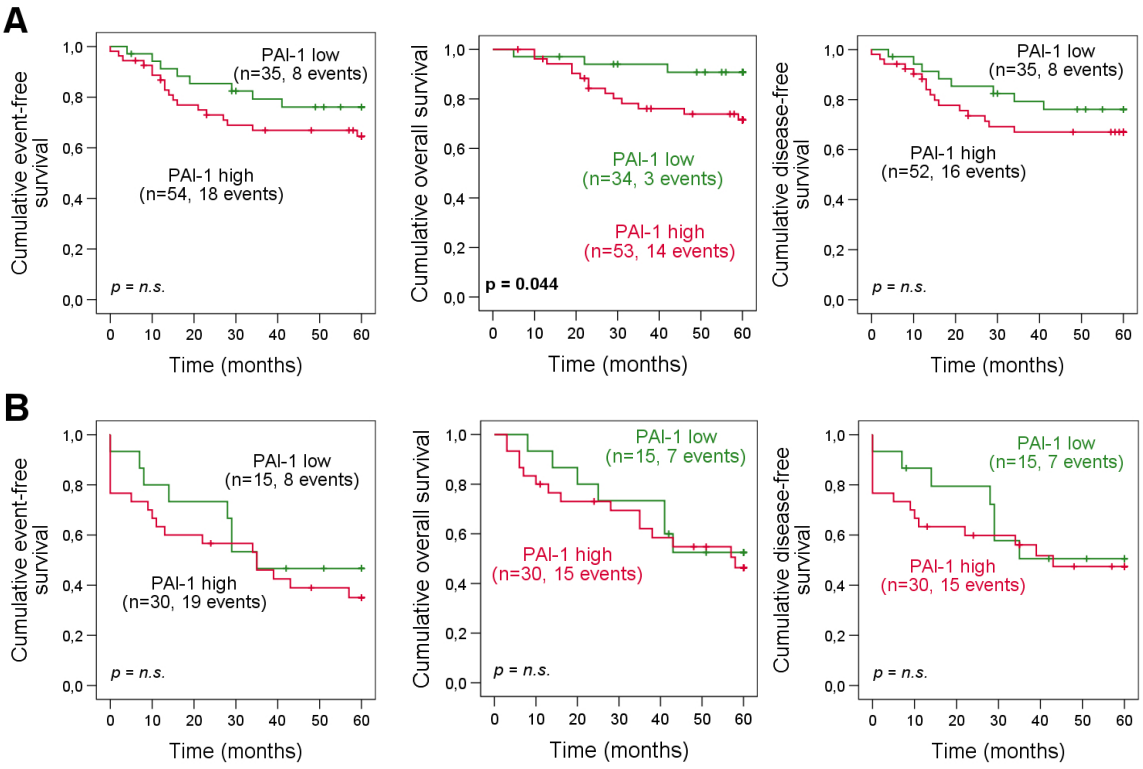

Figure 29. Probability of EFS, OS, and DFS of patients with TNBC as stratified by PAI-1 expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) TNBC patients that have received adjuvant chemotherapy, with elevated PAI-1 expression levels show significantly worse OS (p = 0.044, middle panel) than the group of patients with low PAI-1 expression. **(B)** TNBC patients with high PAI-1 expression levels, that have received no adjuvant chemotherapy, display no difference in EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low PAI-1 expression.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, PAI-1: plasminogen activator inhibitor type-1, TNBC: triple-negative breast cancer.

4.4.2.3 Impact of uPAR expression levels analyzed by IHC on the probability of EFS, OS, and DFS of TNBC patients

Two groups of TNBC patients were differentiated characterized by urokinase plasminogen activator receptor (uPAR) expression in tumor tissue: patients with low levels of uPAR (n = 49) and patients with high levels of uPAR (n = 98). Initially, scoring of the stained tissue resulted in four TNBC patient groups: score 0 (low intensity, n = 6), score 1 (medium-low intensity, n = 43), score 2 (medium-high intensity, n = 72), and score 3 (high intensity, n = 26). Patients with uPAR intensity score 1 were attributed to the low intensity score group; patients with uPAR intensity score 2 were attributed to the high intensity score group. **Figure 30, A** shows the analyzation process of the whole TNBC patient collective. Various subcategories of TNBC patients were analyzed; patients with invasive ductal carcinoma **(Fig. 30, B)**, patients with high-grade TNBC **(Fig. 30, C)**, patients with lymph node-negative TNBC **(Fig. 31, A),** and patients with lymph node-positive TNBC **(Fig. 31, B)**. Also, patients who received adjuvant chemotherapy **(Fig. 32, A)** and patients who did not receive adjuvant chemotherapy **(Fig. 32, B)** were evaluated. **Figures 30-32** show the comparison of generated Kaplan-Meier curves using the log-rank test (Mantel-Cox test).

An analysis of the whole collective showed that TNBC patients with elevated uPAR expression levels tend to have worse EFS, OS, and DFS than patients with low uPAR expression levels. However, statistical significance was not attained. A minimum *p*-value of 0.166 was computed analyzing the impact of high uPAR expression levels on the probability of DFS in patients with invasive ductal TNBC **(Fig. 30, B)**.

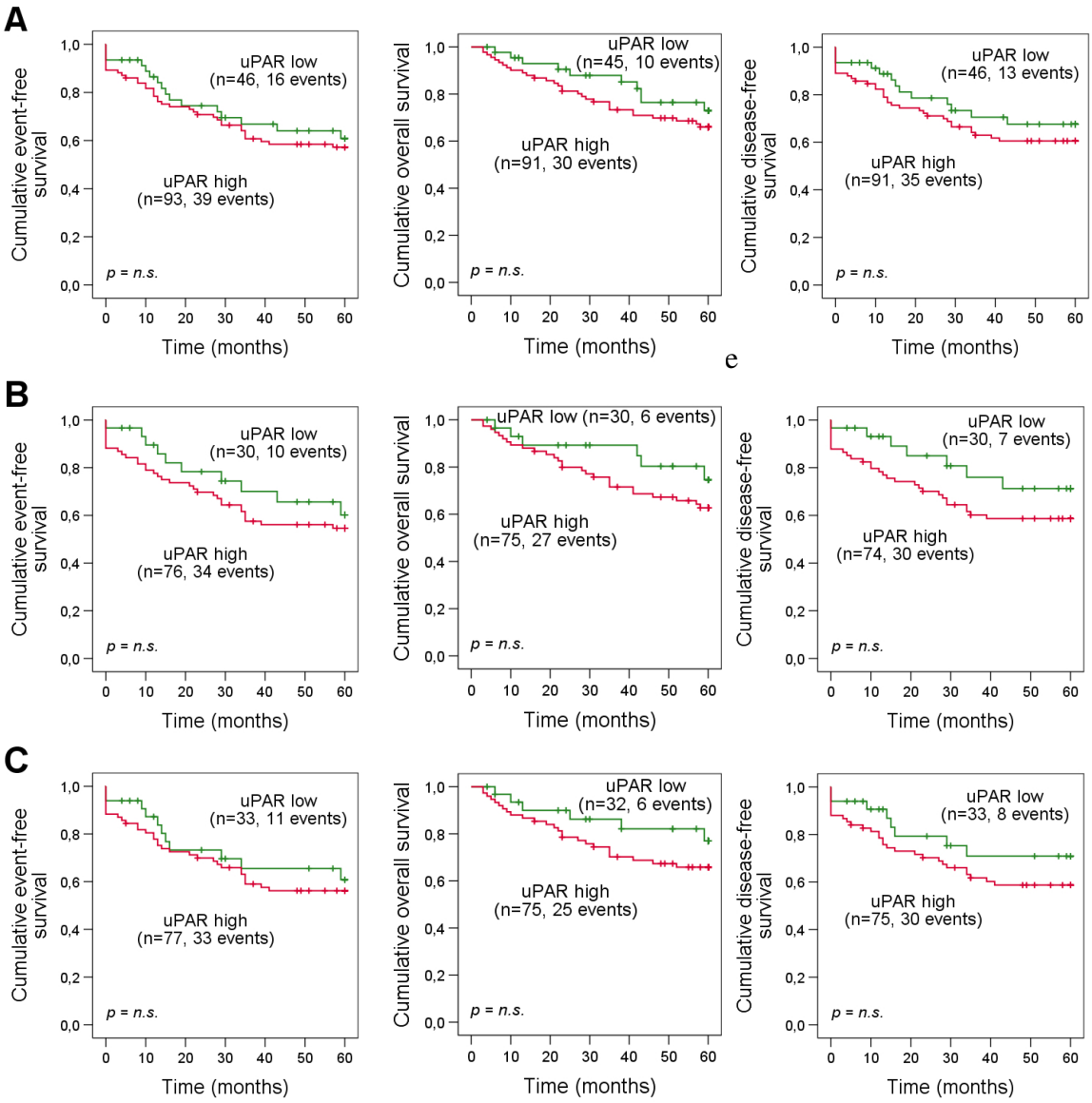

Figure 30. Probability of EFS, OS, and DFS of patients with TNBC as stratified by uPAR expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) TNBC patients with elevated uPAR expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups. **(B)** TNBC patients with invasive ductal carcinoma and elevated uPAR expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups. **(C)** TNBC patients with high-grade tumors and elevated uPAR expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups. Analysis of TNBC patients with low-grade and intermediate-grade tumors is not depicted because of the small number of cases.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

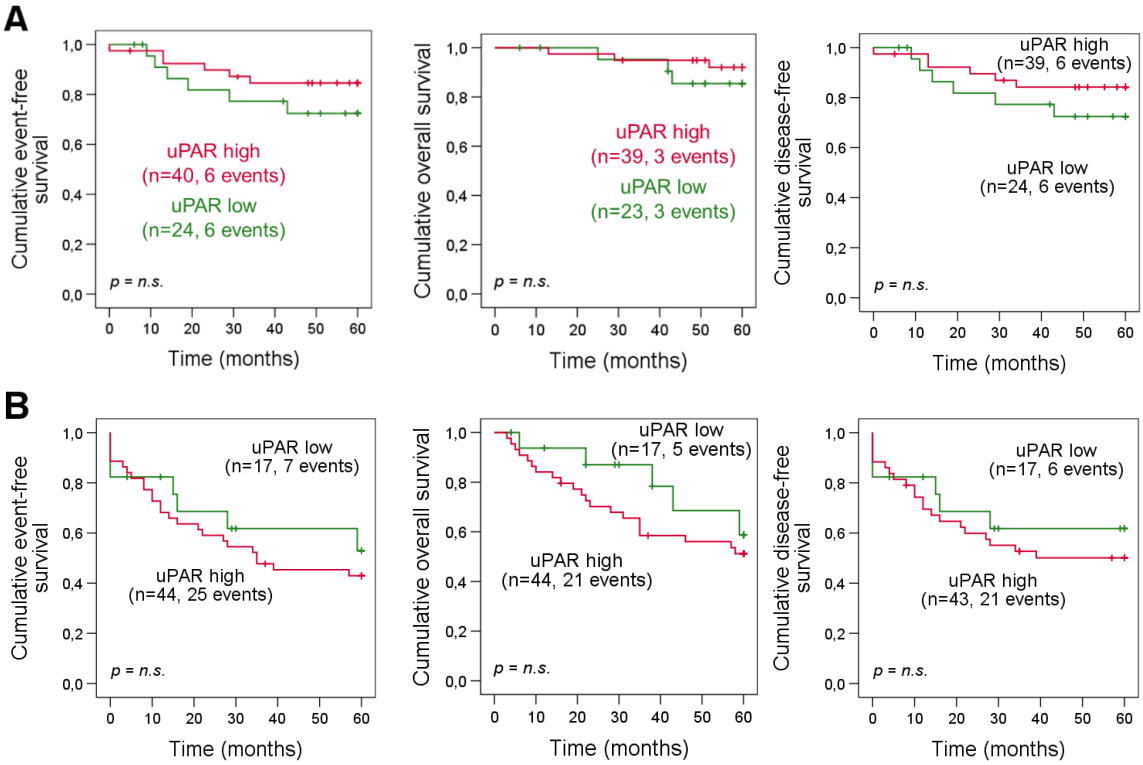

Figure 31. Probability of EFS, OS, and DFS of patients with TNBC as stratified by uPAR expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) Lymph node-negative TNBC patients with elevated uPAR expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups. **(B)** Lymph node-positive TNBC patients with elevated uPAR expression levels tend to show worse EFS (left panel), OS (middle panel), and DFS (right panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

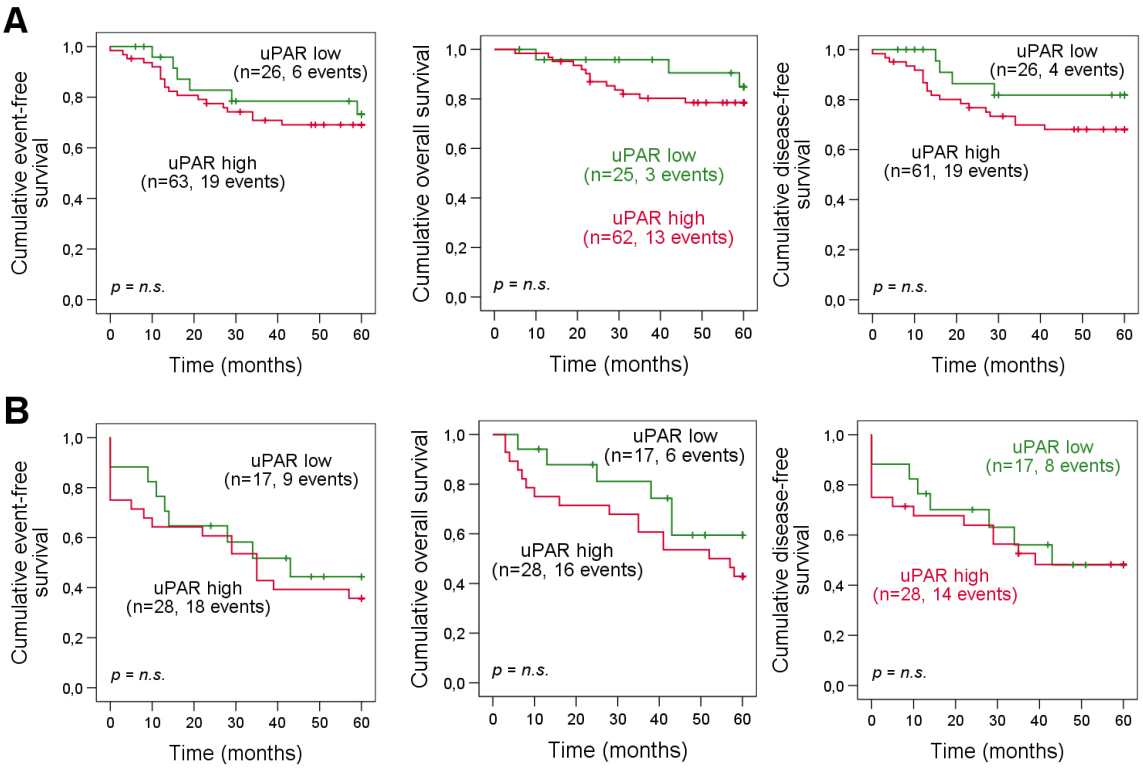

Figure 32. Probability of EFS, OS, and DFS of patients with TNBC as stratified by uPAR expression levels in primary tumor tissues analyzed by IHC (Kaplan-Meier analysis). (A) TNBC patients that have received adjuvant chemotherapy, with elevated uPAR expression levels tend to show worse DFS (right panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups. **(B)** TNBC patients with high uPAR expression levels, that have received no adjuvant chemotherapy, tend to show worse OS (middle panel) than the group of patients with low uPAR expression; however, there is no statistically significant difference between these groups.
DFS: disease-free survival, EFS: event-free survival, IHC: immunohistochemistry, OS: overall survival, TNBC: triple-negative breast cancer, uPAR: urokinase-type plasminogen activator receptor.

5 Discussion

Triple-negative breast cancer (TNBC) is a very challenging breast cancer subtype. Besides surgery and radiation therapy, the only established systemic treatment option is cytotoxic chemotherapy. Chemotherapy is indisputably an effective therapy for TNBC patients. It can prevent disease recurrence and death; breast cancer-related mortality is significantly reduced. Chemotherapy even improves the outcome of TNBC patients to a greater extent compared with patients with estrogen receptor (ER)-positive tumors. Nevertheless, TNBC patients have a poorer survival after chemotherapy than patients with breast cancers of other subtypes *(Foulkes et al. 2010)*.

Cytotoxic agents, such as anthracyclines or taxanes lack specificity. The toxic side effects of chemotherapy are immense and must not be underestimated. Therefore, the decision to use chemotherapy should be made carefully and well-informed. Presently, almost all TNBC patients reflexively are treated with (neoadjuvant) chemotherapy *(Curigliano et al. 2017)*. Only patients with very small ductal triple-negative tumors (≤5 mm) and node-negative disease are potentially spared from chemotherapy. In 2017, the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer recommended against routine clinical adjuvant chemotherapy in pT1a pN0 TNBC patients *(Curigliano et al. 2017)*. The Munich Tumor Center (Tumorzentrum München) recommends the indication of neoadjuvant chemotherapy for cT1b TNBC and adjuvant chemotherapy for pT1b pN0 TNBC *(MunichTumorCenter 2017)*. For well-founded decision making regarding the indication of systemic therapy, prognostic and predictive biomarkers are essential. However, the established parameters for evaluating prognosis and predicting the response to therapy in hormone receptor (HR)-positive breast cancer are presently not recommended in TNBC. For instance, the Munich Tumor Center (Tumorzentrum München) advises performing molecular multigene tests, such as Oncotype DX®, MammaPrint®, Endopredict®, and Prosigna® only in HR-positive breast cancer, but not in TNBC *(MunichTumorCenter 2017)*. Another example is Ki-67. Even though the proliferation marker is significantly correlated with a pathologic complete response, it has no prognostic effect in HR-negative breast cancer. Patients with HR-negative disease and high Ki-67 levels have a rather better outcome because of the high response rate to chemotherapy *(Denkert et al. 2013)*. Furthermore, Ki-67 is also not

recommended as a predictive factor to guide the decision on neoadjuvant chemotherapy in TNBC *(AWMF-S3-Leitlinie 2018)*. The biomarkers, urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1), are currently also only established for evaluating HR-positive breast cancer. Determination of uPA and PAI-1 can indicate chemotherapy in borderline indication cases such as lymph node-negative or G2 breast cancer *(Harris et al. 2016, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. In contrast, the biomarkers uPA and PAI-1 are presently not recommended in TNBC. The American Society of Clinical Oncology (ASCO) advises against the use of uPA and PAI-1 for decision making concerning adjuvant chemotherapy in TNBC *(Harris et al. 2016)*. This recommendation is solely based on the fact that data are missing that evaluate the clinical utility of uPA and PAI-1 in the triple-negative subtype of breast cancer *(Harris et al. 2016)*. Therefore, there is a substantial need to study the prognostic and predictive impact of uPA and PAI-1 in TNBC.

In the present thesis, the development of reproducible immunohistochemical staining procedures enabled the assessment of not only uPA and PAI-1, but also urokinase-type plasminogen activator receptor (uPAR) in a TNBC patient cohort. It was possible to both demonstrate histologic localization of uPA, PAI-1, and uPAR and determine the levels of uPA, PAI-1, and uPAR in TNBC tissue. Furthermore, the correlation of uPA/PAI-1/uPAR expression with follow-up data allowed the evaluation of the prognostic impact of these three biomarkers in a TNBC patient cohort. Two of the three antibodies applied in this study had been used in previous studies for analysis of breast cancer cells: the mAb #3689 directed against uPA and the mAb IID7 directed against uPAR *(Jankun et al. 1993, Costantini et al. 1996, Luther et al. 1997, Schmitt et al. 2008, Hildenbrand et al. 2009, Kotzsch et al. 2010, Lang et al. 2013)*. In contrast, there was no study available using the mAb #3786 directed against PAI-1. Histologic localization of uPA, PAI-1, and uPAR in TNBC cells were consistent with previous findings that analyzed unselected breast cancer cells of all subtypes. On the cellular level, uPA, PAI-1, and uPAR were mainly present in the cytoplasm of triple-negative tumor cells. Several previous studies reported cytoplasmic localization of uPA, PAI-1, and uPAR in unselected breast cancer cells of all subtypes *(Jänicke et al. 1990, Jänicke et al. 1991, Jankun et al. 1993, Costantini et al. 1996, Luther et al. 1997, Dublin et al. 2000, de Bock and Wang 2004, Giannopoulou et al. 2007, Schmitt et al. 2008, Kotzsch et al. 2010, Lang et al. 2013)*. In contrast to the cytoplasm of tumor cells, the concentration of uPA, PAI-1, and uPAR appears to be minimal or zero inside the nuclei of triple-negative tumor cells. Concordant results were found by Jankun et al. when

staining unselected breast cancer cells of all subtypes. Still, they occasionally observed staining in the nucleus using antibodies directed against uPA, PAI-1, and uPAR *(Jankun et al. 1993)*. Corresponding to the membranous localization of uPAR, strong staining of the cell membrane was observed using an anti-uPAR antibody in TNBC cells. Several previous studies also described cell membrane staining for uPAR when analyzing breast cancer cells *(Jankun et al. 1993, Costantini et al. 1996, Kotzsch et al. 2010)*. In fact, uPA, PAI-1, and uPAR were not only detected in triple-negative tumor cells but also in adjacent stromal cells. Slightly increased concentrations of uPA, PAI-1, and uPAR were often noticed in the tissue surrounding the malignant tumor. Similar results were found by previous studies that analyzed staining patterns of unselected breast cancer cells of all subtypes. Diffuse staining of connective tissue was observed in many cases using uPA-/PAI-1-/uPAR-specific antibodies *(Jankun et al. 1993, Costantini et al. 1996, Luther et al. 1997, de Bock and Wang 2004, Giannopoulou et al. 2007, Hildenbrand et al. 2009, Kotzsch et al. 2010)*. Besides evaluating the staining localization, the intensity of the staining was assessed. Previous findings have shown that there is an inverse correlation between uPA/PAI-1/uPAR expression and HR status. Several studies have found that ER- or progesterone receptor (PR)-negative breast cancer tissue express higher levels of uPA and PAI-1 *(Jänicke et al. 1990, Jänicke et al. 1991, Bouchet et al. 1999, Look et al. 2002)*. The same applies to uPAR *(Grøndahl-Hansen et al. 1995, Bouchet et al. 1999, de Witte et al. 2001)*. Since TNBC tests negative for HRs, one could assume that uPA, PAI-1, and uPAR expression is especially high in triple-negative tumor tissue. Results of this thesis verify this inverse correlation between uPAR expression and HR expression in a TNBC cohort. In fact, uPAR was highly expressed in TNBC. The analysis of uPAR levels in TNBC demonstrated that 67% of cases showed high uPAR expression and 33% showed low levels of uPAR. Levels of PAI-1 and uPA in TNBC were also elevated in this study, though not as strong compared with uPAR. In this case, 62% of TNBC showed high levels of PAI-1, and 55% displayed high uPA expression. Results consistent with these findings were published by Xu et al. in 2018, who analyzed PAI-1 via immunohistochemistry (IHC). They found a significantly elevated PAI-1 expression in TNBC patients when tumor tissue was compared with healthy adjacent tissue *(Xu et al. 2018)*. Results of another study were also concordant with our findings. Schmidt et al. used the enzyme-linked immunosorbent assay (ELISA) FEMTELLE® to determine uPA and PAI-1 in TNBC patients and found that 89% of TNBC exhibited high levels of uPA and PAI-1 *(Schmidt et al. 2014)*.

Previous studies have found that increased uPA, PAI-1, and uPAR expression levels of breast cancer patients were associated with poor survival *(Carriero et al. 1994, Duggan et al. 1995, Grøndahl-Hansen et al. 1995, Costantini et al. 1996, Dublin et al. 2000, Guyton et al. 2000, Giannopoulou et al. 2007, Kotzsch et al. 2010)*. While uPA and PAI-1 represent already established prognostic and predictive biomarkers for evaluating HR-positive breast cancer, the impact of uPA, PAI-1, and uPAR on survival amongst TNBC patients remains unknown so far. Thus, we analyzed the impact of uPA, PAI-1, and uPAR in the TNBC subtype. Results of this study emphasize the prognostic impact of all three markers for the risk assessment of breast cancer. In particular, the impact of PAI-1 on the probability of survival was highest compared with uPA and uPAR. Statistical significance was attained after analyzing three patient subgroups: patients with high-grade TNBC (overall survival (OS): $p = 0.033$), accounting for 82% of the patient cohort; patients with invasive ductal tumors (OS: $p = 0.037$), accounting for 78% of the cohort; and patients who have received adjuvant chemotherapy (OS: $p = 0.044$), accounting for 62% of the cohort. Results of this study correspond with data recently published by Zhang et al. in 2018. They observed a significant association of PAI-1 with the prognosis of TNBC patients. High PAI-1 expression was associated with worse relapse-free survival, distant metastasis-free survival, and OS *(Zhang et al. 2018)*. Contrasting results were published by Witzel et al. who observed no association between mRNA PAI-1 expression and survival in TNBC *(Witzel et al. 2014)*. A possible explanation for these conflicting data could be that Witzel et al. used a different method of measurement. In contrast to both the study by Zhang et al. and our study, Witzel et al. determined levels of PAI-1 using microarray analysis instead of IHC. Further studies are needed to clarify the actual prognostic value of PAI-1 in TNBC, but the results of our study suggest that PAI-1 is a valuable biomarker in TNBC. The determination of PAI-1 could generate more information on TNBC patients to enable more personalized therapy. For example, high levels of PAI-1 could eventually indicate chemotherapy in TNBC. Currently, pT1a pN0 TNBC patients are essentially excluded from chemotherapy *(Curigliano et al. 2017, MunichTumorCenter 2017)*. Analysis of PAI-1 could generate additional information to identify those TNBC patients who benefit from chemotherapy despite the small size of their tumors and negative status of their lymph nodes. On the contrary, all TNBC patients with bigger tumors (>pT1a) are treated with chemotherapy. However, does every single one of these patients benefit from chemotherapy? Previous findings suggested that not only breast cancer displays immense heterogeneity but also TNBCs constitute heterogeneous molecular

subtypes of breast cancer. Kalimutho et al. divide TNBC into several subclasses including basal-like, mesenchymal-like, mesenchymal stem-like, luminal androgen receptor expression, and immunomodulatory subtypes. They observed that these molecular subclasses differ in their response to therapy *(Kalimutho et al. 2015)*. Therefore, we need biomarkers that allow a more accurate assessment of TNBC to predict its clinical behavior. Results of this study suggest that determination of PAI-1 could generate valuable additional information to support the indication of chemotherapy in TNBC. On the other hand, TNBC patients with low levels of PAI-1 are possibly a patient group that does not benefit from chemotherapy.

While the results of this study significantly prove the prognostic impact of PAI-1 in the risk assessment of TNBC, the impact of uPA was less powerful. Nevertheless, the results of this study showed, that TNBC patients with elevated uPA expression levels tend to show worse survival than the group of patients with low uPA expression. This effect was highest in G3 TNBC patients, who represent the majority (i.e., 82%) of our patient cohort. The results of analyzing this poorly-differentiated subgroup of TNBC patients indicated that the impact of uPA on disease-free survival (DFS) displayed a trend toward significance (p = 0.097). Furthermore, we observed a moderate impact of uPA in patients who have not received chemotherapy. In contrast, there was absolutely no association between uPA and survival in patients who received adjuvant chemotherapy. The impact of uPA seems to be eliminated by treatment with chemotherapy. In contrast to the results of our study suggesting the possible prognostic potential of uPA in G3 TNBC, the data published by Witzel et al. showed no association between uPA and survival in triple-negative tumors *(Witzel et al. 2014)*. Therefore, further investigation of uPA is needed to clarify its potential prognostic and predictive impact in TNBC.

While the determination of uPA and PAI-1 are established to guide decision making on chemotherapy in lymph node-negative (N0) HR-positive breast cancer, the results of this study showed no association between uPA and PAI-1 levels and survival of N0 TNBC patients. One distinct feature of TNBC could be responsible for this result. In contrast to unselected breast cancer, the triple-negative subtype shows no correlation between tumor size and lymph node status. In fact, 55% of women with small tumors (≤1 cm) present with positive lymph nodes *(Dent et al. 2007, de Ruijter et al. 2011)*. This characteristic of TNBC may explain the lack of a prognostic impact of uPA and PAI-1 in N0 TNBC.

In contrast to lymph node-negative disease, our study results suggest that PAI-1 possibly has an effect on survival in the subgroup of lymph node-positive

TNBC. Patients with elevated PAI-1 expression levels tend to show worse survival than the group of patients with low PAI-1 expression; however, there was no statistically significant difference between these groups. Currently, almost all patients with TNBC receive (neoadjuvant) cytotoxic chemotherapy. Lymph node positivity adds another reason for the recommendation of chemoterapy. Therefore, at present, it is hardly imaginable, that some of these clinically high-risk node-positive TNBC patients could be spared from treatment with cytotoxic chemotherapy in the future. However, the use of molecular multigene tests already allow recommendations against adjuvant chemotherapy in limited node-positive (pN0-1) HR-positive breast cancer, that are at low genomic risk for recurrence. Similar to molecular multigene tests, analysis of PAI-1 expression could eventually generate additional information to enable a more differentiated treatment decision in lymph node-positive TNBC patients. The high expression of PAI-1 could support the recommendation of systemic treatment. In contrast, low levels of PAI-1 could indicate that chemotherapy is possibly not beneficial to the patient. Further research is needed to evaluate the prognostic and predictive impact of PAI-1 in node-positive TNBC.

To date, there is no widespread use of uPA and PAI-1 determination in routine clinical practice *(Lang et al. 2013, Harbeck et al. 2014, Senkus et al. 2015)*. There are several reasons for this. First, the only established method to assess uPA and PAI-1 levels quantitatively in clinical practice is the use of ELISA techniques *(Schmitt et al. 2008, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. The ELISA-derived FEMTELLE® uPA/PAI-1 assay ideally needs about 100-300 mg fresh or frozen tumor material to measure uPA and PAI-1. However, in routine clinical practice, the tumor tissue is usually fixed with formalin and embedded in paraffin. ELISA relies on fresh or frozen material as a source, thereby, limiting the clinical utility of both uPA and PAI-1. Moreover, the rather large amount of tissue that is recommended for determination by ELISA can be a problem. Even though Thomssen et al. demonstrated that it was feasible to determine uPA and PAI-1 in tissue obtained by a preoperative core needle biopsy, analysis of very small amounts of tissue extracts is sometimes technically impossible *(Thomssen et al. 2009)*. A possible solution to these problems is to determine both uPA and PAI-1 by IHC. In contrast to ELISA, IHC-based analysis requires only small amounts of tumor tissue. Moreover, uPA and PAI-1 levels can be determined by IHC in formalin-fixed, paraffin-embedded (FFPE) tumor tissue, which usually is available in routine clinical settings. A disadvantage is that IHC generates semiquantitative data, whereas ELISA is a quantitative method. The scoring of IHC staining by a

pathologist is more subjective than measurement by ELISA. Therefore, scoring should be done by a highly experienced pathologist to ensure consistent and reliable results. Immunohistochemical determination of HR status also relies on scoring by a pathologist and is the standard method worldwide in routine clinical practice. Previous studies have shown a significant association between uPA or PAI-1 IHC scores and respective ELISA levels *(Jänicke et al. 1990, Reilly et al. 1992)*. Lang et al. propose the optimization of IHC via image analysis for more objective scoring results. They demonstrated that the combination of IHC with image analysis is a reliable alternative method for determination of uPA and PAI-1 to ELISA *(Lang et al. 2013)*. Further studies are needed to establish an acceptable alternative method for the determination of both uPA and PAI-1 to enable more widespread use of these markers in clinical practice. Another reason for the limited use of uPA and PAI-1 determination in clinical practice, are doubts about their prognostic and predictive impact. In contrast to guidelines by ASCO, the German AGO Breast Committee, and the Munich Tumor Center (Tumorzentrum München), a German guideline published at the end of 2018 by the AWMF (Association of the Scientific Medical Societies in Germany) abandoned the previous recommendation of uPA and PAI-1 for decision making concerning the indication of adjuvant chemotherapy in HR-positive, human epidermal growth factor receptor 2 (HER2)-negative, lymph node-negative breast cancer. The reasons given for this decision were weak points in the study design of the Chemo-N0 trial. Patients with low uPA and PAI-1 did not receive any systemic treatment. In contrast to current routine clinical practice, low-risk HR-positive patients were not treated with endocrine therapy alone. Furthermore, the HER2 status of the patients included in the Chemo-N0 trial was not determined. However, there is some evidence that the different subtypes of breast cancer differ in their levels of uPA and PAI-1. Schmidt et al. observed higher uPA and PAI-1 expression in HER2-positive or TNBC compared with luminal tumors *(Schmidt et al. 2014)*. The results of our study support the hypothesis that TNBC expresses high levels of both uPA and PAI-1. The prognostic impact of uPA and PAI-1 seems to differ depending on the subtype of breast cancer being analyzed. According to the results of Witzel et al., the prognostic impact of uPA and PAI-1 are limited to HER2-positive breast cancer. They found no association of uPA and PAI-1 with survival in TNBC *(Witzel et al. 2014)*. In contrast, our study results demonstrate that PAI-1 has a significant prognostic effect in TNBC. Moreover, our results indicated a trend toward significance when the prognostic impact of uPA in TNBC was analyzed. In any case, further investigation is needed to evaluate the expression of uPA and PAI-1

in selected groups of patients with breast cancer and analyze its prognostic impact in different breast cancer subtypes.

Besides uPA and PAI-1, previous studies have found that increased uPAR expression levels of breast cancer are associated with poor survival *(Duggan et al. 1995, Grøndahl-Hansen et al. 1995, Costantini et al. 1996, Dublin et al. 2000, Foekens et al. 2000, Guyton et al. 2000, Giannopoulou et al. 2007, Kotzsch et al. 2010)*. Currently, there is no data available investigating the impact of uPAR on the outcome of TNBC patients. This study is one of the first to correlate levels of uPAR in a TNBC cohort with survival. Similar to uPA, the prognostic impact of uPAR in TNBC patients was less strong compared with PAI-1. However, TNBC patients with elevated uPAR expression levels tended to show worse survival than the group of patients with low uPAR expression. At present, there are no other studies available investigating the prognostic or predictive impact of uPAR in TNBC. Further studies are needed to evaluate its potential prognostic impact in TNBC.

Interestingly, we observed that uPA, PAI-1, and uPAR had a no impact on the survival of lymph node-negative (N0) TNBC patients. The determination of all three biomarkers seems to be irrelevant in the risk assessment of N0 TNBC patients. While uPA and PAI-1 are established biomarkers for evaluating the risk of recurrence and prognosis in N0 HR-positive breast cancer patients, the results of our study showed no prognostic effect of uPA and PAI-1 in N0 TNBC. Presently, the nodal status is only an approved parameter to assess the risk of recurrence and metastasis in non-TNBC patients. About 70% of node-negative breast cancer patients are cured by locoregional therapy alone *(Kantelhardt et al. 2011, Criscitiello et al. 2012)*. The determination of uPA and PAI-1 facilitates the identification of those patients who would significantly benefit from chemotherapy, and those who would not since chemotherapy would be overtreatment. Therefore, analysis of uPA and PAI-1 often prevents non-TNBC patients with the borderline indication for chemotherapy from receiving treatment with chemotherapy and suffering subsequent damage. In contrast, irrespective of their nodal status, TNBC patients are usually classified into the high-risk group regarding the risk of recurrence, solely because of their triple-negativity. Accordingly, cytotoxic chemotherapy is recommended in almost every patient with TNBC *(AGOBreastCommittee 2018)*. The only exceptions are N0 patients with very small triple-negative tumors with a size up to 5 mm (T1a). Several guidelines recommend against routine treatment with adjuvant chemotherapy in pT1a pN0 TNBC *(Curigliano et al. 2017, MunichTumorCenter 2017)*. However, the group of patients with TNBC that is spared from chemotherapy because of these guidelines is rather small. Cristello et al.

propose that 60% of node-negative TNBC patients are cured by locoregional therapy alone *(Criscitiello et al. 2012)*. Therefore, it is essential to find new strategies that enable the more precise evaluation of the risk of recurrence in N0 TNBC patients.

The prognostic impact of uPA and uPAR in the risk assessment of TNBC was found to be lower than that of PAI-1. However, the determination of uPA and uPAR enabled the differentiation of two patient subgroups characterized by different survival probabilities. Unfortunately, the differences between these groups were not significant, which may be owing to the relatively low number of patients in this analysis (n = 172). In addition, the classification of the tumors via manual scoring of the stained tissues is a crude method. The scoring by a pathologist led to the differentiation of a maximum of four staining intensity groups. For statistical analysis, some of these groups were combined to form two groups; one group was characterized by low levels of the antigen (uPA, PAI-1, or uPAR) and the other was characterized by high levels of the antigen. However, this approach hinders the definition of an optimal cut point of the antigen for statistical analysis. In contrast, quantitative methods such as ELISA and real-time polymerase chain reaction enable the definition of a more differentiated cut point. Semiquantitative image analysis can be used as a possible method for improving the evaluation of immunohistochemical staining. The application of IHC-based image analysis results in the differentiation of more numerous staining intensity groups, thereby enabling optimization of the antigen cut point.

Besides the possible prognostic impact of uPAR, the exceptionally high uPAR expression in TNBC could be a potential target for the treatment of TNBC. As mentioned previously, at present, the only established systemic treatment option of TNBC is cytotoxic chemotherapy *(Coates et al. 2015, Senkus et al. 2015, Curigliano et al. 2017, AGOBreastCommittee 2018)*. Because of its triple-negativity, personalized therapy options, such as trastuzumab, a monoclonal antibody interfering with HER2, or tamoxifen, a selective ER modulator, is not recommended for the treatment of TNBC *(Joensuu and Gligorov 2012, Harris et al. 2016, MunichTumorCenter 2017, AGOBreastCommittee 2018, AWMF-S3-Leitlinie 2018)*. Several studies already investigated the effect of knockdown of uPAR in unselected breast cancer cells of all subtypes. Downregulation of uPAR resulted in the inhibition of tumorigenesis *(Subramanian et al. 2006, Kunigal et al. 2007, Li et al. 2010)*. Two studies are available that evaluate the inhibition of uPAR in the TNBC subtype. Huber et al. showed that the direct interaction of uPAR with uPA leads to an enhanced malignant potential of TNBC. Moreover, they observed that the

combined inhibition of both uPAR and uPA in TNBC cells significantly reduced cell viability, migration, and invasion *(Huber et al. 2016)*. Hamurcu et al. also managed to suppress uPAR-mediated survival, proliferation, migration, and invasion of TNBC cells. They showed that the knockdown of autophagy-related genes (LC3 (microtubule-associated light chain 3) and Beclin-1) inhibited the expression of uPAR *(Hamurcu et al. 2018)*. These promising results indicate that uPAR has potential as a new target for the treatment of TNBC patients. However, currently, no agent is available that allows the clinical evaluation of uPAR inhibition.

Besides uPAR, the results of our study also suggest a rather high expression of uPA and PAI-1 in TNBC. Both biomarkers could also be potential new targets in the treatment of TNBC. The orally administrable upamostat (WX-671), a prodrug of WX-UK1 that inhibits uPA, has already been investigated in phase II clinical trial. Goldstein et al. compared combination treatment with upamostat and capecitabine with capecitabine monotherapy in HER2-negative metastatic breast cancer. They observed that upamostat significantly improved progression-free survival (PFS) in patients who had received prior adjuvant chemotherapy. Furthermore, they proved that upamostat was safe and well tolerated by patients *(Goldstein et al. 2013)*. These promising results warrant further studies evaluating the inhibition of uPA via upamostat in breast cancer patients. Currently, there are no studies available evaluating the effect of upamostat on TNBC. Since TNBC possibly expresses high uPA levels, the inhibition of uPA could be more effective in the treatment of patients with the triple-negative disease compared with non-triple-negative breast cancer patients.

Besides uPA and uPAR, the potential of PAI-1 as a target was also evaluated in numerous previous studies. Several inhibitors of PAI-1 have been developed *(Rouch et al. 2015)*. Inhibition of PAI-1 was researched both in vitro and in vivo *(Rouch et al. 2015)*. A few studies are available evaluating the inhibition of PAI-1 in breast cancer cells. It was shown that the inhibition of PAI-1 resulted in the decreased metastatic potential of breast cancer cells *(Blake et al. 2009, Fortenberry et al. 2016)*. Now, there are no data available concerning PAI-1 inhibition in TNBC cells. Currently, no PAI-1 inhibitor is available and approved for human therapeutic use. Therefore, the evaluation of PAI-1 in a clinical setting has not been possible yet *(Fortenberry 2013)*.

Further studies are needed to investigate not only PAI-1 but also uPA and uPAR as targets for breast cancer treatment. Previous studies showed promising results evaluating these three factors as targets for breast cancer treatment. Because of the suspected high expression of uPA, PAI-1, and uPAR in TNBC,

targeting these molecules could be especially effective in the triple-negative subgroup of breast cancer.

6 Summary

Breast cancer is a very heterogeneous disease comprising multiple molecular subtypes, such as luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and basal-like breast cancer (BLBC). The various tumor groups strongly differ in their clinicopathological characteristics and response to therapy and patient outcome. In clinical practice, surrogate definitions of molecular subclasses are established and defined by parameters such as hormone receptor (HR) status, HER2 expression, and the level of Ki-67. One challenging breast cancer subtype, triple-negative breast cancer (TNBC), lacks the estrogen receptor (ER), progesterone receptor (PR), and HER2 overexpression. Patients with TNBC have a worse prognosis because of its aggressive clinical behavior. Patients with the triple-negative disease do not benefit from established targeted therapy, such as tamoxifen or trastuzumab. The only established systemic treatment is cytotoxic chemotherapy. Moreover, personalized therapy is hampered by the lack of prognostic and predictive biomarkers that guide making decisions on chemotherapy in TNBC patients.

The urokinase-type plasminogen activator system (uPAS) consists of numerous components, such as urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), and its receptor, the urokinase-type plasminogen activator receptor (uPAR). They are part of a complex system regulating fibrinolysis, cell migration, degradation of the extracellular matrix, and consequently, cancer cell dissemination and metastasis.

Both uPA and PAI-1 are established parameters for evaluating prognosis and predicting the response to therapy in breast cancer. Guidelines advise to determine the status of both uPA and PAI-1 in HR-positive, HER2-negative breast cancer patients to guide the decision on adjuvant systemic therapy in borderline indication cases, such as lymph node-negative or G2 disease *(Harris et al. 2016, MunichTumorCenter 2017, AGOBreastCommittee 2018)*. However, the use of uPA and PAI-1 is currently not recommended in TNBC *(Harris et al. 2016)*. Previous studies mainly evaluated the impact of uPA and PAI-1 in unselected breast cancer, i.e. patient cohorts with heterogeneous molecular subtypes. Therefore, it is essential to investigate these biomarkers specifically in different breast cancer subtypes.

In this thesis, the development of reproducible immunohistochemical staining procedures enabled the assessment of uPA, PAI-1, and uPAR in a TNBC

patient cohort. We observed high expression of uPAR in TNBC as 67% of tumors showed high uPAR levels. The expression of both uPA and PAI-1 was also elevated in this study, though not as strong compared with uPAR. The levels of PAI-1 were high in 62% of TNBC, and 55% displayed high uPA expression. Staining results of uPA, PAI-1, and uPAR were correlated with follow-up data of the TNBC patient cohort. Results of this study underscore the prognostic impact of uPA, PAI-1, and uPAR in the risk assessment of breast cancer. Patients with high immunohistochemistry scoring results of all three investigated components of the uPAS tend to have worse outcomes than do patients with low uPA, PAI-1, or uPAR scoring results. The impact of PAI-1 on the probability of survival was highest compared with uPA and uPAR. Statistical significance was attained after analyzing three patient subgroups: patients with high-grade TNBC (OS: $p = 0.033$), patients with invasive ductal tumors (OS: $p = 0.037$), and patients that have received adjuvant chemotherapy (OS: $p = 0.044$). While the results of this study significantly suggest a prognostic impact of PAI-1 in the risk assessment of TNBC, the impact of uPA and uPAR on survival was less powerful.

The results of this study showed that TNBC patients with elevated uPA expression levels tend to show worse survival than the group of patients with low uPA expression. This effect was highest in G3 TNBC patients, which represent the majority of 82% of our patient cohort. Analyzing this poorly-differentiated subgroup of TNBC patients, the impact of uPA on DFS displayed a trend toward significance ($p = 0.097$). Similar to uPA, the prognostic impact of uPAR in TNBC patients was less strong compared with PAI-1. However, TNBC patients with elevated uPAR expression levels tend to show worse survival than the group of patients with low uPAR expression, but statistically significant results were not attained.

In summary, the results suggest a prognostic impact of PAI-1 in the risk assessment of TNBC. Besides PAI-1, uPA and uPAR also seem to have a potential value for evaluating the prognosis of TNBC patients. Further studies are needed to both verify PAI-1 as a prognostic factor in TNBC and investigate the possible impact of uPA and uPAR. Moreover, the relatively high expression of the three markers, in particular, uPAR, but also uPA and PAI-1, could be an attractive target for therapy of TNBC. Further evaluation of uPA, PAI-1, and uPAR as therapeutic targets is necessary.

7 References

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8 Abbreviations

ABC	Avidin-Biotin Complex
ADP	Adenosine diphosphate
AGO	Arbeitsgemeinschaft Gynäkologische Onkologie
ASCO	American Society of Clinical Oncology
ATF	Amino-terminal fragment
BCT	Breast conservative therapy
BLBC	Basal-like breast cancer
BRCA1/2	Breast cancer 1/2 gen
CD	Cluster of differentiation
CK	Cytokeratins
D	Domain
DAB	3,3‘- diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ESMO	European Society for Medical Oncology
FFPE	Formalin-fixed, paraffin-embedded
GFD	Growth factor domain
GPI	Glycosylphosphatidylinositol
h	Hours
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HER2	Human epidermal growth factor receptor 2
HIER	Heat-induced epitope retrieval
HMW-uPA	High molecular weight urokinase-type plasminogen activator
HR	Hormone receptor
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IHC	Immunohistochemistry
Int	Integrin
IHC	In situ hybridization

LC3	Microtubule-associated light chain 3
LMW-uPA	Low molecular weight urokinase-type plasminogen activator
LOE-1	Level-of-evidence-1
LPBC	Lymphocyte-predominant breast cancer
LSAB	Labeled Streptavidin-Biotin
mAb	Monoclonal antibody
MCR	Munich Cancer Registry
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCCN	National Comprehensive Cancer Network
NNBC-3-trial	Node-Negative Breast Cancer-3 trial
PAI-1	Plasminogen activator inhibitor type-1
PAI-2	Plasminogen activator inhibitor type-2
PARP	Poly(adenosine diphosphate (ADP)-ribose) polymerase
pCR	Pathologic complete response
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
Pl-ogen	Plasminogen
PFS	Progression-free survival
PR	Progesterone receptor
RKI	Robert Koch-Institut
RT	Room temperature
SOP	Standard operating procedure
TBS	Tris-buffered saline
TILs	Tumor-infiltrating lymphocytes
TNM	Tumor size, lymph node status, metastasis
TMA	Tissue microarray
TNBC	Triple-negative breast cancer
tPA	Tissue-type plasminogen activator
TUM	Technical University of Munich
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
uPAS	Urokinase-type plasminogen activator system
VEGF	Vascular endothelial growth factor
VN	Vitronectin
WSG	West German Study Group
°C	Degree Celsius

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