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Molecular investigation of clinically relevant biomarkers as members of the uPA receptor interactome in triple-negative breast cancer

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1. Introduction

Current research in tumor therapy focusses on targeted and personalized therapy. This study refers to the identification of new biomarkers with regard to an optimized and individual therapy in the battle against a particularly aggressive form of breast cancer (BC), the triple-negative breast cancer (TNBC).

1.1 Breast Cancer

1.1.1 Epidemiology

According to the World Health Organization (WHO), BC is the second most common cancer in the world and the most common cancer in females, both in the developing and developed regions. In 2018, an estimated 2.09 million BC cases were newly diagnosed worldwide (Bray et al., 2018). In 2012, approximately 464,000 women were diagnosed with BC in Europe (European Network of Cancer Registries, 2014). In Germany, the average life-time risk for a woman to develop BC is 9.2% i.e. every eleventh woman in Germany is likely to develop BC in her life (Giersiepen et al., 2005; Schön et al., 2004). BC is the leading cause of cancer death amongst women worldwide (Bray et al., 2018). Furthermore, BC incidence is increasing worldwide but simultaneously, BC mortality is decreasing in most of developed countries (Autier et al., 2010).

1.1.2 Risk factors

Multiple risk factors exist for BC. Examples are older age, genetic risk factors, high density of breast tissue, family cancer history and a high number of lifetime menstrual cycles. In addition, being overweight, smoking, alcohol consumption, hormone therapy during and after menopause, and lack of exercise after menopause also increase the risk of developing BC. Factors like multiple and early childbirth and long breast-feeding periods are protective against BC (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019; European Network of Cancer Registries, 2014).

Patients with germline mutations of the breast and ovarian cancer susceptibility genes BRCA1 and BRCA2 have an increased risk for BC (Antoniou et al., 2003; Ford et al., 1998; Narod, 2006). Patients with a family or a personal history of breast and/or ovarian cancer should be counselled, informed, and tested for BRCA1/2 mutations

(Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019; Hanf et al., 2015).

1.1.3 Screening

BC screening is a promising way of detecting BC early and in lower stages, therefore reducing mortality and improving quality of life for BC patients (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019). In developed countries, due to widespread screening programs and reduction of hormonal replacement, the incidence, prognosis, and mortality of BC has been reduced (Glass et al., 2007).

The statuary screening program in Germany includes an annual palpation examination by a gynaecologist for women from the age of 30 years on and a mammography screening every other year between the age of 50 and 69 years. Due to the prevention program, the BC specific mortality and the treatment-dependent morbidity have been reduced significantly (Hanf et al., 2015). BC is more often diagnosed at an earlier stage with better prognosis (Cianfrocca & Goldstein, 2004; Fitzgibbons et al., 2000).

1.1.4 Prognostic and predictive factors in breast cancer

Prognostic factors in BC are (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019):

- TNM stage (tumor size, nodal status, distant metastasis)
- Status of surgical margin
- Histological tumor type and nuclear grade of the tumor
- Peritumoral lymphatic vessel and vascular invasion

Prognostic factors make implications about the natural course of the disease. They are used to foresee patients outcomes independently from the therapy given (Henry & Hayes, 2006). The most important prognostic factor is the nodal status (Cianfrocca & Goldstein, 2004; Fitzgibbons et al., 2000). Other important factors are tumor size and nuclear grading. In patients with node-negative BC, tumor size is the most important prognostic factor and treatment decisions are based on it (Cianfrocca & Goldstein, 2004).

Predictive factors for adjuvant therapy according to the German BC guideline (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019) are:

- Estrogen receptor (ER) and progesterone receptor (PR) status and menopausal status considering systemic endocrine therapy
- Human epidermal growth factor receptor 2 (HER2)-status considering anti-HER2 therapy.

Predictive factors make statements about the expected effectiveness of a certain therapy. They predict the sensitivity or resistance of a tumor regarding a specific therapy. Predictive factors support therapy decision making (Henry & Hayes, 2006).

In patients with early stage, hormone receptor positive, HER2-receptor negative and lymph node negative BC, different tests exist to distinguish between patients benefitting from chemotherapy and those not benefitting from it.

Different multi-gene tests, for example Oncotype DX®, MammaPrint®, Endopredict® and Prosigna® found entrance to the German BC guideline. They assist in therapeutic decision making in cases where conventional markers could not reveal a definite decision. By testing different gene levels, they calculate risk scores detailing how likely the patient will suffer from distant recurrence. In patients with low risk of distant recurrence, chemotherapy might do more harm than good and can be avoided (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019). Prospective studies for Oncotype DX® and MammaPrint® showed long disease-free survival (DFS) in hormone receptor positive, HER2-receptor negative and nodal-negative patients with low recurrence scores (Cardoso et al., 2016; Gluz et al., 2016; Sparano et al., 2015a).

The OPTIMA Prelim Trial compared different gene tests (among others Oncotype DX®, MammaPrint® and Prosigna®) in hormone-receptor positive and HER2-receptor negative patients. The outcome was not conclusive and showed only broad accordance of different tests. This indicates that for individual patients, different tests foresee different risks and make different recommendations regarding chemotherapy treatment (Bartlett et al., 2016).

1.1.5 Standard clinical assessment

TNM Classification

The TNM classification considers size, nodal status, and incidence of metastasis of a patient's tumor. It is an important prognostic marker giving information about an estimated course of the disease (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019). An overview of the TNM classification is added to the Appendix (Table 15).

Histologic grading

Every BC is evaluated histologically. The grading should be performed according to the Bloom and Richardson grading system which was modified by Elston and Ellis in 1991 (Bloom & Richardson, 1957; Elston & Ellis, 1991). An overview of the BC grading system is added to the Appendix (Table 16, Table 17).

1.2 Triple-negative Breast Cancer

1.2.1 Definition

TNBC is defined by the lack of expression of the ER, PR, and the absence of overexpression of the HER2 gene (Dent et al., 2007). The assessment of the steroid ER and PR hormone receptors and the HER2-receptor form part of the routine examinations for every BC patient (Hammond et al., 2010; Wolff et al., 2007). ER and PR receptor statuses are determined by immunohistochemical analysis. They are considered negative if less than 1% of the tumor cells are stained positive for these factors (Hammond et al., 2010). The HER2 status is established either by immunohistochemistry (IHC) or by fluorescence in situ hybridization (FISH).

1.2.2 Epidemiology and clinic

Approximatively 10-20% of all invasive BC are TNBC (Boyle, 2012; Carey et al., 2006; Vona-Davis et al., 2008). TNBC show a poorer prognosis than other BC subtypes (Brown et al., 2008; Carey et al., 2007; Cheang et al., 2008; Nielsen et al., 2004; Sorlie et al., 2001; Sorlie et al., 2003). Patients with TNBC are usually younger than other BC patients and often premenopausal (Bauer et al., 2007a; Carey et al., 2006; Kwan et al., 2009). The tumors occur more often in women of African American and/or Hispanic origin and are associated with a low socioeconomic status (Bauer et al., 2007a; Carey

et al., 2006; Hudis & Gianni, 2011; Kwan et al., 2009; Morris et al., 2007; Stead et al., 2009; Zhang et al., 2016).

At the time of diagnosis, for TNBC, the tumor size is usually larger, and the tumors are less differentiated compared to other BC subtypes (Carey et al., 2006; Dent et al., 2007; Rakha et al., 2007). TNBC are aggressive in their behavior and are detected late and at an advanced stage of the disease. The tumors are less likely to be diagnosed via the regular screening programs, but more likely to be diagnosed in between them. This is either due to the rapid growth of the tumor, to the higher density of the breast, or the occurrence of the tumor; henceforward screening is recommended (Anders & Carey, 2009; Collett et al., 2005; Dent et al., 2007; Hudis & Gianni, 2011).

TNBC are more likely to be nodal-positive than other BC and tend to spread to viscera (Criscitiello et al., 2012; Dent et al., 2007; Hudis & Gianni, 2011; Liedtke et al., 2008; Lin et al., 2008; Rodriguez-Pinilla et al., 2006; Smid et al., 2008). In histology, TNBC often show pushing borders, central necrosis, and lymphocytic infiltrates (Foulkes et al., 2010).

Patients with TNBC present with poor survival independent of their cancer stage (Bauer et al., 2007a; Kumar & Aggarwal, 2016). Time to local recurrence, time to distant recurrence, and median time to death is shorter in TNBC compared to other BC subtypes (Dent et al., 2007). The survival time after distant metastasis is shorter compared to other BC types (Dent et al., 2007; Hudis & Gianni, 2011; Kumar & Aggarwal, 2016). Interestingly, there is a peak in distant recurrence between one to three years after diagnosis, thereafter incidence of distant recurrence diminishes (Dent et al., 2007; Jatoi et al., 2011; Kumar & Aggarwal, 2016). Therefore, patients with TNBC are more likely to die from their cancer in the first five years after diagnosis but show, if is survived, a lower tendency of BC death approximatively seven years after diagnosis (Dent et al., 2007; Jatoi et al., 2011; Kumar & Aggarwal, 2016).

1.2.3 Heterogeneity in triple-negative breast cancer

BC and especially TNBC is a heterogeneous, diverse, and complex disease. TNBC is known to be the most heterogeneous type consisting of multiple different subtypes (Fleisher et al., 2016; Lehmann et al., 2011). TNBC do not share an evident target for therapy or phenotypic features but are linked by the absence of these (Marme & Schneeweiss, 2015; Witzel & Muller, 2015).

The complexity and poor prognosis of TNBC led to extensive research in this area. The focus of research lies on gene expression profiling and the similarity of TNBC with familial BC. Gene expression profiling and immunohistochemical markers allow BC patients to be subgrouped according to therapeutic targets that they express. These subgroups are intended to be a guide for physicians in clinical decision making (Haffty et al., 2006; Perou, 2010). In 2000, Perou et al. were the first ones to subgroup BC based on gene expression profiling using cDNA microarrays (Perou et al., 2000). The newest and current classification defines five BC subtypes and the normal breast-like subtype: luminal A, luminal B, HER2-enriched, basal-like, and claudin-low. Tumors of the luminal subtypes are steroid hormone receptor-positive (Perou, 2010). In luminal B tumors, proliferation genes are stronger expressed than in luminal A tumors (Prat & Perou, 2011). The HER2-enriched subtype consists of tumors which present a HER2-enriched gene expression pattern. Surprisingly, there are HER2-enriched tumors that are not clinically HER2-positive (Perou, 2010). Basal-like tumors have a poor prognosis. They express cytokeratines like CK 5, 6, and 17. TNBC form the major part of the basal-like subtype, but both subtypes are not synonymous (Bertucci et al., 2008; Foulkes et al., 2010; Nielsen et al., 2004; Prat et al., 2010). Most of the claudin-low tumors are TNBC. They tend to have a poor prognosis (Prat et al., 2010).

1.2.4 Therapy

Surgery

Like other invasive BC, TNBC are treated either with breast conserving surgery followed by radiotherapy or by mastectomy (Kumar & Aggarwal, 2016; Pan et al., 2015). The goal of the surgical procedures is to create tumor-free margins (Hanf et al., 2015; Pilewskie et al., 2014). Today, the standard of care is the breast conserving surgery (Gnant et al., 2015).

The gold standard for patients with clinically negative lymph node status is the sentinel lymph node biopsy (SLNB) (Gnant et al., 2015; Reimer et al., 2014). SLNB distinguishes between nodal-negative patients not requiring any further surgery of the axilla and nodal-positive patients needing a dissection of the axilla (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019; Hanf et al., 2015).

Systemic therapy

Enormous progress has been made regarding systemic therapy of BC, primarily due to a better understanding of the tumor biology. In TNBC, druggable targets like ER, PR, or HER2 are missing and therefore targeted therapeutic options are limited and remain a challenge (Witzel & Muller, 2015). Until new treatment options are available, chemotherapy remains the gold standard for TNBC patients despite its inevitable toxicity and the risk of developing chemoresistance (Zhang et al., 2016).

Chemotherapy regimens have dramatically changed recently. In the past, a regimen of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) used to be the standard of care (Tancini et al., 1983). In 1998, the International Consensus Panel on Treatment of Primary BC stated that anthracycline-based regimens should be favored over CMF-regimens due to their shorter application. In 2001, the International Consensus Panel on Treatment of Primary BC underlined this statement (Goldhirsch et al., 1998). Later, it was shown that adding a taxane to an anthracycline-regimen improved DFS and overall survival (OS) significantly (Goldhirsch et al., 2001; Henderson et al., 2003).

In 2015, the International Expert Consensus on the Primary Treatment of Early BC published recommendations based on the different BC subtypes. The variability among TNBC patients responding differently to chemotherapy remains a challenge (Criscitiello et al., 2012). Currently, for TNBC, a regimen containing anthracyclines and taxanes is considered optimal, both for the neoadjuvant and for the adjuvant setting (Denduluri et al., 2016; Gnant et al., 2015; Mobus, 2016; Stover & Winer, 2015; Untch et al., 2015). An alternative anthracycline-free regimen is a combination of docetaxel and cyclophosphamide or CMF (Denduluri et al., 2016). The Arbeitsgemeinschaft Gynäkologische Onkologie (AGO) recommends platinum-based chemotherapy for neoadjuvant treatment, especially for patients with TNBC and BRCA mutations or for patients with a family history of breast or ovarian cancer. This does not apply for the adjuvant setting or for the therapy of early TNBC (Byrski et al., 2010; Collignon et al., 2016; Hanf et al., 2015; Isakoff et al., 2015; Jackisch et al., 2015; Petrelli et al., 2014; Sikov et al., 2015; Silver et al., 2010; Stover & Winer, 2015).

1.3 Biomarker

According to the Biomarkers Definition Working Group, a biomarker is defined as a „measurable indicator that is used to distinguish precisely, reproducibly, and objectively either a normal biological state from a pathological state, or the response

to a specific therapeutic intervention“ (de Gramont et al., 2015).

Biomarkers are needed to tailor individual therapies and make treatment decisions in favor of the patient and of the medical costs. They can deliver prognostic and/or predictive statements (Henry & Hayes, 2006; Kaufmann & Pusztai, 2011; Simon et al., 2009).

The Tumor Marker Utility Gradings System (TMUGS) serves as an objective and standardized evaluation for biomarkers. Standardization of biomarkers is important because lack of consistent guidelines can lead to unnecessary treatments, side effects, and costs (Hayes et al., 1996). The level of evidence (LOE) scale of the TMUGS represents a ranking of the validity and strength of a biomarker for clinical decision making. An overview of the LOE classification is added to the Appendix (Table 18).

To establish a new biomarker for clinical practice, several requirements must be achieved. The new biomarker must provide additional information for routine clinical assessment or must provide equal information at lower expenses, be less invasive, be easier to handle, or be less risky to determine (Van Poznak et al., 2015).

The road to the establishment of a new biomarker is long. Standardization concerning preanalytical, analytical, and postanalytical steps is required (de Gramont et al., 2015). Different criteria must be met: the setting and the utility of the biomarker must be defined precisely; its magnitude and reliability must be proven. To evaluate the magnitude of the study, the outcome or treatment effect must differ in patients with positive test results from patients with negative test results. The test must be reliable and reproducible, and sensitivity and specificity must be appropriate. Cutoff points must be comprehensible and validated (Henry & Hayes, 2006; Simon et al., 2009). Finally, to introduce a biomarker into clinical practice, the best way is to show its potential in a prospective randomized controlled clinical trial (Ginsburg & Kuderer, 2012; Harbeck et al., 2002a; Polley et al., 2013).

1.4 Tumor-associated proteolysis

The transformation of a locally growing tumor into an invading, spreading and metastasizing tumor is what makes it dangerous. Proteolytic activity of extracellular matrix (ECM) components or non-ECM components play key roles in tumor growth, invasion, angiogenesis and metastasis (Liotta & Kohn, 2001).

The metastatic process consists of different steps: invasion of the local environment, intravasation in lymphatic or blood vessels, survival and locomotion in the vessels, extravasation and tumor growth at the metastatic site (Chiang et al., 2016; Duffy, 1992). In this process, several natural cell barriers, basement membranes and interstitial connective tissues have to be overcome (Chiang et al., 2016).

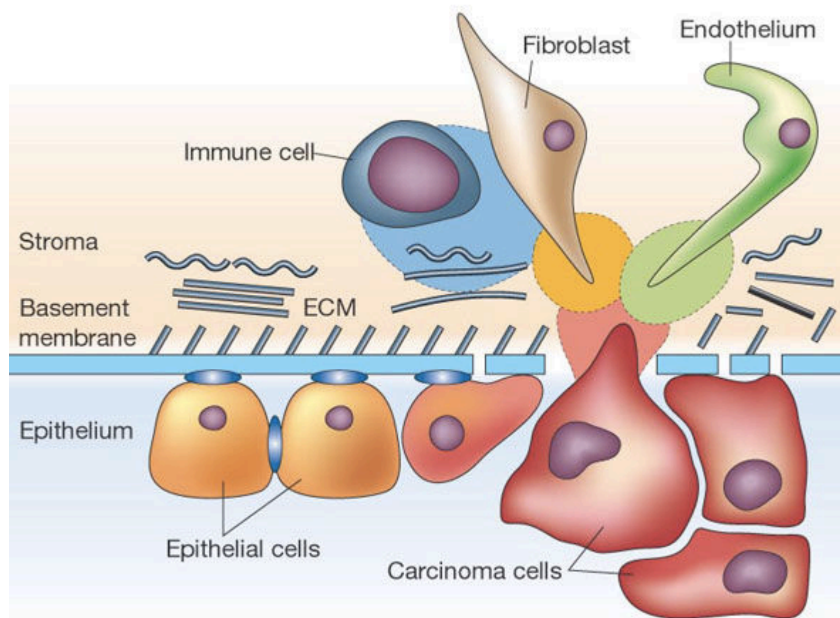


Figure 1. Tumor invasion in the ECM (taken from Liotta & Kohn, 2001)
The process of invasion of cancer cells in the metastatic site involves communication between different cells and the epithelial stroma. Stromal cells and premalignant epithelium stand in close contact. Different cells like host fibroblasts, immune cells and endothelial cells are activated. They communicate via different enzymes, leading to a modification of the ECM and the basement membrane and to the degradation of normal tissue boundaries.

Local invasion begins with initial signaling pathways controlling the cytoskeletal behavior, afterwards proteases reverse cell-matrix and cell-cell junctions and tumor cells migrate into proximal tissue. As seen in Figure 1, metastasis begins when they find access to basement membrane, lymphatic or blood vessels (Friedl & Alexander, 2011; Liotta & Kohn, 2001). Tumor cells and the surrounding environment stand in close contact and regulate each other (Duffy, 1992). Cancer cells have the ability to adapt to the environment. Not only the tumor cells but also the stroma cells around them undergo profound changes in the metastatic process and the microenvironment plays a significant role in it (Clark & Vignjevic, 2015). The microenvironment dominated by proteases consists of ECM remodeling by proteolysis and proteolytic processing of chemokines, growth factors and their receptors. Stromal cells around the tumor play an important role in tumor progression. Fibroblasts, endothelial cells and macrophages are activated by the growing tumor cells. In response to the tumor growth, the stroma

activates cytokines, growth factors and ECM components and thereby reorganizes the structure of the microenvironment of the tumor. Protease systems play an important role both in the stromal and tumor cells. They lead to tumor invasion and progression through different mechanisms. Cell surface proteases lead to proteolysis of structural ECM proteins. This generates epitopes of ECM components with adhesion- or migration-promoting effects and the structure remodeling leading the way for invading tumor cells. Also, proteases secreted to the cell surface process and activate other proteases, the cell surface receptors. This accounts for adaptive changes of receptor availability on tumor and stromal cells. Secreted proteases regulate the availability of growth factors (Friedl & Alexander, 2011).

Tumor cells imitate non-neoplastic cell behavior and mechanisms of healthy tissue (Johnsen et al., 1998). Some essential differences exist: under physiological circumstances proteolysis always stays controlled and self-limited whereas in cancer and metastasis the controlling mechanisms are lost (Duffy, 1992).

1.5 The uPA system

The uPA system consists of the serine proteases Urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR), plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). Figure 2 illustrates the signal pathways of the uPA system. Proteolytic enzymes like serine proteases hydrolyze peptide bonds or other ester bonds.

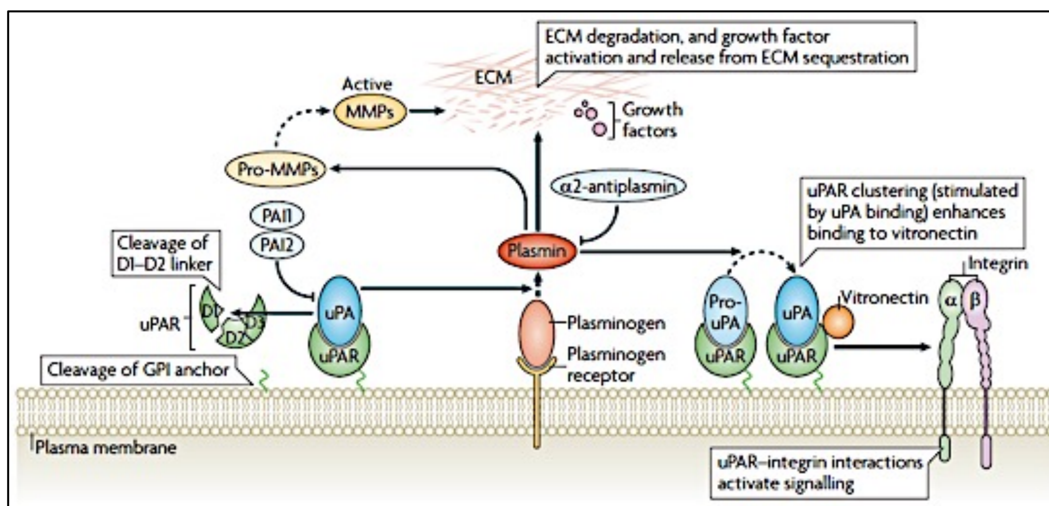


Figure 2. The uPA-receptor interactome (taken from Smith & Marshall, 2010)
The uPA-receptor binds uPA both in its inactive and in its activated form. Plasminogen can be activated to the serine protease plasmin by uPA. Plasmin can reciprocally activate pro-uPA. Plasmin can activate zymogen forms of matrix metalloproteinases and growth factors, leading to degradation of the ECM. PAI-1, PAI-2 and α2-antiplasmin are serine protease inhibitors and can antagonize the proteolytic capability of uPA and Plasmin. uPAR is also able to Vitronectin and integrins.

uPA is a serine protease secreted by different cell types in an almost inactive form, the zymogen pro-uPA. The uPA-receptor binds uPA both in its inactive and in its activated form with high affinity (Dano et al., 2005). PAI-1 is a serine protease inhibitor and belongs to the serpin superfamily. PAI-1 can form a complex with proteinases such as uPA and thereby inactivates them. Under physiological conditions, PAI-1 blocks uPA during blood coagulation. In cancer tissue, PAI-1 is involved in tumor invasion and metastasis (Harbeck et al., 2001). uPAR is located on the cell membrane and is attached to it by a glycosyl phosphatidylinositol anchor (Eden et al., 2011). Since the receptor lacks a transmembranous or an intracellular domain, it depends on multiple ligands, co-receptors, and pathways to transport its signals into the cell (Smith & Marshall, 2010). uPAR expression can be found in many different healthy tissues but also in a variety of cancer tissues (Blasi & Sidenius, 2010). Amongst others, uPAR is able to bind uPA, vitronectin (VN), and integrins (Andreasen et al., 1997).

The function of the uPA system under physiological conditions is important, especially in remodeling of tissues in the context of wound healing, lysis of blood clots, and during early pregnancy (Blasi & Sidenius, 2010; Smith & Marshall, 2010). The uPA system also acts under pathological conditions like infections, development of atherosclerosis, restenosis, inflammation, and cancer (Binder et al., 2007). In the latter cases especially in migration of tumor cells and invasion to other tissues (Blasi & Sidenius, 2010).

1.6 The uPA-receptor interactome

The uPAR interactome forms a system around uPAR and its interactive partners (Figure 2). uPAR plays the key role in the uPAR interactome. Numerous highly organized interactions between uPAR, serpin inhibitors, zymogens, active proteins, and its co-receptors form a complex mechanism playing an important role in the human body (Andreasen et al., 1997). In summary, the uPAR interactome determines pericellular proteolysis and therefore degradation of the ECM in healthy or cancer tissue (Binder et al., 2007; Dano et al., 2005).

uPA and PAI-1 are approved biomarkers in node-negative BC patients and are members of the uPA-receptor interactome (Krop et al., 2017). Surprisingly, not only increased levels of uPA but also of its inhibitor PAI-1 are associated with cancer cells. PAI-1 shields the tumor from tumor-associated proteases, thereby ensuring the matrix formation, and preventing tumor cell proteolysis (Dublin et al., 2000; Schmitt et al., 1997). uPA-PAI-1-complex formation plays an important role in regulating tumor cell

adhesion and migration by binding uPAR with subsequent cellular internalization (Look et al., 2002; Stefansson & Lawrence, 1996).

uPAR is not expressed in healthy tissue or benign tumors of the breast but only in cancer (Grondahl-Hansen et al., 1993). It is present not only in tumor cells, but also in stromal cells surrounding the tumor (Boonstra et al., 2011). uPAR binds uPA, and therefore leads uPA to the points were proteolysis happens. uPAR is higher expressed in metastatic cancer than in benign or primary cancer. Additionally, it is expressed highest at the invading tumor margins. In the short term, it may make predictions about disease progression and therapeutic decision making. In the long term, the specific molecular role has to be identified and targeted with specific medication (de Bock & Wang, 2004).

VN is an adhesive glycoprotein and is mainly produced by the liver. It is present in platelets of the blood plasma and in the ECM (Andreasen et al., 1997). VN is a ligand of uPAR and operates through redistribution of uPAR on the cell surface. By relocalizing uPAR, it influences proteolysis of or adhesion to the ECM (Eden et al., 2011). PAI-1 also binds to VN. The connection with VN keeps PAI-1 in its active form (Andreasen et al., 1997). Also, PAI-1 binding to VN causes dimerization of uPAR, therefore leading to cell anchorage (Eden et al., 2011).

1.6.1 Plasminogen

Plasminogen is a zymogen and is mainly produced in the liver (Andreasen et al., 1997; Dano et al., 2005; Raum et al., 1980). It is present in blood plasma and extravascular fluids (Curino et al., 2002). Plasminogen can be activated to the serine protease plasmin by different proteases, including uPA and the tissue-type plasminogen activator (tPA). uPA is mainly responsible for tissue remodeling processes, tPA plays an important role in thrombolysis (Dano et al., 2005). Plasmin plays a key role in the activation of the fibrinolytic system (Syrovets et al., 2012) and is important for fibrinolysis during physiological hemostasis (Mazar, 2008; Smith & Marshall, 2010). Plasmin is also involved in other physiological and pathological processes, for example wound healing (Creemers et al., 2000), cell migration, angiogenesis (Castellino & Ploplis, 2005), inflammation (Ploplis et al., 1998), tissue remodeling (Lund et al., 2000), tumor growth, and metastasis (Ranson et al., 1998). It has a broad spectrum of substrates and can degrade many non-collagenous proteins of the ECM such as fibronectin, VN, and fibrin (Andreasen et al., 1997; Binder et al., 2007; Foekens et al.,

2000; Liotta et al., 1981). It can activate zymogen forms of matrix metalloproteinases and release growth factors (Andreasen et al., 1997; Gouri et al., 2016; Mazar, 2008).

Plasminogen receptors are a heterogeneous group of proteins and bind plasminogen and plasmin on the cell surface (Didiasova et al., 2014). Numerous cells, including tumor cells (Durliat et al., 1992; Hembrough et al., 1995) express receptors for plasminogen, leading to its activation on the cell surface, and degradation of surrounding proteins (Plow et al., 1986). Cells with plasminogen receptors usually also express receptors for uPA and tPA (Plow et al., 1986). Miles et al. found that plasminogen activation is more effective when plasminogen is bound to the cell surface (Miles & Plow, 1985). Plasminogen receptors play a role in tumor development and progression in numerous types of cancer (Didiasova et al., 2014). For cellular plasminogen activation, three requirements are needed: uPA bound to its receptor, plasminogen located around the cell surface, and direct interaction of uPA and plasminogen (Andronicos & Ranson, 2001; Didiasova et al., 2014). Stonelake et al. reported that presence of plasminogen and uPA will lead to degradation of the tumor basement membrane and that blockage of plasminogen activation decreased the degradation (Stonelake et al., 1997).

uPA and plasminogen form a reciprocal activation system. uPA activates plasminogen to plasmin (Foekens et al., 2000). Plasmin, on the other hand, converts pro-uPA to active uPA forming an amplification loop (Ellis, 1996). Active uPA is also known as high molecular weight-uPA (HMW-uPA) and can be processed to low molecular weight-uPA (LMW-uPA). Besides this being its main role, uPA is also capable of cleaving other proteins as well (Duffy, 1996; Gold et al., 1989; Mars et al., 1993; Mengele et al., 2010).

1.6.2 Integrins

Integrins are a group of cell surface adhesion receptors, each being a heterodimer and consisting of an α and a β domain. 18 α and 8 β variants are known today, combining to form 24 integrins in total (Hynes, 2002). Integrins are responsible for numerous cell-cell- and cell-ECM-interactions. They are transmembranous receptors and can mediate signals bi-directionally across cell membranes (Barczyk et al., 2010; Conroy et al., 2016; Hynes, 1992). They can regulate cell adhesion, proliferation, migration, and differentiation, both under physiological and pathological circumstances (Tabatabai et al., 2010). These qualities make them key factors in tumor growth and

metastasis (Desgrosellier & Cheresh, 2010).

The ECM stands in close contact with adjacent cells and incessant communication between the ECM and the cells is needed for the hemostasis of the tissue. Integrins are mainly responsible for these cell-ECM interactions. They can also connect with the cytoskeleton. In the present study, the focus was based on integrin $\alpha v\beta 3$, which is present on numerous different cell types, including cancer cells (Takada et al., 2007) and interacts with multiple different ligands (Meyer et al., 1998). It interacts, for instance, with VN. It binds to the same binding site as PAI-1, with PAI-1 having a higher affinity than integrin $\alpha v\beta 3$ (Stefansson & Lawrence, 1996). Integrin $\alpha v\beta 3$ and VN are known to be important for cell migration, but if PAI-1 binds to VN instead of integrin $\alpha v\beta 3$, cell migration is inhibited. Binding of uPA to PAI-1 changes the PAI-1 conformation (Shore et al., 1995) and decreases the affinity of PAI-1 to VN (Stefansson et al., 1996) and promotes cell migration (Stefansson & Lawrence, 1996).

Integrin $\alpha v\beta 3$ is expressed in high concentrations on endothelial cells in remodeling tissues, in tumor tissue (Brooks et al., 1995) and on tumor cells (Schneider et al., 2011). It plays a key role in tumor angiogenesis, supporting the development of newly formed vessels (Brooks et al., 1995; Weis & Cheresh, 2011). A tumor requires a vascular architecture to grow and metastasize. Integrins play an important role in this process (Ruegg & Mariotti, 2003).

From a tumor size of 1- 2mm^3 on, a tumor depends on newly sprouting vessels to grow (Max et al., 1997). Integrin $\alpha v\beta 3$ occurs at a higher level in newly proliferating tumor vasculature of different organs, for example in BC tissue. It is triggered by hypoxia (Cowden Dahl et al., 2005). Integrin $\alpha v\beta 3$ leads to extravasation and transendothelial migration of tumor cells (Bauer et al., 2007b) and to the production of matrix metalloproteinases (Baum et al., 2007), both involved in metastasis.

Integrin $\alpha v\beta 3$ is often present in numerous cancers like melanoma, BC, prostate cancer, cervical cancer, glioma, ovarian cancer, and pancreatic cancer (Carter, 2010; Rolli et al., 2003). In its activated state, integrin $\alpha v\beta 3$ encourages metastasis in BC (Rolli et al., 2003). In BC but also in many other cancers, the density of newly proliferating vessels correlates with the metastatic potential and prognosis (Gasparini & Harris, 1995). High integrin $\alpha v\beta 3$ expression is common in BC and is associated with disease progression and metastasis (Felding-Habermann et al., 2001). In brain and bone metastases, integrin $\alpha v\beta 3$ expression is higher than in the primary BC tissue

(Vogetseder et al., 2013).

1.7 Biomarkers as members of the uPA system in breast cancer

In 1988, Duffy et al. presented data showing that BC patients with high levels of uPA enzymatic activity presented with significantly shorter DFS (Duffy et al., 1988). Jänicke et al. proved shortly after that uPA antigen and PAI-1 had a prognostic impact in node-negative and in node-positive BC patients (Harbeck et al., 2001; Jänicke et al., 1989).

Later, it was shown by further investigations that uPA is a prognostic factor independent of tumor size, axillary node status, and ER status (Duffy et al., 1990; Foekens et al., 1992; Grondahl-Hansen et al., 1993). The clinical role of uPA and PAI-1 as prognostic factors was endorsed by Jänicke et al. (Jänicke et al., 2001). They showed in a prospective multicenter therapy trial in node-negative patients that these biomarkers correlated with DFS and therapy response. They reported that within this trial, lymph node-negative BC patients showing high levels of uPA and PAI-1 benefited from an adjuvant CMF-chemotherapy. They showed as well that patients with low levels of uPA and PAI-1 had a smaller risk of disease recurrence and that adjuvant chemotherapy could be avoided (Harbeck et al., 2002a; Harbeck et al., 2002b; Jänicke et al., 2001). While uPA has a stronger prognostic impact in the first three years after diagnosis, the prognostic impact of PAI-1 increases over time (Schmitt et al., 1997).

The fact that uPA and PAI-1 are statistically independent prognostic biomarkers in primary BC was confirmed by Look et al. for BC in general and by Jänicke et al. for node-negative BC patients (Jänicke et al., 2001; Look et al., 2002). In patients where both PAI-1 and uPA are low, the risk of disease recurrence is $<5\%$ within the first 5 years after diagnosis (Harbeck et al., 1999; Harbeck et al., 2001). The combination of uPA and PAI-1 shows a greater prognostic impact than tumor size, nuclear grade, steroid hormone receptors ER/PR, or menopausal status (Harbeck et al., 2002b).

In 2007, uPA and PAI-1 were designated as biomarkers with the highest LOE (LOE-1) in BC according to the TMUGS (Schmitt et al., 2010). They were recommended by the AGO and the American Society for Clinical Oncology (ASCO) (Harris et al., 2007). uPA and PAI-1 can not only foresee poor patient outcome but also foresee if node-negative BC patients would benefit from adjuvant chemotherapy or not, thus providing prognostic and predictive clinically useful information.

Other than uPA and PAI-1, uPAR is not yet validated as a biomarker in BC. Similar to uPA and PAI-1, with uPAR being present, tendencies for worse survival were shown in BC and different other cancer types (Boonstra et al., 2011; Grondahl-Hansen et al., 1993). Until now, no validated tests for the expression exist, therefore test results differ extremely (de Bock & Wang, 2004). Also, uPAR is present in various other pathological states. Confounding variables should be considered (Boonstra et al., 2011). On the other hand, an advantage of uPAR is that it exists in a soluble form, suPAR, and is expressed in urine and plasma. This makes it easier to measure and could be used in the future for follow-up testing (Boonstra et al., 2011).

2. Aim of the Study

TNBC is a high-risk BC. It concerns primarily young patients and the outcome is significantly worse than in other BC types. Therapy options remain limited. The aim of current studies is to develop an individual and precise targeted and personalized therapy for TNBC patients. Therefore, new targets either for the application of existing or newly developed therapies must be investigated. This study engages with the question if plasminogen and/or integrin αvβ3 expression can serve as targets for these potential therapies and can predict a patient's clinical outcome.

The Aim of the Study was:

- to assess plasminogen and integrin αvβ3 expression in formalin-fixed paraffin-embedded (FFPE) tissues of TNBC patients
- to standardize the immunohistochemical methods of detection for these potential biomarkers
- to define clinically relevant cutoff values by state-of-the-art statistics
- to relate expression of plasminogen and integrin αvβ3 expression to the uPAR interactome in TNBC and evaluate whether they make a prediction of the clinical outcome and survival of TNBC patients
- to correlate the occurrence of the proteins with clinical course of the disease and different other markers of TNBC patients
- to evaluate plasminogen and integrin αvβ3' s potential to function as biomarkers in TNBC patients
- to identify by these proteins high- and low-risk patients, to determine which patient could benefit from a certain therapy and therefore to prevent patients from unnecessary overtreatments and associated harmful side effects.

3. Materials and Methods

3.1 Patients and Tissue Collection

The study included 185 patients with TNBC that were treated in the Klinik und Poliklinik für Frauenheilkunde of the Klinikum rechts der Isar, Technical University of Munich, between 1987 and 2007. Patients from the age of 27 to 96 were included. The median follow-up was 67.4 months in a range of minimum 1 month to maximum 244 months. All patients had given their written informed consent and the local Ethics Committee approved the study. The patients were treated either with breast conserving surgery or mastectomy. During surgery, the tumors were removed and directly transported to the in-house Department of Pathology. FFPE tissue blocks were routinely produced. The routine procedure consisted of a haematoxylin/eosin staining to evaluate the tumor and immunohistochemical staining of ER, PR, and HER2. Tumor samples were archived in the biobank of the Klinik und Poliklinik für Frauenheilkunde of the Klinikum rechts der Isar, Technical University of Munich.

In collaboration with the Department of Pathology at the Helmholtz Zentrum Munich, tissue microarrays (TMA) of FFPE blocks were constructed. The tissue samples of the patients investigated were assembled on nine TMAs.

Patient cohort (n=185)

Table 1. Patient cohort

Information		Number	%
Age at diagnosis in years (n=185)			
	<50	59	31.9
	≥50	126	68.1
Median age (range) in years	57 (27-96)		
Menopausal Status (n=185)			
	premenopausal	49	26.5
	perimenopausal	8	4.3
	postmenopausal	128	69.2
Histological subtype (n=178)			
	Invasive ductal	145	81.5
	Medullary	18	10.1
	Invasive lobular	9	5.1
	Other	6	3.4
Primary Tumor (pT) (n=175)			
	pT1	54	30.9
	pT2	88	50.3
	pT3	14	8.0
	pT4	19	10.9
Nodal status (pN) (n=162)			
	pN0	85	52.5
	pN1	56	34.6
	pN2	16	9.9
	pN3	5	3.1
Metastasis (M) (n=183)			
	M0	129	70.5
	M1	54	29.5
Histological grade (G) (n=178)			
	G1	3	1.7
	G2	22	12.4
	G3	153	86.0
Surgical procedure (n=184)			
	Breast Conserving Surgery	108	58.7
	Mastectomy	76	41.3
Adjuvant chemotherapy (n=178)			
	Yes	110	61.8
	No	68	38.2
Adjuvant endocrine therapy (n=176)			
	Yes	24	13.6
	No	152	86.4
Neoadjuvant chemotherapy (n=183)			
	Yes	17	9.3
	No	166	90.7
Locoregional disease recurrence (n=180)			
	Yes	33	18.3
	No	147	81.7
OS (n=149)			
	Deceased	67	45.0
	Alive	82	55.0

Legend:
T: Primary Tumor; T1: Tumor ≤20 mm in greatest dimension; T2: Tumor >20 mm but ≤50 mm in greatest dimension; T3: Tumor >50 mm in greatest dimension; T4: Tumor of any size with direct extension to the chest wall and/or to the skin
N: Regional lymph nodes; pN0: no regional lymph node metastasis identified histologically; pN1: micrometastases or metastases in 1-3 axillary/internal mammary lymph nodes; pN2: metastases in 4-9 axillary lymph nodes; pN3: metastases in ≥10 axillary lymph nodes
M: Distant metastasis; M0: no clinical or radiographic evidence of distant metastasis; M1: distant detectable metastasis
G: Histologic grade; G1: low histologic grade (favorable); G2: intermediate histologic grade (moderately favorable); G3: high histologic grade (unfavorable)

Taken from: http://emedicine.medscape.com/article/2007112-overview

Neoadjuvant and adjuvant chemotherapy regimens in the patient cohort

Table 2. Neoadjuvant chemotherapy regimen in the patient cohort

Neoadjuvant chemotherapy	
	Number of patients
Yes	17
No	166
Unknown	5
Chemotherapy regimen	**Number of patients**
Anthracycline-containing regimen	6
Anthracycline-containing regimen + taxane	4
Taxane only	2
Anthracycline-containing regimen + CMF	1
Anthracycline-containing regimen + CMF + taxane	1
Other chemotherapy regimen	1
Unknown chemotherapy regimen	2
Legend: CMF: cyclophosphamide, methotrexate, and 5-fluorouracil	

Table 3. Adjuvant chemotherapy regimen in the patient cohort

Adjuvant Chemotherapy	
	Number of patients
Yes	110
No	68
Unknown	10
Chemotherapy regimen	**Number of patients**
Anthracycline-containing regimen	48
CMF	28
Anthracycline-containing regimen + taxane	17
Anthracycline-containing regimen + CMF	8
Other chemotherapy regimen	2
Unknown chemotherapy regimen	7
Legend: CMF: cyclophosphamide, methotrexate, and 5-fluorouracil	

In total, 120 patients received chemotherapy. 17 patients received neoadjuvant and 110 patients received adjuvant chemotherapy. 7 patients received both adjuvant and neoadjuvant chemotherapy. In the adjuvant setting, chemotherapy was applied from 1990 until 2006. Between 1991 and 2006 some of the patients received neoadjuvant chemotherapy as well. Chemotherapy regimens containing anthracyclines were given

starting in 1994, containing taxanes up from 2000. Anthracycline-containing regimens were given between 1990 and 2006, the combination of anthracyclines and CMF was given between 1994 and 2006, the combination of anthracyclines and taxanes between 1997 and 2006. CMF alone was given between 1992 and 2004. The chemotherapy regimens were always adjusted to the current guidelines.

3.2 Construction of tissue microarrays

The idea of a TMA was first invented by Battifora in 1986 (Battifora, 1986). In 1998, Kononen et al. developed a technique to produce TMAs (Kononen et al., 1998). A TMA is a collection of multiple tissue samples arranged in a recipient paraffin block. A TMA consists of multiple tissues in the shape of a cylinder and harbours a preferred size of 1mm in diameter (Camp et al., 2008). To construct a TMA, different FFPE TNBC tissues were evaluated and assembled. From each block, 3μm thick cuts were produced and stained with haematoxylin/eosin to identify representative tumor sections. Cylindrical core samples were extracted from the donor block using the Manual Tissue Arrayer MTA-1 (Alpha Metrix GmbH, Rödermark, Germany) and then transferred to the recipient block. Tissues from other organ systems like kidney, liver, or placenta were included in the TMA block as reference tissues.

3.3 Immunohistochemistry

3.3.1 General information

IHC is a widely-used technique to visualize proteins in tissue samples. It is based on the interaction of tissue antigens with antibodies (ab). This technique allows to show the localization of proteins in cells and tissues in a semi-quantitative manner. IHC stainings is rated by a scaled immunoreactive score (IRS). IHC has become increasingly popular because of the availability of reactive ab. The procedure arose first in 1941 and is nowadays routinely used in the laboratories. It plays an important role in cancer diagnostics and research. IHC is widely available and easy to practise. In this study, standard operating procedures (SOP) were created for the staining of plasminogen and integrin $\alpha v\beta 3$. All tissue samples were collected and fixed in FFPE tissue samples according standardized procedures in the Department of Pathology of the Klinikum rechts der Isar, Technical University of Munich. The stainings and the IRS were all assessed applying the same laboratory protocols and by the same personnel.

3.3.2 Reagents and Instruments

Table 4. Reagents

Reagents	
Ab directed to integrin $\alpha v\beta 3$	Millipore (Merck), Darmstadt, Germany: Mouse monoclonal IgG1 ab Concentration: 100 µg at 0.1 mg/ml Immunogen: human and rabbit CD51/61 Clone: 23C6; REF: CBL544; Lot: NG1857572
Ab directed to plasminogen	Abcam, Cambridge, UK: Mouse monoclonal IgG1 ab Concentration: 500 µg at 2 mg/ml Immunogen: human plasminogen and plasmin molecules Clone: 3C2; REF: Ab10178; Lot: GR176771-1
Ab diluent	Zytomed Systems, Berlin, Germany: Ab Diluent, 500 ml, ready to use REF: ZUC025-500; Lot: J726
Citrate buffer Preparation: Create 3 l of a solution of 1 mmol/l citric acid monohydrate. Add 1N NaOH until a pH of 6 is reached.	Sigma-Aldrich, St. Louis, USA: Citric acid monohydrate Molecular weight: 210.14 g/mol REF: C1909
Diaminobenzidine (DAB) Substrate Kit	Zytomed Systems, Berlin, Germany: DAB Substrate Buffer (High Contrast), 500 ml REF: DAB5000plus; Lot: 201-DA DAB Chromogen (liquid DAB concentrate), 30ml REF: DAB5000plus; Lot: 201-DA
Ethylenediaminetetraacetic acid **(**EDTA) buffer Preparation: Create 3 l of a solution of 0.1 mmol/l EDTA. Add 1N NaOH until a pH of 8 is reached.	Sigma-Aldrich, St. Louis, USA: REF: E-5134; Lot: 033K0005 Molecular weight: 372.24 g/mol
Haematoxylin	AppliChem, Darmstadt, Germany: Mayer´s Haemalaun – Solution for Microscopy, 1000 ml REF: A0884; Lot: 2O009349
Hydrogen peroxide (H_2O_2)	Merck/Millipore, Darmstadt, Germany: Hydrogen peroxide 30% (v/v), 250 ml, Emsure ISO REF: 1.07210.0250; Lot: K44176710 321
Pertex	Medite GmbH, Burgdorf, Germany: Pertex Eindeckmedium REF 41-4012-00
Polymer detection system	Zytomed Systems, Berlin, Germany: ZytoChem Plus Horseradish Peroxidase (HRP) One-Step Polymer anti-mouse/rabbit/rat, 100 ml, ready to use REF: ZUC053-100; Lot: K727
Tris-buffered saline (TBS) Preparation of 3 L of concentrated stock solution: Create 2,1 l of a solution of 0.7 mol/l Trizma®-Base. Add 2 N 37% (v/v) hydrochloric acid (12.0 mol/l) until a pH of 7.6 is reached. Add 270 g of NaCl into the solution and fill up to 3,000 ml. To produce the washing buffer, dilute 1:10 of the stock solution with distilled water.	Sigma-Aldrich, St. Louis, USA: Trizma® base REF: T-1503 Molecular weight: 121.14 g/mol Merck/Millipore, Darmstadt, Germany: NaCl REF: 1.06404 Molecular weight: 58.449 g/mol

Table 5. Instruments

Instruments	
Cooking plate	Rommelsbacher, Dinkelsbühl, Germany Model: AK 2080
Cover glas	R. Langenbrinck, Labor- und Medizintechnik, Emmendingen, Germany: Size: 24x32 mm/24x40 mm/24x50 mm Thickness: 0.13-0.16 mm
Humidity chamber	TissueGnostics, Medical and Biotech Solutions, Vienna, Austria
Incubator	Memmert, Schwabach, Germany: REF: D06060; Model: 400
Magnetic stirrer	IKA Labortechnik, Staufen, Germany: Model: RCT basic
Microscope slides	R. Langenbrinck, Labor- und Medizintechnik, Emmendingen, Germany: Super Frost Plus, 25x75x1.0 mm REF: 03-0060
pH meter	Schott, Mainz, Germany
pH meter, reference buffer pH 4.00+/- 0.01 at 25 °C pH 7.00+/- 0.01 at 25 °C	Sigma-Aldrich, Saint Louis, USA: REF: B5020, Lot: 115K0101, 500ml REF: B4770, Lot: 066K0026, 500ml
Pipette	Eppendorf, Hamburg, Germany: Eppendorf Research® - 2.5 µl – REF 3816843 - 10 µl – REF 2376724 - 20 µl – REF 2196524 - 100 µl – REF 3002963 - 200 µl – REF 2299314 - 1000 µl – REF 3173323 Greiner Bio-One, Frickenhausen, Germany: Serological Pipette, steril, Cellstar 10 ml REF: 607160; Lot: 12110251
Pressure cooker	WMF, Geislingen/Steige, Germany: Model: Ideal
Scale	Sartorius, Göttingen, Germany: Model: BP310S
Slide scanner Scanning software	Hamamatsu Photonics, Hamamatsu, Japan NanoZoomer 2.0 HAT slide scanner NDP.view2 Viewing software NanoZoomer Digital Pathology

3.3.3 Staining protocol for plasminogen- and integrin αvβ3-directed antibodies

Dewaxing, hydration, and tissue pre-treatment

The TMA slides were labelled with the date, the ab employed, and its dilution. The slides were heated at 58 °C over night to remove the paraffin. In order to hydrate and further dewax the slides, they were put in alcohol baths with diminishing concentrations of 100%, 96% and 70% (Dako et al., 2013). Formaldehyde modifies the antigen molecules by inducing intermolecular cross-linkages with proteins. These cross-links can be broken down by Heat Induced Epitope Retrieval. For this, a pressure cooker was used. The slides were put in boiling EDTA buffer (for the ab directed to plasminogen) or in boiling citrate buffer (for the ab directed to integrin αvβ3) for four min. Endogenous peroxidase activity was blocked by using hydrogen peroxide 3% (v/v) for 20 min at room temperature thus preventing unspecific background staining.

Primary ab

The primary ab was diluted in ab diluent. For the ab directed to plasminogen, the dilution was 1:35 (stock solution: 2 mg/ml) and for the integrin αvβ3-directed ab, the dilution was 1:50 (stock solution: 0.1 mg/ml). The slides were then each covered with 120 µl of the ab dilution. They were then placed in a humidified chamber and incubated over night at 4 °C.

Secondary ab

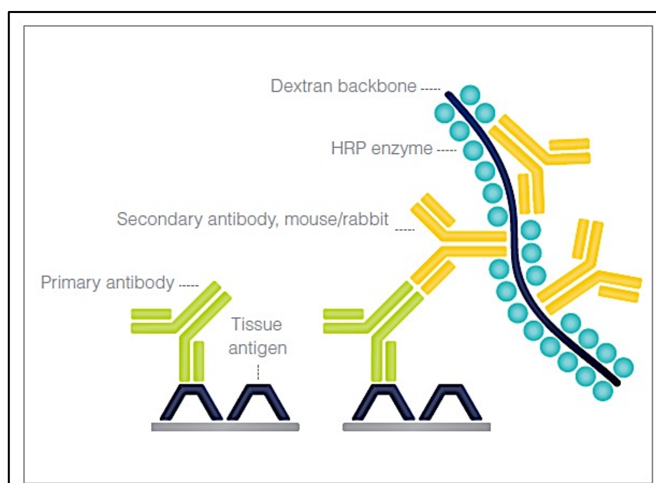


Figure 3. Two-step polymer method (EnVision^{TM}) taken from Dako et al., 2013
After application of the primary ab, primary andibody binds to tissue antigen. Secondary ab labelled with a dextrane backbone conjugated with Horseradish Peroxidase (HRP) enzyme is applied for visualization. The secondary ab uses the enzymatic activity of HRP to visualize the chromogenic substrate.

Primary ab are not labelled themselves. Therefore, the localization of the primary ab needs to be visualized indirectly through the application of a secondary ab binding the primary ab. In this case, the secondary ab consists of a dextran backbone conjugated with HRP enzymes and ab against mouse and rabbit. The secondary ab uses the enzymatic activity of HRP to visualize the chromogenic substrate, which represents the brown color on the slides (Figure 3).

The slides were covered with 120 μl of the secondary ab. The secondary ab was incubated on the TMA for 30 min at room temperature. To visualize the ab/antigen complex, the slides were covered with 3´3´Diaminobenzidine (DAB) tetrachloride for 8 min at room temperature.

Counterstaining of the tissue

To stain the nuclei and the tissue architecture, counterstaining was performed with haematoxylin for 50 sec. After this, the slides were rinsed with tab water for 5 min. Shortly, the slides were put in a bath of distilled water. The slides were placed in alcohol baths of increasing concentrations (70%, 96%, 100%) to dehydrate them. Finally, each slide was covered with a xylene based mounting medium and a cover glas slip and dried for at least 24 h.

Production of control tissues

In order to prove the staining specificity, the following measures were taken:

- tissue controls were included: liver was stained as positive control for plasminogen (Raum et al., 1980); kidney was stained as positive control for integrin αvβ3 (Ponten et al., 2008).
- control slides were realized using pure ab diluent omitting the primary ab.

3.4 Scanning of the tissue

The slides were scanned at high resolution using the *Hamamatsu NanoZoomer HAT slide scanner* (Hamamatsu Photonics, Hamamatsu, Japan) placed at the Klinik und Poliklinik für Frauenheilkunde of the Klinikum rechts der Isar, Technical University of Munich, and visualized using the *NPD.view2 NanoZoomer Digital Pathology Viewing software*. The scanning was performed according to the operator´s manual with a magnification of 40.

3.5 Scoring system

The evaluation of the staining intensity was performed at the Department of Pathology at the Helmholtz Zentrum München by the director of the Department Prof. Dr. med. Axel Karl Walch and by its former vice director, Prof. Dr. rer. nat. Michaela Aubele.

The IRS was performed in an objective way. The pathologists were blinded regarding the patient´s course of disease when evaluating the tumor stainings.

For the evaluation of the staining procedures, using either the ab directed to plasminogen or integrin $\alpha v\beta 3$, the scoring system was categorized into four levels (Remmele & Stegner, 1987).

0 - no staining
1 - weak staining
2 - moderate staining
3 - strong staining

In order to guarantee high specificity and sensitivity of the staining, control tissues expressing a high amount of plasminogen or integrin $\alpha v\beta 3$ or lacking plasminogen or integrin $\alpha v\beta 3$ were produced. Concerning the control tissues, it was important that the visualized antigen was present at a constant and well-established level.

For the control stainings, tissues were incubated in the absence of the primary ab. Instead of the primary ab pure ab diluent was applied on the slides. All other steps were performed according to the described protocol (see Table 7 and Table 8). Control stainings were important to rule out nonspecific background staining and to ensure specificity of the staining (Dako et al., 2013).

Table 6. IRS according to Remmele and Stegner, 1987
The IRS is calculated by multiplication of points of percentage of positive cell nuclei with points of staining intensity. The strongest intensity within a tissue is considered.

IRS according to Remmele and Stegner, 1987		
Percentage of positive cell nuclei	**Staining intensity**	**IRS-points**
No positive nuclei: 0 points	No staining reaction: 0 points	Points of percentage of positive cell nuclei multiplied by Points of staining intensity (0-12 points)
<10% positive nuclei: 1 point	Weak staining reaction: 1 point	
10-50% positive nuclei: 2 points	Intermediate staining reaction: 2 points	
51-80% positive nuclei: 3 points	Strong staining reaction: 3 points	
>80% positive nuclei: 4 points		

IRS-points	IRS-classification
0-1	0 = negative
2-3	1 = positive, weak expression
4-8	2 = positive, mild expression
9-12	3 = positive, strong expression

Taken from Remmele & Stegner, 1987

3.6 Statistical methods

The statistical analysis regarding the patient data and the scoring results of the plasminogen and integrin $\alpha v\beta 3$ results were performed using the IBM SPSS Statistics software Version 24. A p-value of ≤ 0.05 was considered statistically significant.

DFS was considered as the time from diagnosis of the cancer until tumor progression. OS was considered as the time from diagnosis of the cancer until death of the patient by the cancer or any other cause.

Pearson´s Chi-square (χ^2)-Test was performed to seek for associations between clinical parameters and researched factors. The Pearson´s Chi-square (χ^2)-test analyzes the probability of independence of categorical data. It measures how likely an observed distribution is due to chance. A p-value of ≤ 0.05 indicates that the categorical data are dependent, because the likelihood of this exact distribution is seen below 0.05% of the time.

The levels of plasminogen and integrin $\alpha v\beta 3$ staining intensity were associated with DFS and OS using Cox regression analysis. In survival analysis, several factors can influence a patient´s prognosis and potentially affect the survival. Cox regression can assess the effects of several covariates on survival time simultaneously. A hazard ratio of greater than 1 indicates that the factor is positively associated with the probability of the event and therefore negatively associated with the length of survival. A hazard ratio of lower than 1 indicates a reduction of the hazard and is therefore a good prognostic factor. A hazard ratio of 1 indicates no effect. These calculations were made for all of the patients and for subgroups of patients treated with chemotherapy, including traditional factors like nodal status, tumor size, menopausal status, nuclear grade, and adjuvant chemotherapy.

Survival curves were plotted with the SPSS software in accordance with the Kaplan-Meier method for OS and DFS. Survival was represented on the x-axis in months, the

probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (DFS, OS), vertical tick-marks represent a drop-out out of the study.

4. Results

In this study, IHC staining protocols were established for a plasminogen-directed ab and an integrin $\alpha v\beta 3$-directed ab. The goal was to create a reproducible and specific staining for FFPE TNBC TMAs slides.

The establishment of an optimal staining protocol contained different steps: finding the optimal ab, the right dilution for a specific staining, the optimal incubation time and temperature, the right heat induced antigen retrieval with the right buffer and the optimal secondary ab. Some steps were taken from the routine procedure of the laboratory of the Klinik und Poliklinik für Frauenheilkunde of the Klinikum rechts der Isar, Technical University of Munich. They were combined with manufacturers advice and tests according to Dako´s Manual for IHC (Dako et al., 2013).

4.1 Manual staining protocol for plasminogen-directed ab

The staining protocol for the plasminogen-directed ab10178, Abcam can be found in Table 7. Some of the steps were taken from the laboratory routine of the Klinik und Poliklinik für Frauenheilkunde of the Klinikum rechts der Isar, Technical University of Munich. These steps contained the dewaxing protocol at the beginning of the staining, intermitting baths with washing buffer, application of DAB-substrate, counterstaining with haematoxylin, and the dehydration protocol at the end of the staining. Other steps of the protocol were newly tested and optimized. We checked different incubation temperatures (from +4 °C to room temperature), different ab dilutions from 1:400 to 1:35, different incubation times (from one hour to 24 h), different secondary ab (polymer vs. LSAB), and application of heat induced antigen retrieval with different buffers (citrate or EDTA buffer). The protocols were tested on full tissue slides of BC, test TMA slides of different BC, full liver slides and slides of skeletal muscle. The final tests were then approved by Prof. Schmitt and Prof. Aubele. With their permission, the final TNBC TMAs were stained.

Table 7. SOP: plasminogen-directed ab10178
Immunohistochemical staining protocol for plasminogen-directed ab10178 using heat induced antigen retrieval in boiling EDTA buffer, overnight incubation time at +4 °C, using Polymer method and DAB-substrate

SOP: plasminogen-directed ab (ab10178, Abcam)
Deparaffinising and rehydration in descending alcohol row: a. Place in a bath of xylene for 10 min b. Repeat step a with a second xylene bath c. Place in a bath of 100% (v/v) isopropanol for five min d. Repeat step c with a second bath of 100% (v/v) isopropanol e. Place in a bath of 96% (v/v) ethanol for five min f. Place in a bath of 70% (v/v) ethanol for five min
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Pre-treatment in pressure cooker in boiling EDTA buffer (pH 8.0) for four min
Rinse in cold tab water for five min
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Block with 3 % (v/v) hydrogen peroxide (45 ml of distilled water + 5 ml 30% (v/v) H_2O_2), incubate for 20 min at room temperature
Rinse in cold tab water for five min
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Application of primary ab: **plasminogen-directed ab10178 (Lot No. GR176771-1),** diluted 1:35 (stock solution: 2 mg/ml) with ab diluent, cover each slide with 120 µl of the solution, incubate over night at +4 °C in a sealed box
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Application of secondary ab (Polymer, Zytomed®): cover each slide with 120 µl of the solution, incubate for 30 min at room temperature
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Application of DAB-substrate: cover each slide with 120 µl of the solution, (1 ml of the washing buffer + 50 µl DAB substrate), incubate for 8 min at room temperature
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Place in a bath of **Maier´s haematoxylin** for 50 sec
Rinse in cold tab water for five min
Transfer the slides to distilled water
Dehydration in ascending alcohol row: a. Place in a bath of 70% (v/v) ethanol for three min b. Place in a bath of 96% (v/v) ethanol for three min c. Place in a bath of 100% (v/v) isopropanol for three min d. Repeat step c with a second bath of 100% (v/v) isopropanol e. Place in a bath of xylene for three min f. Repeat step e with a second xylene bath
Cover with Pertex® mounting medium

4.2 Manual staining protocol for integrin αvβ3-directed ab

Table 8. SOP: integrin αvβ3-directed ab CBL544
Immunohistochemical staining protocol for integrin αvβ3-directed ab CBL544 using heat induced antigen retrieval in boiling citrate buffer, overnight incubation time at +4 °C, using Polymer method and DAB-substrate

SOP: integrin αvβ3 ab-directed (CBL544, Millipore)
Deparaffinising and rehydration in descending alcohol row: a. Place in a bath of xylene for 10 min b. Repeat step a with a second xylene bath c. Place in a bath of 100% (v/v) isopropanol for five min d. Repeat step c with a second bath of 100% (v/v) isopropanol e. Place in a bath of 96% (v/v) ethanol for five min f. Place in a bath of 70% (v/v) ethanol for five min
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Pre-treatment in pressure cooker in boiling citrate buffer (pH 6.0) for four min
Rinse in cold tab water for five min
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Block with 3% (v/v) hydrogen peroxide (45 ml of distilled water + 5 ml 30 % (v/v) H_2O_2), incubate for 20 min at room temperature
Rinse in cold tab water for five min
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Application of primary ab: **integrin αvβ3-directed ab CBL544 (Lot No. NG1857572)**, diluted 1:50 (stock solution: 0.1 mg/ml). with ab diluent, cover each slide with 120 µl of the solution, incubate over night at +4 °C in a sealed box
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Application of secondary ab (Polymer, Zytomed®): cover each slide with 120 µl of the solution, incubate for 30 min at room temperature
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Application of DAB-substrate: cover each slide with 120 µl of the solution, (1 ml of the washing buffer + 50 µl DAB substrate), incubate for 8 min at room temperature
Place in a bath of washing buffer for five min, exchanging the buffer after two and a half min
Place in a bath of **Maier´s haematoxylin** for 50 sec
Rinse in cold tab water for five min
Transfer to distilled water
Dehydration in ascending alcohol row: a. Place in a bath of 70% (v/v) ethanol for three min b. Place in a bath of 96% (v/v) ethanol for three min c. Place in a bath of 100% (v/v) isopropanol for three min d. Repeat step c with a second bath of 100% (v/v) isopropanol e. Place in a bath of xylene for three min Repeat step e with a second xylene bath
Cover with Pertex® mounting medium

The staining protocol for αvβ3-directed ab CBL544, Millipore can be found in Table 8. Some of the steps were taken from the laboratory routine of the Klinik und Poliklinik für

Frauenheilkunde of the Klinikum rechts der Isar, Technical University of Munich. These steps contained the dewaxing protocol at the beginning of the staining, intermitting baths with washing buffer, application of DAB-substrate, counterstaining with haematoxylin, and the dehydration protocol at the end of the staining. Other steps of the protocol were newly tested and optimized. We checked different incubation temperatures (from +4 °C to room temperature), different ab dilutions from 1:200 to 1:50, different incubation times (from one hour to 24 h), different secondary ab (polymer vs. LSAB), and application of heat induced antigen retrieval with different buffers (citrate or EDTA buffer). The protocols were tested on full tissue slides of BC, test TMA slides of different BC, full kidney slides and lymph node slides. The final tests were then approved by Prof. Schmitt and Prof. Aubele. With their permission, the final TNBC TMAs were stained.

4.3 Assessment of plasminogen expression by IHC

After various staining tests on different full tissue and TMA slides, immunohistochemical staining with plasminogen-directed ab10178 was performed on TNBC TMA according to the established and abovementioned protocol (Table 7). The aim was to score the staining intensity of plasminogen in our TNBC patients and compare the staining results with different clinical and histomorphological factors. An optimal staining protocol shows maximum specific staining with the least amount of unspecific background staining. The figures below show the assessment of plasminogen expression by IHC of TNBC primary tumor tissues. Plasminogen-directed ab10178 (Lot No. GR176771-1) was applied on TNBC tissues using the Polymer method and applying pressure cooking with EDTA buffer.

The staining was assessed in malignant epithelial tumor cells. Positive immunostaining (brown color) was located in the cytoplasm of the tumor cells (Figure 4 A, B, C, D). Additionally to the cytoplasmic staining, nuclear plasminogen staining was found in several cases (Figure 4 A, B, C, D). The staining was often heterogenous throughout the tumor with some negative areas within the positively stained tumors. The IRS was calculated out of the intensity of the staining and the percentage of stained cells. The strongest intensity within a tumor was considered for the IRS. Stromal cells were sometimes positive and unspecific background staining appeared in some cases, but the staining was never as intense as the one seen in tumor (Figure 4 A, B, C, D).

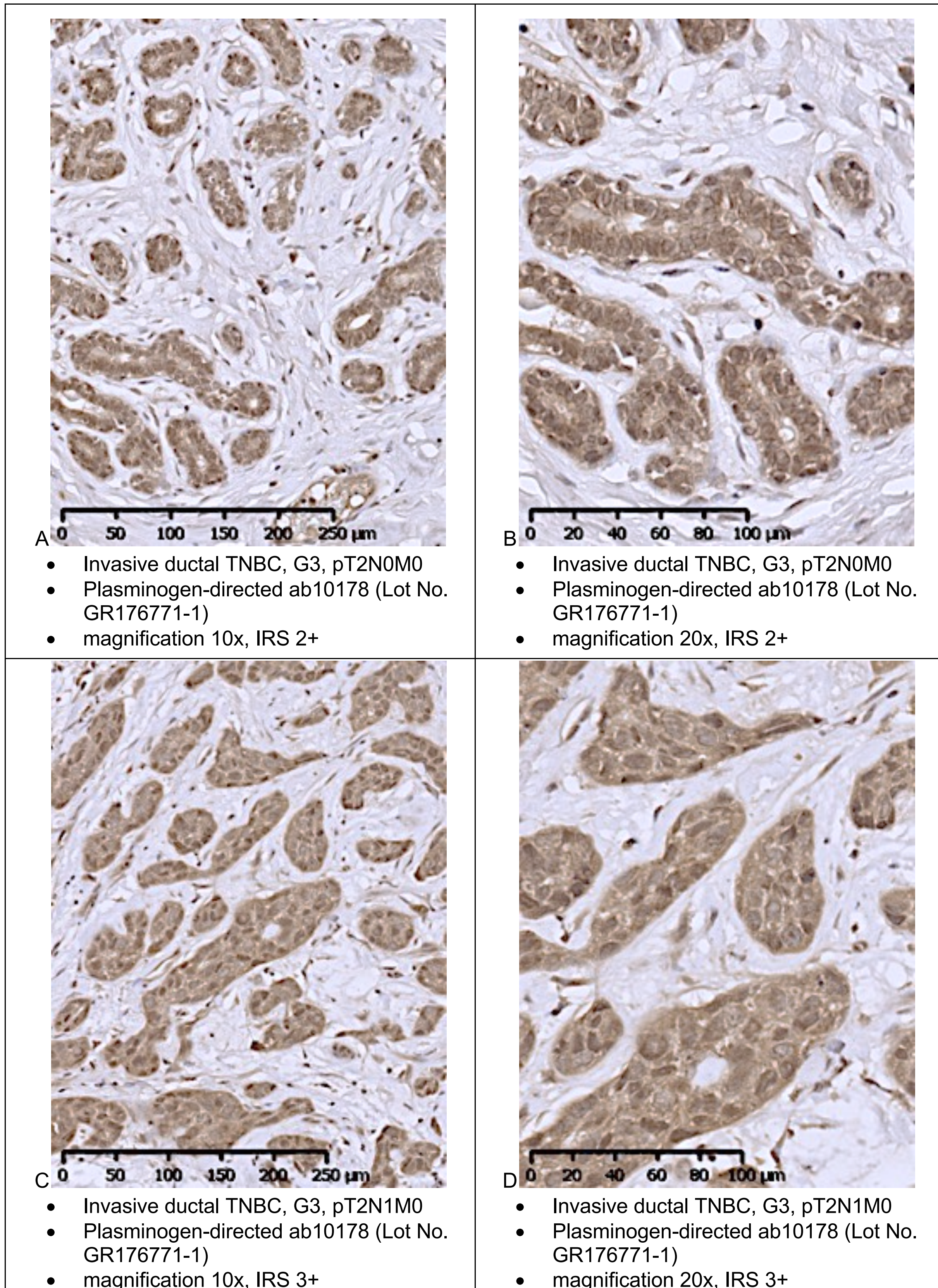


Figure 4. Assessment of plasminogen by IHC

IHC staining with plasminogen-directed ab10178 showing moderate staining results in G3 invasive ductal TNBC with a magnification of 10x (A); moderate staining in G3 invasive ductal TNBC with a magnification of 20x (B); strong staining results in G3 invasive ductal TNBC with a magnification of 10x (C); and strong staining results in a G3 ductal TNBC with a magnification of 20x (D)

4.4 Assessment of integrin αvβ3 expression by IHC

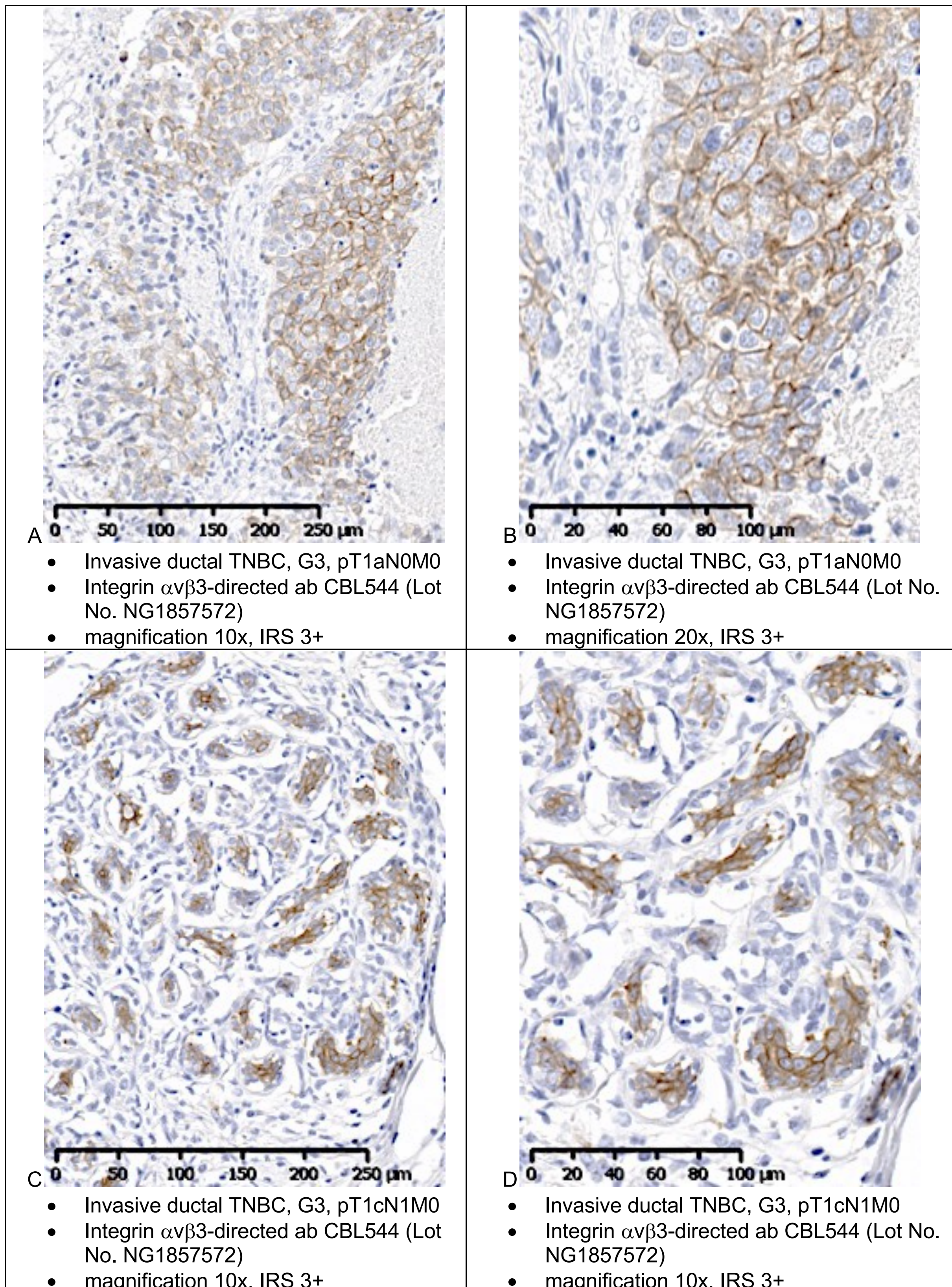


Figure 5. Assessment of integrin αvβ3 by IHC
IHC staining with integrin αvβ3-directed ab CBL544 showing strong staining results in G3 invasive ductal TNBC with a magnification of 10x (A); strong staining in G3 invasive ductal TNBC with a magnification of 20x (B); strong staining results in G3 invasive ductal TNBC with a magnification of 10x (C); and strong staining results in a G3 ductal TNBC with a magnification of 20x (D)

After various staining tests on different full tissue and TMA slides, immunohistochemical staining with integrin αvβ3-directed ab CBL544 was performed on TNBC TMA according to the established and abovementioned protocol (Table 8). The aim was to identify the staining intensity of integrin αvβ3 in our TNBC patients and compare the staining results to different clinical and histomorphological factors. An optimal staining protocol shows maximum specific staining with the least amount of unspecific background staining. The figures below show the assessment of integrin αvβ3 expression by IHC of TNBC primary tumor tissues. Integrin αvβ3-directed ab CBL544 (Lot No. NG1857572) was applied on TNBC tissues using the Polymer method and applying pressure cooking with citrate buffer.

The staining was assessed in malignant epithelial tumor cells. Positive immunostaining (brown color) was mostly found in the cellular membrane of tumor cells (Figure 5 A, B). Some of the tumors showed weak cytoplasmic staining (Figure 5 C, D). Staining results were often heterogenous (Figure 5 A, C) showing weak and strong staining results within one tumor. The IRS was calculated out of the intensity of the staining and the percentage of stained cells. The strongest intensity within a tumor was considered for the IRS. Stromal cells were sometimes positive, but the staining was never as intense as the one seen in the tumor (Figure 5 A, B). Occasional staining of blood vessel walls was also seen. The vasculature in the tumor showed high expression of integrin αvβ3.

4.5 Tissue controls

To guarantee high specificity and sensitivity of the staining, positive controls, negative controls, and control slides without primary ab were stained (Figure 6). Tissues with known presence of plasminogen or integrin αvβ3 were used for positive control stainings. Negative control stainings were made on tissues knowing not to contain the protein to rule out nonspecific staining. Control stainings were stained according to the SOP but omitting the primary ab.

4.5.1 Control stainings for plasminogen

Tissues with known presence of plasminogen were used as control staining slides. In case of plasminogen, liver was used as positive control (Figure 6 A). Plasminogen is produced in the liver therefore this organ is known to contain high amounts of the protein. Positive controls were stained according to the same SOPs as TNBC tissues

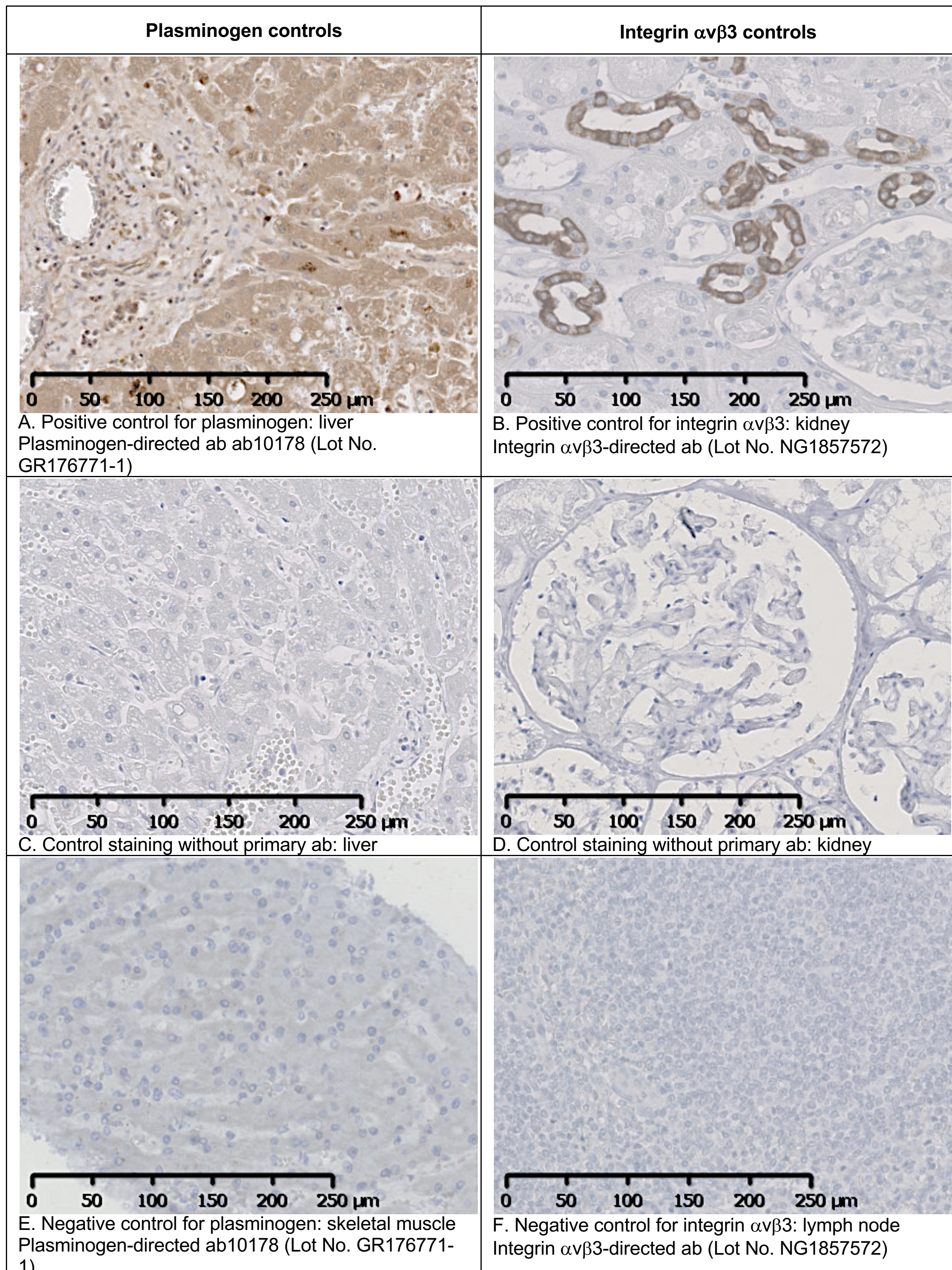


Figure 6. Positive and negative controls
IHC staining with plasminogen ab 10178 (A, C, E) showing strong positive staining in liver tissue (A), no positive staining in the control liver tissue without application of primary ab (B) and no staining in skeletal muscle tissue (E). IHC staining with integrin αvβ3-directed ab CBL544 (B, D, F) showing strong positive staining in kidney tissue (B), no positive staining in the control kidney tissue without application of primary ab (D) and no staining in lymph node tissue (F).

(Table 7). In the positive control for plasminogen staining, a liver lobule is shown. Brown color shows the presence of plasminogen in hepatocytes. Negative controls were made on tissues knowing not to contain the protein to rule out nonspecific staining. Negative controls were stained according to the same SOPs as TNBC tissues (Table 7). In case of plasminogen, skeletal muscle was selected. The cutout shows human skeletal muscle (Figure 6 E). Skeletal muscle shows no staining of plasminogen and no unspecific background staining. Control slides were stained according to the SOP but omitting the primary ab. Instead of applying primary ab diluted in ab diluent, pure ab diluent was applied on the slides. Except for that, every other step of the SOP was followed (Table 7). This control is important to rule out nonspecific background staining and to ensure specificity of the staining (Dako et al., 2013).

4.5.2 Control stainings for integrin αvβ3

Tissues with known presence of integrin αvβ3 were used as control staining slides. Kidney is known to contain high amounts of integrin αvβ3, therefore it was used as positive control (Figure 6 B) (Ponten et al., 2008). Positive controls were stained according to the same SOPs as TNBC tissues (Table 8). On the kidney slide, several renal tubules and a glomerulus are shown. The slide shows heterogenous staining with the glomerulus being negative and the renal tubules showing predominantly strong staining results for integrin αvβ3. Brown color shows the presence of αvβ3 in the tubuli (Figure 6 B). Negative controls were made on tissues knowing not to contain the protein to rule out nonspecific staining. Negative controls were stained according to the same SOPs as TNBC tissues (Table 8). In case of integrin αvβ3, lymph node was selected. The cutout shows human lymph node (Figure 6 F). Lymph node shows no staining of integrin αvβ3 and no unspecific background staining. Control slides were stained according to the SOP but omitting the primary ab. Instead of applying primary ab diluted in ab diluent, pure ab diluent was applied on the slides. Except for that, every other step of the SOP was followed (Table 8). This control is important to rule out nonspecific background staining and to ensure specificity of the staining (Dako et al., 2013).

4.6 Association of plasminogen with clinical and histomorphological factors

Table 9. Association of plasminogen staining with clinical and histomorphological factors
Association of plasminogen staining with clinical and histomorphological factors like age, menopausal status, tumor size, nodal status, nuclear grade, locoregional disease recurrence, OS and application of adjuvant chemotherapy using the Pearson's Chi-square (χ^2)-Test with level of significance (p) determined in TMA of TNBC patients.

Parameter	Number of patients	Plasminogen (low / high)	p-value
Age	**161**		0.178
< 50 years	53	28/25	
≥ 50 years	108	69/39	
Menopausal status	**159**		0.144
Pre-/perimenopausal	50	26/24	
postmenopausal	109	70/39	
Tumor size (pT)	**151**		0.472
pT1 + pT2	121	72/49	
pT3 + pT4	30	20/10	
Nodal status	**138**		0.524
Negative	72	42/30	
Positive	66	42/24	
Nuclear grade	**153**		0.611
G1 + G2	22	12/10	
G3	131	79/52	
Locoregional disease recurrence	**155**		0.746
Yes	32	20/12	
No	123	73/50	
OS	**130**		0.235
Deceased	60	42/18	
Alive	70	42/28	
Adjuvant chemotherapy	**153**		0.702
Treated	95	56/39	
Not treated	58	36/22	

Plasminogen expression assessed by IHC:
- Low: IRS 0-2+ (combined negative, weak, and moderate staining results by IHC)
- High: IRS 3+ (strong staining)

Cutoff values:
- Age: patients <50 years vs. patients ≥50 years
- Menopause: Pre- or perimenopausal patients vs. postmenopausal patients
- Tumor size: tumors ≤50 mm in greatest dimension vs. tumors >50 mm in greatest dimension or tumors of any size with direct extension to the chest wall and/or to the skin
- Nodal status: no regional lymph node metastasis identified vs. regional lymph node metastasis identified
- Nuclear grade: low (favorable) and intermediate (moderately favorable) histologic grade vs. high (unfavorable) histologic grade
- Locoregional disease recurrence: Locoregional disease recurrence vs. no locoregional disease recurrence
- OS: deceased patients vs. alive patients
- Adjuvant chemotherapy: patients treated with adjuvant chemotherapy vs. patients not treated with adjuvant chemotherapy

p-Value:
- Assessed by Pearson´s Chi-square (χ^2)-Test

Taken from: http://emedicine.medscape.com/article/2007112-overview

Scoring results of the plasminogen-directed ab stainings on TMA of TNBC patients were associated to clinical and histomorphological factors using the Pearson's Chi-

square (χ^2)-Test. Factors like the patients age at diagnosis (<50 vs. ≥ 50 years), menopausal status (pre- and perimenopausal vs. postmenopausal), tumor size (pT1+2 vs. pT3+4), nodal status (negative vs. positive), nuclear grade (G1+2 vs. G3), locoregional disease recurrence (yes vs. no), OS (deceased or alive) and application of adjuvant chemotherapy (yes vs. no) were included.

Plasminogen expression was divided into two subgroups. The best cutoff value of the IRS was calculated and defined as low (IRS 0-2+) versus high (IRS 3+). 98 patients expressed plasminogen at a low level (IRS 0-2+), 64 patients expressed plasminogen at a high level (IRS 3+). The distribution of clinical parameters was analyzed according to this cutoff.

The staining results were associated with clinical factors such as age (<50 vs. ≥ 50 years), menopausal status (pre- and perimenopausal vs. postmenopausal), disease recurrence (yes vs. no), OS (deceased vs. alive), and adjuvant chemotherapy treatment (treated vs. not treated). They were also associated with histomorphological factors such as tumor size (pT1 and pT2 vs. pT3 and pT4), nodal status (negative vs. positive), and nuclear grade (G1 and G2 vs. G3). No statistically significant associations were found for TNBC between these factors and plasminogen expression levels, indicating a comparatively uniform distribution within the subgroups.

4.7 Association of integrin αvβ3 with clinical and histomorphological factors

Scoring results of the integrin αvβ3-directed ab stainings on TMA of TNBC patients were associated to clinical and histomorphological factors using the Pearson's Chi-square (χ^2)-Test. Factors like the patients age at diagnosis (<50 vs. ≥ 50 years), menopausal status (pre- and perimenopausal vs. postmenopausal), tumor size (pT1+2 vs. pT3+4), nodal status (negative vs. positive), nuclear grade (G1+2 vs. G3), locoregional disease recurrence (yes vs. no), OS (deceased or alive) and application of adjuvant chemotherapy (yes vs. no) were included.

Integrin αvβ3 expression was divided into two subgroups. The best cutoff value of the IRS was calculated and defined as low (IRS 0-1+) versus high (IRS 2+-3+). 130 patients expressed integrin αvβ3 at a low level (IRS 0-1+), 42 patients expressed integrin αvβ3 at a high level (IRS 2+-3+). The distribution of clinical parameters was analyzed according to this cutoff.

Table 10. Association of integrin $\alpha v\beta 3$ staining with clinical and histomorphological factors
Association of integrin $\alpha v\beta 3$ staining with clinical and histomorphological factors like age, menopausal status, tumor size, nodal status, nuclear grade, locoregional disease recurrence, OS and application of adjuvant chemotherapy using the Pearson's Chi-square (χ^2)-Test with level of significance (p) determined in TMA of TNBC patients.

Parameter	Number of patients	Integrin αvβ3 (low / high)	p-value
Age	**170**		**0.013**
< 50 years	56	49/7	
≥ 50 years	114	80/34	
Menopausal status	**169**		**0.031**
Pre-/perimenopausal	53	46/7	
postmenopausal	116	83/33	
Tumor size (pT)	**161**		0.953
pT1 + pT2	130	100/30	
pT3 + pT4	31	24/7	
Nodal status	**148**		**0.028**
Negative	77	64/13	
Positive	71	48/23	
Nuclear grade	**162**		**0.024**
G1 + G2	23	13/10	
G3	139	109/30	
Locoregional disease recurrence	**164**		0.634
Yes	33	26/7	
No	131	98/33	
OS	**138**		0.115
Deceased	63	44/19	
Alive	75	61/14	
Adjuvant chemotherapy	**163**		0.978
Treated	100	76/24	
Not treated	63	48/15	

Integrin αvβ3 expression assessed by IHC:
- Low: IRS 0-1+ (combined negative and weak staining results by IHC)
- High: IRS 2+-3+ (combined moderate and strong staining results by IHC)

Cutoff values:
- Age: patients <50 years vs. patients ≥50 years
- Menopausal status: Pre- or perimenopausal patients vs. postmenopausal patients
- Tumor size: tumors ≤50 mm in greatest dimension vs. tumors >50 mm in greatest dimension or tumors of any size with direct extension to the chest wall and/or to the skin
- Nodal status: no regional lymph node metastasis identified vs. regional lymph node metastasis identified
- Nuclear grade: low (favorable) and intermediate (moderately favorable) histologic grade vs. high (unfavorable) histologic grade
- Locoregional disease recurrence: Locoregional disease recurrence vs. no locoregional disease recurrence
- OS: deceased patients vs. alive patients
- Adjuvant chemotherapy: patients treated with adjuvant chemotherapy vs. patients not treated with adjuvant chemotherapy

p-Value:
- Assessed by Pearson´s Chi-square (χ^2)-Test

Taken from: http://emedicine.medscape.com/article/2007112-overview

The staining results were associated with clinical factors such as age (<50 vs. ≥ 50 years), menopausal status (pre- and perimenopausal vs. postmenopausal), disease recurrence (yes vs. no), OS (deceased vs. alive), and adjuvant chemotherapy treatment (treated vs. not treated). They were also associated with histomorphological

factors such as tumor size (pT1 and pT2 vs. pT3 and pT4), nodal status (negative vs. positive), and nuclear grade (G1 and G2 vs. G3).

Age (p=0.013), menopausal status (p=0.031), nodal status (p=0.028), and nuclear grade (p=0.024) were significantly associated with integrin αvβ3 expression.

For tumor size, disease recurrence, OS, and adjuvant chemotherapy treatment no statistically significant associations were found for TNBC between these factors and integrin αvβ3 expression levels, indicating a comparatively uniform distribution within the subgroups.

4.8 Cox regression analysis

Univariate and multivariable Cox regression analyses for OS for plasminogen- and integrin αvβ3-directed ab

Univariate and multivariable Cox regression analyses were performed to assess the clinical impact of plasminogen expression levels and integrin αvβ3 expression levels on a patient’s OS.

Univariate Cox regression analysis was performed for OS to examine whether plasminogen and integrin αvβ3 might constitute predictive marker in TNBC. The strength of association between traditional clinical parameters, plasminogen and integrin αvβ3 expression levels with patients´ OS by univariate and multivariable analyses is summarized in Table 11. Clinical and histomorphological factors as well as the expression levels of plasminogen and integrin αvβ3 were considered in the calculations, including nodal status (negative vs. positive), tumor size (pT1 and pT2 vs. pT3 and pT4), menopausal status (pre- and perimenopausal vs. postmenopausal), nuclear grade (G1 and G2 vs. G3), adjuvant chemotherapy treatment (treated vs. not treated), and expression levels for plasminogen (low vs. high) and integrin αvβ3 (low vs. high).

Multiple established prognostic factors were confirmed by our univariate analysis. Nodal status (p<0.001; HR: 3.27; 95% CI 1.84-5.81) and tumor size (p<0.001; HR: 3.86; 95% CI 2.29-6.50) were of statistical significance predicting the probability of OS by univariate analysis. Menopausal status (p=0.025; HR: 1.96; 95% CI 1.09-3.54) and adjuvant chemotherapy treatment (p<0.001; HR: 2.54; 95% CI 1.55-4.14) also contributed statistically significant information by univariate analysis regarding OS.

Table 11. Cox regression analysis for OS for plasminogen- and integrin αvβ3-directed ab

Variable	Number of patients	Univariate		Multivariable	
		HR [95% CI]	p	HR [95% CI]	p
Nodal status	**148**		**< 0.001**		**0.002**
Negative	75	**1**		**1**	
Positive	73	**3.27 [1.84-5.81]**		**2.57 [1.39-4.73]**	
Tumor size	**159**		**< 0.001**		**0.008**
pT1 + pT2	128	**1**		**1**	
pT3 + pT4	31	**3.86 [2.29-6.50]**		**2.31 [1.25-4.28]**	
Menopausal status	**167**		**0.025**		0.237
Pre-/peri	53	**1**		1	
Post	114	**1.96 [1.09-3.54]**		1.54 [0.75-3.17]	
Nuclear grade	**160**		0.369		
G1 + G2	25	1			
G3	135	1.38 [0.68-2.81]		---	
Adjuvant CTX	**163**		**< 0.001**		**0.005**
Treated	101	**1**		1	
Not treated	62	**2.54 [1.55-4.14]**		2.28 [1.29-4.04]	
Plasminogen	**144**		0.078		
low	87	1			
high	57	0.61 [0.35-1.06]		---	
Integrin αvβ3	**154**		0.079		
low	117	1			
high	37	1.61 [0.95-2.74]		---	

Legend:

Plasminogen expression assessed by IHC:
- Low: IRS 0-2+ (combined negative, weak, and moderate staining results by IHC)
- High: IRS 3+ (strong staining)

Integrin αvβ3 expression assessed by IHC:
- Low: IRS 0-1+ (combined negative and weak staining results by IHC)
- High: IRS 2+-3+ (combined moderate and strong staining results by IHC)

Cutoff values:
- Nodal status: no regional lymph node metastasis identified vs. regional lymph node metastasis identified
- Tumor size: tumors ≤50 mm in greatest dimension vs. tumors >50 mm in greatest dimension or tumors of any size with direct extension to the chest wall and/or to the skin
- Menopausal status: Pre- or perimenopausal patients vs. postmenopausal patients
- Nuclear grade: low (favorable) and intermediate (moderately favorable) histologic grade vs. high (unfavorable) histologic grade
- Adjuvant chemotherapy: patients treated with adjuvant chemotherapy vs. patients not treated with adjuvant chemotherapy

HR [95% CI]:
- Hazard ratio with a confidence interval of 95%

p-Value:
- Assessed by Cox regression analysis

Taken from: http://emedicine.medscape.com/article/2007112-overview

Among the established clinical and histomorphological factors, only nuclear grade did not show a statistical significance by univariate Cox regression analysis predicting the probability of OS in our patient cohort (p=0.369; HR: 1.38; 95% CI 0.68-2.81).

Plasminogen levels were dichotomized in low (IRS 0-2+) vs. high (3+) and did not represent a significant predictive factor for OS (p=0.078; HR: 0.61; 95% CI 0.35-1.06). Integrin αvβ3 expression levels were dichotomized in low (0-1+) vs. high (2+-3+) was equally not associated with OS (p=0.079; HR: 1.61; 95% CI 0.95-2.74).

Multivariable analysis was calculated for OS by gradually including the established clinicopathological factors nodal status, tumor size, menopausal status, and adjuvant chemotherapy treatment as covariates for testing whether they constitute statistically significant independent variables.
In a multivariable analysis of the patient cohort using nodal status, tumor size, menopausal status and adjuvant chemotherapy treatment as covariates, nodal status (p=0.002; HR: 2.57; 95% CI 1.39-4.73), tumor size (p=0.008; HR: 2.31; 95% CI 1.25-4.28) and adjuvant chemotherapy treatment (p=0.005; HR: 2.28; 95% CI 1.29-4.04) remained independent markers for poor OS. Menopausal status (p=0.237; HR: 1.54; 95% CI 0.75-3.17) could not be confirmed as an independent marker for poor OS by multivariable analysis.

Univariate and multivariable Cox regression analyses for DFS for plasminogen- and integrin αvβ3-directed ab

Univariate and multivariable Cox regression analyses were performed to assess the clinical impact of plasminogen expression levels and integrin αvβ3 expression levels on a patient's DFS.

To analyze whether plasminogen and integrin αvβ3 might constitute predictive marker in TNBC, univariate Cox regression analysis was performed for DFS. The strength of association between traditional clinical parameters, plasminogen and integrin αvβ3 expression levels with patients´ DFS by univariate und multivariable analyses is summarized in Table 12. Clinical and histomorphological factors as well as the expression levels of plasminogen and integrin αvβ3 were considered in the calculations, including nodal status (negative vs. positive), tumor size (pT1 and pT2 vs. pT3 and pT4), menopausal status (pre- and perimenopausal vs. postmenopausal), nuclear grade (G1 and G2 vs. G3), adjuvant chemotherapy treatment (treated vs. not treated), and expression levels for plasminogen (low vs. high) and integrin αvβ3 (low vs. high).

Table 12. Cox regression analysis for DFS for plasminogen- and integrin $\alpha v\beta 3$-directed ab

Variable	Number of patients	Univariate		Multivariable	
		HR [95% CI]	p	HR [95% CI]	p
Nodal status	**148**		**0.001**		**0.011**
Negative	76	**1**		**1**	
Positive	72	**2.45 [1.44-4.15]**		**2.07 [1.18-3.61]**	
Tumor size	**160**		**< 0.001**		**0.001**
pT1 + pT2	129	**1**		**1**	
pT3 + pT4	31	**3.34 [2.01-5.56]**		**2.70 [1.49-4.90]**	
Menopausal status	**170**		0.237		
Pre-/peri	54	1			
Post	116	1.36 [0.82-2.24]		---	
Nuclear grade	**162**		0.453		
G1 + G2	25	1			
G3	137	1.29 [0.66-2.53]		---	
Adjuvant CTX	**165**		**0.001**		0.054
Treated	101	**1**		1	
Not treated	64	**2.16 [1.36-3.42]**		1.71 [0.99-2.94]	
Plasminogen	**147**		0.178		
low	89	1			
high	58	0.71 [0.43-1.17]		---	
Integrin $\alpha v\beta 3$	**157**		0.116		
low	118	1			
high	39	1.50 [0.91-2.49]		---	

Legend:

Plasminogen expression assessed by IHC:
- Low: IRS 0-2+ (combined negative, weak, and moderate staining results by IHC)
- High: IRS 3+ (strong staining)

Integrin $\alpha v\beta 3$ expression assessed by IHC:
- Low: IRS 0-1+ (combined negative and weak staining results by IHC)
- High: IRS 2+-3+ (combined moderate and strong staining results by IHC)

Cutoff values:
- Nodal status: no regional lymph node metastasis identified vs. regional lymph node metastasis identified
- Tumor size: tumors $\leq$50 mm in greatest dimension vs. tumors >50 mm in greatest dimension or tumors of any size with direct extension to the chest wall and/or to the skin
- Menopausal status: Pre- or perimenopausal patients vs. postmenopausal patients
- Nuclear grade: low (favorable) and intermediate (moderately favorable) histologic grade vs. high (unfavorable) histologic grade
- Adjuvant chemotherapy: patients treated with adjuvant chemotherapy vs. patients not treated with adjuvant chemotherapy

HR [95% CI]:
- Hazard ratio with a confidence interval of 95%

p-Value:
- Assessed by Cox regression analysis

Taken from: http://emedicine.medscape.com/article/2007112-overview

Multiple established prognostic factors were confirmed by our univariate analysis. Nodal status (p=0.001; HR: 2.45; 95% CI 1.44-4.15) and tumor size (p<0.001; HR: 3.34; 95% CI 2.01-5.56) were of statistical significance predicting the probability of DFS by univariate analysis. Adjuvant chemotherapy treatment (p=0.001; HR: 2.16; 95% CI 1.36-3.42) also contributed statistically significant information by univariate analysis

regarding DFS. Among the established clinical and histomorphological factors, only nuclear grade (p=0.453; HR: 1.29; 95% CI 0.66-2.53) and menopausal status (p=0.237; HR: 1.36; 95% CI 0.82-2.24) did not show a statistical significance by univariate Cox regression analysis predicting the probability of DFS in our patient cohort. Plasminogen levels were dichotomized in low (IRS 0-2+) vs. high (3+) and did not represent a significant predictive factor for DFS (p=0.178; HR: 0.71; 95% CI 0.43-1.17). Integrin αvβ3 expression dichotomized in low (0-1+) vs. high (2+-3+) was equally not associated with DFS (p=0.116; HR: 1.50; 95% CI 0.91-2.49).

Multivariable analysis was calculated for DFS by gradually including the established clinicopathological factors nodal status, tumor size, and adjuvant chemotherapy treatment as covariates for testing whether they constitute statistically significant independent variables.

In a multivariable analysis of the patient cohort using nodal status, tumor size, and adjuvant chemotherapy treatment as covariates, nodal status (p=0.011; HR: 2.07; 95% CI 1.18-3.61) and tumor size (p=0.001; HR: 2.70; 95% CI 1.49-4.90) remained independent marker for poor DFS. Adjuvant chemotherapy treatment (p=0.054; HR: 1.71; 95% CI 0.99-2.94) could not be confirmed as an independent marker for poor DFS by multivariable analysis.

Univariate and multivariable Cox regression analyses for OS in patients treated with chemotherapy- Integrin αvβ3

Univariate and multivariable Cox regression analyses were performed on a subgroup of the patient cohort treated either with neoadjuvant and/or with adjuvant chemotherapy to assess the clinical impact of integrin αvβ3 expression levels on OS of this patient subgroup.

Univariate Cox regression analysis was performed for OS to examine whether integrin αvβ3 expression levels might constitute a predictive marker in TNBC patients treated with chemotherapy. The strength of association between traditional clinical parameters, and integrin αvβ3 expression levels with patients´ OS by univariate and multivariable analyses is summarized in Table 13. Clinical and histomorphological factors as well as the integrin αvβ3 expression levels were considered in the calculations, including nodal status (negative vs. positive), tumor size (pT1 and pT2 vs. pT3 and pT4), menopausal status (pre- and perimenopausal vs. postmenopausal),

nuclear grade (G1 and G2 vs. G3), and expression levels for integrin αvβ3 (low vs. high).

Table 13. Integrin αvβ3: Cox regression analysis for OS for patients treated with chemotherapy

Variable	Number of patients	Univariate		Multivariable	
		HR [95% CI]	p	HR [95% CI]	p
Nodal status	**94**		**0.006**		**0.010**
Negative	47	**1**		**1**	
Positive	47	**3.46 [1.44-8.32]**		**3.82 [1.38-10.59]**	
Tumor size	**98**		**0.026**		**0.021**
pT1 + pT2	87	**1**		**1**	
pT3 + pT4	11	**2.82 [1.13-7.01]**		**3.08 [1.18-8.00]**	
Menopausal status	**105**		0.162		
Pre-/peri	43	1			
Post	62	1.74 [0.80-3.75]		---	
Nuclear grade	**100**		0.471		
G1 + G2	8	1			
G3	92	1.71 [0.40-7.29]		---	
Integrin αvβ3	**96**		**0.048**		0.386
low	73	**1**		1	
high	23	**2.12 [1.01-4.47]**		1.48 [0.61-3.55]	

Legend:

Integrin αvβ3 expression assessed by IHC:
- Low: IRS 0-1+ (combined negative and weak staining results by IHC)
- High: IRS 2+-3+ (combined moderate and strong staining results by IHC)

Cutoff values:
- Nodal status: no regional lymph node metastasis identified vs. regional lymph node metastasis identified
- Tumor size: tumors ≤50 mm in greatest dimension vs. tumors >50 mm in greatest dimension or tumors of any size with direct extension to the chest wall and/or to the skin
- Menopausal status: Pre- or perimenopausal patients vs. postmenopausal patients
- Nuclear grade: low (favorable) and intermediate (moderately favorable) histologic grade vs. high (unfavorable) histologic grade

HR [95% CI]:
- Hazard ratio with a confidence interval of 95%

p-Value:
- Assessed by Cox regression analysis

Taken from: http://emedicine.medscape.com/article/2007112-overview

Multiple established prognostic factors were confirmed by our univariate analysis. Nodal status (p=0.006; HR: 3.46 95% CI 1.44-8.32) and tumor size (p=0.026; HR: 2.82; 95% CI 1.13-7.01) were of statistical significance predicting the probability of OS by univariate analysis. Integrin αvβ3 expression dichotomized in low (0-1+) vs. high (2+-3+) also contributed statistically significant information by univariate analysis regarding OS (p=0.048; HR: 2.12; 95% CI 1.01-4.47). Among the established clinical and histomorphological factors, nuclear grade (p=0.471; HR: 1.71; 95% CI 0.40-7.29) and menopausal status (p=0.162; HR: 1.74; 95% CI 0.80-3.75) did not show statistical

significance by univariate Cox regression analysis predicting the probability of OS in this patient cohort.

Multivariable analysis was calculated for OS by gradually including the established clinicopathological factors nodal status, tumor size, and integrin $\alpha v\beta 3$ expression as covariates for testing whether they constitute statistically significant independent variables.
In a multivariable analysis of the patient cohort using nodal status, tumor size, and integrin $\alpha v\beta 3$ expression as covariates, nodal status (p=0.010; HR: 3.82; 95% CI 1.38-10.59) and tumor size (p=0.021; HR: 3.08; 95% CI 1.18-8.00) remained independent marker for poor OS. Integrin $\alpha v\beta 3$ expression (p=0.386; HR: 1.48; 95% CI 0.61-3.55) could not be confirmed as an independent marker for poor OS by multivariable analysis in this patient subgroup.

Univariate and multivariable Cox regression analyses for DFS in patients treated with chemotherapy- Integrin $\alpha v\beta 3$

Univariate and multivariable Cox regression analyses were performed on a subgroup of the patient cohort treated either with neoadjuvant and/or with adjuvant chemotherapy to assess the clinical impact of integrin $\alpha v\beta 3$ expression levels on DFS of this patient subgroup.

Univariate Cox regression analysis was performed for DFS to examine whether integrin $\alpha v\beta 3$ expression levels might constitute a predictive marker in TNBC patients treated with chemotherapy. The strength of association between traditional clinical parameters and integrin $\alpha v\beta 3$ expression levels with patients DFS by univariate and multivariable analyses is summarized in Table 14. Clinical and histomorphological factors as well as the integrin $\alpha v\beta 3$ expression levels were considered in the calculations, including nodal status (negative vs. positive), tumor size (pT1 and pT2 vs. pT3 and pT4), menopausal status (pre- and perimenopausal vs. postmenopausal), nuclear grade (G1 and G2 vs. G3), and expression levels for integrin $\alpha v\beta 3$ (low vs. high).

Multiple established prognostic factors were confirmed by our univariate analysis. Nodal status (p=0.005; HR: 2.94 95% CI 1.39-6.23) and tumor size (p=0.004; HR: 3.23; 95% CI 1.46-7.14) were of statistical significance predicting the probability of DFS by univariate analysis.

Table 14. Integrin $\alpha v\beta 3$: Cox regression analysis for DFS for patients treated with chemotherapy

Variable	Number of patients	Univariate		Multivariable	
		HR [95% CI]	p	HR [95% CI]	p
Nodal status	**94**		**0.005**		**0.025**
Negative	48	**1**		**1**	
Positive	46	**2.94 [1.39-6.23]**		**2.52 [1.12-5.65]**	
Tumor size	**98**		**0.004**		**0.007**
pT1 + pT2	87	**1**		**1**	
pT3 + pT4	11	**3.23 [1.46-7.14]**		**3.30 [1.39-7.82]**	
Menopausal status	**107**		0.735		
Pre-/peri	43	1			
Post	64	1.11 [0.60-2.08]		---	
Nuclear grade	**101**		0.392		
G1 + G2	8	1			
G3	93	1.87 [0.48-7.83]		---	
Integrin αvβ3	**98**		**0.031**		0.201
low	73	**1**		1	
high	25	**2.07 [1.07-4.02]**		1.67 [0.76-3.67]	

Legend:

Integrin αvβ3 expression assessed by IHC:
- Low: IRS 0-1+ (combined negative and weak staining results by IHC)
- High: IRS 2+-3+ (combined moderate and strong staining results by IHC)

Cutoff values:
- Nodal status: no regional lymph node metastasis identified vs. regional lymph node metastasis identified
- Tumor size: tumors ≤50 mm in greatest dimension vs. tumors >50 mm in greatest dimension or tumors of any size with direct extension to the chest wall and/or to the skin
- Menopausal status: Pre- or perimenopausal patients vs. postmenopausal patients
- Nuclear grade: low (favorable) and intermediate (moderately favorable) histologic grade vs. high (unfavorable) histologic grade

HR [95% CI]:
- Hazard ratio with a confidence interval of 95%

p-Value:
- Assessed by Cox regression analysis

Taken from: http://emedicine.medscape.com/article/2007112-overview

Integrin αvβ3 expression dichotomized in low (0-1+) vs. high (2+-3+) also contributed statistically significant information by univariate analysis regarding DFS (p=0.031; HR: 2.07; 95% CI 1.07-4.02). Among the established clinical and histomorphological factors, nuclear grade (p=0.392; HR: 1.87; 95% CI 0.48-7.83) and menopausal status (p=0.735; HR: 1.11; 95% CI 0.60-2.08) did not show statistical significance by univariate Cox regression analysis predicting the probability of DFS in this patient cohort.

Multivariable analysis was calculated for DFS by gradually including the established clinicopathological factors nodal status, tumor size, and integrin αvβ3 expression as

covariates for testing whether they constitute statistically significant independent variables.

In a multivariable analysis of the patient cohort using nodal status, tumor size, and integrin $\alpha v\beta 3$ expression as covariates, nodal status (p=0.025; HR: 2.52; 95% CI 1.12-5.65) and tumor size (p=0.007; HR: 3.30; 95% CI 1.39-7.82) remained independent marker for poor DFS. Integrin $\alpha v\beta 3$ expression (p=0.201; HR: 1.67; 95% CI 0.76-3.67) could not be confirmed as an independent marker for poor DFS by multivariable analysis in this patient subgroup.

4.9 Impact on survival of plasminogen and integrin $\alpha v\beta 3$ expression levels

The association of plasminogen expression and integrin $\alpha v\beta 3$ expression levels in TNBC patients assessed by IHC with OS and DFS is visualized by the respective Kaplan Meier survival curves.

4.9.1 Plasminogen

A. Probability of OS by plasminogen expression level

The Kaplan Meier survival curve depicted in Figure 7 visualizes the clinical impact of plasminogen expression level assessed by IHC on the probability of OS in TNBC patients over a time period of 250 months. Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (OS), vertical tick-marks represent a drop-out out of the study. In total, 144 patients were included in the calculations. The patients were dichotomized by plasminogen expression level in low (IRS 0-2+; n=87; blue color) vs. high (3+; n=57; green color).

The number of patients showing high plasminogen levels (green color) at the beginning of the study was 57. They showed 18 events over a time period of 250 months, indicating that 18 patients died over this time period of any cause not necessarily related to the TNBC. After 150 months, 8 patients with high plasminogen levels were still alive, after 250 months none of the patients was still alive. The remaining patients (n=39) dropped out of the study because of other reasons.

The number of patients showing low plasminogen levels (blue color) at the beginning of the study was 87. They showed 42 events over a time period of 250 months,

indicating that 42 patients died over this time period of any cause not necessarily related to the TNBC. After 150 months, 5 patients with low plasminogen levels were still alive, after 250 months none of the patients was still alive. The remaining patients (n=45) dropped out of the study because of other reasons.

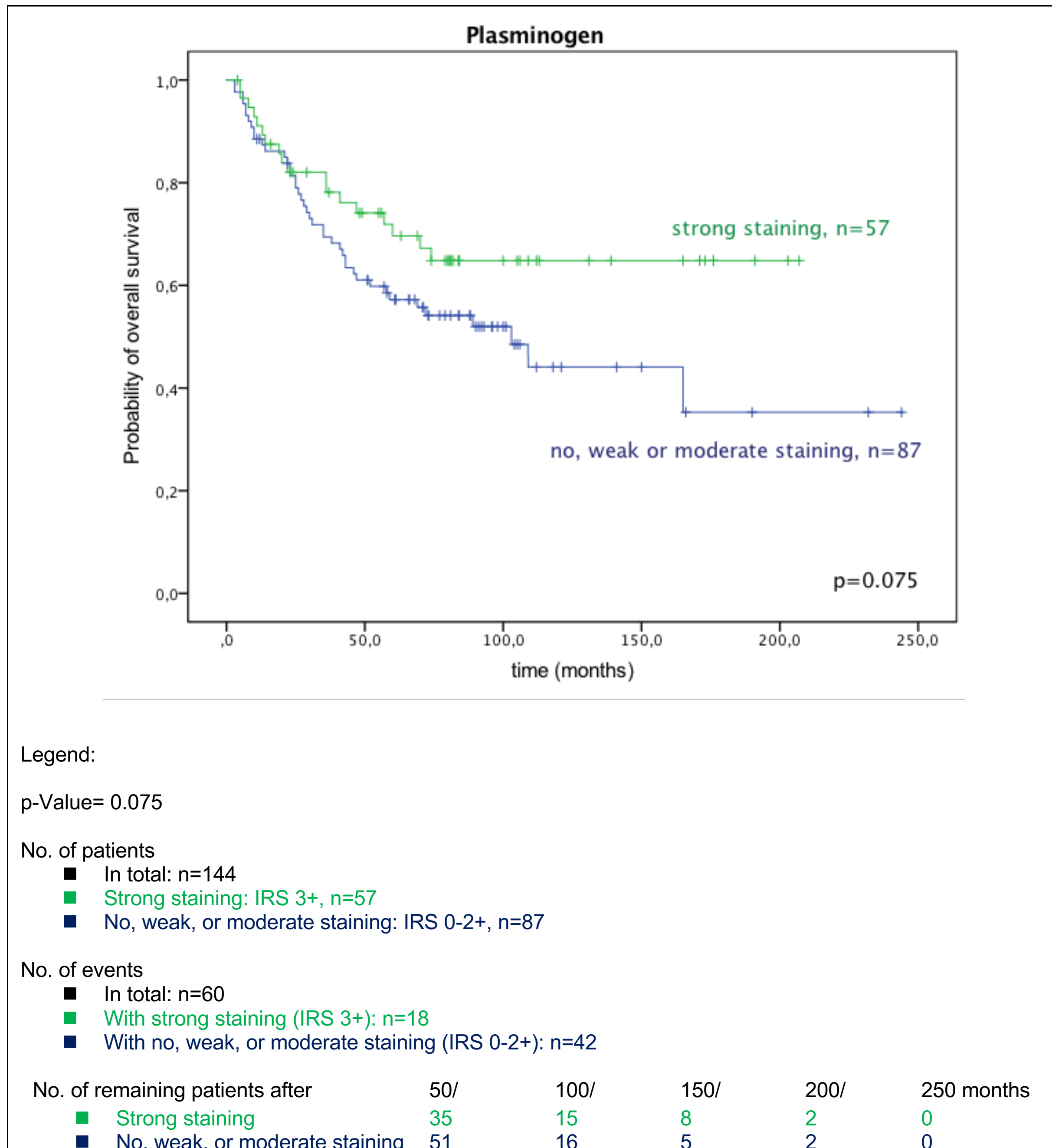


Figure 7. Probability of OS by plasminogen expression level

In summary, this Kaplan Meier survival curve shows that patients with low plasminogen expression level tended to show lower probability of OS and that patients with high plasminogen expression level tended to show higher probability of OS over a time period of 250 months without being statistically significant (p=0.075).

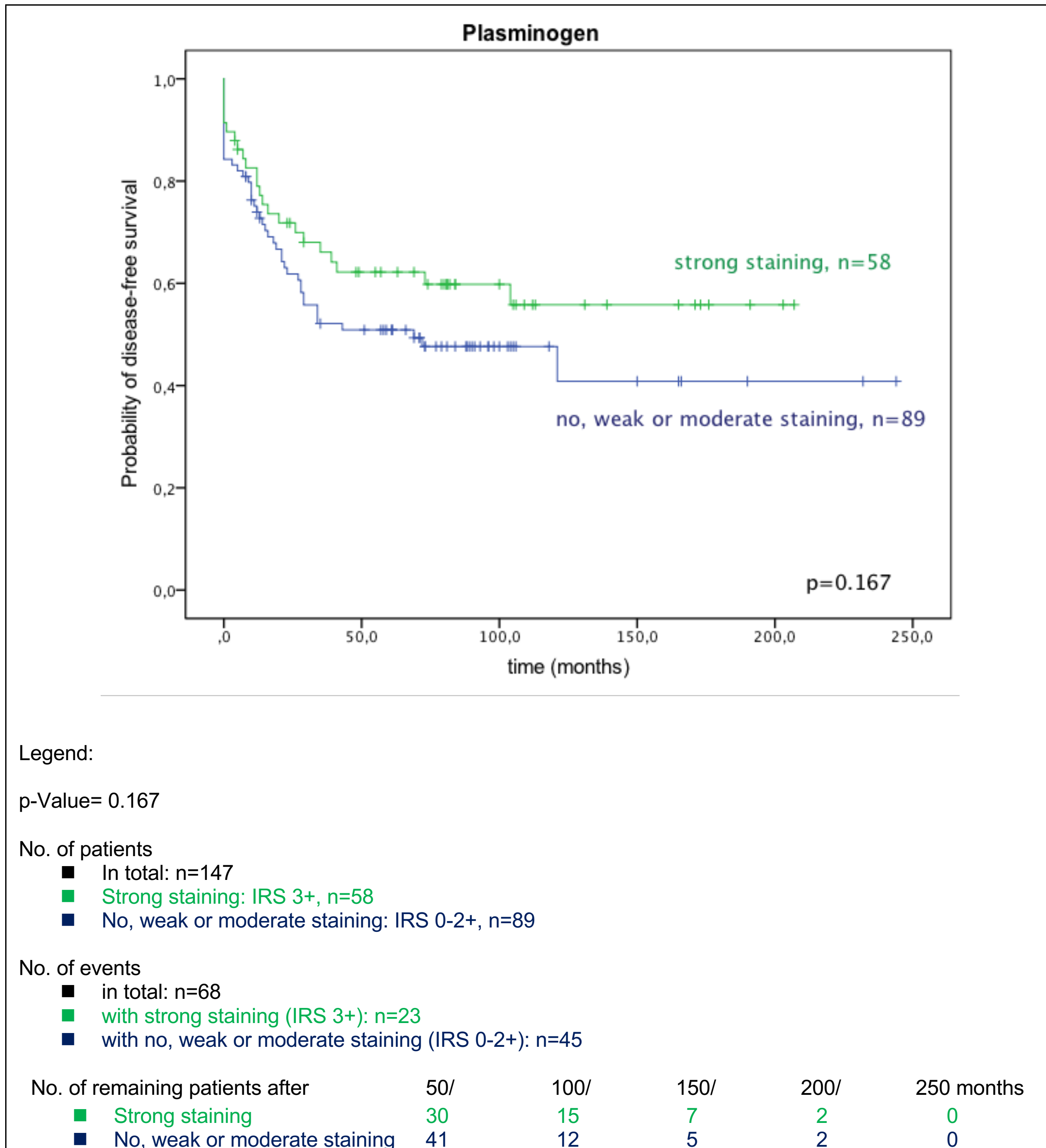
B. Probability of DFS by plasminogen expression level

The Kaplan Meier survival curve depicted in Figure 8 visualizes the clinical impact of plasminogen expression level assessed by IHC on the probability of DFS in TNBC patients over a time period of 250 months. Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (DFS), vertical tick-marks represent a drop-out out of the study. In total, 147 patients were included in the calculations. The patients were dichotomized by plasminogen expression level in low (IRS 0-2+; n=89; blue color) vs. high (3+; n=58; green color).

The number of patients showing high plasminogen levels (green color) at the beginning of the study was 58. They showed 23 events over a time period of 250 months, indicating that 23 patients experienced tumor progression. After 150 months, 7 patients with high plasminogen levels remained without tumor progression, after 250 months none of the patients remained without tumor progression. The remaining patients (n=35) dropped out of the study because of other reasons.

The number of patients showing low plasminogen levels (blue color) at the beginning of the study was 89. They showed 45 events over a time period of 250 months, indicating that 45 patients experienced tumor progression. After 150 months, 5 patients with low plasminogen levels remained without tumor progression, after 250 months none of the patients remained without tumor progression. The remaining patients (n=44) dropped out of the study because of other reasons.

In summary, this Kaplan Meier survival curve shows that patients with low plasminogen expression level tended to show lower probability of DFS and that patients with high plasminogen expression level tended to show higher probability of DFS over a time period of 250 months without being statistically significant (p=0.167).



Legend:

p-Value= 0.167

No. of patients

- In total: n=147
- Strong staining: IRS 3+, n=58
- No, weak or moderate staining: IRS 0-2+, n=89

No. of events

- in total: n=68
- with strong staining (IRS 3+): n=23
- with no, weak or moderate staining (IRS 0-2+): n=45

No. of remaining patients after	50/	100/	150/	200/	250 months
Strong staining	30	15	7	2	0
No, weak or moderate staining	41	12	5	2	0

Figure 8. Probability of DFS by plasminogen expression level

C. Probability of OS by plasminogen expression level over a period of 5 years

The Kaplan Meier survival curve depicted in Figure 9 visualizes the clinical impact of plasminogen expression level assessed by IHC on the probability of OS in TNBC patients over a time period of 60 months. This time frame was chosen based on the fact that TNBC patients usually relapse during the first three years after diagnosis (Dent et al., 2007). Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (OS), vertical tick-marks represent a drop-out out of the

study. In total, 144 patients were included in the calculations. The patients were dichotomized by plasminogen expression level in low (IRS 0-2+; n=87; blue color) vs. high (3+; n=57; green color).

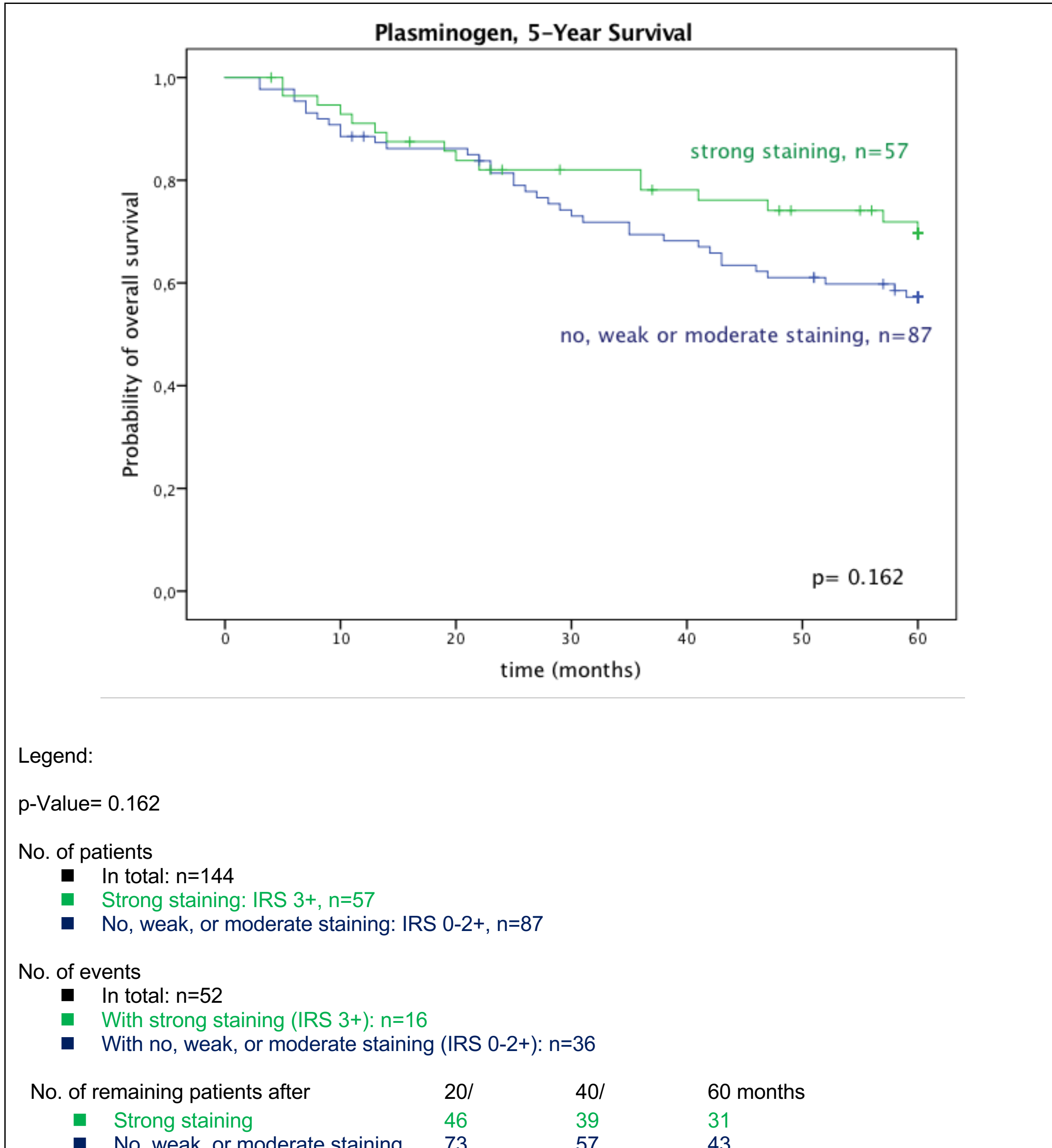


Figure 9. Probability of OS by plasminogen expression level, 5 years

The number of patients showing high plasminogen levels (green color) at the beginning of the study was 57. They showed 16 events over a time period of 60 months, indicating that 16 patients died over this time period of any cause not necessarily related to the TNBC. After 60 months, 31 patients with high plasminogen levels were still alive. The remaining patients (n=10) dropped out of the study because of other reasons.

The number of patients showing low plasminogen levels (blue color) at the beginning of the study was 87. They showed 36 events over a time period of 60 months, indicating that 36 patients died over this time period of any cause not necessarily related to the TNBC. After 60 months, 43 patients with low plasminogen levels were still alive. The remaining patients (n=8) dropped out of the study because of other reasons.

In summary, this Kaplan Meier survival curve shows that patients with low plasminogen expression level tended to show lower probability of OS and that patients with high plasminogen expression level tended to show higher probability of OS over a time period of 60 months without being statistically significant (p=0.162).

D. Probability of DFS by plasminogen expression level over a period of 5 years

The Kaplan Meier survival curve depicted in Figure 10 visualizes the clinical impact of plasminogen expression level assessed by IHC on the probability of DFS in TNBC patients over a time period of 60 months. This time frame was chosen based on the fact that TNBC patients usually relapse during the first three years after diagnosis (Dent et al., 2007). Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (DFS), vertical tick-marks represent a drop-out out of the study. In total, 147 patients were included in the calculations. The patients were dichotomized by plasminogen expression level in low (IRS 0-2+; n=89; blue color) vs. high (3+; n=58; green color).

The number of patients showing high plasminogen levels (green color) at the beginning of the study was 58. They showed 21 events over a time period of 60 months, indicating that 21 patients experienced tumor progression. After 60 months, 27 patients with high plasminogen levels remained without tumor progression. The remaining patients (n=10) dropped out of the study because of other reasons.

The number of patients showing low plasminogen levels (blue color) at the beginning of the study was 89. They showed 42 events over a time period of 60 months, indicating that 42 patients experienced tumor progression. After 60 months, 36 patients with low plasminogen levels remained without tumor progression. The remaining patients (n=11) dropped out of the study because of other reasons.

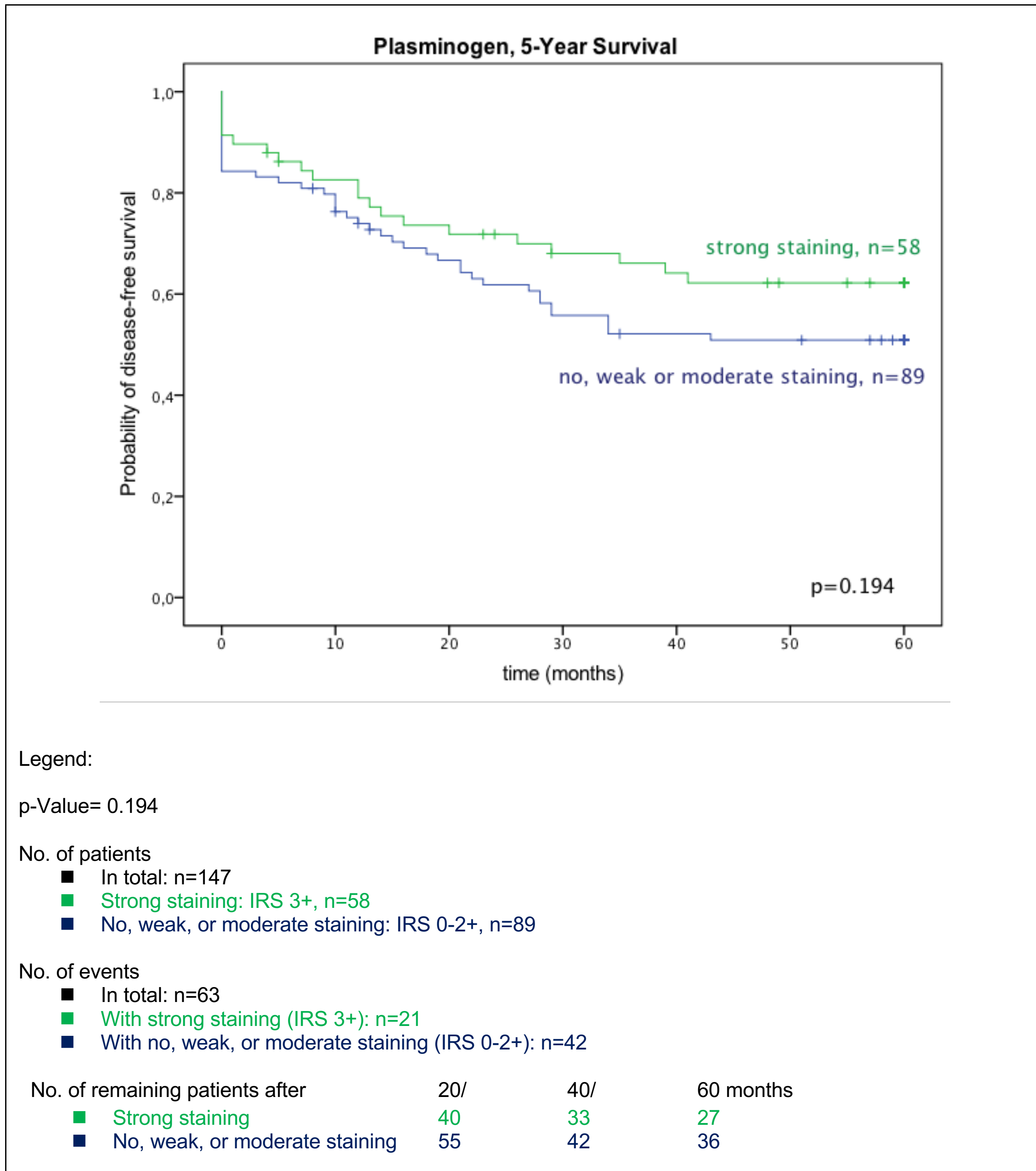


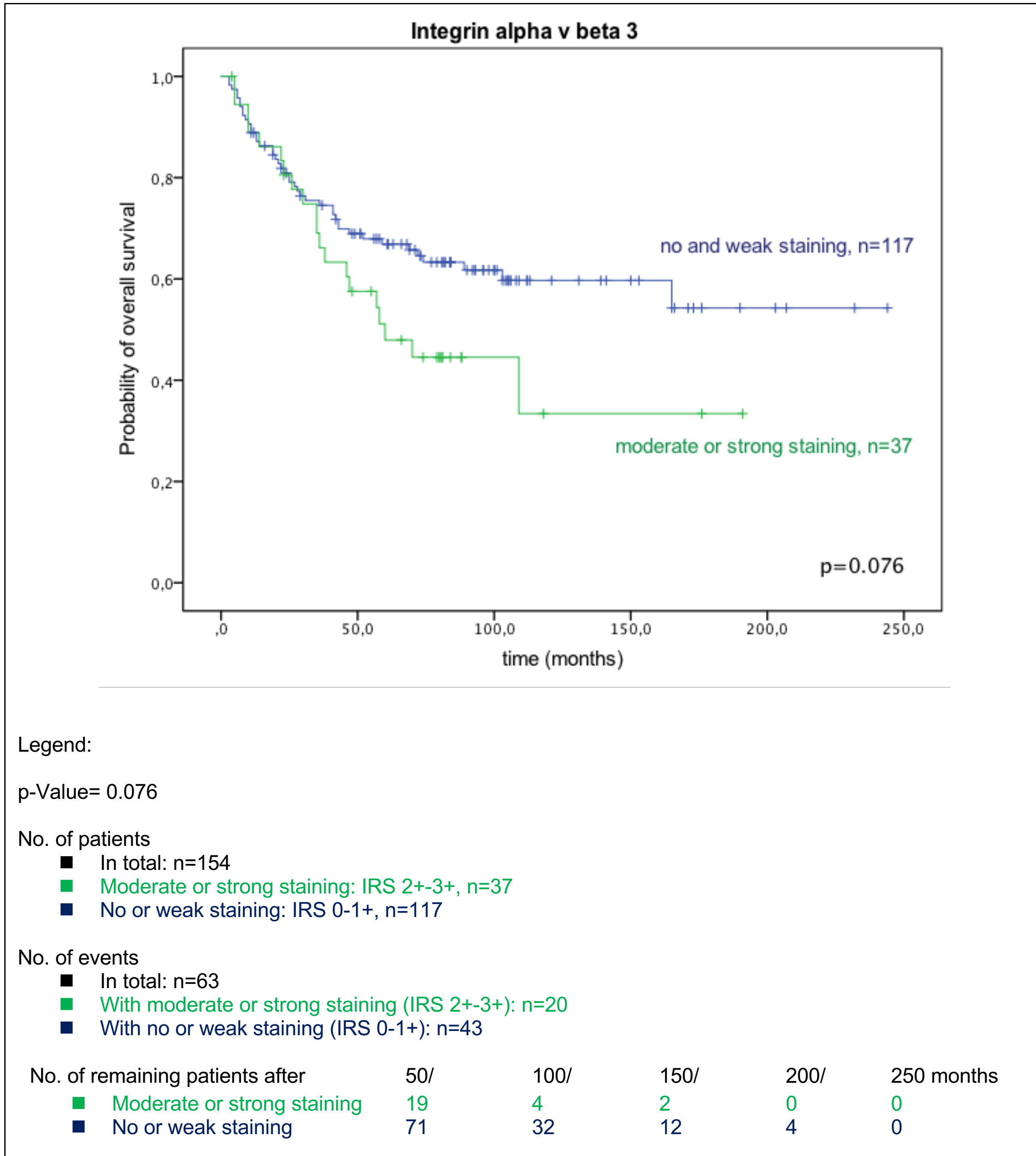
Figure 10. Probability of DFS by plasminogen expression level, 5 years

In summary, this Kaplan Meier survival curve shows that patients with low plasminogen expression level tended to show lower probability of DFS and that patients with high plasminogen expression level tended to show higher probability of DFS over a time period of 60 months without being statistically significant (p=0.194).

Further survival plots were calculated. These survival plots include different cutoff values of plasminogen expression levels, other end points like metastasis-free survival and different patient subgroups. Since these plots did not show relevant information they are illustrated in the Appendix.

4.9.2 Integrin $\alpha v\beta 3$

A. Probability of OS by integrin $\alpha v\beta 3$ expression level



Legend:

p-Value= 0.076

No. of patients

- In total: n=154
- Moderate or strong staining: IRS 2+-3+, n=37
- No or weak staining: IRS 0-1+, n=117

No. of events

- In total: n=63
- With moderate or strong staining (IRS 2+-3+): n=20
- With no or weak staining (IRS 0-1+): n=43

No. of remaining patients after	50/	100/	150/	200/	250 months
Moderate or strong staining	19	4	2	0	0
No or weak staining	71	32	12	4	0

Figure 11. Probability of OS by integrin $\alpha v\beta 3$ expression level

The Kaplan Meier survival curve depicted in Figure 11 visualizes the clinical impact of integrin $\alpha v\beta 3$ expression level assessed by IHC on the probability of OS in TNBC patients over a time period of 250 months. Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (OS), vertical tick-marks represent a drop-out out of the study. In total, 154 patients were included in the calculations. The

patients were dichotomized by integrin αvβ3 expression level in low (IRS=0-1+, n=117; blue color) vs. high (IRS=2+-3+, n=37; green color).

The number of patients showing high integrin αvβ3 expression level (green color) at the beginning of the study was 37. They showed 20 events over a time period of 250 months, indicating that 20 patients died over this time period of any cause not necessarily related to the TNBC. After 150 months, 2 patients with high integrin αvβ3 expression level were still alive, after 250 months none of the patients was still alive. The remaining patients (n=17) dropped out of the study because of other reasons.

The number of patients showing low integrin αvβ3 expression level (blue color) at the beginning of the study was 117. They showed 43 events over a time period of 250 months, indicating that 43 patients died over this time period of any cause not necessarily related to the TNBC. After 150 months, 12 patients with low integrin αvβ3 expression level were still alive, after 250 months none of the patients was still alive. The remaining patients (n=74) dropped out of the study because of other reasons.

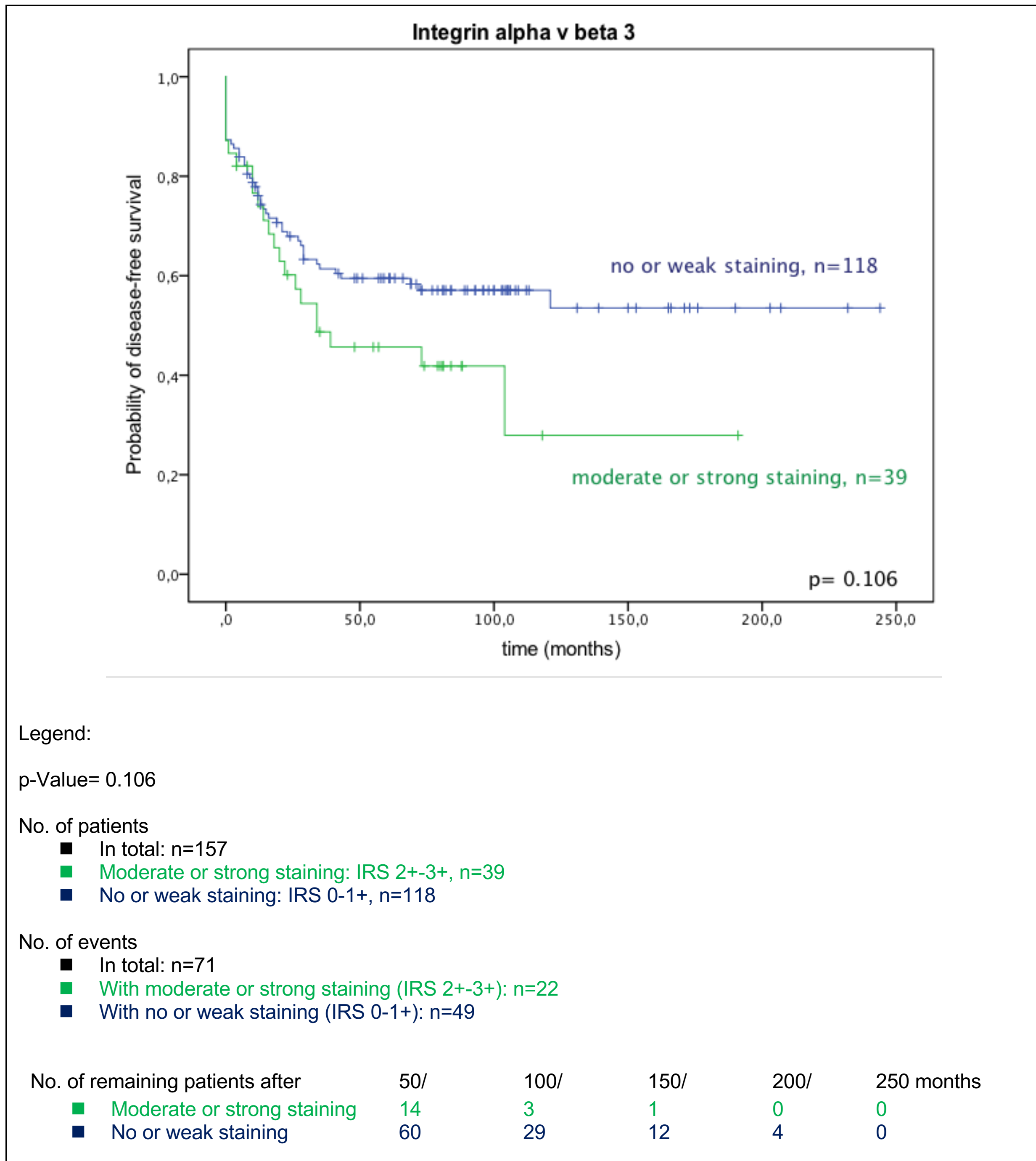
In summary, this Kaplan Meier survival curve shows that patients with low integrin αvβ3 expression level tended to show higher probability of OS and that patients with high integrin αvβ3 expression level tended to show lower probability of OS over a time period of 250 months without being statistically significant (p=0.076).

B. Probability of DFS by integrin αvβ3 expression level

The Kaplan Meier survival curve depicted in Figure 12 visualizes the clinical impact of integrin αvβ3 expression level assessed by IHC on the probability of DFS in TNBC patients over a time period of 250 months. Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (DFS), vertical tick-marks represent a drop-out out of the study. In total, 157 patients were included in the calculations. The patients were dichotomized by integrin αvβ3 expression level in low (IRS=0-1+, n=118; blue color) vs. high (IRS=2+-3+, n=39; green color).

The number of patients showing high integrin αvβ3 expression level (green color) at the beginning of the study was 39. They showed 22 events over a time period of 250 months, indicating that 22 patients experienced tumor progression. After 150 months, 1 patient with high plasminogen levels remained without tumor progression, after 250

months none of the patients remained without tumor progression. The remaining patients (n=17) dropped out of the study because of other reasons.



Legend:

p-Value= 0.106

No. of patients

- In total: n=157
- Moderate or strong staining: IRS 2+-3+, n=39
- No or weak staining: IRS 0-1+, n=118

No. of events

- In total: n=71
- With moderate or strong staining (IRS 2+-3+): n=22
- With no or weak staining (IRS 0-1+): n=49

No. of remaining patients after	50/	100/	150/	200/	250 months
Moderate or strong staining	14	3	1	0	0
No or weak staining	60	29	12	4	0

Figure 12. Probability of DFS by integrin $\alpha v\beta 3$ expression level

The number of patients showing low integrin αvβ3 expression level (blue color) at the beginning of the study was 118. They showed 49 events over a time period of 250 months, indicating that 49 patients died over this time period because of the TNBC. After 150 months, 12 patients with low plasminogen levels were still alive, after 250 months none of the patients was still alive. The remaining patients (n=57) dropped out of the study because of other reasons.

In summary, this Kaplan Meier survival curve shows that patients with low integrin $\alpha v\beta 3$ expression level tended to show higher probability of DFS and that patients with high integrin $\alpha v\beta 3$ expression level tended to show lower probability of DFS over a time period of 250 months without being statistically significant (p=0.106).

C. Probability of OS in patients treated with chemotherapy by integrin $\alpha v\beta 3$ expression level

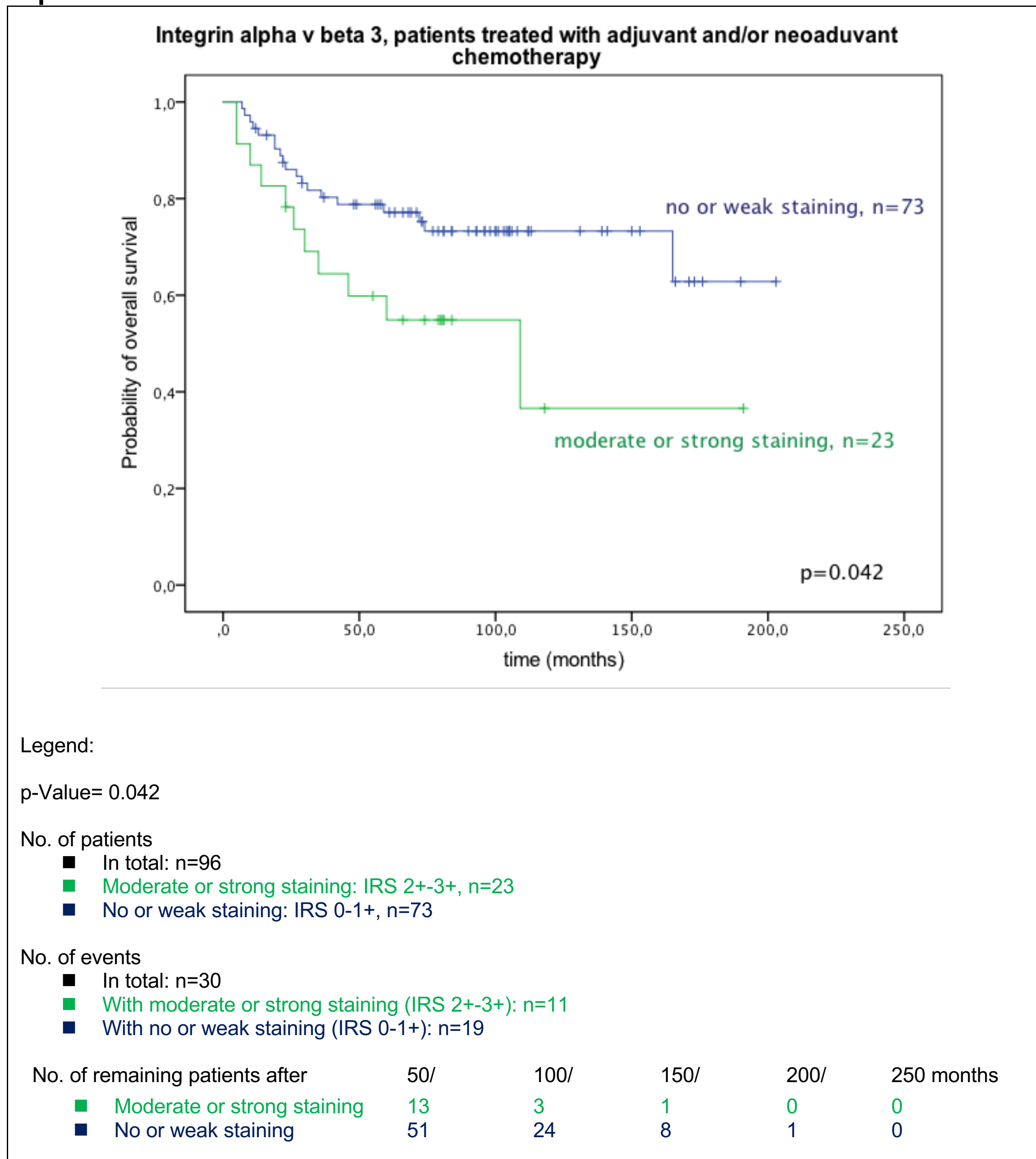


Figure 13. Probability of OS by $\alpha v\beta 3$, Chemotherapy

The Kaplan Meier survival curve depicted in Figure 13 visualizes the clinical impact of integrin $\alpha v\beta 3$ expression level assessed by IHC on the probability of OS over a time

period of 250 months in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy. Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (OS), vertical tick-marks represent a drop-out out of the study. In total, 96 patients were included in the calculations. The patients were dichotomized by integrin αvβ3 expression level in low (IRS=0-1+, n=73; blue color) vs. high (IRS=2+-3+, n=23; green color).

The number of patients showing high integrin αvβ3 expression level (green color) at the beginning of the study was 23. They showed 11 events over a time period of 250 months, indicating that 11 patients died over this time period of any cause not necessarily related to the TNBC. After 150 months, 1 patient with high integrin αvβ3 expression level was still alive, after 250 months none of the patients was still alive. The remaining patients (n=12) dropped out of the study because of other reasons.

The number of patients showing low integrin αvβ3 expression level (blue color) at the beginning of the study was 73. They showed 19 events over a time period of 250 months, indicating that 19 patients died over this time period because of the TNBC. After 150 months, 8 patients with low integrin αvβ3 expression level were still alive, after 250 months none of the patients was still alive. The remaining patients (n=54) dropped out of the study because of other reasons.

In summary, this Kaplan Meier survival curve shows in a subgroup of patients treated with adjuvant and/or neoadjuvant chemotherapy that patients with low integrin αvβ3 expression level showed significantly higher probability of OS and that patients with high integrin αvβ3 expression level showed significantly lower probability of OS over a time period of 250 months ($p=0.042$).

D. Probability of DFS in patients treated with chemotherapy by integrin αvβ3 expression level

The Kaplan Meier survival curve depicted in Figure 14 visualizes the clinical impact of integrin αvβ3 expression level assessed by IHC on the probability of DFS over a time period of 250 months in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy. Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (DFS), vertical tick-marks represent a drop-out out of the study. In total, 98 patients were included in the calculations. The patients were

dichotomized by integrin $\alpha v\beta 3$ expression level in low (IRS=0-1+, n=73; blue color) vs. high (IRS=2+-3+, n=25; green color).

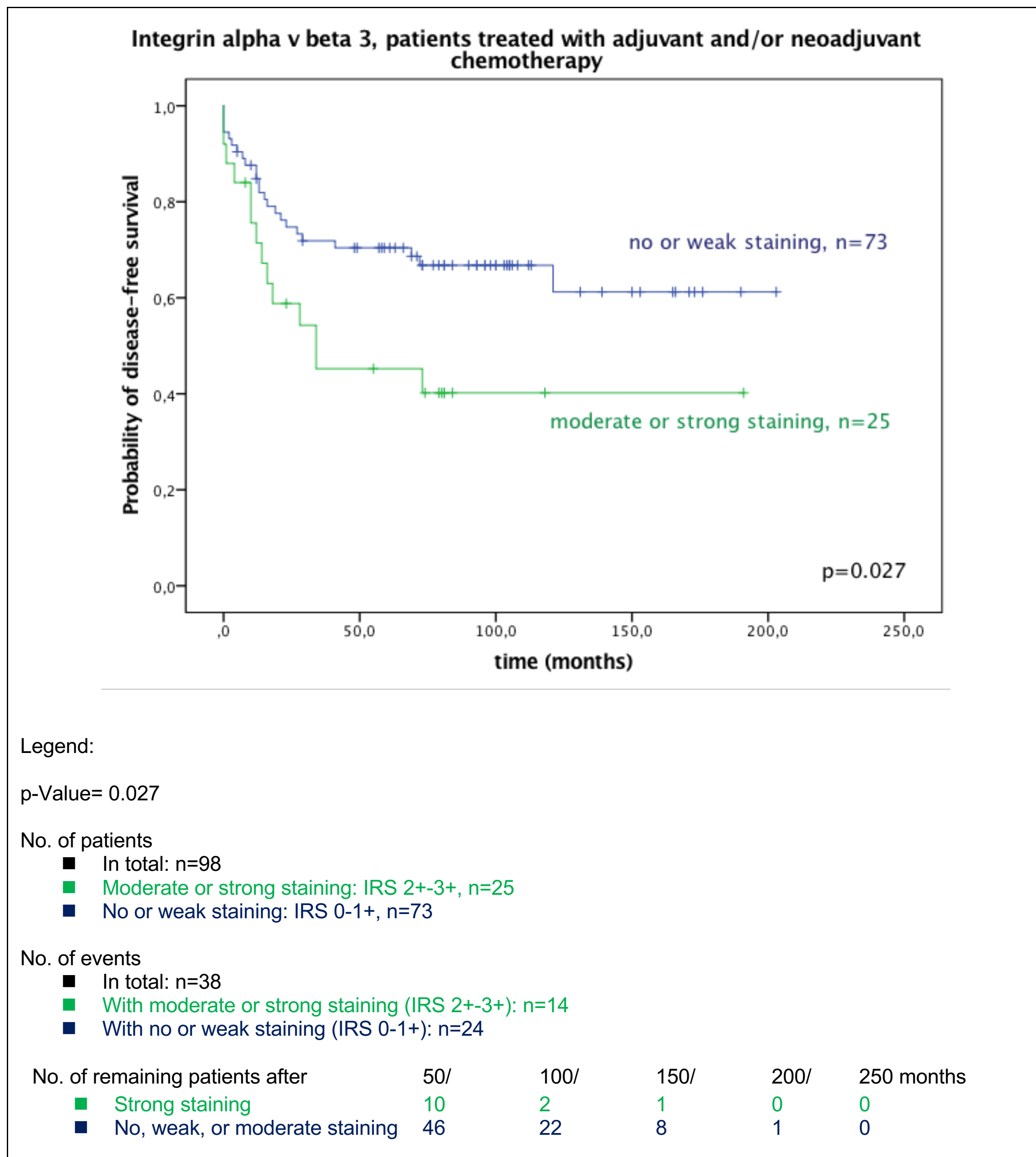


Figure 14. Probability of DFS by integrin $\alpha v\beta 3$ expression level, Chemotherapy

The number of patients showing high integrin $\alpha v\beta 3$ expression level (green color) at the beginning of the study was 25. They showed 14 events over a time period of 250 months, indicating that 14 patients experienced tumor progression. After 150 months, 1 patient with high integrin $\alpha v\beta 3$ expression level remained without tumor progression, after 250 months none of the patients remained without tumor progression. The remaining patients (n=11) dropped out of the study because of other reasons.

The number of patients showing low integrin αvβ3 expression level (blue color) at the beginning of the study was 73. They showed 24 events over a time period of 250 months, indicating that 24 patients experienced tumor progression. After 150 months, 8 patients with low integrin αvβ3 expression level remained without tumor progression, after 250 months none of the patients remained without tumor progression. The remaining patients (n=49) dropped out of the study because of other reasons.

In summary, this Kaplan Meier survival curve shows in a subgroup of patients treated with adjuvant and/or neoadjuvant chemotherapy that patients with low integrin αvβ3 expression level showed significantly higher probability of DFS and that patients with high integrin αvβ3 expression level showed significantly lower probability of DFS over a time period of 250 months (p=0.027).

E. Probability of OS in patients treated with chemotherapy by integrin αvβ3 expression level over a period of 5 years

The Kaplan Meier survival curve depicted in Figure 15 visualizes the clinical impact of integrin αvβ3 expression level assessed by IHC on the probability of OS over a time period of 60 months in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy. This time frame was chosen based on the fact that TNBC patients usually relapse during the first three years after diagnosis (Dent et al., 2007). Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (OS), vertical tick-marks represent a drop-out out of the study. In total, 96 patients were included in the calculations. The patients were dichotomized by integrin αvβ3 expression level in low (IRS=0-1+, n=73; blue color) vs. high (IRS=2+-3+, n=23; green color).

The number of patients showing high integrin αvβ3 expression level (green color) at the beginning of the study was 23. They showed 10 events over a time period of 60 months, indicating that 10 patients died over this time period of any cause not necessarily related to the TNBC. After 60 months, 11 patients with high integrin αvβ3 expression level were still alive. The remaining patients (n=2) dropped out of the study because of other reasons.

The number of patients showing low integrin αvβ3 expression levels (blue color) at the beginning of the study was 73. They showed 16 events over a time period of 60 months, indicating that 16 patients died over this time period because of the TNBC.

After 60 months, 46 patients with low integrin αvβ3 expression level were still alive. The remaining patients (n=11) dropped out of the study because of other reasons.

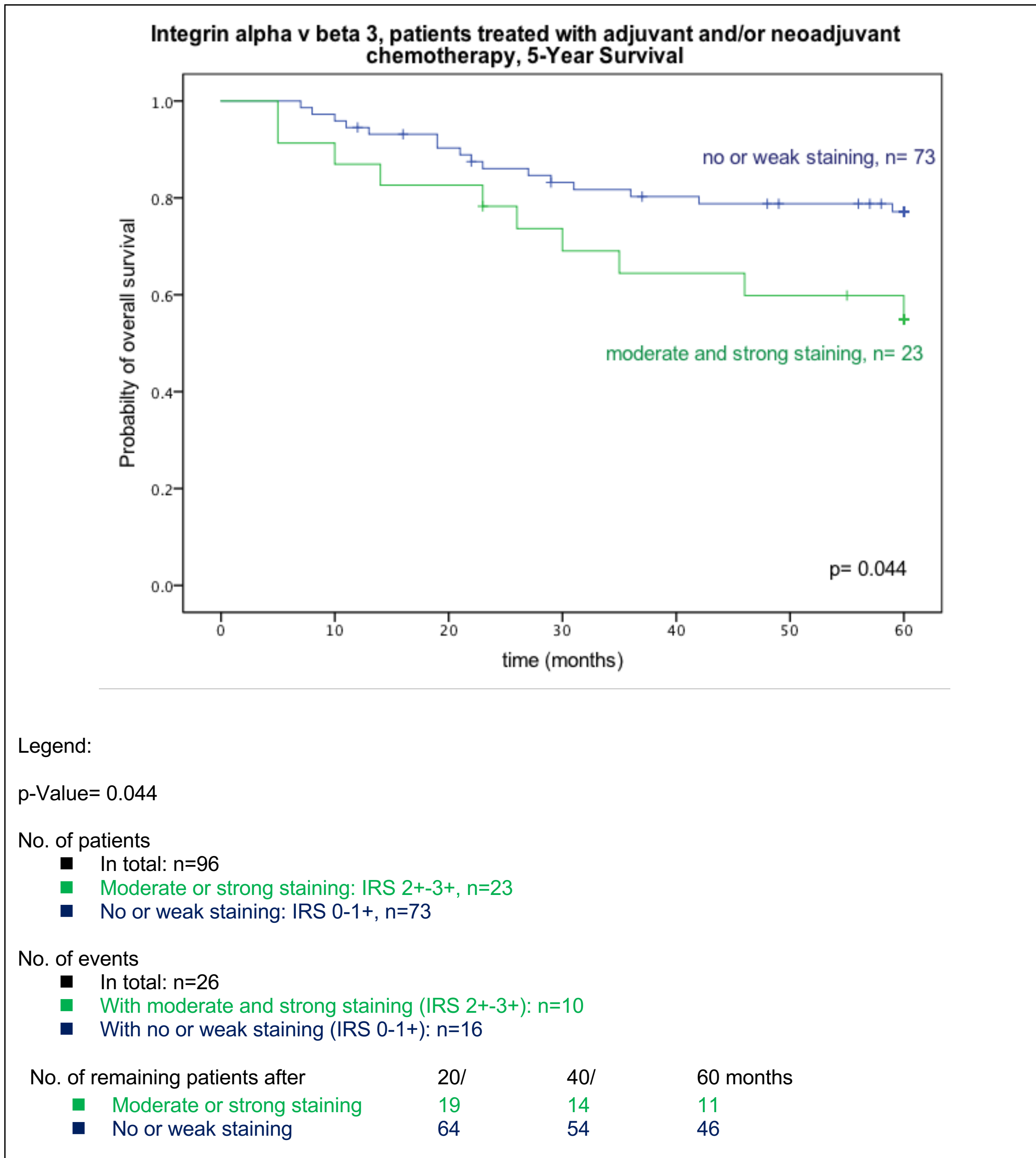
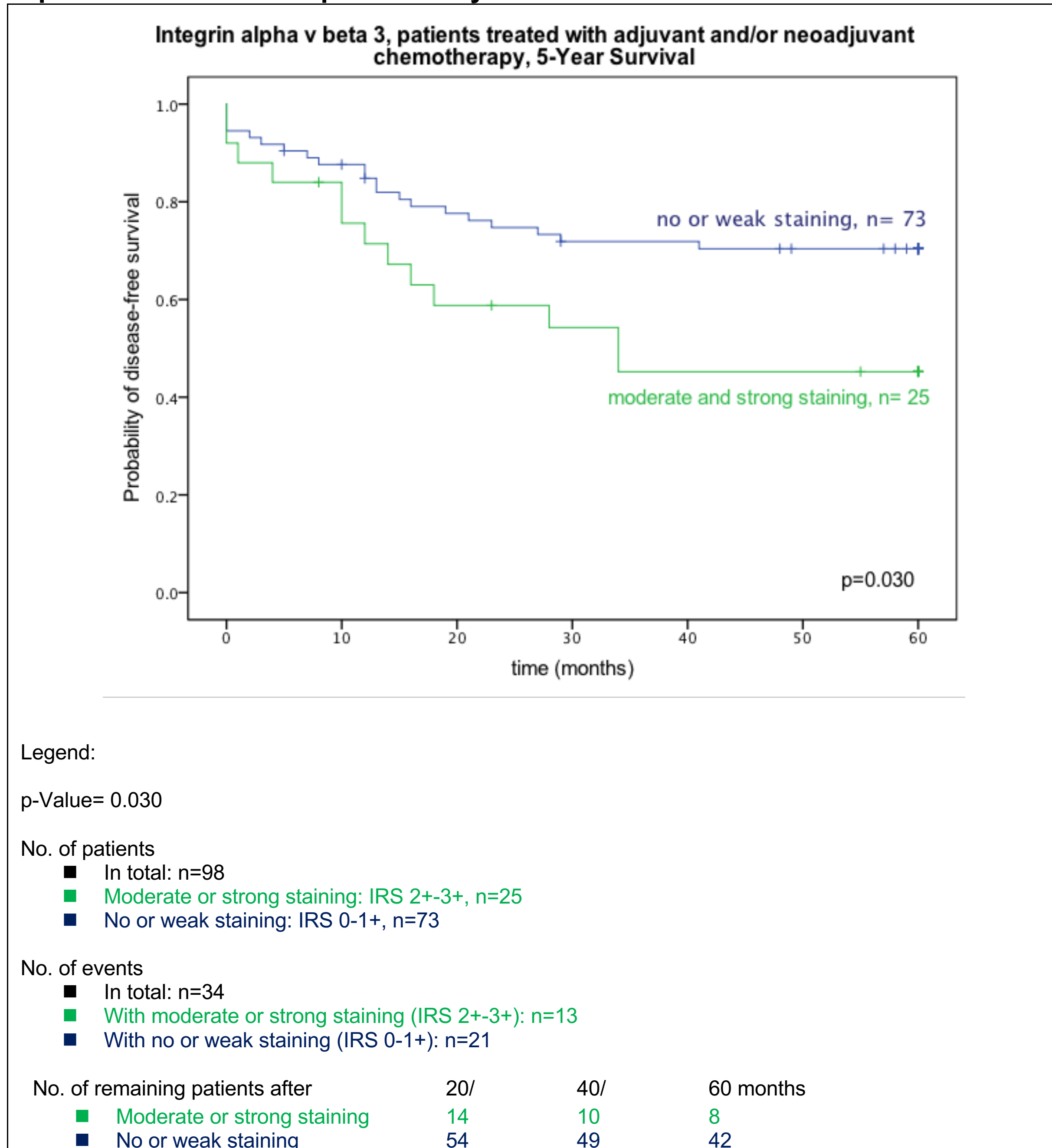


Figure 15. Probability of OS by integrin αvβ3 expression level, Chemotherapy, 5 years

In summary, this Kaplan Meier survival curve shows in a subgroup of patients treated with adjuvant and/or neoadjuvant chemotherapy that patients with low integrin αvβ3 expression level showed significantly higher probability of OS and that patients with high integrin αvβ3 expression level showed significantly lower probability of OS over a time period of 60 months (p=0.044).

F. Probability of DFS in patients treated with chemotherapy by integrin $\alpha v\beta 3$ expression level over a period of 5 years



Legend:

p-Value= 0.030

No. of patients

- In total: n=98
- Moderate or strong staining: IRS 2+-3+, n=25
- No or weak staining: IRS 0-1+, n=73

No. of events

- In total: n=34
- With moderate or strong staining (IRS 2+-3+): n=13
- With no or weak staining (IRS 0-1+): n=21

No. of remaining patients after	20/	40/	60 months
Moderate or strong staining	14	10	8
No or weak staining	54	49	42

Figure 16. Probability of DFS by integrin $\alpha v\beta 3$ expression level, Chemotherapy, 5 years

The Kaplan Meier survival curve depicted in Figure 16 visualizes the clinical impact of integrin $\alpha v\beta 3$ expression level assessed by IHC on the probability of DFS over a time period of 60 months in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy. This time frame was chosen based on the fact that TNBC patients usually relapse during the first three years after diagnosis (Dent et al., 2007). Time of survival was represented on the x-axis in months, the probability of survival was shown on the y-axis in percent. Steps on the survival curves stand for a patient´s event (DFS), vertical tick-marks represent a drop-out out of the study. In total, 98 patients were

included in the calculations. The patients were dichotomized by integrin $\alpha v\beta 3$ expression level in low (IRS=0-1+, n=73; blue color) vs. high (IRS=2+-3+, n=25; green color).

The number of patients showing high integrin $\alpha v\beta 3$ expression level (green color) at the beginning of the study was 25. They showed 13 events over a time period of 60 months, indicating that 13 patients experienced tumor progression. After 60 months, 8 patients with high integrin $\alpha v\beta 3$ expression level remained without tumor progression. The remaining patients (n=4) dropped out of the study because of other reasons.

The number of patients showing low integrin $\alpha v\beta 3$ expression level (blue color) at the beginning of the study was 73. They showed 21 events over a time period of 60 months, indicating that 21 patients experienced tumor progression. After 60 months, 42 patients with low integrin $\alpha v\beta 3$ expression level remained without tumor progression. The remaining patients (n=10) dropped out of the study because of other reasons.

In summary, this Kaplan Meier survival curve shows in a subgroup of patients treated with adjuvant and/or neoadjuvant chemotherapy that patients with low integrin $\alpha v\beta 3$ expression level showed significantly higher probability of DFS and that patients with high integrin $\alpha v\beta 3$ expression level showed significantly lower probability of DFS over a time period of 60 months (p=0.030).

Further survival plots were calculated. These survival plots include different cutoff values of integrin $\alpha v\beta 3$ expression level and other end points like metastasis-free survival. Since these plots did not show relevant information they are illustrated in the Appendix.

5. Discussion

The current study investigates new potential biomarkers related to the biology of an aggressive subtype of BC, the TNBC. Therapeutic options are limited due to lack of knowledge in tumor biology. Although intense research has been conducted in this area, no clinically validated predictive biomarkers exist for TNBC. Biomarkers related to the uPAR interactome seem to be a possible clue. The uPAR interactome plays a crucial role in many different cancer types. The uPAR interactome should be of interest concerning predictive biomarkers, because its members play a strong role in the degradation of the ECM, tumor invasion, tumor growth, cell migration, and angiogenesis and metastasis (Dublin et al., 2000; Look et al., 2002; Mazar, 2008). Since 2007, uPA and PAI-1 are biomarkers with LOE-1 for BC (Schmitt et al., 2010). Regarding the uPAR interactome, published findings apply to BC in general but are not specific for TNBC. It was stated by Witzel et al. that the prognostic and/or predictive value of uPA and PAI-1 may vary between the different BC subtypes (Witzel et al., 2014). To our knowledge, this investigation is the first one to evaluate clinically relevant biomarkers as members of the uPAR interactome in a patient cohort of TNBC. The present study was considered a pilot project, evaluating whether the proteins should be considered for further research.

5.1 Study design and clinical parameters

This study was designed as a retrospective study. Ideally, the clinical availability of new biomarkers should be assessed by a prospective randomized study (Harbeck et al., 2002a; Polley et al., 2013) but if designed properly, retrospective studies can also provide evidence in favor of a biomarker (Polley et al., 2013). However, it is often difficult to retrace confounding variables in hindsight (Harbeck et al., 2002a). The patient cohort was collected between the years 1987 and 2007. This time period allowed the assembly of a large cohort of TNBC patients. Yet, preanalytical, analytical, and postanalytical steps as well as treatment guidelines have significantly changed over the years (Simon et al., 2009).

All patients were treated in the Klinikum rechts der Isar, Technical University of Munich. This guaranteed a uniform standard of procedures. The Klinikum rechts der Isar, Technical University of Munich owns one of the largest TNBC biobanks. Well-organized and structured biobanks form the keystone of well-grounded immunohistochemical cancer research (Saini et al., 2015; Vora & Thacker, 2015). Due

to ethical reasons, no patient was left untreated. All of the patients were operated and most of them received some form of chemotherapy. Deny of a therapy in favor of research was not allowed, therefore no control groups existed. Prognostic statements could therefore not be made.

Nine TMAs containing TNBC cases were stained by IHC using the abovementioned protocols (Table 7 and Table 8). Multiple studies have compared the results and concluded that TMAs are often representative for whole tumor sections. This is ensured by putting at least two core samples of the same tumor on each TMA (Camp et al., 2000; Torhorst et al., 2001). IHC has got various advantages. It is widely available, relatively cheap, and easy to practice but it is known to lack general standardization. Even today, international guidelines for the detection of ER, PR, and HER2 are not harmonized (Metzger-Filho et al., 2012).

We evaluated data of 185 patients. The median age at diagnosis was 57. Patients age varied between 27 and 96 years. In the cohort studied here, 30.8% of the patients were pre- or perimenopausal at time of diagnosis. TNBC is known to affect younger patients compared to other subtypes (Liedtke et al., 2015). This characteristic was proven by this collective. Patients were on average younger than patients in other collectives with other subtypes and comparable in age with patients of other TNBC collectives (Bauer et al., 2007a; Davies et al., 2011; Thike et al., 2010).

The WHO recently renamed the group of invasive ductal carcinoma in invasive carcinoma not otherwise specified. Both of the names can be used as synonyms (Sinn & Kreipe, 2013). In our cohort, 81.5% of the tumors were invasive ductal carcinomas/invasive carcinomas not otherwise specified. In the literature, invasive ductal carcinomas/invasive carcinoma not otherwise specified take 85% of all BC (Eheman et al., 2009) and approximatively 85% of TNBC (Plasilova et al., 2016).

In our cohort, 5.1% of the tumors were invasive lobular carcinomas. Looking at BC in general, invasive lobular carcinoma are the second biggest group averaging 15% (McCart Reed et al., 2015). This differs in TNBC with invasive lobular carcinoma being approximately 1% of all TNBC (Plasilova et al., 2016). In our cohort more often than usual tumors were medullary BC (10.1%). This differs from other large TNBC cohorts showing approximately 1% of the tumors being medullary (Plasilova et al., 2016).

In accordance with the literature, this patient collective showed mostly undifferentiated tumors. This is known for TNBC (Albergaria et al., 2011). In this patient collective, 86%

of the tumor were grade 3 tumors. Only 1.7% of the patients were diagnosed with grade 1 tumors and 12.4% with grade 2 tumors. The tumors were not only of higher grade but were also bigger at the time of diagnosis. 69.1% of the tumors were larger than 2 cm in diameter. Only 30.9% of the patients were diagnosed with tumors smaller than 2 cm in diameter. Bauer et al. stated that tumor size is significantly larger in patients with TNBC (22mm vs. 17mm) and that 76% of the tumors were poorly differentiated in comparison with 28% in other BC subgroups (Bauer et al., 2007a). This is also in line with Dent et al. who found out that TNBC patients were more likely to have grade 3 tumors (66% vs. 25%) and that tumor size was larger in the TNBC subgroup (3.0 vs. 2.1) (Dent et al., 2007). Our findings are therefore in line with the literature (Foulkes et al., 2010).

Regarding the tendency of tumor cells to spread to lymph nodes, axillary lymph node involvement was common. 47.5% of the patients showed lymph node involvement. On the other hand, 52.5% of the patients did not show any lymph node involvement at time of diagnosis. In other studies, lower percentage of lymph node involvement was shown (Dent et al., 2009; Si et al., 2014). Patients with TNBC are more likely to die from the cancer than patients with other subtypes of BC. In this patient cohort, the rate was 45% in accordance with Dent et al. who reported a frequency of 43% (Dent et al., 2009).

The established clinical factors like nodal status, tumor size, menopausal status, nuclear grade, and adjuvant chemotherapy treatment were analyzed using univariate and multivariable Cox regression analysis. Tumor size and nodal status showed statistical significance in univariate and multivariable analysis regarding OS and DFS. Carter et al. showed in a systematic review that two of the most important independent prognostic indicators for BC are tumor size and extent of axillary lymph node involvement (Carter et al., 1989). These findings also apply to TNBC patients. A high significant association between tumor size and axillary lymph node involvement and cancer progression or mortality was also shown in large TNBC patient cohorts (Urru et al., 2018). Adjuvant treatment with chemotherapy showed statistical significance in univariate and in multivariable analysis regarding OS and in univariate analysis regarding DFS in this study. In a long-term follow-up of 1000 TNBC patients, Sparano et al. showed significantly improved OS and DFS in patients who had received adjuvant chemotherapy (Sparano et al., 2015b). In this study, menopausal status also showed statistical significance in univariate analysis regarding OS but failed it in multivariable analysis regarding OS and in univariate analysis regarding DFS.

On the other hand, nuclear grade did not show statistical significance in univariate analysis regarding OS or DFS. This means that no significant associations with mortality were found for histologic grade. This states the opposite of histologic grade being one of the best-established prognostic factors for BC in general (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019). Urru et al. found similar results in their cohort of 841 TNBC patients. Histologic grade had no role in survival outcome, probably due to the usually high histological grade in TNBC patients. In their cohort, 1.5% of the patients were G1, similar to this study, where 1.7% of the patients were G1 (Urru et al., 2018).

Next to the established histomorphological factors, two distant members of the uPAR interactome were investigated more specifically. Plasminogen and integrin $\alpha v \beta 3$ expressions were assessed manually by IHC on TMAs prepared from FFPE primary TNBC tissues. The scoring results were associated to clinical and histomorphological patient data. Univariate and multivariable Cox regression analyses and Kaplan Meier survival plots were calculated.

5.2 Plasminogen

Scoring results of the plasminogen-directed ab stainings on TMA of TNBC patients were associated to clinical and histomorphological factors using the Pearson's Chi-square (χ^2)-Test. The staining results were associated with clinical factors such as age (<50 vs. ≥ 50 years), menopausal status (pre- and perimenopausal vs. postmenopausal), disease recurrence (yes vs. no), OS (deceased vs. alive), and adjuvant chemotherapy treatment (treated vs. not treated). They were also associated with histomorphological factors such as tumor size (pT1 and pT2 vs. pT3 and pT4), nodal status (negative vs. positive), and nuclear grade (G1 and G2 vs. G3). No statistically significant associations were found for TNBC between these factors and plasminogen expression levels, indicating a comparatively uniform distribution within the subgroups.

The univariate analysis of plasminogen expression showed that OS (p=0.078) and DFS (p=0.178) failed statistical significance. In Kaplan Meier plots, strong plasminogen staining showed a tendency of higher probability of OS and DFS but without being statistically significant. Considering the entire follow-up process, high plasminogen expression was correlated with higher probability of OS (p=0.075) and DFS (p=0.167).

Considering a period of 60 months after diagnosis, high plasminogen expression was also correlated with higher probability of OS (p=0.162) and DFS (p=0.194).

The KM plotter analysis of published data regarding plasminogen mRNA expression showed similar results to our data (Gyorffy et al., 2010). The KM plotter program is an online Kaplan-Meier plotter including a large dataset with over 6000 BC samples. The purpose of this program is a meta-analysis-based discovery and validation of biomarkers. This program includes multiple databases and shows with statistical significance (p=0.018) in basal-like BC that high expression of plasminogen is associated with a better DFS. Unfortunately, the KM plotter results for plasminogen considering OS are not statistically significant (p=0.075) and the patient cohort studied was relatively small (n=76) (Gyorffy et al., 2010). In order to make reliable conclusions, bigger patient cohorts would need to be investigated.

In our study, plasmin(ogen) protein being highly expressed in TNBC patients´ tumors, showed longer OS and DFS than when plasmin(ogen) was expressed at a lower level. However, statistical significance just failed.

To my knowledge, until today, no study has addressed the impact of tumor-associated plasminogen expression in TNBC patients. According to Curino et al., the analysis of the plasminogen-activation system in relation to tumor progression is difficult due to the numerous components and the broad substrate specificity of plasmin, the active form of plasminogen, which is able to degrade and activate a multitude of proteins (Curino et al., 2002). With plasminogen and plasmin being omnipresent, the exact measurements of these proteins can be difficult and its potential to serve as a biomarker is limited. Supporting this remark, in the present study TNBC patients without plasmin(ogen) expression in their tumors did not exist.

Some research was done considering plasmin(ogen) and BC models. In a metastatic BC model, Ranson et al. showed that cells that overexpress uPA and uPAR also bind significantly more plasminogen on their cell surface and activate more plasminogen to plasmin than non-metastatic BC cell lines. Ranson et al. suggested that these features contribute to metastatic capability (Ranson et al., 1998). These findings were supported by Stonelake et al.. They showed that with plasminogen being present, metastatic BC cell lines degraded the human endothelial basement membrane but that inhibitors of uPA and plasmin blocked this process (Stonelake et al., 1997). This proves the important role of plasminogen in the uPAR interactome.

Castellino and Ploplis stated that the ability of plasminogen to degrade not only fibrin clots but also other matrix proteins implicated the important role of plasmin, for example in tumor dissemination. According to Castellino et al., blocking plasmin could be a therapeutic approach in different diseases, including cancer (Castellino & Ploplis, 2005). Stonelake et al. suggested that inhibition of uPA and plasmin(ogen) activation could potentially reduce the metastatic potential of BC cells (Stonelake et al., 1997).

Plasmin(ogen) expression has been studied in other cancer types as well. Curino et al. found that the presence of plasmin(ogen) leads to tumor growth, reduction of tumor-infiltrating macrophages, and angiogenesis (Curino et al., 2002). Bugge et al. inoculated metastatic Lewis lung carcinoma expressing high uPA levels in plasminogen-deficient mice and in control mice. All mice developed tumors but Lewis lung sarcoma in plasminogen-deficient mice were smaller in size, hemorrhaged rarely, took longer to spread to the lymph nodes, and showed a better survival. On the other hand, it was also shown that tumor growth and dissemination also occurred in plasminogen-deficient mice but at a lower level. This suggests, that plasmin(ogen) plays an important role in proteolysis leading to tumor cell dissemination and metastasis but plasminogen-deficient mice still generate sufficient proteolytic activity for tumor development (Bugge et al., 1997).

In an animal model with plasminogen-deficient mice, Bugge et al. stated that fibrinolysis might be the fundamental physiological role of plasmin(ogen). Mice with a plasminogen-deficiency suffer for example from severe thrombosis (Bugge et al., 1996). In 1998, Bugge et al. showed that Polyoma virus-induced BC in plasminogen-deficient mice showed significantly less lung metastases. This indicates that plasmin(ogen) is an important factor for tumor progression and metastasis in BC (Bugge et al., 1998). Perides et al. investigated the role of plasmin in the development of brain metastasis in an experimental metastatic melanoma model. They studied plasminogen-deficient mice which were inoculated with metastatic mouse melanoma cells. They demonstrated that plasmin encourages metastasis to the brain and that inhibition of the fibrinolytic system could play a role in cancer therapy (Perides et al., 2006).

In conclusion, data about plasmin(ogen) in BC and especially in TNBC is extremely limited. Known results are confounding, with plasminogen on the one hand leading to metastatic potential, and on the other hand being omnipresent in different cohorts

without being able to make significant statistical statements. To further characterize the impact of plasmin(ogen) in TNBC patients, a larger cohort should be investigated. It may also be helpful to include other BC subgroups to detect whether plasminogen is omnipresent in other BC subgroups as well.

5.3 Integrin αvβ3

Integrins play an omnipresent role in cancer. They are required for tumor progression, tumor cell detachment from the primary tumor site, tumor cell migration, and tumor cell adhesion (Schneider et al., 2011). Integrins cause interactions between tumor cells and the ECM or stromal cells and the metastatic site (Schneider et al., 2011). Besides its prominent role in cancer, integrin αvβ3 is involved in neoangiogenesis, platelet aggregation, and osteoclast function (Schneider et al., 2011; Takayama et al., 2005). It is a marker of neovascularization in breast tumors and is mostly expressed in tumor blood vessels but is also expressed on the cell membrane of tumor cells (Hariharan et al., 2007).

Scoring results of the integrin αvβ3-directed ab stainings on TMA of TNBC patients were associated to clinical and histomorphological factors using the Pearson's Chi-square (χ^2)-Test. The staining results were associated with clinical factors such as age (<50 vs. ≥ 50 years), menopausal status (pre- and perimenopausal vs. postmenopausal), disease recurrence (yes vs. no), OS (deceased vs. alive), and adjuvant chemotherapy treatment (treated vs. not treated). They were also associated with histomorphological factors such as tumor size (pT1 and pT2 vs. pT3 and pT4), nodal status (negative vs. positive), and nuclear grade (G1 and G2 vs. G3). Age (p=0.013), menopausal status (p=0.031), nodal status (p=0.028), and nuclear grade (p=0.024) were significantly associated with integrin αvβ3 expression. For tumor size, disease recurrence, OS, and adjuvant chemotherapy treatment no statistically significant associations were found for TNBC between these factors and integrin αvβ3 expression levels, indicating a comparatively uniform distribution within the subgroups. Our univariate analysis of integrin αvβ3 expression showed that OS (p=0.079) and DFS (p=0.116) failed statistical significance for the whole patient cohort. When looking at a distinct group of patients who had been treated with chemotherapeutics, integrin αvβ3 expression was significantly associated with OS (p=0.048) and DFS (p=0.031) in the univariate analysis and failed statistical significance in multivariate analysis for OS (p= 0.386) and DFS (p=0.201).

In this study, with integrin αvβ3 being highly expressed in tumors of TNBC patients´, OS and DFS were shorter than when integrin αvβ3 was expressed at a lower level. In the whole patient cohort, integrin αvβ3 expression just failed statistical significance in OS (p=0.076) and DFS (p=0.106). Considering only patients that had received adjuvant and/or neoadjuvant chemotherapy, OS (p=0.042) and DFS (p=0.027) were significantly shorter with integrin αvβ3 being highly expressed over a time period of 250 months and also significantly shorter with integrin αvβ3 being highly expressed in OS (p=0.044) and DFS (p=0.030) over a period of 60 months.

These results are in line with the results of the KM plotter program. The KM plotter program showed statistically significant results for integrin αvβ3 expression in basal-like BC (Gyorffy et al., 2010). This program shows with a statistical significance of p=0.0056 in basal-like BC that high mRNA expression of integrin αv is associated with poor DFS.

Gasparini et al. found that breast tumors of different subtypes that had metastasized showed higher levels of integrin αvβ3 (Gasparini et al., 1998). Felding-Habermann et al. showed that integrin αvβ3 was necessary for BC metastasis (Felding-Habermann et al., 2001). Those patients expressing high levels of integrin αvβ3 had a poor prognosis compared to patients with low levels of integrin αvβ3. They showed in multivariable analysis, that integrin αvβ3 was an independent marker both in node-negative and node-positive patients treated with adjuvant therapy (Gasparini et al., 1998). In our patient cohort, this statement could not be confirmed. According to our data, integrin αvβ3 expression was not statistically significant when looking at multivariate analysis, meaning that it could not be confirmed to be an independent marker for OS or DFS. On the other hand, when looking at a patient subgroup who had been treated with adjuvant and/or neoadjuvant chemotherapeutics, integrin expression was significantly associated with OS (p=0.048) and DFS (p=0.031) in the univariate analysis. These differences could be explained by different BC subtypes. In this study, only TNBC tumors were investigated, compared to Gasparini et al. who investigated BC in general.

In this study, with integrin αvβ3 being highly expressed in tumors of TNBC patients´, OS and DFS were shorter than when integrin αvβ3 was expressed at a lower level. In the whole patient cohort, integrin αvβ3 expression just failed statistical significance in

OS (p=0.076) and DFS (p=0.106). Considering only patients that had received adjuvant and/or neoadjuvant chemotherapy, OS (p=0.042) and DFS (p=0.027) were significantly shorter with integrin αvβ3 being highly expressed over a time period of 250 months and also significantly shorter with integrin αvβ3 being highly expressed in OS (p=0.044) and DFS (p=0.030) over a period of 60 months.

Integrin αvβ3 is involved in metastasis and is highly expressed in BC cells disseminated to the bone (Schneider et al., 2011; Sloan et al., 2006; Takayama et al., 2005). According to Kwakwa and Sterling, integrin αvβ3 is a major stimulus of bone metastasis. Integrin αvβ3 mediates cell adhesion to the ECM resulting in bone metastasis (Kwakwa & Sterling, 2017). In this study, only the primary tumor tissues were investigated. Therefore, we could not make statements about the site of metastasis in this patient cohort. According to Liapis et al. the expression of integrin αvβ3 is higher in bone metastasis (Liapis et al., 1996).

Interestingly, in this patient cohort statistical significance for integrin αvβ3 expression was only reached when looking at a subgroup of the patients that had received adjuvant and/or neoadjuvant chemotherapy. OS (p=0.042) and DFS (p=0.027) were significantly shorter with integrin αvβ3 being highly expressed over a time period of 250 months and also significantly shorter with integrin αvβ3 being highly expressed in OS (p=0.044) and DFS (p=0.030) over a period of 60 months.
This suggests firstly, that TNBC tumors with high expression of integrin αvβ3 may not respond to adjuvant and/or neoadjuvant chemotherapeutics and therefore the OS and DFS are poorer compared to tumors with low integrin αvβ3 expression. Secondly, this suggests that integrin αvβ3 may be a valuable therapeutic target in combination with conventional adjuvant and/or neoadjuvant therapy. According to Piva et al. drug resistance is one of the most important obstacles in cancer therapeutics. The interaction between the microenvironment and the tumor cells forms the opportunity for the cancer cells to adapt to the chemotherapeutical stress. It was shown that integrins play an important role in this process by helping the cancer cells to survive chemotherapeutic treatments (Piva et al., 2017). Drug resistance plays an important role in TNBC. Recent findings show that the contact between tumor cells and the ECM plays a crucial role in this process. Meads et al. suggest targeting these interfering points during initial treatment to prevent the development of drug resistance (Meads et al., 2009). Aoudjit et al. reviewed several studies, all supporting the idea of

ECM/integrin signaling in development of chemoresistance. Targeting this pathway could significantly improve anticancer therapy and patient survival. Lack of attachment of endothelial and epithelial cells to the EMC induces a form of apoptosis, called anoikis (Aoudjit & Vuori, 2012). Luo et al. suggested that inactivation of integrin αvβ3 could serve as a novel approach to reverse cisplatin resistance in BC cells and that knockdown of integrin αv and β3 leads to substantially sensitized tumor cells to cisplatin (Luo et al., 2018). Nair et al. showed that integrin β3 signaling inhibits apoptosis induced by the chemotherapeutic epirubicin in cell cultures with TNBC cells. Integrin β3 signaling is an important pathway used by BC cells to evade chemotherapy induced stress (Nair et al., 2016). According to Long et al. expression of integrin αvβ3 was associated to resistance to vinblastine in renal cell carcinoma. They suggested, that targeted restrain of integrin αvβ3 in combination with vinblastine may be beneficial in primary renal cell carcinoma (Long et al., 2013).

To our knowledge, until now no study has addressed the impact of integrin αvβ3 in TNBC patients. Integrin αvβ3 is found in multiple different cancers like human malignant melanoma (Felding-Habermann et al., 1992), in human gliomas, in non-small-cell lung carcinoma, in some ovarian cancer cell lines (Goodman et al., 2012), and in human BC in general (Gasparini et al., 1998). In many cancer types, high expression of integrin αvβ3 is associated with the potential to metastasize. Lorger et al. showed that tumor cells expressing integrin αvβ3 promote brain metastasis, leading to angiogenesis, independently of hypoxia (Lorger et al., 2009).

A lot of effort has been put into using integrins as therapeutic or imaging targets. Integrin αvβ3 is not only being used as a potential target to treat cancer, but is also used as an imaging target, for example for non-small-cell lung cancer and its metastases (Chen et al., 2005). The goal in developing Positron emission tomography (PET)-based molecular imaging is to detect cancer or cardiovascular disease early, to monitor disease progression, to evaluate therapeutic response by visualization of the biological processes noninvasively and in vivo. Detecting integrin αvβ3 has the advantage that it is specifically expressed in tumor angiogenesis, metastasis, ischemic heart disease, atherosclerosis and osteoclast-mediated bone resorption. Several tracers have been developed and are currently investigated in clinical studies (Cai & Conti, 2013; Chen et al., 2016).

5.4 Therapeutic outlook

Biomarkers are needed because they predict the course of a disease and/or response to cancer therapy and therefore may prevent patients from unnecessary therapies and foresee which patients do most likely benefit from the treatment and who more likely might react with side effects without any benefit. Clinical intention is to increase the effectiveness of treatment and thereby decrease side effects and costs (Duffy & Crown, 2008; Duffy et al., 1988; Look et al., 2002; Powles, 1997; Schmitt et al., 2010). The first goal in defining cancer biomarkers is to establish validated tests with high reproducibility and robustness. In practice, this means that variation of preanalytical and analytical tests should be minimized (Simon et al., 2009). In this study, the focus was laid on looking for predictive biomarkers which are members of the uPAR interactome to allow monitoring the clinical course of TNBC. They are necessary to create personalized medicine, to select those patients who would benefit from a certain therapy and to avoid unnecessary side-effects and costs in patients who would not profit from the treatment (de Gramont et al., 2015). Personalized precision medicine must fulfill two essential criteria: it must be able to assess a cancer patient´s risk (follow-up) and predict patients´ response to a certain therapy (Harbeck et al., 2002a). Being able to tailor a specific therapy for an individual patient, a clinician should have precise knowledge about the biology of the cancer (Polley et al., 2013). This applies especially to TNBC since not enough information is available for this disease in order to personalize cancer therapy yet.

Despite multiple newly arising therapeutic options, chemotherapy remains state of the art. Other options are only available in clinical studies or in combination with chemotherapy. Multiple potential targeted therapies are thoroughly being researched. Until now, no targeted therapy for TNBC does exist. Research focusses on different potential targets (Marme & Schneeweiss, 2015). New therapeutic options are among others antiangiogenic agents, poly(ADP-ribose) polymerase (PARP)-inhibitors, checkpoint inhibitors and antiandrogen medication.

Antiangiogenic agents

Bevacizumab is a humanized anti-vascular endothelial growth factor-A monoclonal ab. In Germany, bevacizumab is used in HER2-negative metastasized BC patients in combination with chemotherapy. The combination of bevacizumab with chemotherapy

represents an alternative to polychemotherapy. The ECOG 2100 trial showed better progression-free survival when combining paclitaxel with bevacizumab. However, the median OS was similar between both groups (Miller et al., 2007). The RIBBON-1 trial showed that capecitabine plus bevacizumab significantly improved PFS compared to capecitabine alone. These trials could neither show improvement in OS (Robert et al., 2011).

PARP inhibition

PARP inhibitors found their way into the therapy of TNBC with BRCA mutations. PARP is involved in the base excision repair and is responsible for repairing single-strand breaks. Inhibiting PARP leads to numerous double-strand breaks and to the lethality of tumor cells (Marme & Schneeweiss, 2015). Olaparib was tested in a non-comparative multicenter study to assess efficacy and safety in advanced BRCA associated BC. The results show positive proof of concept for PARP inhibition in BRCA-deficient BC patients (Tutt et al., 2010). The OlympiAD trial is a randomized, phase 3 trial comparing olaparib monotherapy with standard therapy with single-agent chemotherapy of the physician´s choice in metastatic BC patients with germline BRCA mutations. It was shown that olaparib monotherapy provided a significant benefit over standard therapy (Robson et al., 2017).

Immunotherapy

New therapeutic options exist when looking at checkpoint inhibitors. The programmed cell death protein 1 (PD-1) pathway regulates immune response and inhibits activation of immune cells. PD-1 is an inhibitory immune checkpoint receptor and is expressed on different immune cells. PD-L1, a PD-1 ligand is located on tumor cells. The binding leads to hiding of the tumor cells from the immune system. Inhibition of the PD-1 is used in tumor therapy of different solid tumors and leads to activation of the immune system (Nanda et al., 2016). Agents targeting PD-1 or PD-L1 have shown promising results in antitumor activity in treatment of solid tumors. Most of the immunotherapy studies in BC were made in TNBC. Pembrolizumab was investigated in patients with PD-L1-positive chemotherapy resistant metastatic TNBC in the Keynote-012 phase Ib trial. Pembrolizumab showed an overall response rate of 18.5% (Nanda et al., 2016). Avelumab was studied in women with metastatic TNBC unselected for PD-L1 expression and showed a modest overall response rate of 8.6% (Dirix et al., 2018).

The IMpassion study showed that adding atezolizumab to chemotherapy in patients with PD-L1-positive metastatic or inoperable locally advanced TNBC increased the OS significantly for 9.5 months (Schmid et al., 2018). Further studies are needed to identify the optimal combination of immune- and chemotherapy and the optimal setting of adjuvant or neoadjuvant application (Marra et al., 2019).

Antiandrogen medication

Approximately 30% of TNBC express the androgen receptor. It seems to be a potential therapeutic target (Wang et al., 2016). Gucalp et al. investigated in a phase II study if bicalutamide had a positive effect in patients with TNBC and androgen receptor expression. He showed that Bicalutamid lead to median progression free survival of 12 weeks and was well tolerated (Gucalp et al., 2013). Bonnefoi et al. showed in a phase II study with extensively pretreated women with TNBC and androgen receptor positivity that abiraterone acetate lead to a progression free survival of 2.8% (Bonnefoi et al., 2016).

Further approaches

When establishing cancer biomarkers for clinical decision making, an important challenge arises: the biomarker should be able to classify the patients into subgroups for which different treatment options apply (Polley et al., 2013). The hope is to find new targets for individual therapy within these groups, for example to create personalized cancer treatment for TNBC patients. Predictive biomarkers are essential, because only patients expressing the biomarker will benefit from the targeted therapy (Polley et al., 2013; Witzel & Muller, 2015). Members of the uPAR interactome seem like a suitable target in cancer therapy. uPAR expression is mostly expressed in tumor cells but not in adjacent tissue (Li & Cozzi, 2007). Considering targeting the uPAR interactome, it is important to note, that several animal models have shown that some members of the interactome are not vitally important for survival of healthy individuals but could be used as target when overexpressed in cancer (Schmitt et al., 2011; Schmitt et al., 2010).

Targeting the uPAR interactome

Different approaches to target distinct members of the uPAR interactome do exist. The two most investigated practices are inhibitors targeting the catalytic activity of uPA or

peptides and ab preventing uPA binding to uPAR (Duffy et al., 2014). One example of a synthetic uPA-inhibitor is Mesupron. Mesupron and its precursor WX-UK I are small-sized synthetic serine-protease inhibitors blocking both uPA and plasmin activity. They show promising preclinical effects on primary tumor cells and metastatic tumor cells in rats with BC (Setyono-Han et al., 2005). A randomized, open label Phase I study showed in inoperable pancreatic cancer that Mesupron had positive effects on tumor responses and on 1-year survival rates (Heinemann et al., 2010). In metastatic BC promising results were shown considering DFS and overall response rates in Phase I and Phase II studies. No serious side effects occurred (Goldstein, 2008; Goldstein et al., 2013; Schmitt et al., 2011).

Ab against both uPA and uPAR showed promising results in preclinical trials. Ossowski showed that in nude mice with human squamous cell carcinoma treated with anti-uPA ab local invasion of the tumor was reduced compared to control mice (Ossowski et al., 1991). Xu et al. showed that a monoclonal ab against uPAR inhibited metastasis, proliferation, and survival of tumor cells in different animal xenograft models of different solid tumors (Xu et al., 2014). Montuori et al. also showed promising results in targeting uPAR. They found a polyclonal ab that was able to block important uPAR interactions and led to inhibition of cell adhesion and migration (Montuori et al., 2016).
A different approach was that overexpression of PAI-2 in human metastatic melanoma cell lines showed interesting results. Cell lines where PAI-2 expression was clearly higher showed significantly less or no metastasis (Mueller et al., 1995). In an in vivo melanoma mouse experiment, Ma et al. showed significantly less metastasis in mice injected with adenovirus-mediated PAI-1 (Ma et al., 1997). At last, Tarighi et al. showed in MDA-MB-231 BC cell line that an antagonist peptide of uPAR inhibited proliferation and induced BC cell death (Tarighi et al., 2015).

Further research considering uPAR/integrin interactions revealed that blockage of uPAR/integrin interaction reduced the progression of metastatic BC cells in vivo (van der Pluijm et al., 2001). LeBeau et al. showed in xenograft models with TNBC tumors, that tumor growth could be reduced by using monoclonal anti-uPAR ab 2G10. Additionally, they showed that using anti-uPAR ab 2G10 conjugated to a radionuclide were effective in tumor regression (LeBeau et al., 2013). Bauer et al. showed that the combination of gemcitabine with anti-uPAR ab ATN-658 had a better outcome than

treatment with gemcitabine alone in orthotropic mouse models of human pancreatic carcinoma (Bauer et al., 2005).

Targeting integrins

Integrins are involved in the pathology of many diseases. Pharmacological inhibition of integrins is of great interest for diseases like thrombosis, immune diseases, osteoporosis, acute coronary heart syndrome and cancer. Additionally, integrins are located on the cell surface, making them accessible as pharmacological targets (Millard et al., 2011).

The role of integrins is versatile, therefore different therapeutic approaches for different illnesses were made. The general opinion leads to the notion that targeting integrins would antagonize tumor growth and metastasis. Actually, several clinical attempts of targeting integrins in vivo have been made. The goal is to regulate the tumor vasculature and making it more accessible for chemotherapy or radiation (Tabatabai et al., 2010).

It was shown that targeting integrin $\alpha v\beta 3$ for example with systemic application of LM609 ab results in suppression of angiogenesis. LM609 is an anti-integrin $\alpha v\beta 3$ ab. The inhibition of integrin $\alpha v\beta 3$ led to apoptosis in newly proliferating vessels, tumor cells and endothelial cells but did not inhibit preexisting vessels (Gasparini et al., 1998; Kumar et al., 2001; Strömblad et al., 1996). Nevertheless, the effect of anti-integrin drugs on normal cells remains unclear and should be the subject of further research (Carter, 2010).

Abciximab, a monoclonal ab against integrin $\alpha IIb\beta 3$, was developed to reduced ischemic events in patients with acute coronary symptoms. It is nowadays restricted to high risk patients (Cox et al., 2010). Natalizumab is a monoclonal ab used in patients with multiple sclerosis, that binds to the $\alpha 4$ integrin subunit. It is used with a risk management strategy due to severe and potentially fatal side effects of progressive multifocal leukoencephalopathy (Cox et al., 2010). Etaracizumab is a monoclonal ab against $\alpha v\beta 3$ currently studied for treatment of solid cancers and melanoma in early phases. It was tested in a randomized multicenter phase II study in human stage IV melanoma patients. Patients received etaracizumab with or without darcabazine. It did not show a survival benefit to darcabazine, which now is obsolete (Hersey et al., 2010). An orally available $\alpha v\beta 3$ antagonist called MK 0429 is currently investigated for

prostate cancer (Cox et al., 2010).

The small molecule inhibitor S247 inhibits integrin αvβ3 directly. It showed promising results in mouse models. The number of bone metastasis could significantly be reduced with application of the inhibitor (Harms et al., 2004). The broad spectrum integrin receptor antagonist GLPG0187 did not show convincing results in a phase I study in glioma patients (Cirkel et al., 2016).

Eptifibatide and tirofiban, two small molecule inhibitors against integrin αIIbβ3 were developed to reduce ischemic events in patients with acute coronary symptoms. They are restricted to high risk patients (Cox et al., 2010).

Several studies engaged with cilengitide, the first antiangiogenic agent targeting integrins αvβ3, αvβ5 and α5β1. This small molecule inhibits angiogenesis and induces apoptosis of endothelial cells by blocking the contact between integrins with the ECM (Mas-Moruno et al., 2010). Since both tumor cells and newly proliferating vessels express integrins, both cell types are targeted by this therapy (Carter, 2010). Cilengitide was tested in several tumors and showed promising results (Albert et al., 2006; Hariharan et al., 2007; Oliveira-Ferrer et al., 2008). Unfortunately, the phase III study CENTRIC showed that addition of cilengitide to temozolomide chemoradiotherapy could not improve outcomes in glioblastoma patients (Stupp et al., 2014). Burke et al. showed promising results in BC models. The combination of cilengitide and radiotherapy improved survival without adding toxicity to the treatment (Burke et al., 2002). Integrin αvβ3 could therefore be a target to prevent bone metastasis by BC cells. This seems very important, because treatment of bone metastasis in BC patients at present remains mostly palliative (Sloan et al., 2006).

Hill et al. showed that high levels of integrin αvβ3 were found in a subgroup of TNBC, the mesenchymal subtype. They targeted it with a novel peptide called φRGDechi. They indicated that targeting integrin αvβ3 diminished adhesion, migration, invasion and capacity of building vascular-like structures (Hill et al., 2019). Zhong et al. showed that αvβ3-targeted micellar mertansine prodrug mediates highly potent and targeted treatment of integrin αvβ3 overexpressing TNBC xenografts in nude mice. The drug showed high affinity to the tumor cells, high accumulation in the tumor, insignificant systemic toxicity and effective inhibition of tumor growth (Zhong et al., 2017). Ross et al. utilized integrin αvβ3 as a molecular target for enhancing drug delivery to the tumor cells in the bone. They showed that bone metastasis could be reduced significantly

and with less toxicity than equimolar doses of free docetaxel (Ross et al., 2017). Menendez et al. showed that in the presence of integrin αvβ3 antagonists taxol-induced cell death had a strong synergism. This suggests that targeting integrin αvβ3 may simultaneously prevent BC angiogenesis, growth and chemoresistance. (Menendez et al., 2005).

Another aspect of targeted therapy is the targeted delivery of therapy. Integrins were studied to deliver chemotherapeutics, oncolytic viruses, pro-apoptotic peptidases and radionucleotides to tumor cells and its surroundings. One of the studies showed that delivery of doxorubicin to integrin αvβ3-positive tumor vasculature inhibited the growth of metastasis (Desgrosellier & Cheresh, 2010).

Integrin inhibition initially showed high potential. In the meantime successful therapeutic inhibition of integrins is difficult because of the important functions of integrins in physiological processes. Additionally, side effects occurred, which should not be underestimated (Cox et al., 2010).

5.5 Conclusion

Knowledge of the important role of the uPAR interactome in BC lead to the idea of exploring members of the uPAR interactome in TNBC. The primary goal of the present study was to investigate whether two members of the uPAR interactome plasmin(ogen) and integrin αvβ3 serve as potential new biomarkers in TNBC patients. Statements about the validity of plasmin(ogen) expression in TNBC are hard to make because of the omnipresence of the protein. In the present cohort, TNBC patients without plasmin(ogen) expression in their tumors did not exist. In Kaplan Meier plots, strong plasminogen staining showed a tendency of higher probability of OS and DFS but without being statistically significant. Further studies are needed to find out more about the role of plasmin(ogen) in TNBC tumors. Patients with high integrin αvβ3 expression that had been pretreated with adjuvant and/or neoadjuvant chemotherapy, were found to have poor outcome. Our results suggest that integrin αvβ3 could be a predictive marker in patients with TNBC who receive adjuvant and/or neoadjuvant chemotherapy. Thus, our study provides convincing evidence that integrin αvβ3 could be a potential predictive biomarker for TNBC´s cells. Taken together, this study clearly indicates that integrin αvβ3 could regulate drug sensitivity in TNBC cells. Additionally, this study could not only reveal the potential predictive biomarker in TNBC but targeting integrin

$\alpha v\beta 3$ could be an effective therapeutic strategy in integrin $\alpha v\beta 3$ overexpressing TNBC tumors as well.

Further studies will be necessary in order to test whether consistent results for integrin $\alpha v\beta 3$ can be obtained in larger TNBC cohorts. If this is the case, integrin $\alpha v\beta 3$ would be a highly valuable marker as it could influence therapeutic decisions in TNBC patients. Nevertheless, integrin $\alpha v\beta 3$ could have the potential to support clinical therapy decisions by helping to identify those patients who could be possible candidates for alternative therapeutic approaches such as a combination of conventional chemotherapy and targeted integrin $\alpha v\beta 3$ therapy. In future, members of the uPAR interactome could serve as a biomarkers that help to further dissect the heterogeneity of TNBC and as a therapy target, allowing individualised treatment.

6. Abstract

TNBC is a subgroup of BC, compared to other BC subgroups often affecting younger patients, showing aggressive behavior, and leading to early death. In consideration of the poor prognosis of TNBC patients, predictive biomarkers are urgently needed to make statements about individualized treatment options. The uPAR interactome consists of multiple interacting proteins which play a key role in tumor cell spread and metastasis. Previously, the important role of the uPAR interactome in BC was proven for uPA and PAI-1, being accepted LOE-1 biomarkers transferred to clinical routine application. In this thesis, two additional important factors of the uPAR interactome, plasmin(ogen) and integrin αvβ3, were analyzed for their potential to serve as biomarkers to assess a TNBC patient´s risk profile. The Klinikum rechts der Isar, Technical University of Munich maintains a large biobank of tissue samples of TNBC patients. 185 patients were retrieved and the patients´ course of disease were followed over a median period of 67.4 months. 10.9% of the patients were diagnosed with metastases at time of diagnosis, 18.3% of the patients relapsed during the observation time and 45.0% of the patients died. IHC stainings were performed employing plasminogen- and integrin αvβ3-directed ab. Statistical analyses were performed using the IBM SPSS Statistics. Cox regression analysis showed that protein expression of integrin αvβ3 and plasminogen failed statistical significance both in univariate and multivariable analyses considering OS and in DFS. Different from that, considering only TNBC patients treated with chemotherapy, it showed that expression of integrin αvβ3 was of statistical significance predicting the probability of OS ($p=0.048$) and DFS ($p=0.031$) in the univariate analysis but failed statistical significance in multivariable analysis. In this subgroup, Kaplan Meier plots showed statistical significance. Considering the entire follow-up process, low integrin αvβ3 expression was correlated with higher probability of OS ($p=0.042$) and DFS ($p=0.027$). Considering a period of 60 months after diagnosis, low integrin αvβ3 expression was also correlated with higher probability of OS ($p=0.044$) and DFS ($p=0.030$).

In summary, evidence is presented that especially integrin αvβ3 as a member of the uPAR interactome, may play an important role in the subgroup of TNBC patients and might therefore be a potential novel predictive cancer biomarker and therapy target, allowing individualised treatment.

7. Appendix

7.1 TNM staging system for breast cancer

Table 15. TNM staging system for BC

TNM Classification, Part I	
Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤ 20 mm in greatest dimension
T1mi	Tumor ≤ 1 mm in greatest dimension
T1a	Tumor > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumor > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumor > 10 mm but ≤ 20 mm in greatest dimension
T2	Tumor > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumor > 50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Clinical Regional Lymph Nodes (N)	
NX	Regional lymph node cannot be assessed
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastases in ipsilateral infraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)

Table 15. TNM staging system for BC

TNM Classification, Part II	
Pathologic Regional Lymph Nodes (pN)	
pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis identified histologically
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by SNLB but not clinically detected
pN1a	Metastases in 1-3 axillary lymph nodes, at least one metastasis greater than 2.0 mm
pN1b	Metastases in internal mammary nodes with micrometastases or macrometastases detected by SNLB but not clinically detected
pN1c	Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by SNLB but not clinically detected
pN2	Metastases in 4-9 axillary lymph nodes; or in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4-9 axillary lymph nodes (at least one tumor deposit greater than 2.0 mm)
pN2b	Metastases in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in 10 or more axillary lymph nodes; or in infraclavicular lymph nodes; or in clinically detected ipsilateral internal mammary lymph nodes in the presence of one or more positive level I, II axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by SLNB but not clinically detected; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in 10 or more axillary lymph nodes (at least one tumor deposit greater that 2.0 mm); or metastases to the infraclavicular nodes
pN3b	Metastases in clinically detected ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by SLNB but not clinically detected
pN3c	Metastases in ipsilateral supraclavicular lymph nodes
Distant Metastases (M)	
M0	No clinical or radiographic evidence of distant metastases
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

Taken from: https://cancerstaging.org/references-tools/quickreferences/Documents/BreastLarge.pdf

7.2 Histologic grading of breast cancer

Table 16. Histologic grading of BC

Feature	Score
Tubule and gland formation	
Majority of tumour (>75%)	1
Moderate degree (10-75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
0-5/10 HPF	1
6-11/10 HPF	2
>12/10 HPF	3
	Total score 3-9
Legend: HPF= High power field, these data are valid for a field diameter of 0,45mm and a field number of 18	

Taken from Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019

Table 17. Scoring of the histologic grading of BC

Total Score	Malignancy	Grading score	Definition
3-5	Low	1	Well differentiated
6-7	Moderate	2	Moderately differentiated
8-9	High	3	Poorly differentiated

Taken from Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften, 2019

The grading system includes three tumor characteristics: tubule formation, the mitotic index, and nuclear pleomorphism. Each characteristic is evaluated by a score of 1-3. The tubule formation is a measure for the differentiation of the tumor cells. Nuclear size and shape in comparison to normal breast tissue is evaluated in the score of nuclear pleomorphism. The mitotic index must be evaluated precisely. The field is divided in HPF and the number of mitoses is counted per 10 HPF (WHO 2003).

7.3 Tumor Marker Utility Grading System

Table 18. TMUGS

Level	Type of evidence
I	Evidence from a single, high-powered, prospective controlled study that is specifically designed to test marker, or evidence from well-done meta-analysis and/or overview of level I studies. Ideally, the study is a prospective, randomized controlled trial in which diagnostic and/or therapeutic clinical decisions in one arm are determined at least in part based on marker results, and diagnostic and/or therapeutic clinical decisions in the control arm are made independently of marker results, or Evidence from overview of LOE II studies addressing specific use.
II	Evidence from study in which marker data are determined in relationship to prospective therapeutic trial that is performed to test therapeutic hypothesis but not specifically designed to test marker utility. Specimen collection for marker study and statistical analysis are prospectively determined in protocol as secondary objectives.
III	Evidence from large but retrospective studies from which variable numbers of samples are available or selected. Statistical analysis for tumor marker was not dictated prospectively at time of therapeutic trial design.
IV	Evidence from small retrospective studies that do not have prospectively dictated therapy, follow-up, specimen selection, or statistical analysis.
V	Evidence from small pilot studies designed to determine or estimate distribution of marker levels in sample populations.

Taken from Hayes et al., 1996

7.4 Statistics

Kaplan Meier plots: Plasminogen

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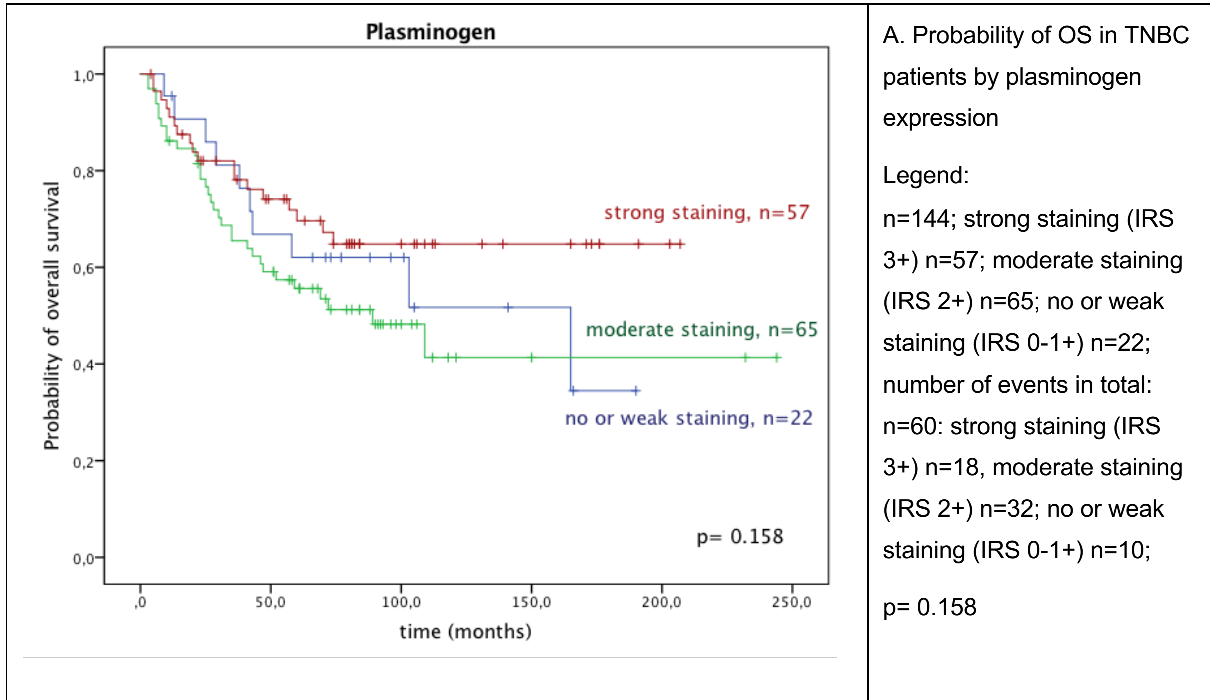
<td>
</td>
<td>A. Probability of OS in TNBC patients by plasminogen expression

Legend:
n=144; strong staining (IRS 3+) n=57; moderate staining (IRS 2+) n=65; no or weak staining (IRS 0-1+) n=22; number of events in total: n=60: strong staining (IRS 3+) n=18, moderate staining (IRS 2+) n=32; no or weak staining (IRS 0-1+) n=10;

p= 0.158</td>
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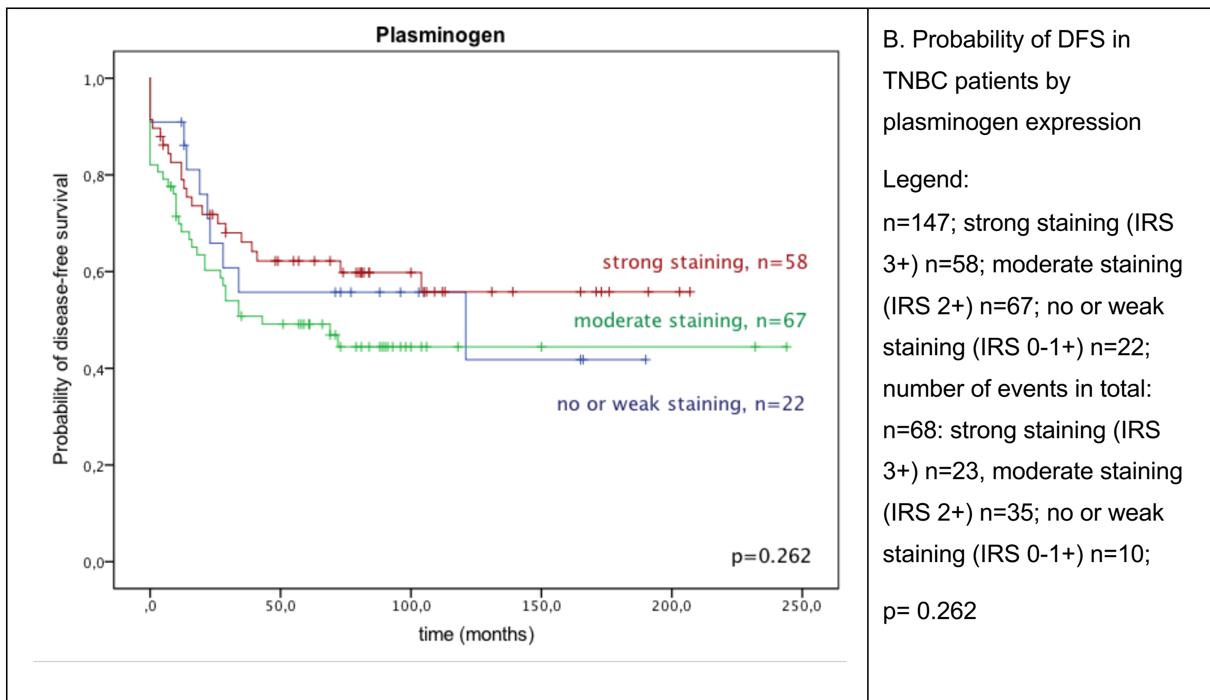
<td>
</td>
<td>B. Probability of DFS in TNBC patients by plasminogen expression

Legend:
n=147; strong staining (IRS 3+) n=58; moderate staining (IRS 2+) n=67; no or weak staining (IRS 0-1+) n=22; number of events in total: n=68: strong staining (IRS 3+) n=23, moderate staining (IRS 2+) n=35; no or weak staining (IRS 0-1+) n=10;

p= 0.262</td>
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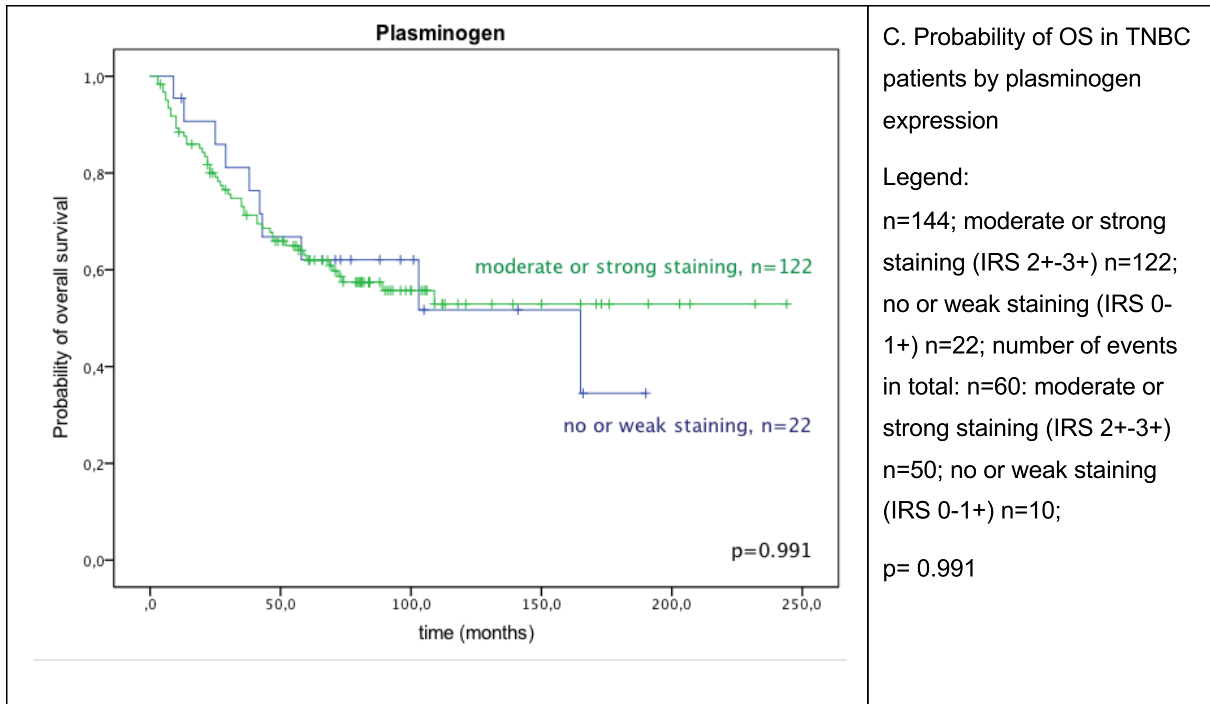
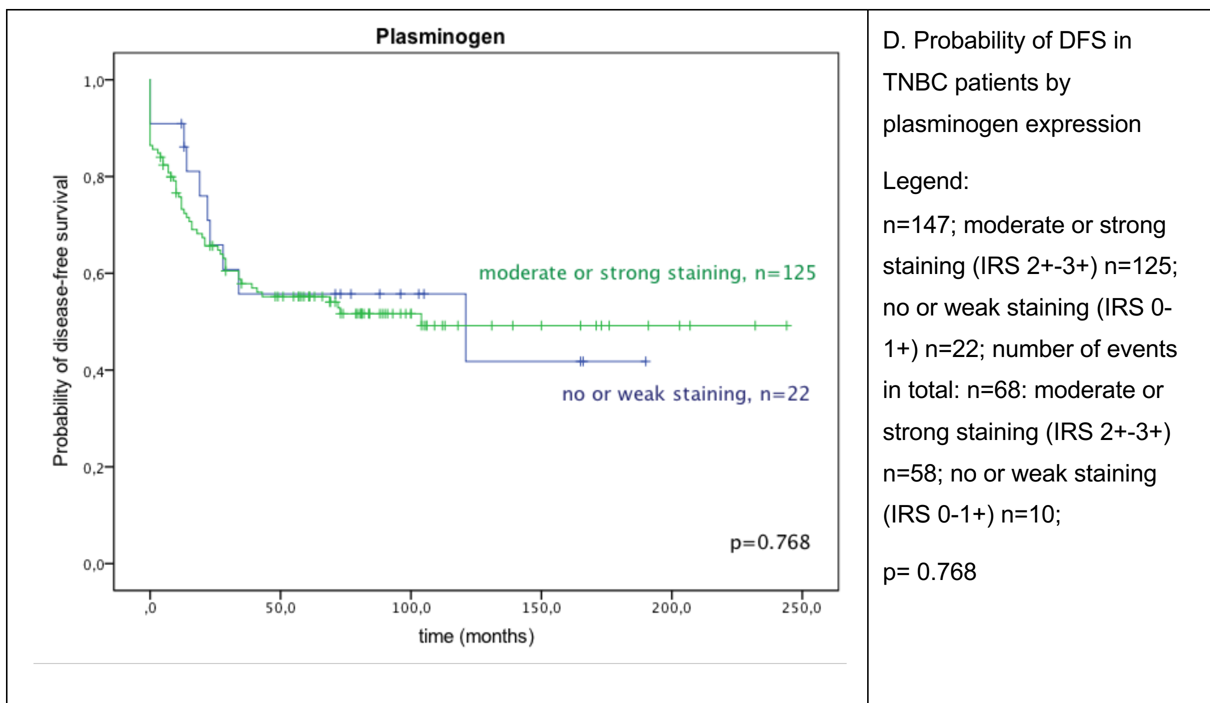


C. Probability of OS in TNBC patients by plasminogen expression

Legend:

n=144; moderate or strong staining (IRS 2+-3+) n=122; no or weak staining (IRS 0-1+) n=22; number of events in total: n=60: moderate or strong staining (IRS 2+-3+) n=50; no or weak staining (IRS 0-1+) n=10;

p= 0.991

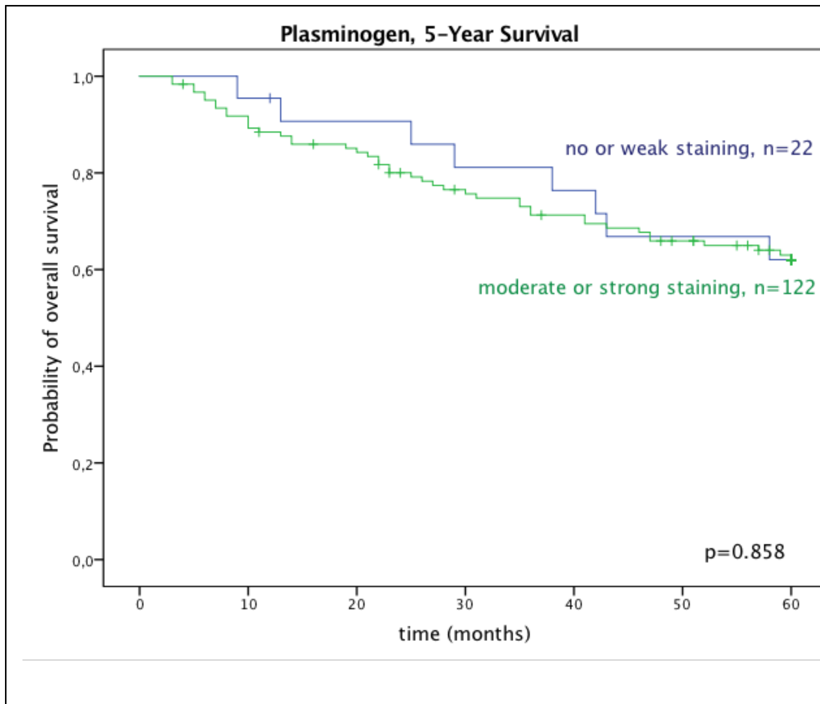


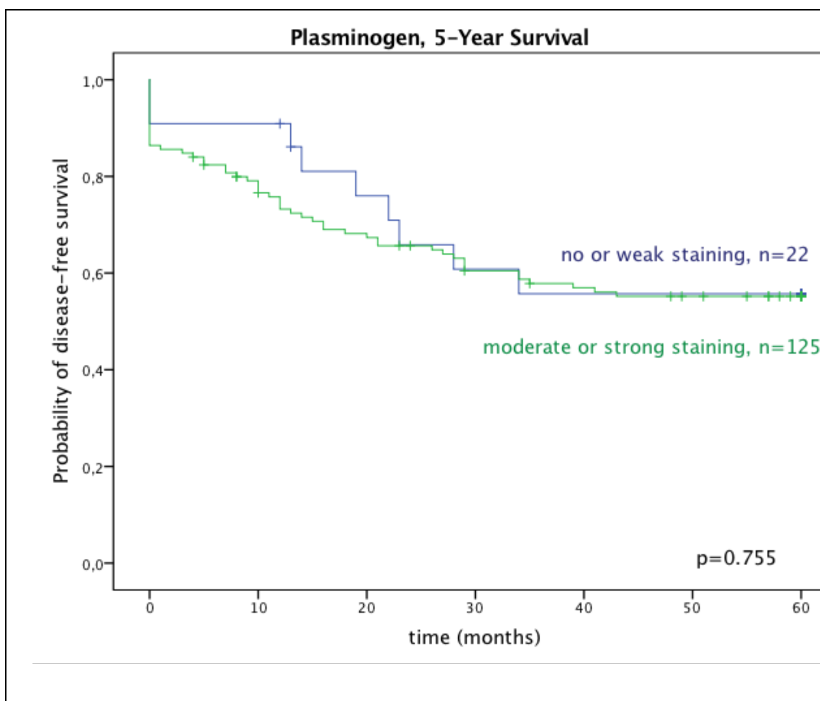
D. Probability of DFS in TNBC patients by plasminogen expression

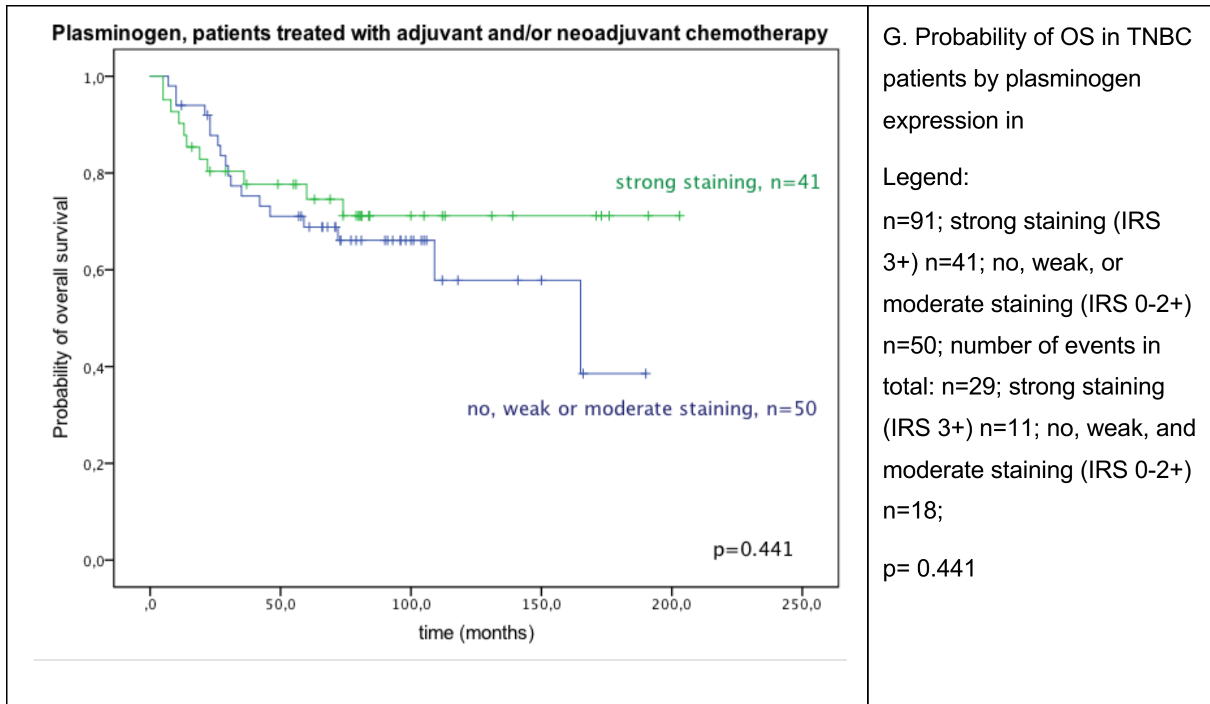
Legend:

n=147; moderate or strong staining (IRS 2+-3+) n=125; no or weak staining (IRS 0-1+) n=22; number of events in total: n=68: moderate or strong staining (IRS 2+-3+) n=58; no or weak staining (IRS 0-1+) n=10;

p= 0.768

	E. Probability of OS over 5 years in TNBC patients by plasminogen expression Legend: n=144; moderate or strong staining (IRS 2+-3+) n=122; no or weak staining (IRS 0-1+) n=22; number of events in total: n=52: moderate or strong staining (IRS 2+-3+) n=44; no or weak staining (IRS 0-1+) n=8; p= 0.858

	F. Probability of DFS over 5 years in TNBC patients by plasminogen expression Legend: n=147; moderate or strong staining (IRS 2+-3+) n=125; no or weak staining (IRS 0-1+) n=22; number of events in total: n=63: moderate or strong staining (IRS 2+-3+) n=54; no or weak staining (IRS 0-1+) n=9; p= 0.755

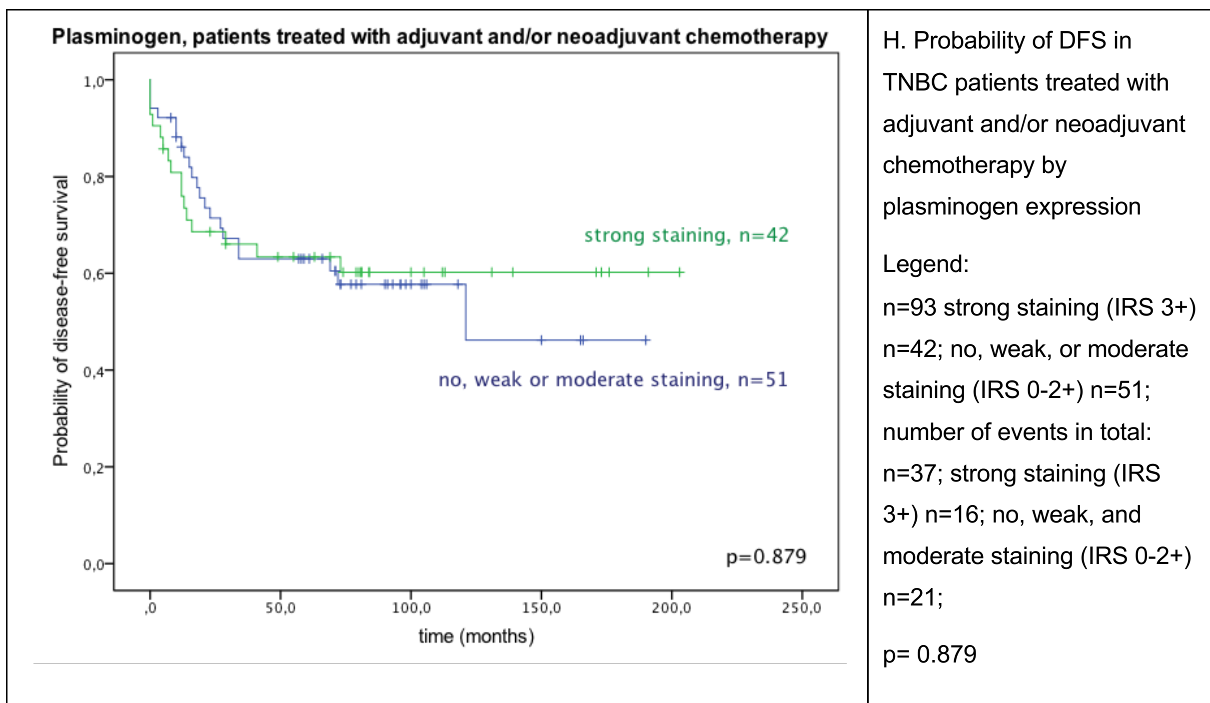


G. Probability of OS in TNBC patients by plasminogen expression in

Legend:

n=91; strong staining (IRS 3+) n=41; no, weak, or moderate staining (IRS 0-2+) n=50; number of events in total: n=29; strong staining (IRS 3+) n=11; no, weak, and moderate staining (IRS 0-2+) n=18;

p= 0.441

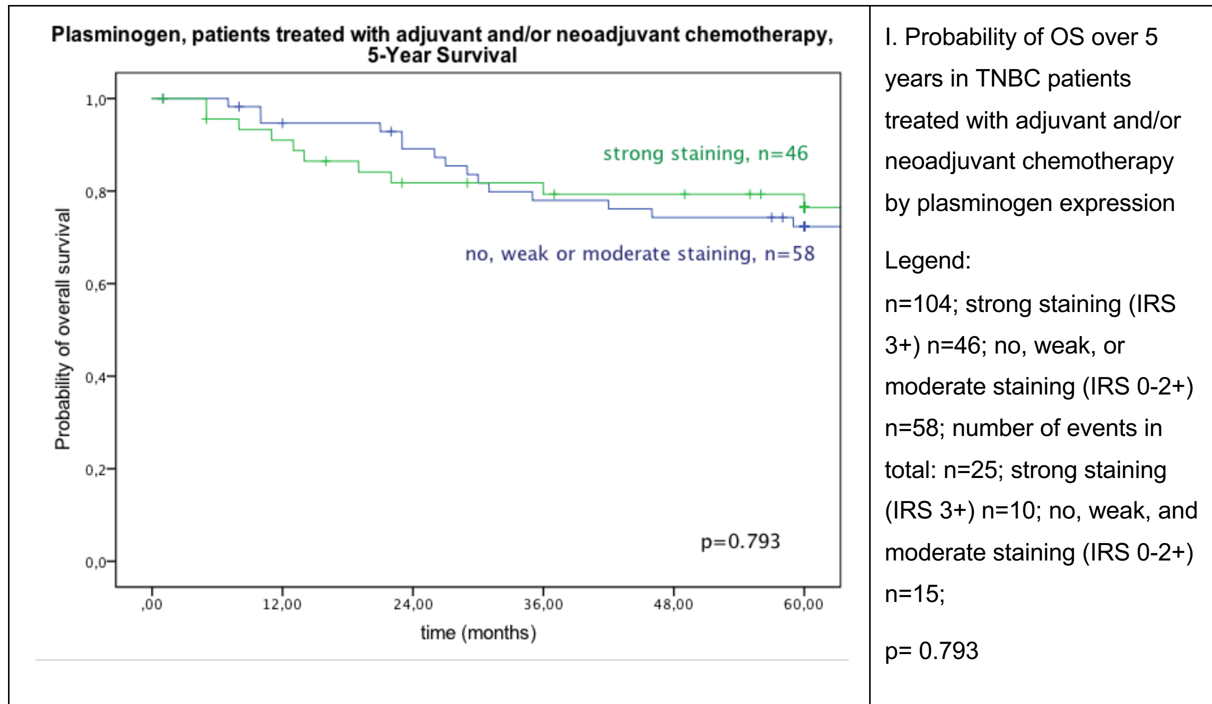


H. Probability of DFS in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

Legend:

n=93 strong staining (IRS 3+) n=42; no, weak, or moderate staining (IRS 0-2+) n=51; number of events in total: n=37; strong staining (IRS 3+) n=16; no, weak, and moderate staining (IRS 0-2+) n=21;

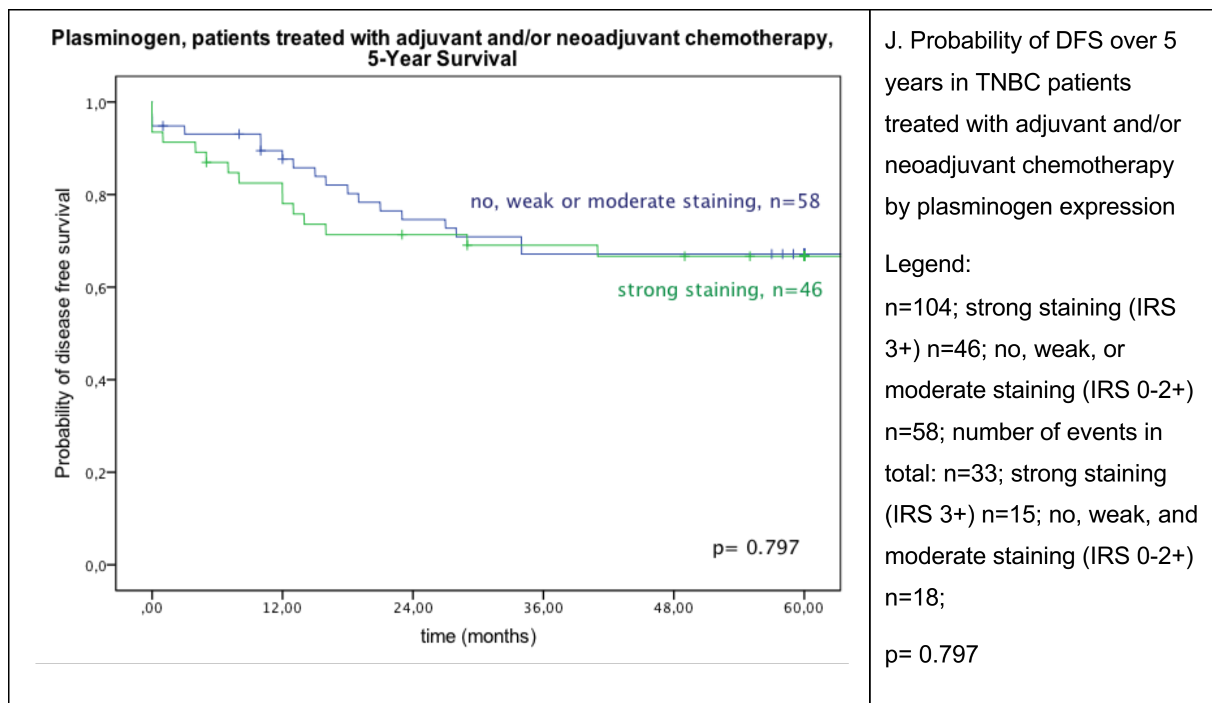
p= 0.879



I. Probability of OS over 5 years in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

Legend:
n=104; strong staining (IRS 3+) n=46; no, weak, or moderate staining (IRS 0-2+) n=58; number of events in total: n=25; strong staining (IRS 3+) n=10; no, weak, and moderate staining (IRS 0-2+) n=15;

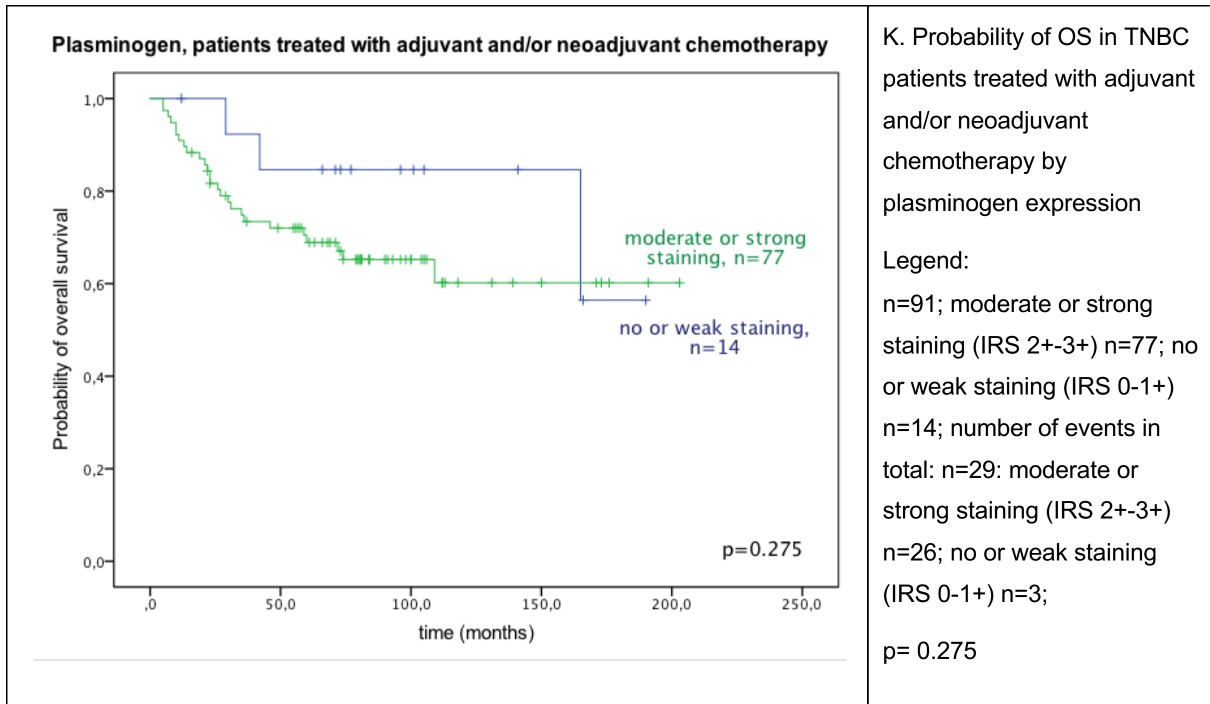
p= 0.793



J. Probability of DFS over 5 years in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

Legend:
n=104; strong staining (IRS 3+) n=46; no, weak, or moderate staining (IRS 0-2+) n=58; number of events in total: n=33; strong staining (IRS 3+) n=15; no, weak, and moderate staining (IRS 0-2+) n=18;

p= 0.797

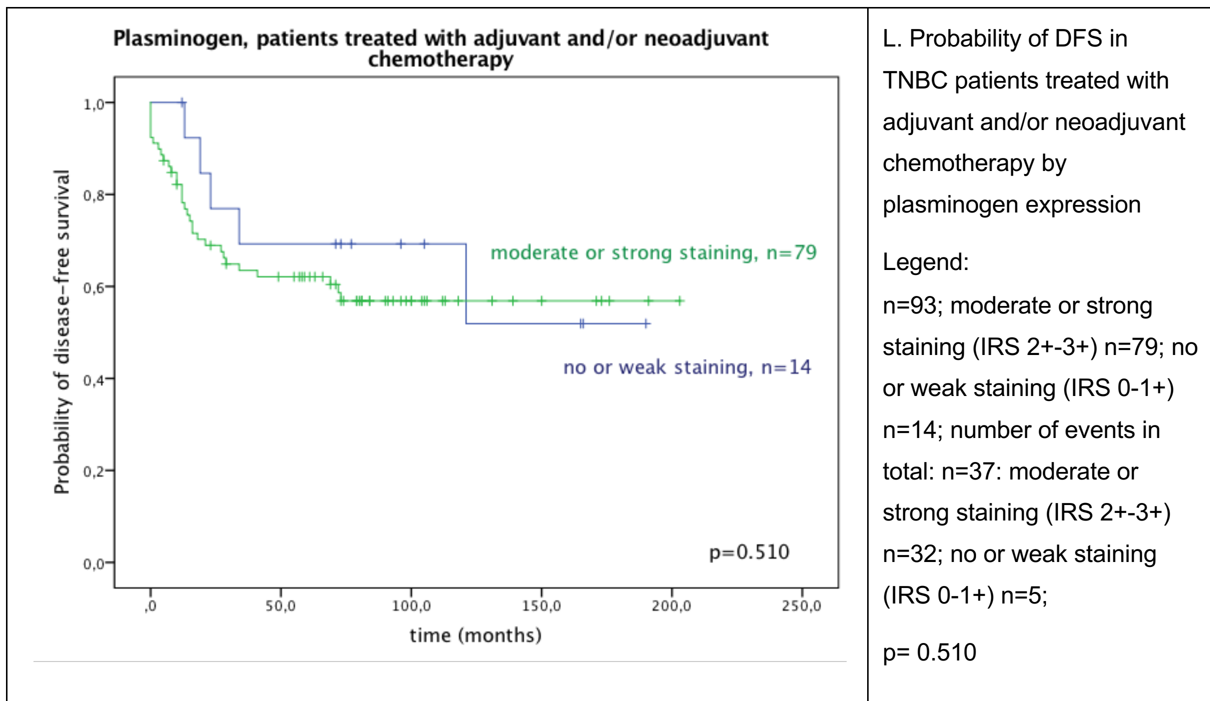


K. Probability of OS in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

Legend:

n=91; moderate or strong staining (IRS 2+-3+) n=77; no or weak staining (IRS 0-1+) n=14; number of events in total: n=29: moderate or strong staining (IRS 2+-3+) n=26; no or weak staining (IRS 0-1+) n=3;

p= 0.275

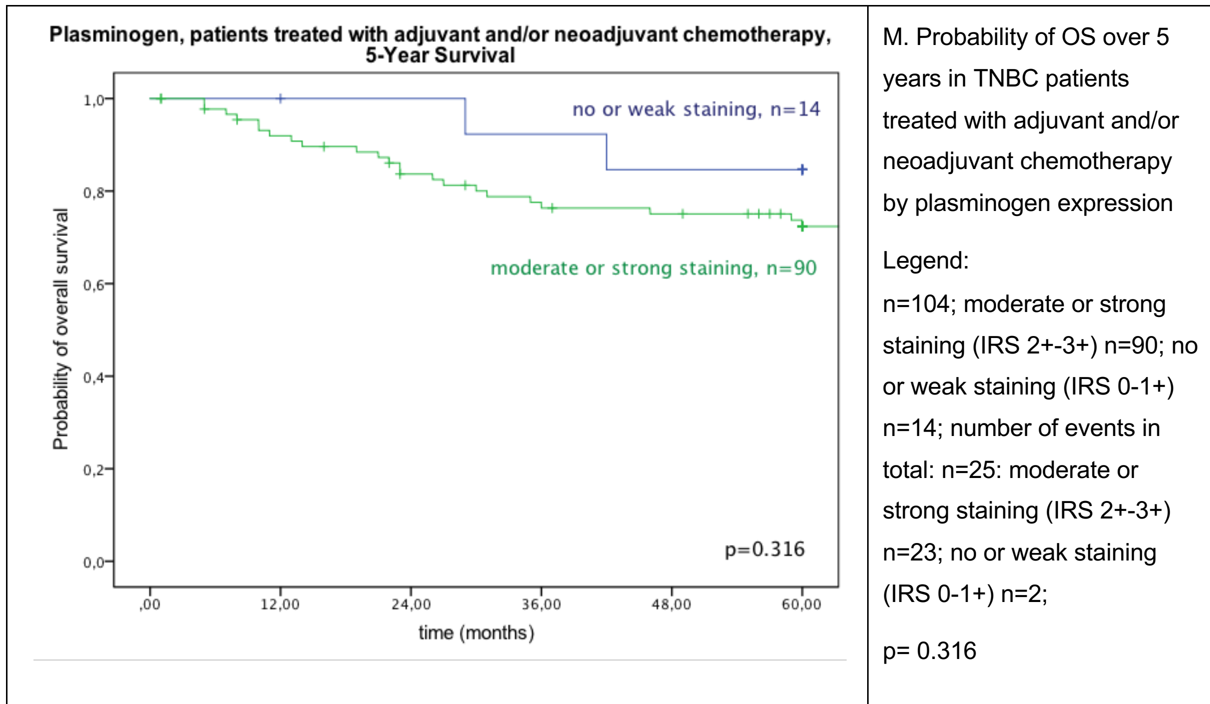


L. Probability of DFS in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

Legend:

n=93; moderate or strong staining (IRS 2+-3+) n=79; no or weak staining (IRS 0-1+) n=14; number of events in total: n=37: moderate or strong staining (IRS 2+-3+) n=32; no or weak staining (IRS 0-1+) n=5;

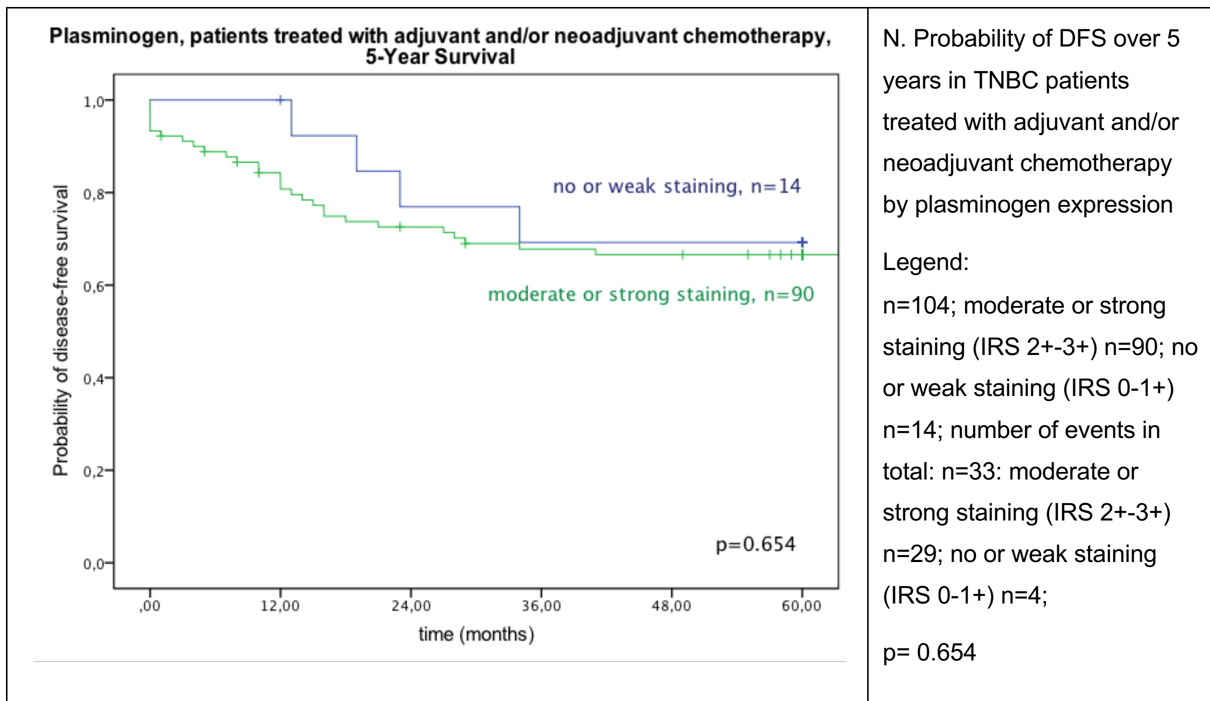
p= 0.510



M. Probability of OS over 5 years in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

Legend:
n=104; moderate or strong staining (IRS 2+-3+) n=90; no or weak staining (IRS 0-1+) n=14; number of events in total: n=25: moderate or strong staining (IRS 2+-3+) n=23; no or weak staining (IRS 0-1+) n=2;

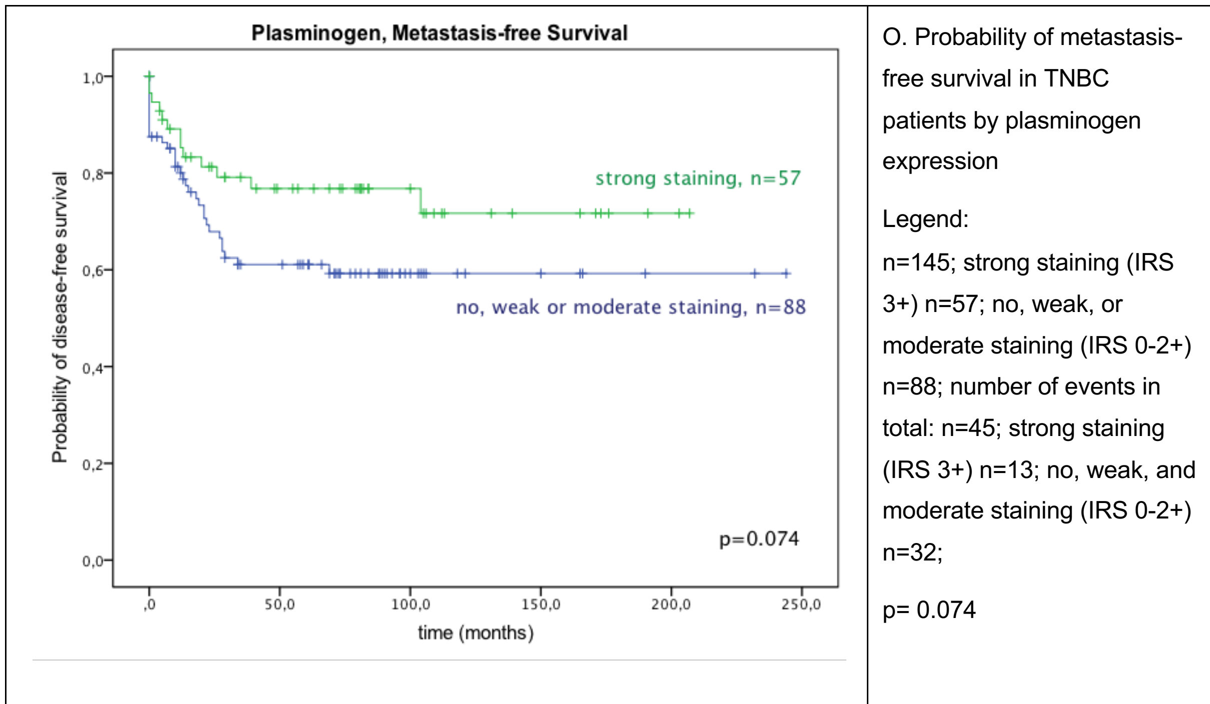
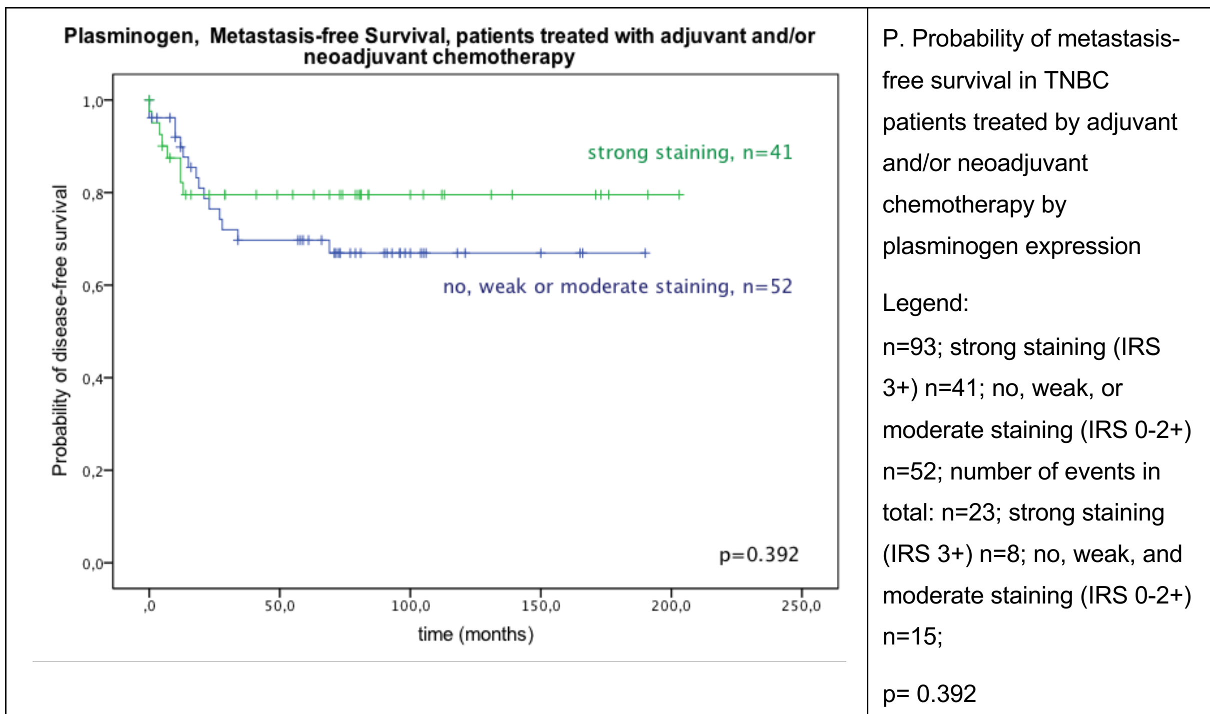
p= 0.316



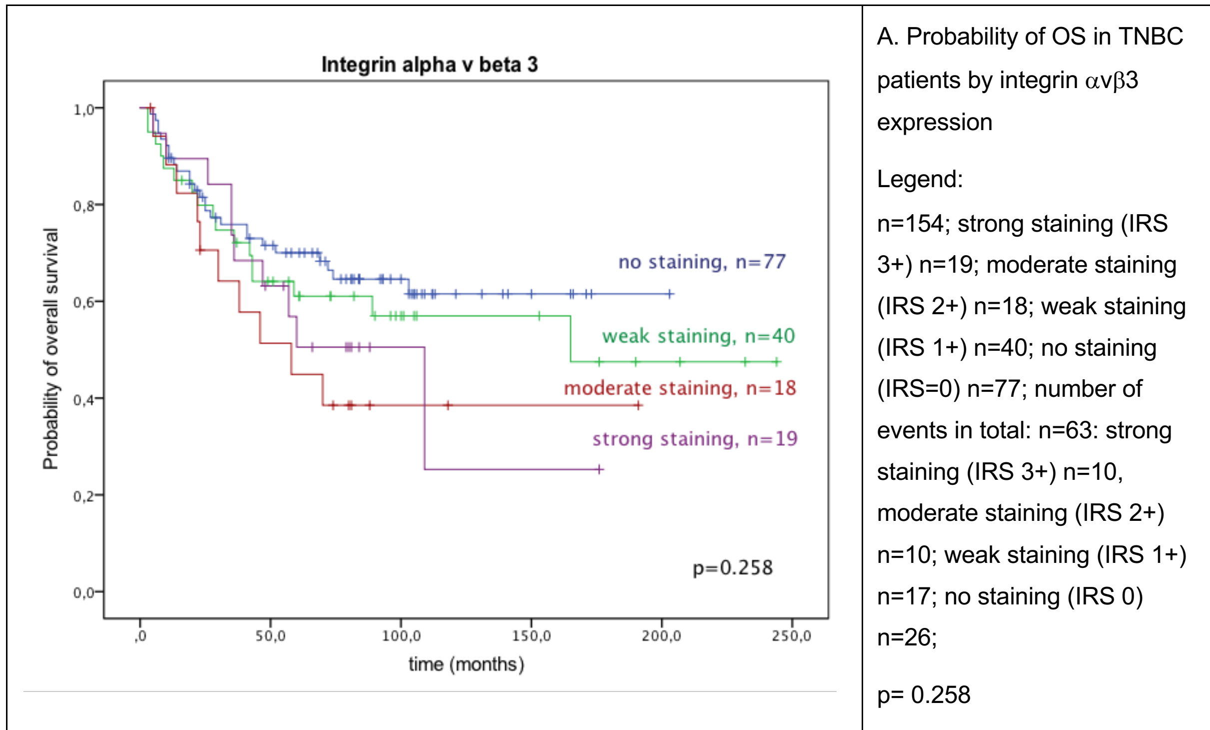
N. Probability of DFS over 5 years in TNBC patients treated with adjuvant and/or neoadjuvant chemotherapy by plasminogen expression

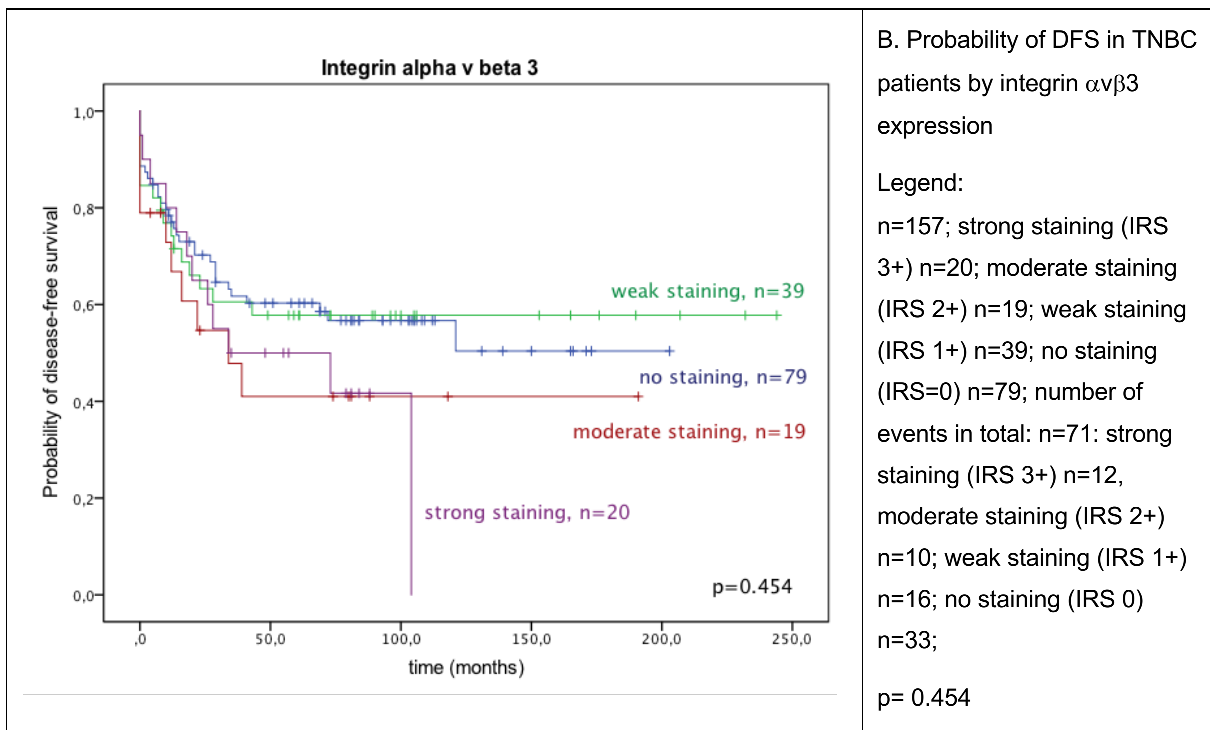
Legend:
n=104; moderate or strong staining (IRS 2+-3+) n=90; no or weak staining (IRS 0-1+) n=14; number of events in total: n=33: moderate or strong staining (IRS 2+-3+) n=29; no or weak staining (IRS 0-1+) n=4;

p= 0.654

 	O. Probability of metastasis-free survival in TNBC patients by plasminogen expression Legend: n=145; strong staining (IRS 3+) n=57; no, weak, or moderate staining (IRS 0-2+) n=88; number of events in total: n=45; strong staining (IRS 3+) n=13; no, weak, and moderate staining (IRS 0-2+) n=32; p= 0.074
 	P. Probability of metastasis-free survival in TNBC patients treated by adjuvant and/or neoadjuvant chemotherapy by plasminogen expression Legend: n=93; strong staining (IRS 3+) n=41; no, weak, or moderate staining (IRS 0-2+) n=52; number of events in total: n=23; strong staining (IRS 3+) n=8; no, weak, and moderate staining (IRS 0-2+) n=15; p= 0.392

Kaplan Meier plots: Integrin $\alpha v\beta 3$

	A. Probability of OS in TNBC patients by integrin $\alpha v\beta 3$ expression Legend: n=154; strong staining (IRS 3+) n=19; moderate staining (IRS 2+) n=18; weak staining (IRS 1+) n=40; no staining (IRS=0) n=77; number of events in total: n=63: strong staining (IRS 3+) n=10, moderate staining (IRS 2+) n=10; weak staining (IRS 1+) n=17; no staining (IRS 0) n=26; p= 0.258

	B. Probability of DFS in TNBC patients by integrin $\alpha v\beta 3$ expression Legend: n=157; strong staining (IRS 3+) n=20; moderate staining (IRS 2+) n=19; weak staining (IRS 1+) n=39; no staining (IRS=0) n=79; number of events in total: n=71: strong staining (IRS 3+) n=12, moderate staining (IRS 2+) n=10; weak staining (IRS 1+) n=16; no staining (IRS 0) n=33; p= 0.454

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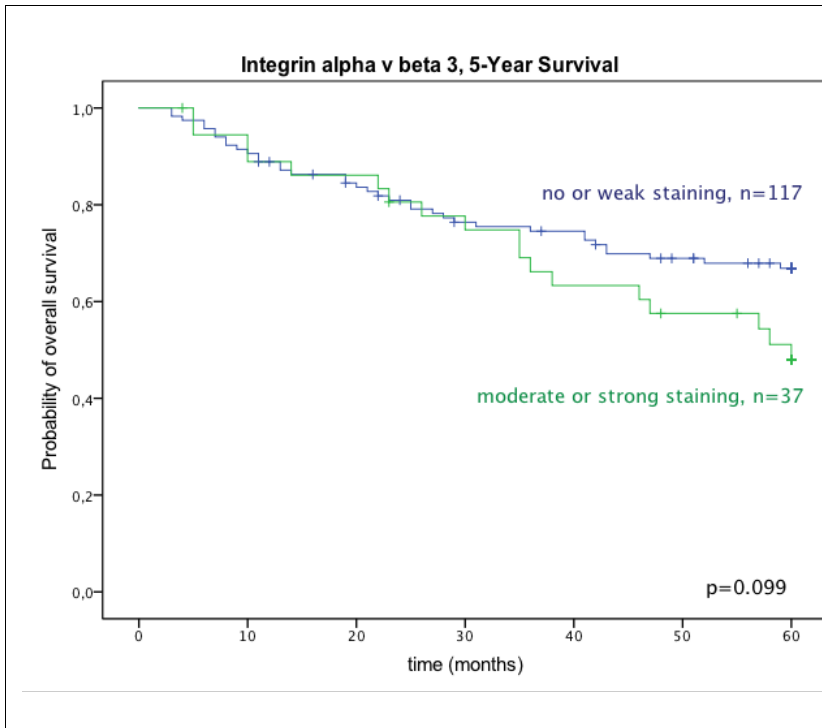
<td>
</td>
<td>C. Probability of OS over 5 years in TNBC patients by integrin αvβ3 expression

Legend:
n=154; moderate or strong staining (IRS 2+-3+) n=37; no or weak staining (IRS 0-1+) n=117; number of events in total: n=55: moderate or strong staining (IRS 2+-3+) n=18; no or weak staining (IRS 0-1+) n=37;

p= 0.099</td>
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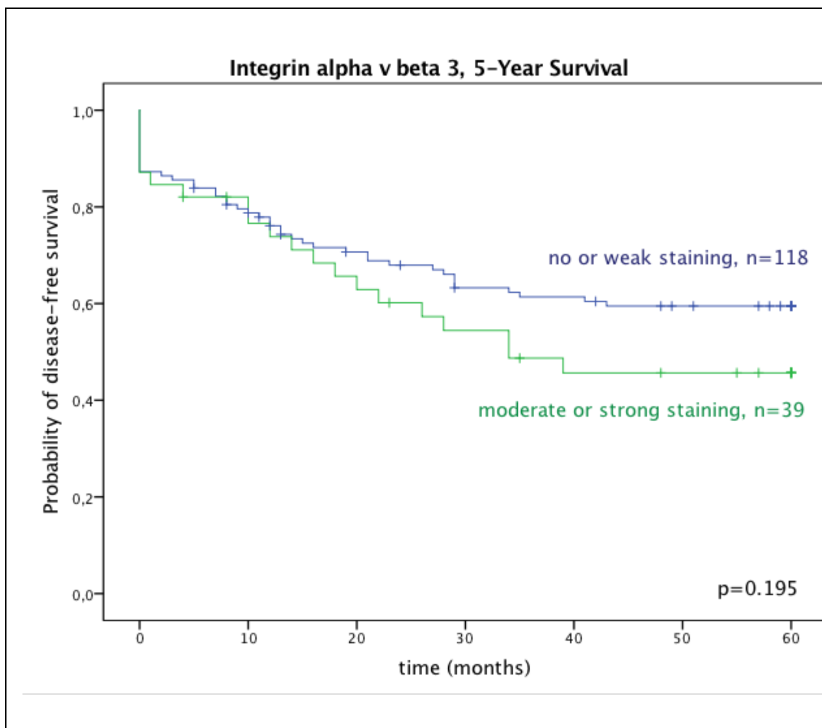
<td>
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<td>D. Probability of DFS over 5 years in TNBC patients by integrin αvβ3 expression

Legend:
n=157; moderate or strong staining (IRS 2+-3+) n=39; no or weak staining (IRS 0-1+) n=118; number of events in total: n=66: moderate or strong staining (IRS 2+-3+) n=20; no or weak staining (IRS 0-1+) n=46;

p= 0.195</td>
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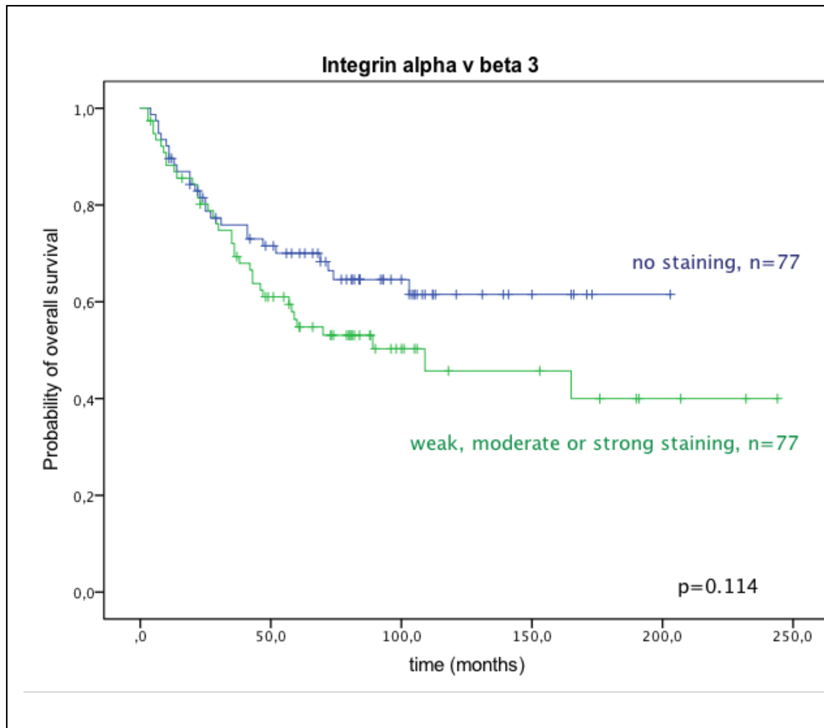
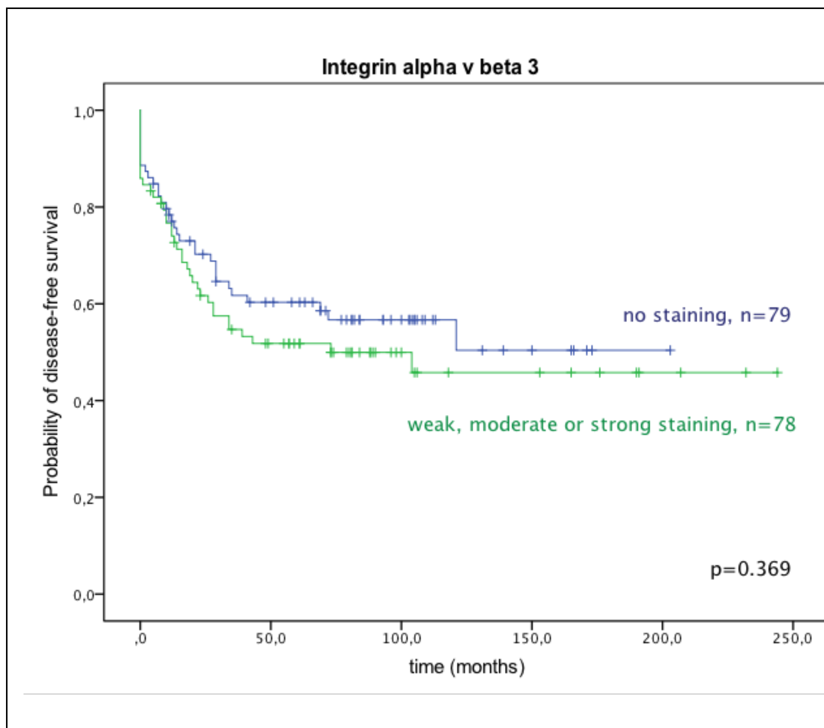


E. Probability of OS in TNBC patients by integrin αvβ3 expression

Legend:
n=154; weak, moderate, or strong staining (IRS 1+-3+) n=77; no staining (IRS 0) n=77; number of events in total: n=63: weak, moderate, or strong staining (IRS 1+-3+) n=37; no staining (IRS 0) n=26;

p= 0.114



F. Probability of DFS in TNBC patients by integrin αvβ3 expression

Legend:
n=157; weak, moderate, or strong staining (IRS 1+-3+) n=78; no staining (IRS 0) n=79; number of events in total: n=71: weak, moderate, or strong staining (IRS 1+-3+) n=38; no staining (IRS 0) n=33;

p= 0.369

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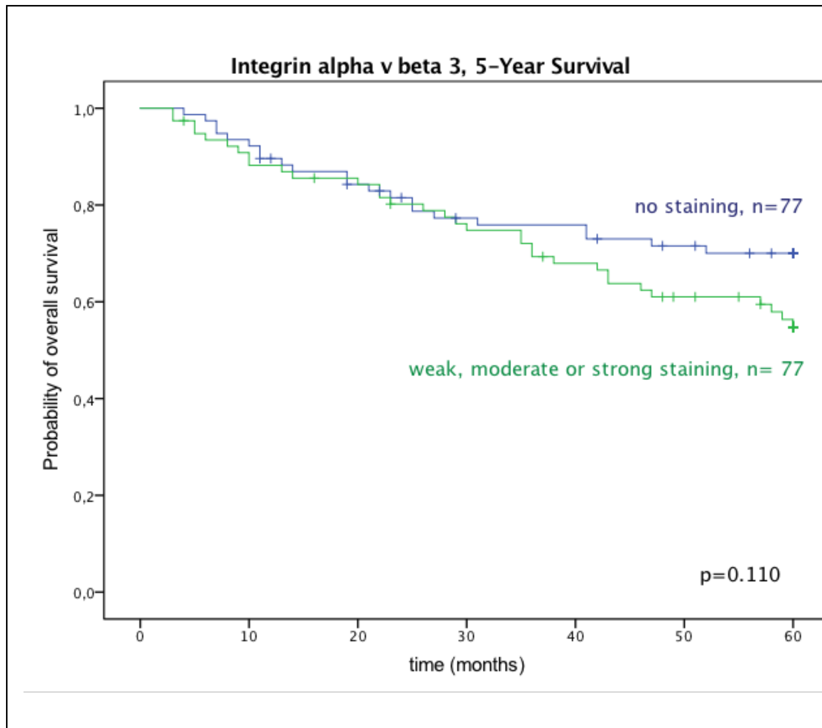
<tr><td>
</td><td>G. Probability of OS over 5 years in TNBC patients by integrin $\alpha v\beta 3$ expression

Legend:
n=154; weak, moderate, or strong staining (IRS 1+-3+) n=77; no staining (IRS 0) n=77; number of events in total: n=55: weak, moderate, or strong staining (IRS 1+-3+) n=23; no staining (IRS 0) n=22;

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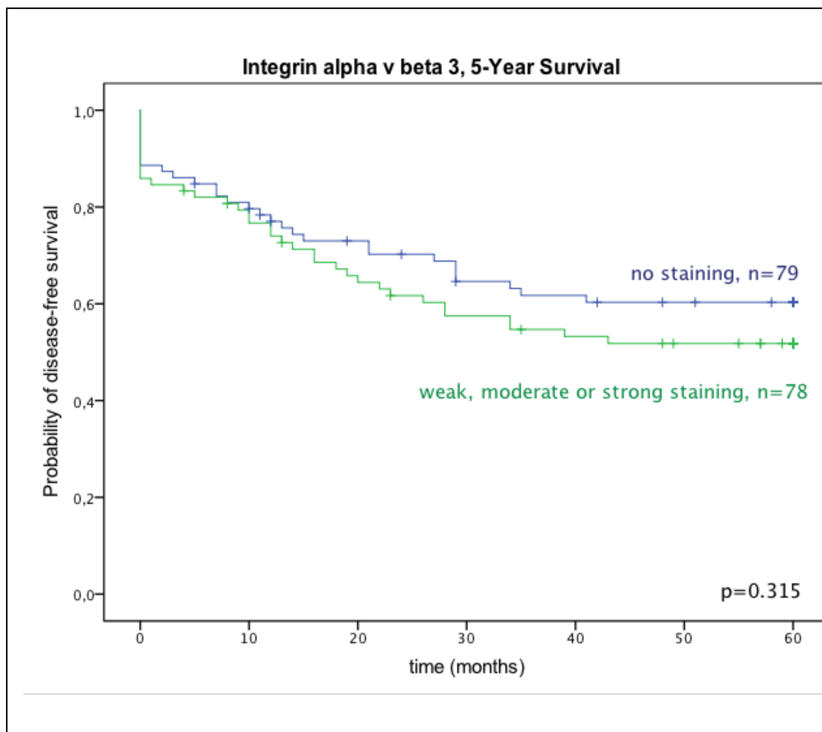
<tr><td>
</td><td>H. Probability of DFS over 5 years in TNBC patients by integrin $\alpha v\beta 3$ expression

Legend:
n=157; weak, moderate, or strong staining (IRS 1+-3+) n=78; no staining (IRS 0) n=79; number of events in total: n=66: weak, moderate, or strong staining (IRS 1+-3+) n=36; no staining (IRS 0) n=30;

p= 0.315</td></tr>
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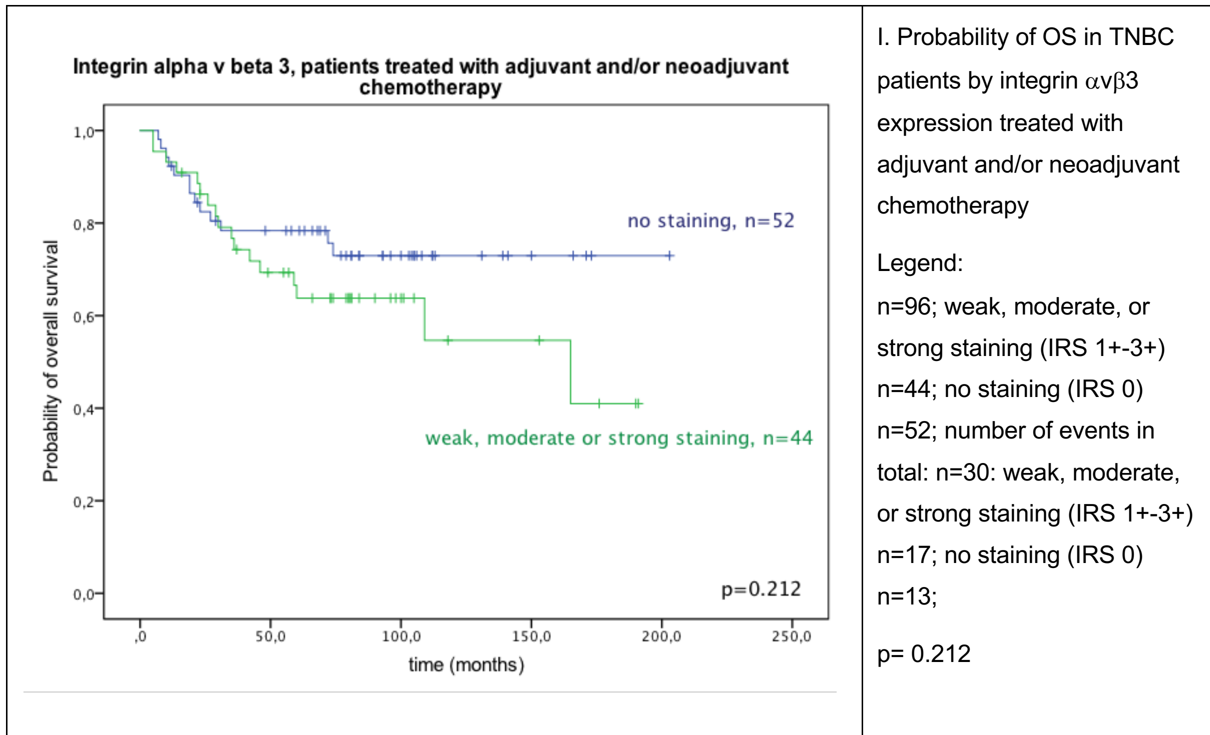
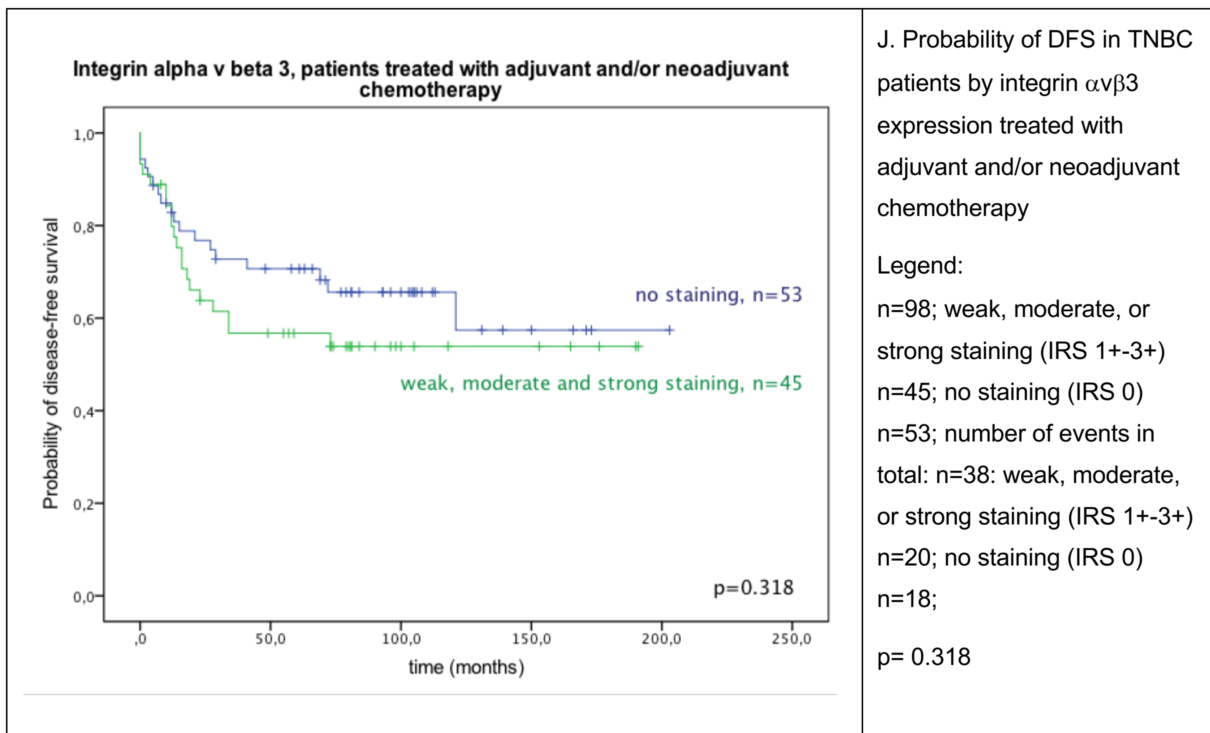


I. Probability of OS in TNBC patients by integrin αvβ3 expression treated with adjuvant and/or neoadjuvant chemotherapy

Legend:

n=96; weak, moderate, or strong staining (IRS 1+-3+) n=44; no staining (IRS 0) n=52; number of events in total: n=30: weak, moderate, or strong staining (IRS 1+-3+) n=17; no staining (IRS 0) n=13;

p= 0.212

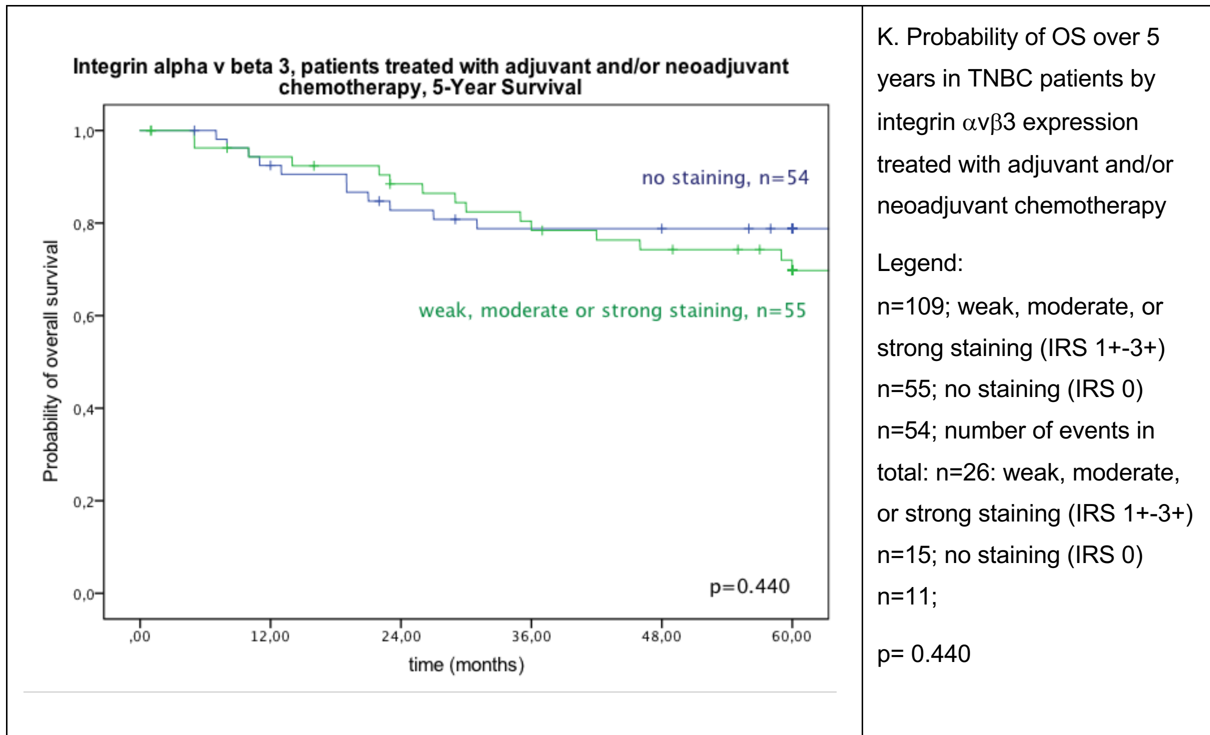
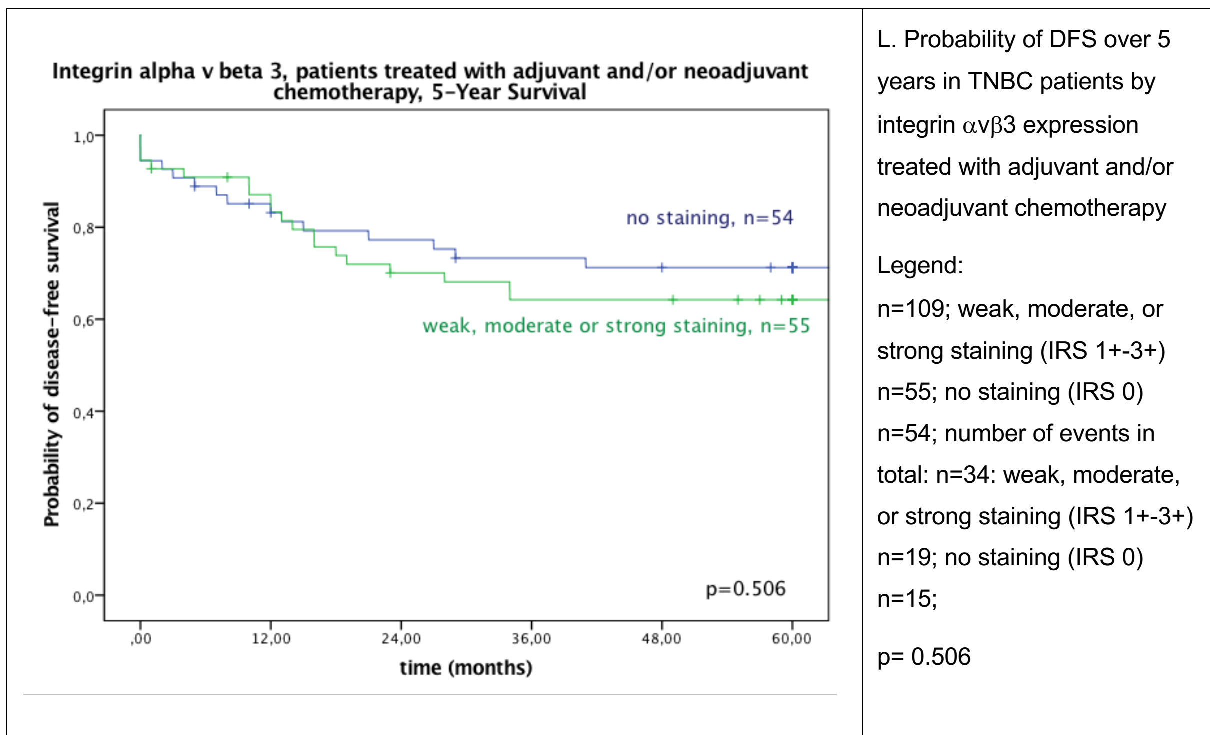


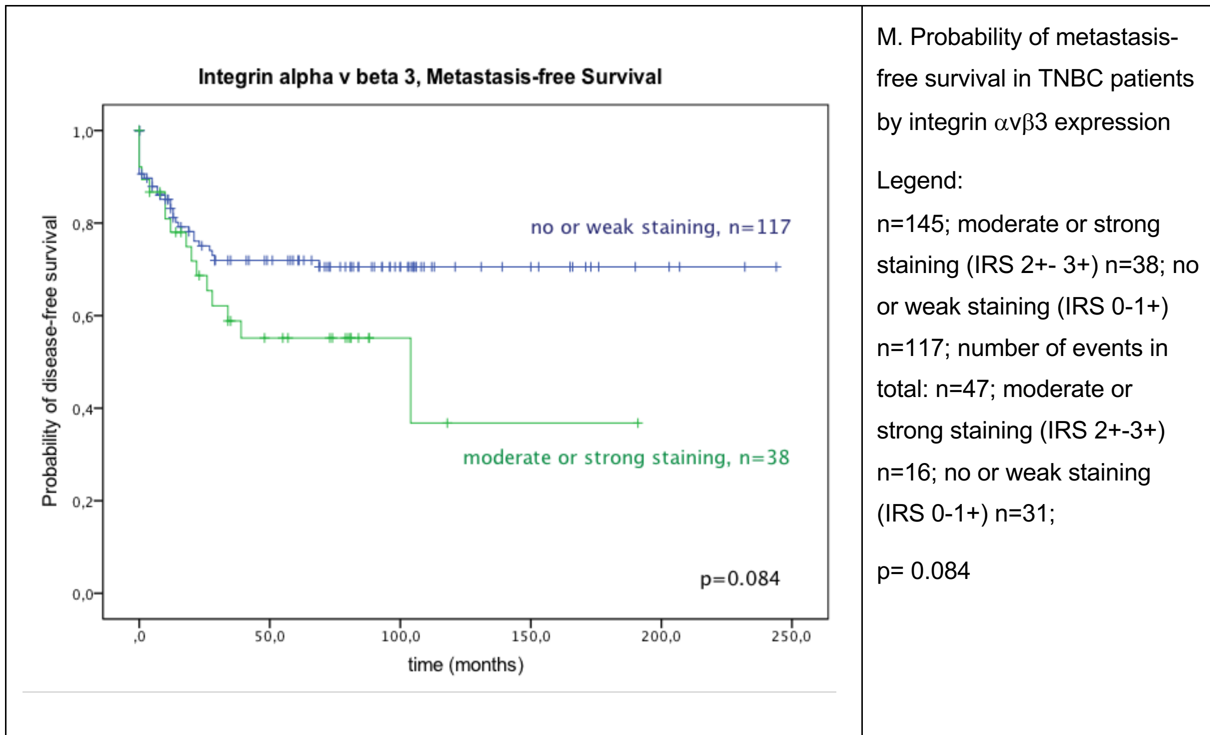
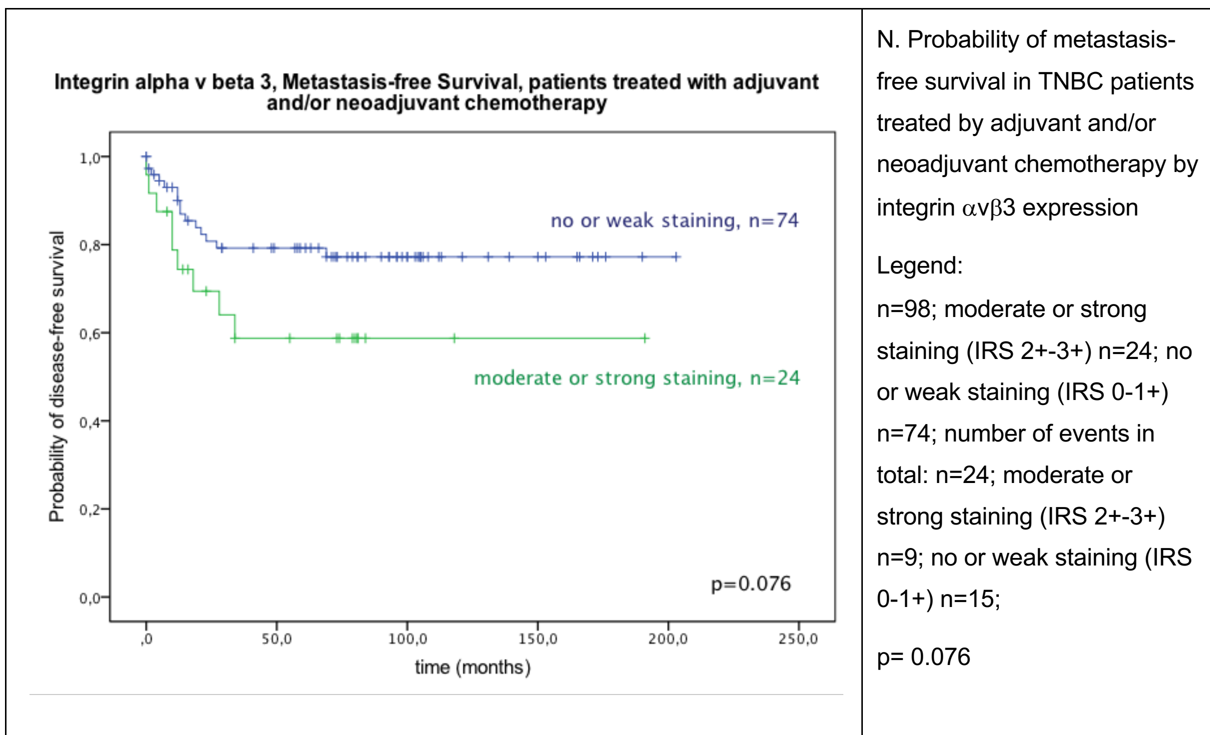
J. Probability of DFS in TNBC patients by integrin αvβ3 expression treated with adjuvant and/or neoadjuvant chemotherapy

Legend:

n=98; weak, moderate, or strong staining (IRS 1+-3+) n=45; no staining (IRS 0) n=53; number of events in total: n=38: weak, moderate, or strong staining (IRS 1+-3+) n=20; no staining (IRS 0) n=18;

p= 0.318

	K. Probability of OS over 5 years in TNBC patients by integrin αvβ3 expression treated with adjuvant and/or neoadjuvant chemotherapy Legend: n=109; weak, moderate, or strong staining (IRS 1+-3+) n=55; no staining (IRS 0) n=54; number of events in total: n=26: weak, moderate, or strong staining (IRS 1+-3+) n=15; no staining (IRS 0) n=11; p= 0.440
	L. Probability of DFS over 5 years in TNBC patients by integrin αvβ3 expression treated with adjuvant and/or neoadjuvant chemotherapy Legend: n=109; weak, moderate, or strong staining (IRS 1+-3+) n=55; no staining (IRS 0) n=54; number of events in total: n=34: weak, moderate, or strong staining (IRS 1+-3+) n=19; no staining (IRS 0) n=15; p= 0.506

	M. Probability of metastasis-free survival in TNBC patients by integrin $\alpha v\beta 3$ expression Legend: n=145; moderate or strong staining (IRS 2+- 3+) n=38; no or weak staining (IRS 0-1+) n=117; number of events in total: n=47; moderate or strong staining (IRS 2+-3+) n=16; no or weak staining (IRS 0-1+) n=31; p= 0.084
	N. Probability of metastasis-free survival in TNBC patients treated by adjuvant and/or neoadjuvant chemotherapy by integrin $\alpha v\beta 3$ expression Legend: n=98; moderate or strong staining (IRS 2+-3+) n=24; no or weak staining (IRS 0-1+) n=74; number of events in total: n=24; moderate or strong staining (IRS 2+-3+) n=9; no or weak staining (IRS 0-1+) n=15; p= 0.076

7.5 Abbreviations

ab .... *Antibody*
AGO .... *Arbeitsgemeinschaft Gynäkologische Onkologie*
ASCO .... *American Society for Clinical Oncology*
BC .... *breast cancer*
BRCA1 .... *Breast cancer 1, early onset*
BRCA2 .... *Breast cancer 2, early onset*
cDNA .... *Complementary desoxyribonucleic acid*
CK .... *Cytokeratin*
CMF .... *Cyclophosphamide, methotrexate, fluorouracil*
DAB .... *3´3´Diaminobenzidine*
DFS .... *Disease-free survival*
ECM .... *Extracellular matrix*
EDTA .... *Ethylenediaminetetraacetic acid*
ER .... *Estrogen receptor*
FFPE .... *Formalin-fixed paraffin-embedded*
h .... *Hours*
HER2 .... *Human epidermal growth factor receptor 2*
HMW-uPA .... *High molecular weight-urokinase-type plasminogen activator*
HPF .... *High power fields*
HRP .... *Horseradish peroxidase*
IHC .... *Immunohistochemistry*
IRS .... *Immunoreactive score*
LMW-uPA .... *Low molecular weight-urokinase-type plasminogen activator*
LOE .... *Level of evidence*
min .... *Minutes*
MTA .... *Manual tissue arrayer*
OS .... *Overall survival*
PAI .... *Plasminogen activator inhibitor*
PARP .... *Poly(ADP-ribose) polymerase*
PET .... *Positron Emission Tomography*
PR .... *Progesterone receptor*
sec .... *Seconds*
SLNB .... *Sentinel lymph node biopsy*
SOP .... *Standard Operating Procedure*
SPSS .... *Statistical Package for the Social Sciences*
TMA .... *Tissue microarray*
TMUGS .... *Tumor Marker Utility Grading System*
TNBC .... *Triple-negative breast cancer*
TNM .... *Tumor size, nodal status, distant metastasis*

tPA .... *Tissue-type plasminogen activator*
uPA .... *Urokinase-type plasminogen activator*
uPAR .... *Urokinase-type plasminogen activator receptor*
VN .... *Vitronectin*
WHO .... *World Health Organization*

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7.8 References

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