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Abbreviations:
DD = dose-dense chemotherapy
HD = high-dose-chemotherapy
MMN = multiple marker negative
ER = estrogen receptor
PR = progesterone receptor

Abstract

Purpose: The goal of this analysis was to characterize the survival impact of angiogenesis in patients with high-risk breast cancer (HRBC), particularly the predictive impact on benefit from dose intensification of adjuvant chemotherapy.

Methods: Formalin-fixed tissue sample of **152** patients treated as part of the WSG AM-01 trial by either high-dose (HD) or conventional dose-dense (DD) chemotherapy were analyzed. Angiogenic activity was measured using microvessel count (MVC) and vascular surface area (VSA) determined by the expression of vascular markers CD31 (n=128) and CD105/endoglin (n=130). Protein molecular breast cancer subclasses were analyzed by k-means clustering(k=5). The univariate impact of factors on event-free (EFS) and overall survival (OS) was tested by log-rank statistics and quantified by univariate Cox analysis. Multivariate survival analysis included including factors significant in univariate analysis, as well as interactions was performed for EFS.

Results: Both VSA/CD31 (p=0.004) and VSA/CD105 (p=0.003) were significantly higher among cases with increased Ki-67. A significant association with molecular subtypes was also found for VSA/CD105: In patients with basal-like/Her-2 subtypes, mean was 1.72 vs. 1.24 in patients with other subtypes (p<0.001). Elevated VSA/CD105 was associated with both significantly decreased EFS (p=0.01) and OS (p=0.02). Increased tumor size and positive Her-2 status were also prognostic for poorer EFS. The benefit of dose intensification for EFS was seen in those low-VSA/CD105 patients. The result was evident both in univariate and in multivariate survival analysis including all factors that were significant at the univariate level.

Discussion: Expression of angiogenesis markers may mirror or confer resistance to chemotherapy in patients with breast cancer, particularly within the context of dose intensified chemotherapy. Highly angiogenic tumors may not derive sufficient benefit from dose intensification of chemotherapy alone. Our findings may serve as a rationale for further exploring anti - angiogenic treatment options in patients with such highly angiogenic tumor subtypes.

Introduction

High-risk primary breast cancer (HRBC) is characterized by extensive axillary lymph node involvement. Ten-year mortality of patients with very high lymph node involvement (i.e., more than 10 positive nodes) is around four times as high as in node-negative patients[1]. Their unfavorable outcome persists despite improvement in drug selection and scheduling in adjuvant chemotherapy of breast cancer (BC). To date, there is only limited data regarding the survival impact of factors within this high-risk population. A small number of studies have reported poor outcome among this subgroup of patients in association with increased tumor size, poor grade, estrogen/progesterone receptor negativity or overexpression of Her-2 and p53 [2-4]

Dose intensification and densification of adjuvant chemotherapy remains one of the most controversial issues in breast cancer systemic therapy. The WSG AM-01 trial, using rapidly cycled tandem high-dose (HD) compared with dose-dense (DD) conventional design, is the only study resulting in a significant improvement of event free survival (EFS) and overall survival (OS) in patients with more than nine positive lymph nodes [5].

Recently, molecular breast cancer subtypes based on microarray analysis have been shown to carry a significant prognostic impact [6, 7]. We previously stratified patients with HRBC into molecular subgroups based on a set of immunohistochemical markers and identified poor outcome of patients with basal-like and Her-2 subtypes [8, 9]. The most pronounced benefit of HD chemotherapy was seen in patients with both highly-proliferating triple-negative and/or high-grade tumors (assessed by conventional immunhistochemistry) and with basal-like and/or Her-2 subtypes (defined by clustering analysis). Moreover, in an independent analysis based on the same study population, an increased efficacy of HD was found among patients with YB-1 positive, i.e. potentially drug-resistant tumors [10]

Several studies as well as comprehensive meta-analyses have shown that the angiogenetic potential of BC as assessed by tumor microvessel density (MVD) correlates with progression and metastasis; it thus predicts for clinical outcome [11-13]. MVD or vascular surface area (VSA) can be evaluated by applying immunohistochemistry to tissue sections using different antibodies. In the past, Factor VIII as well as CD31 have been extensively used to analyze tumor vascularisation. CD 31 is a cellular adhesion molecule (PECAM-1), which has been

shown to reliably detect neoangiogenesis in both malignant and benign tissue, despite the fact that it also labels other hematopoetic cells such as plasma cells. Another marker to detect angiogenetic activity is CD34. However, this molecule also labels fibroblasts as well as hematopoetic stem cells and thus may not be useful under all circumstances for angiogenesis detection. CD105, known as endoglin, is a member of the TGF-receptor family[14]. CD105 is expressed by vascular endothelial cells and plays an important role in angiogenesis of breast cancer[15] and other malignancies [16] In contrast to other angiogenic markers, CD 105 / endoglin seems to be more specific for malignant angiogenesis[14, 16, 17]. Several studies have shown that increased vascular density as assessed by a CD 105 antibody as well as elevated serum CD 105 were associated with poorer overall und disease free survival in different types of cancer [14, 16, 18, 19]. as well poorer survival in patients with higher vascular counts (measured by CD 31 antibody) treated by HD chemotherapy[20]

Although the impact of these markers is still a matter of debate, consensus conferences on tumor angiogenesis have supported the notion that all of these molecules are useful for evaluating angiogenesis[21, 22].

The objective of the present investigation was to identify a hypothetical clinically relevant prognostic and/or predictive signature of angiogenesis factors among HRBC patients treated by different adjuvant chemotherapy dose regimens. To this end, the impact of angiogenesis parameters (CD 105 and CD 31, measured retrospectively) on event-free survival in different therapy arms of a randomized clinical trial was studied using univariate and multivariate interaction analysis.

Material and Methods

The West German Study Group (WSG) AM-01 trial and tumor samples

In the present study, paraffin-embedded breast cancer specimens from 181 randomized HRBC patients with more than nine affected axillary LN were available. These patients were previously enrolled in the prospective multi-center WSG AM-01 trial comparing tandem HD with DD conventional chemotherapy. Details of this trial were described previously[23]. The present study is based on archived tissue samples from the clinical trial, as described elsewhere[2]. The final study population

consisted of 152 cases (84%) after exclusion of 29 cases without sufficient remaining material for accurate morphometric analysis.

After immunostaining, 128 (70%) and 132 (73%) cases were available for analysis of CD31 and CD105, respectively (See consort diagram, figure 1).

Immunohistochemistry (IHC):

Immunostaining for vascular markers CD31, CD105 (Endoglin).

3 µm sections were cut and stained for the vascular markers. Briefly, sections were dewaxed and nonspecific binding was blocked by incubation with 3% H_2O_2 and subsequent incubation with 20% goat serum in phosphate buffered saline. Microwave antigen retrieval (citrate buffer pH 6.0 microwave; 320 watt for 30 min in 0.1 mol/l citrate buffer, pH 6.0) or pressure cooker (120°C, 5min) was utilized. Staining for CD31 and CD105 were performed manually. CD105 (DAKO Cytomation, Germany) was detected using the CSA amplification kit (DAKO Cytomation, Germany) following the manufacturers' protocol without antigen retrieval. CD31 (DAKO Cytomation, Germany) staining was performed by using an antigen retrieval solution (DAKO Cytomation, Germany) for 20 min at 120°C (pressure cooker). After intermediate washing steps, the primary antibody was incubated overnight at 4°C. Subsequently, sections were incubated with a biotinylated secondary antibody and connected with Avidin-Biotin coupled with Alkaline Phosphatase or Peroxidase. Finally, staining was developed with Fast Red reagent or DAB; the reaction was stopped under microscopic control. Paraffin sections from colon carcinomas served as positive controls. As negative controls, primary antibodies were omitted or replaced by non - specific immunoglobulins.

Evaluation of vascular markers by morphometry

Immunohistochemical staining was independently assessed by at least two different observers (A.G.; and H. M. or E. E. or V. A. for either CD105 or CD31). For each case with excellent staining quality (at least three invasive tumor containing areas), up to 6 areas of invasive carcinoma with the highest vascular density were identified by low power magnification (100 X; Zeiss axiophot microscope, (Zeiss AG, Germany)) in the tumor center as well as the tumor periphery based on the criteria of Weidner [24].[24]. The selected areas were digitally photographed at high

magnification (200x) with the AxioVision software (rel 4.5) (Zeiss AG, Germany). Subsequently, every picture was printed and the microvessel density (MVD) and vascular surface area (VSA) were assessed. The latter parameter was evaluated by using a transparent reticule, which was overlayed over every individual print; the vessel area was counted and expressed as percent of the whole area.

Microvessels and vascular surface were assessed in a 0.141 mm^2 area.

Mean and median values of vascular markers were calculated for each individual tumor; the median was used in further analysis. In order to analyze inter-observer variability, 10% randomly selected cases were re-evaluated without knowledge of the primary results by an experienced surgical pathologist (A.G.) for all three markers.

Statistical Methods

Bivariate correlations of continuous variables were assessed by Spearman's correlation; associations among discrete variables were assessed by Fisher's exact test. Associations between continuous and discrete variables were analyzed by the t-test or by one-way ANOVA. The primary endpoint for survival analysis was event-free survival **(defined as time from the randomization to first relapse, secondary malignancy or death**); overall survival was considered to be a secondary endpoint. In survival analysis, variables were classified as follows: age (<50 vs. ≥50 years old), tumor size (≥3 cm vs. <3 cm), tumor grade (3 vs.1 and 2), therapy arm (HD vs. DD), **centrally measured** expression of ER, PR, Her-2, ki-67/MIB-1 (positive (>10% strong nuclear staining) vs. negative), and markers for angiogenesis such as VSA/CD 105 (< sample median vs. ≥ sample median). **Molecular subtypes definition based on expression of 24 proteins was described previously**[8, 9]. The Kaplan-Meier method was used to estimate cumulative survival time probabilities. The log-rank test ($p<0.05$) was applied to test for survival differences by treatment arm (possibly stratified by factors). The study was performed in accordance to the REMARK criteria[25]

A preliminary univariate Cox analysis for EFS was performed on each individual marker; those markers with significant univariate impact, as well as treatment arm, were entered into multivariate forward stepwise Cox analysis for EFS, with main effects in the first block and (factor times therapy) interactions in the second block. To test the biological hypothesis that low levels of angiogenesis markers are predictive for response to HD, the appropriate coding in Cox analysis was zero if ≥ sample median and one if < sample median. Confidence intervals are reported at the 95% level. All statistical calculations were performed using the statistical software package SPSS 17.0 for Windows.

Results

Patients, follow up and treatment arms

The treatment groups of the 152 patients were well balanced in terms of baseline characteristics. Median age of patients was 48 years. Median tumor size was 3.0 cm; patients had median of 15 involved lymph nodes; 42 % were G3 tumors. There was no significant difference in the distribution of characteristics between the entire study population **(median age: 47.5 years, median tumor size: 3.0 cm; median of 15 involved lymph nodes; poor grade: 59 % by decentral and 42% by central assessment)** and the subset presented here.

Of our study population, 81 patients (53 %) were randomized to the HD arm and 71 patients (47 %) to the DD arm. In this collective, median follow-up was 65.5 months (range: 4 - 121 months); (HD: median 72.5 months; DD: median 60 months).

CD31 and CD105 Scoring

Evaluation of MVD revealed a median of 13 microvessels (mean 13.7 ± 5.26) by CD31 and of 11.25 microvessels (mean 12.9 ± 6.66) by CD105. Assessment of VSA showed a median of 2.52 % (mean 2.67 ± 1.14) for CD31 and 1.30 (mean 1.43 ± 0.72) for CD105. For both parameters, the sample median levels (i.e. in CD31: 13 (MVD) and 2.52 % (VSA)) were selected as cut-offs to define two distinct groups (low vs. high vessel density).
Figure 1 shows representative examples (maginification 200x) of tissue specimens with low (A: CD31, C: CD105) and high microvessel count (B: CD31, D: CD105).

For both CD31 and CD105, a significant positive correlation was found between increased VSA and MVD ($p<0.001$ in each case). Conversely, for both VSA and MVD, a significant positive correlation was found between CD31 and CD105 ($p<0.001$ in each case).

Correlations and associations

In bivariate correlation analysis between clinical-pathological variables and angiogenic markers, no correlation was found among age, tumor size, number of involved lymph nodes, tumor grade, hormone receptors (ER/PR) and MVD or VSA (for both CD31 and CD105). However, increased proliferation as determined by MIB-1/Ki-67 expression was significantly associated with increased VSA for CD105 (mean 1.58 vs. 1.19; p=0.003) and CD31 (mean 2.92 vs. 2.32; p=0.004), **but not for MVD of both markers**. A significant association with molecular subtypes was also found for VSA/CD105: In patients with “basal-like” or “Her-2” subtypes, the mean was 1.72, compared to 1.24 in patients with luminal A, luminal B, or multiple marker negative subtypes (p<0.001).

Univariate influence of markers on survival

According to Kaplan-Meier estimates on the entire collective, after median follow up of 65.5 months, VSA CD105 was the only significant angiogenesis factor for EFS (median EFS for VSA CD105 high vs. low: 81 months vs. 46 months, p=0.01) and OS (median OS high vs. low: 104 months vs. 87 months, p=0.02) (Figures 3, 4).

Figures:

Figure 3: Prognostic impact of VSA/CD105 on event free survival and 4. overall survival.

In univariate Cox analysis for EFS, the significant markers for poorer EFS were VSA CD105, as well as the established markers tumor size (≥3 cm) and positive Her-2 status (Table 1).

Table 1. Univariate Cox analysis for EFS (markers and therapy arm).

HD therapy, positive ER and/or PR status and negative Her-2 and low proliferation index as measured by MIB-1/Ki-67 status were favorable predictors for OS (data not shown).

Therapy response

In this studied collective, a trend for increased EFS (median EFS HD vs. DD: 81 vs. 52 months, p=0.08) and significantly better OS (median OS HD vs. DD: 113 months vs. 77 months, p=0.026) in favor of HD was shown after 98 months of median follow up.

EFS and OS by therapy arm in angiogenesis subgroups

Both MVD/VSA of angiogenesis markers were tested for their interaction with therapy but only VSA CD 105 was found to be a significant predictor for efficacy of different chemotherapies.

HD was significantly superior to DD in CD105 VSA low tumors for both EFS (median EFS HD vs. DD: 99 vs. 57 months, p=0.02) and OS (median OS HD vs. DD: not reached vs. 80 months, p=0.01, fugure 6), but not in high CD105 VSA tumors (median EFS HD vs. DD: 41 vs. 49 months, p=0.69, median OS HD vs. DD: not reached vs. 80 months, p=0.38, figure 5)

Figure 5: Event free survival in high-VSA/CD105 tumors by chemotherapy arm

Figure 6: Event free survival in low-VSA/CD105 tumors by chemotherapy arm

Multivariate survival analysis for EFS with interactions

Comparison of treatment effects in high-VSA/CD105 tumors (Fig. 4) versus low-VSA/CD105 tumors (Fig. 5) illustrates the qualitatively different treatment impacts on EFS in these subgroups. To test the biological hypothesis that low levels of the angiogenesis marker VSA/CD105 are predictive for benefit from HD compared to DD, VSA/CD105 was coded as described above (zero if $\geq$ sample median). Thus, the interaction term is 1 for patients with low-VSA/CD105 receiving HD, zero otherwise (either high low-VSA/CD105 or DD).

Consistent with stratified Kaplan-Meier analysis, the benefit of dose intensification for EFS in low-VSA/CD105 patients was also significant in multivariate interaction analysis. In the resulting model [including age, therapy, Her-2 , tumor size, ER, PR, Ki-67, VSA CD 105 and interaction of factors significant in the univariate analysis (Her-2, tumor size and VSA CD/105) with therapy] only the interaction of low VSA and HD therapy (p=0.014; HR=0.367, 95% CI: 0.166-0.815) and positive ER status (p=0.027; HR=0.553, 95% CI: 0.327-0.934) were significant (favorable) factors for EFS.

Discussion:

Endoglin (CD 105) is an integral protein of the TGF-β receptor family, which plays an essential role in regulating cell differentiation and proliferation in malignant tissues through smad proteins as well in haematopoeisis and cardiogenesis and strongly associated with stem cell phenotype of breast cancer cells correlating with basal-like or Her-2 tumor types[26]. TGF-β is expressed almost exclusively on endothelial cells of both peri- and intratumoral blood vessels and on tumor stromal components as shown by de Caestecker et al[27]. CD 105 is a co-receptor of TGF-β-1 and -2 and antagonizes inhibitory effects of TGF-β on proliferation and migration, thus promoting growth and migration of tumor cells[28]. Unlike other TGF-β receptors, endoglin was found to be expressed almost exclusively by vascular endothelial cells. Similarly, CD 31 has been discussed as a potential pan-angiogenesis marker, being expressed also by plasma cells, macrophages and neutrophils [29]. Thus, CD31 seems to be

less specific than CD105 regarding malignant angiogenesis, rather representing angiogenesis in both benign and malignant tissues.

In this study, we have investigated the predictive role of the well established angiogenesis parameter CD31 and CD105 in primary tumor specimens regarding patient outcome in a collective of 152 HRBC patients treated by either conventional DD or tandem HD chemotherapy within the randomized WSG AM-01 trial as reported previously [5]. The results of the trial support the significant benefit of rapidly cycled tandem HD over conventional dose DD EC-CMF with regard to both EFS and OS in HRBC. However, despite these findings, HD and DD remains a controversial issue in adjuvant breast cancer therapy due to higher toxicity, costs, and inhomogeneous patient collectives treated within the respective trial. Despite incorporation of modern highly effective agents (such as taxanes)[30], the outcome in this patient group remains poor. Moreover, no predictive marker for benefit from dose-density or dose-increase has been established for clinical use so far.

We now show for the first time, that VSA measured by CD 105 is significantly associated with increased proliferation as well as basal-like and Her-2 molecular subtypes, which are known to impact unfavorably on disease outcome [7, 8]. In our analysis, VSA/CD105 was an independent prognostic marker for decreased survival both by univariate and multivariate analysis.

Moreover, our study revealed a predictive value of VSA/CD 105 with regard to efficacy of dose-intensification. In patients with low angiogenetic activity, a significant benefit of HD was observed: Median EFS in this subgroup was 99 months (compared to 57 months for high angiogenesis tumors) in favor of HD. In contrast, in the VSA CD105 high subgroup, median EFS rates were similarly short in both therapy arms.

The impact of VSA/CD 105 on efficacy of HD for improved EFS persisted in interaction analysis: patients with highly angiogenic tumors derived no significant benefit from dose-intensification of chemotherapy in this collective. Thus, highly dysregulated angiogenesis was associated with apparent resistance to both chemotherapy regimens: In our study, patients with low angiogenic tumors (a prognostically favorable factor) treated by HD had a HR of 0.448 for recurrence as compared to those with high angiogensis treated by any regimen. In contrast, in the collective as a whole[2], high tumor grade, which is a prognostically unfavorable factor, was predictive for response to HD.

Poor outcome of patients with increased angiogenesis as measured by CD 105 is in line with previously published studies in breast cancer and other malignancies[31]. Kumar et al. showed shorter disease-free and overall survival in patients with higher microvessel density in a study with 106 breast cancer patients[14]. Dales et al. reported decreased survival rates in patients with increased counts for CD105 and CD 31 positive microvessels. However, a statistically significant effect on DFS was only observed for CD105 in their multivariate analysis[32] **mirroring CD 31 as less specific marker for tumor angiogenesis[16], what is in line with with our study. Despite a significant correlation of CD31 with CD105, , no prognostic impact of CD31 was found in our study in contrast to previously reported significant negative prognostic impact of MVD measured only by CD 31 in similar collective of patients treated by HD[20]. However this study supports our data in term of relative chemotherapy resistance of highly angiogeneic tumors.**

Elevated endoglin levels in serum have also been associated both with presence of metastatic disease[33] as well as decreased response to endocrine therapy and poorer survival in metastatic breast cancer[34]. The predictive effect of CD 105 with regard to dose-dependent efficacy of chemotherapy correlates also with the data of neoadjuvant CEF chemotherapy as reported by Beresford et al[35]. In this study, breast cancer patients with lower CD 105 tumor counts experienced increased chemotherapy benefit compared to patients with higher levels. Similar to our report, global angiogenesis as measured by CD 34 or CD 31 did not reach statistic significance in this study.

Based on our results, we hypothesize that angiogenesis and hypoxia are key factors in mediating chemotherapy resistance. Hypoxia has been shown to induce endoglin expression in vitro and in vivo[36, 37]. Functionally, endoglin causes anti-apoptotic signals in hypoxic cells. But, as shown both in our investigation and in other studies, altered (mostly up-regulated) angiogenesis reflects distinct, more aggressive tumor types (e.g. basal-like or Her-2 subtypes[38]) and may be associated with or even cause chemoresistance. **The reason that only tumors with low VSA better respond to therapy may be explained by the fact that slower growing tumors induce a less chaotic angiogenic network compared to high angiogenic tumors and thus facilitate a better response to intense chemotherapy regimens. In fact it is already known that histologic high vessel count does not per se indicate a pathophysiological functional dense vascular network susceptible for therapy**

and disrupting of neovasculature could improve chemotherapy delivery to tumor cells [39, 40]. However, in addition, methodic aspects may in part explain the different results when comparing CD105 and CD31.

Targeting of TGF- β signaling leads to depression of angiogenesis (among others through endoglin) and can reverse a stem cell phenotype to a more-differentiated luminal phenotype[41] These effects can be potentially inhibited by dual blockade of TGF-beta and endoglin pathways[42]. Moreover, anti-endoglin antibodies as single agents as well as CD 105 as target for oral DNA vaccine have also been reported as effective in suppressing or preventing tumor progression and prolonging survival in in-vitro models [43, 44]..

Anti-angiogenic therapies are emerging agents for advanced BC (e.g. VEGF antibody bevacizumab) treatment and are currently investigated in (neo)-adjuvant breast cancer clinical trials. These substances are reported to prolong progression-free survival with so far marginal effect on OS rates [45]. This illustrates that optimal use of antiangiogenic agents, preferably in pre-selected patient collectives, are urgently needed for substantial improvement of therapy efficacy in HRBC.

Despite the randomized nature of the patient collective analyzied in our study, the present investigation constitutes a retrospective study performed on archived material and is thus subject to potential biases; in particular, n=29 cases needed to be excluded due to poor immunohistochemical staining quality for both markers. This problem is most likely explained by different fixation protocols of the fifty contributing pathology departments.

In summary, our study suggests an improved efficacy of chemotherapy dose-intensification in HRBC in the low-angiogenesis group, as determined by VSA CD105. The VSA/CD105 high phenotype is associated with the aggressive basal-like and Her-2 subtypes. It seems to be resistant to adjuvant chemotherapy of its good overall efficacy in these subtypes with mostly poorly differentiated tumors. To the best of our knowledge, this is the first report on the importance of VSA in a randomized HRBC collective. Our data indicates that increased angiogenesis may be involved in chemoresistance in aggressive BC despite of the overall high chemosensitivity of this breast cancer subtype[46]. These hypothesis-generating findings warrant validation in future prospective trial concepts.

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Figure 1. Consort diagramm

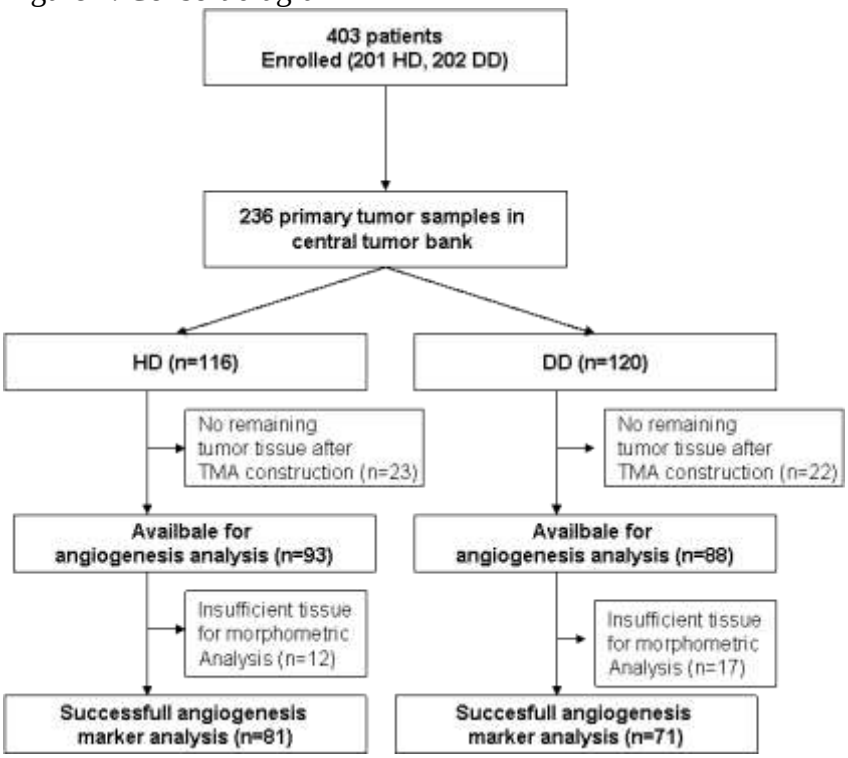

Figure 2.

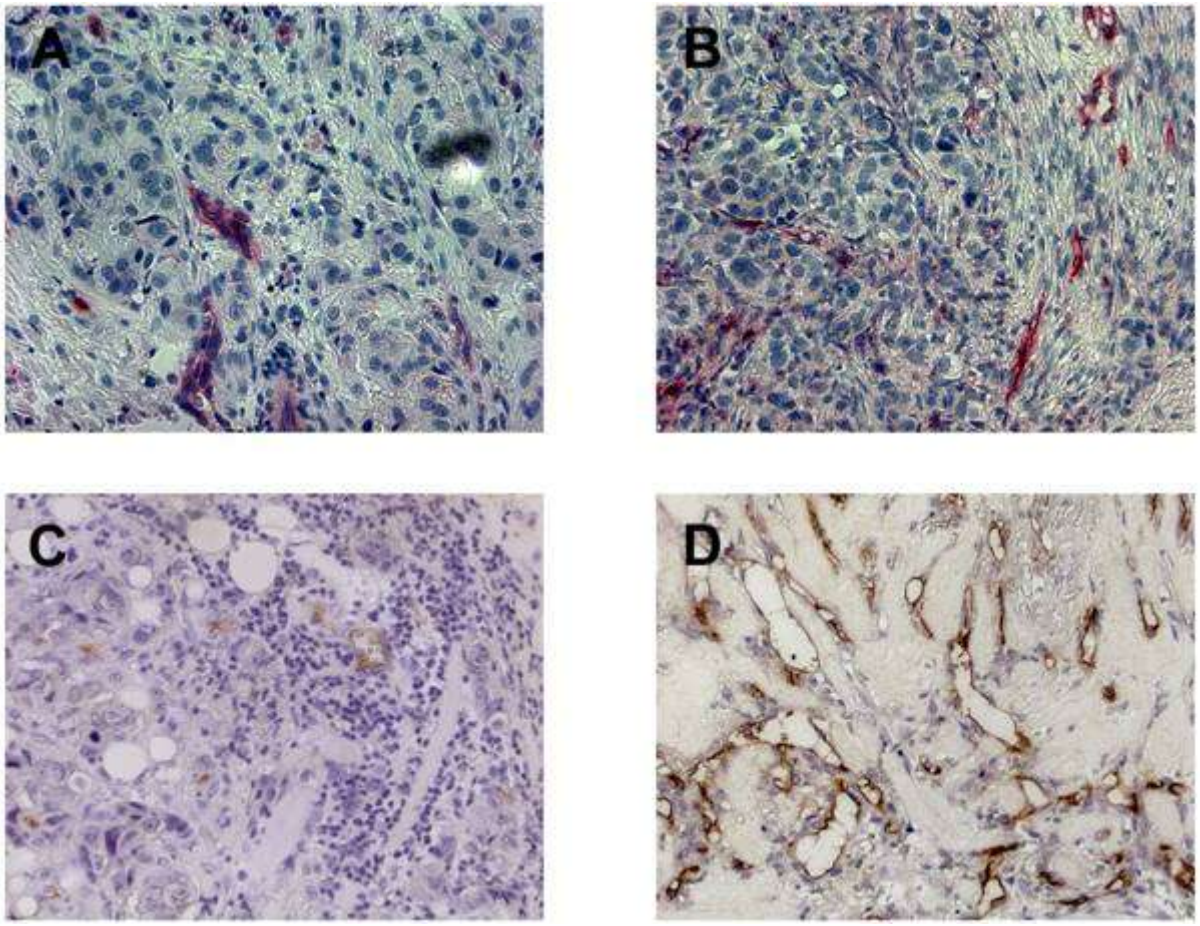
A
B
C
D

Figure 3.

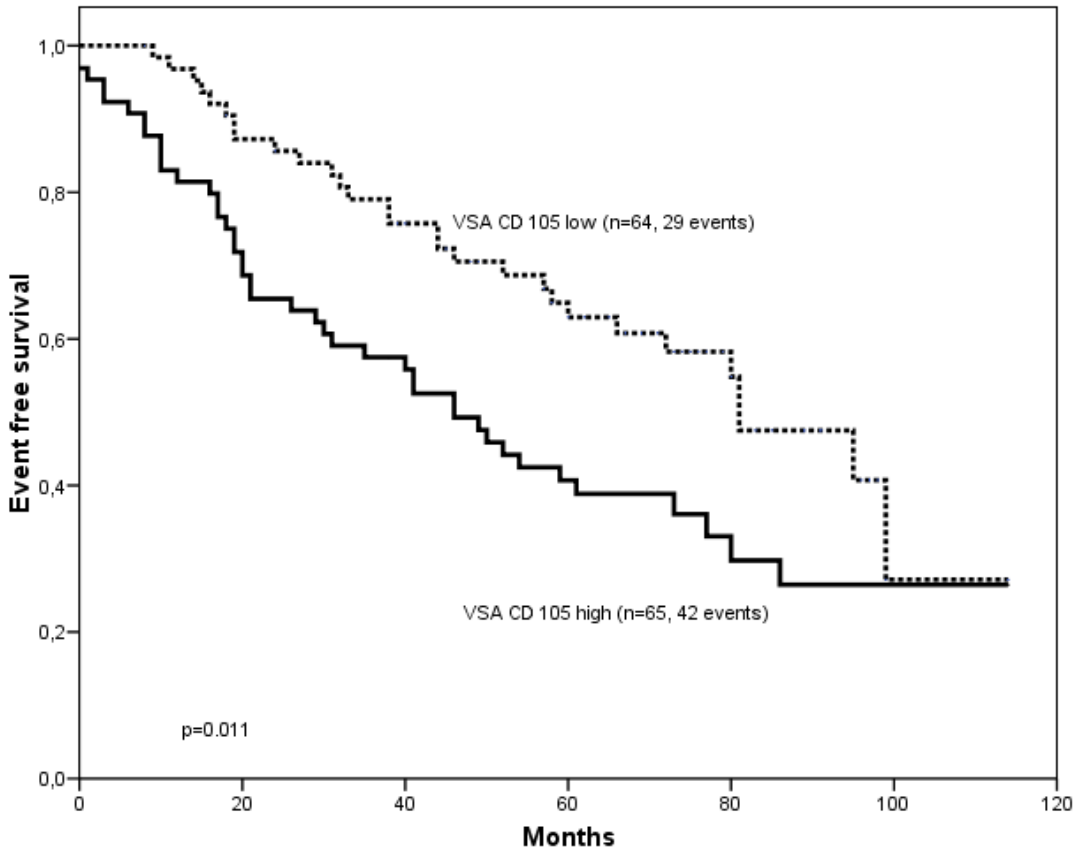
1,0
0,8
0,6
0,4
0,2
0,0
Event free survival
VSA CD 105 low (n=64, 29 events)
VSA CD 105 high (n=65, 42 events)
p=0.011
0
20
40
60
80
100
120
Months

Figure 4.

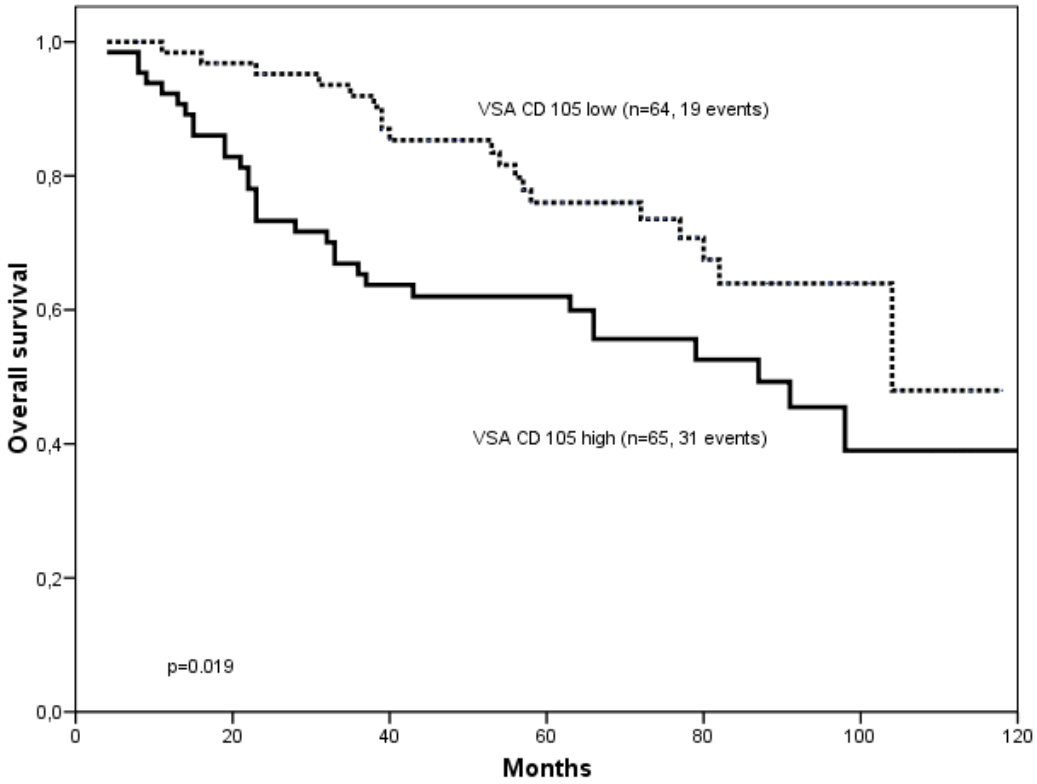

Figure 5.

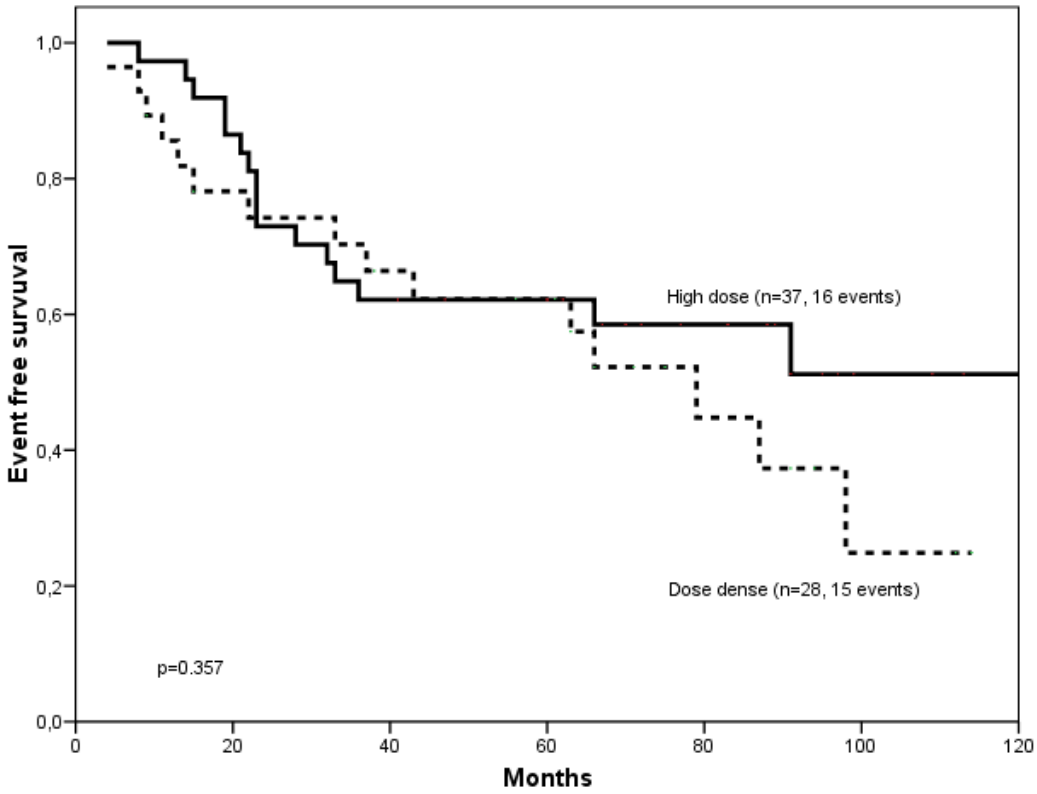

Figure 6

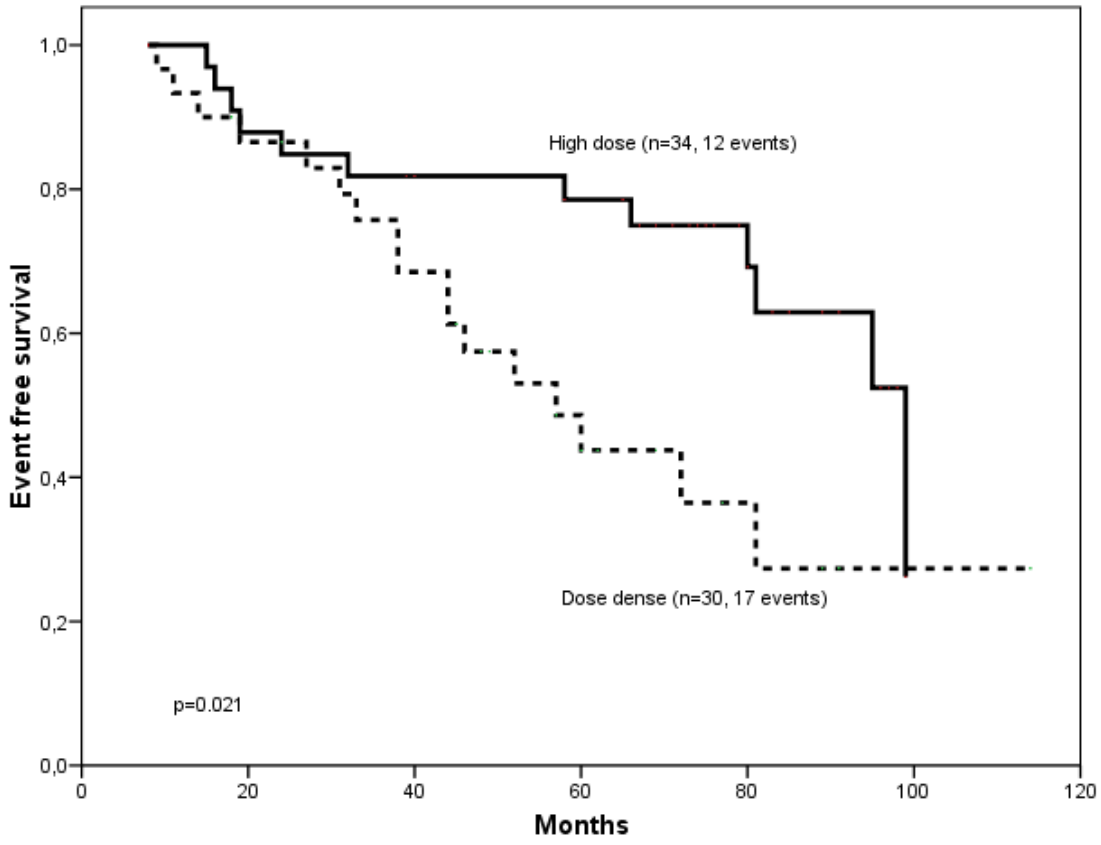

Table 1.

Event Free Survival

		Univariate	
Factor	Coding	p	Hazard ratio[§] [95%-CI]
Therapy	HD vs. DD	0.08	0.68 [0.45-1.05]
Tumor size	≥3cm vs. <3cm	0.037	1.58 [1.03-2.41]
VSA/CD105	Negative vs. Positive	0.01	0.55* [0.34-0.88]
ER	Positive vs. Negative	n.s.	0.68 [0.43-1.07]

PR	Positive vs. Negative	n.s.	0.76 [0.48-1.21]
Grade	G3 vs. G1/2	n.s.	1.07 [0.67-1.67]
Her-2	Positive vs. Negative	0.025	1.76 [1.07-2.89]
Ki-67/MIB-1	Positive vs. Negative	n.s.	1.46 [0.93-2.31]

* lower VSA CD 105 levels are associated with reduced risk for relapse.

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