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A low nuclear-to-cytoplasmic ratio of VDR expression is an independent prognostic marker in breast cancer

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Abstract: The aim of this retrospective study was to analyze the prognostic value of cytoplasmic versus nuclear expression of the vitamin D receptor (VDR) in breast cancer (BC) tissue samples and to relate the results to clinicopathological parameters. VDR expression was assessed in 319 primary breast cancer patients using the Remmele and Stegner immunoreactive scoring (IRS) system. Follow-up data were obtained from the Munich Cancer Registry. The correlation with overall survival (OS) and disease-free survival (DFS) was calculated using univariate and multivariate analyses. Correlation analysis revealed a correlation between nuclear VDR expression and improved outcomes for both OS (p=0.004) and DFS (p=0.001). Conversely, cytoplasmic VDR expression was significantly associated with a shorter OS (p=0.003) and DFS (p<0.001). Additionally, both cytoplasmic and nuclear VDR expression were found to be independent markers of DFS (p<0.001; p=0.021) when examined alongside clinicopathological parameters. Moreover, nuclear VDR expression was positively associated with lower lymph node invasion (pN; p=0.01). For triple-negative patients, cytoplasmic VDR expression was found to have a significant inverse correlation with DFS (p<0.001). Lastly, the ratio of VDR nuclear/cytoplasmic was identified as an auxiliary independent marker of DFS and OS. These findings strongly indicate that the subcellular localization of VDR is crucial in determining BC prognosis. The expression of nuclear VDR appears to have a protective effect, while cytoplasmic VDR is associated with a more aggressive disease course. The data may help identify subgroups of patients with high-risk BC, possibly leading to specific options for targeted tumor therapy.

K**eywords:** Breast cancer; Vitamin D receptor; Subcellular localization; Immunohistochemistry; Prognosis; Overall survival; Disease-free survival

1. Background

More than 2.3 million cases of breast cancer (BC) occur each year, making it the most common cancer among adults. In 95% of all countries, BC is the first or second leading cause of female cancer deaths [1].

BC diagnostics, management, and treatment are multifarious and diverge based on clinical tumor subtypes (Tao et al., 2015; Harbeck et al., 2019). The possibilities of BC therapy have expanded at a breathtaking speed over the past decades, presenting a variety of therapeutic approaches depending on the therapy intention, such as in adjuvant, neoadjuvant, or metastatic settings. Therapeutic regimens comprise surgical interventions, radiation, and systematic procedures, i.e., chemotherapy and endocrine therapy (Cauley et al., 2001; Fisher et al., 2005; Goss et al., 2011). Despite the tremendous development of personalized BC therapies in recent years, including aromatase inhibitors, hormone receptor modulators, and monoclonal antibodies targeting human epidermal growth factor receptor 2 (HER2), consistently high mortality rates due to tumor metastasis persist (Fisher et al., 1998; Khazal and Hill, 2015). Therapies targeting nuclear receptors (NRs), such as the estrogen receptor (ER) and the progesterone receptor (PR), are very effective therapeutic options used both for prevention and treatment (Muscat et al., 2013). Endocrine therapy regimens account for the almost 30% decrease in BC-associated mortality, making it necessary for the treatment of hormone receptor-positive (HR+) BC (Shaikh et al., 2015; Giordano et al., 2018; AWMF). Several clinical studies have already demonstrated a strong correlation between the expression of steroid hormone receptors, such as ER and PR, and disease progression (Ditsch et al., 2012; Welsh, 2017; Lang et al., 2017; Zhang et al., 2017; Reinert et al., 2018). To date, however, some tumors are resistant to these therapeutic options, and the identification of new potential therapeutic targets is a necessary research topic (Liu et al., 2017).

NRs function primarily as transcription factors in the nucleus when activated by binding lipophilic hormones (Escriva et al., 2004; Dawson and Xia, 2012). Besides the well-known ER and PR, NRs such as vitamin D receptor (VDR), retinoid X receptor (RXRα), thyroid hormone receptors (THRs), peroxisome proliferator-activated receptor (PPAR), and others are notably involved in the pathophysiology of BC and other cancer entities (Hua et al., 2009; Zehni et al., 2019; Ditsch et al., 2020). Analysis of NR expression in different intracellular compartments indicates a specific prognostic value that depends on subcellular localization (Shao et al., 2020). In a previous work, we showed a significant correlation between the expression of cytoplasmic RXRα and a shorter outcome in terms of overall survival (OS) and disease-free survival (DFS) in BC, whereas nuclear RXRα expression appears to be a protective factor (Ditsch et al., 2020). In addition, nuclear THR has been confirmed to have cancer-promoting activities in BC development (Shao et al., 2020). In epithelial ovarian cancer, however, high nuclear THR localization was identified to be a positive predictive factor for OS (Ditsch

et al., 2020). Further research in ovarian cancer demonstrated a direct link between the cytoplasmic localization of VDR and reduced OS (Czogalla et al., 2020).

Vitamin D and its receptor became significant in the past when it was demonstrated that this signaling pathway was involved in cardiovascular disease, diabetes, and cancer (Garland et al., 2006; Wang et al., 2008; Pittas et al., 2010). A reduced risk of developing certain cancer types, i.e., breast, ovarian, colorectal, gastric, hematological, kidney, lung, pancreatic, liver, prostate, and skin cancer, is associated with high circulating levels of vitamin D (Deuster et al., 2017). 1α-25 Dihydroxyvitamin D3 (calcitriol) is a seco-steroid hormone and the biologically active metabolite of vitamin D that regulates calcium and phosphate levels in bone metabolism and homeostasis (Holick and Chen, 2008) and affects proliferation and differentiation in carcinoma cells (Kim et al., 2005).

Calcitriol exerts its functions on different tissues by binding to nuclear VDR (Christakos et al., 2003), which interacts with other transcription factors. The best-studied one is probably RXR (Zhang et al., 2011). The interaction of VDR with RXR suggests that RXR could have a regulatory effect on gynecological cancers. Supporting this hypothesis, overexpression of RXR and VDR has been demonstrated in BRCA1-mutated breast cancer cases, predicting OS (Ferlay et al., 2015). The anti-proliferative impacts of calcitriol are believed to be mediated via the nuclear pathway by binding the activated receptor to vitamin D-responsive elements (VDRE) (Cross et al., 1997; Friedich et al., 1999; Omdahl et al., 2002; Carlberg, 2003). VDR has been found in 30 different tissues, where it influences gene expression. VDR expression in the mammary gland undulates during the maturation of the female body, beginning during puberty and peaking during pregnancy and lactation (Welsh, 2017). However, in BC, the expression of VDR is inversely linked to higher cancer incidence, disease progression, and worse prognosis (Ditsch et al., 2012).

Considering the major role of VDR in the etiology of cancer, it appeared necessary to further investigate its behavior in BC. Until now, there has been no analysis of VDR subcellular localization as a prognostic factor in human BC specimens. New knowledge may be promising regarding individualized targeted BC therapy. This survey aimed to outline the prognostic role of cytoplasmic versus nuclear expression of VDR in BC and to correlate the results with clinicopathological criteria.

2. Materials and Methods

2.1. Patient collective

The TC (total collective) (Table 1) of this study included 319 primary BC patients who underwent surgery at the Department of Gynecology and Obstetrics of the Ludwig Maximillian University in Munich, Germany, between 2000-2002. Follow-up data were retrieved from the Munich Cancer

Registry after an observation period of 10 years. The median age of the TC was 59.09, with a standard deviation of ± 13.1. Tumor focality was determined by using clinical diagnostics such as ultrasound, X-ray, or magnetic resonance imaging. Tumor grading was determined according to the common Bloom and Richardson grading system (Elston and Ellis, 1991). According to the Union for International Cancer Control (UICC), the TNM staging at initial diagnosis was defined for each patient (Benson et al., 2003). Hereby, the primary tumor size (pT), lymph node involvement (pN), and distant metastasis (pM) were assessed. VDR expression was microscopically evaluated by using the immune-reactive scoring system of Remmele and Stegner (IRS) (Giordano et al., 2018).

Table 1. Patient characteristics of the total collective; pT = size of the tumor, pN = spread of cancer to nearby lymph nodes, pM = spread of cancer from one part of the body to another, VDR = vitamin D receptor

Patient characteristics	*n* (%)
Age (years)	Median 59.1 ± 13.1 (S.D.)
Tumor foci	Unifocal 173 (54.2) Multifocal 146 (45.7)
Histology	No special Type (NST) 188 (61.4) Non-NST 118 (38.5)
Tumor grading	G1 or G2 165 (52.2) G3 151 (47.7)
pT	pT1 197 (64.3) pT2-pT4 109 (35.6)
pN	pN0 166 (54.2) pN1-pN3 140 (45.7)
pM	pM0 239 (78.1) pM1 67 (21.8)
Nuclear VDR	Negative 124 (38.9) Positive 168 (52.7)
Cytoplasmic VDR	Negative 155 (48.6) Positive 143 (44.8)

2.2. Patient treatment

The treatment of this cohort was previously published in other research works of this study group (Zehni et al., 2019, 2020, 2021a,b; Weissenbacher et al., 2010, 2013). The primary surgical treatment involved depended on disease progress: breast-conserving therapy or modified radical mastectomy. In the case of lymph node involvement, patients received chemotherapy according to the guidelines of the Cancer Treatment Center of Munich at that time. The chemotherapy regimen consisted of six cycles of CMF

every 21 days (cyclophosphamide: 600 mg/m^2 body-surface area; methotrexate: 40 mg/m^2; and 5-fluorouracil: 600 mg/m^2). Adjuvant endocrine therapy with tamoxifen 20-30 mg/day was prescribed for the postmenopausal, hormone receptor-positive patients in this study. When Gonadotropin-releasing hormone (GnRH) analogs came on the market in the later years of the follow-up period, the premenopausal cohort was supplemented with GnRH analogs. In the case of contraindications for the abovementioned substances, patients received aromatase inhibitors. Since the guidelines for BC treatment have substantially changed within the follow-up period of the study, the authors did not include further oncological treatment details.

2.3. Immunochemistry

The tissue samples gathered for immunohistochemistry to identify VDR expression were stained according to previously published methods, described in brief below (Ditsch et al., 2012; Heublein et al., 2017; Zehni et al., 2019; Czogalla et al., 2020). The formalin-fixed and paraffin-embedded sections were dewaxed for 15 minutes with xylol and then rehydrated in three ascending steps in 70-100% concentrations of alcohol. Subsequently, the sections were exposed for 10 min in a pressure cooker using sodium citrate buffer (pH 6.0), containing 0.1 M sodium citrate in distilled water and 0.1 M citric acid for epitope retrieval. After cooling, the slides were washed twice in phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (H_2O_2; Merck, Darmstadt, Germany) in methanol for 20 min. At room temperature for 20 minutes, nonspecific binding of the primary antibodies was prevented by incubating the sections with diluted normal serum (10 mL PBS containing 150 µl horse serum; Vector Laboratories). After incubation with the primary antibodies (anti-VDR, mouse IgG2a, monoclonal, Serotec, Puchheim) for one hour at room temperature, the PBS washing steps were repeated. For 30 minutes, the slides were then incubated with diluted biotinylated anti-serum secondary antibody (10 ml PBS containing 50 µl horse serum, Vector Laboratories, CA) at room temperature. Then, the cells were incubated with the avidin-biotin-peroxidase complex (diluted in 10 mL PBS; Vector Laboratories) for 30 minutes. After repeated PBS washing steps, visualization was achieved with substrate and chromogen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 8–10 min. As a last step, slides were counterstained with Mayer 's acidic hematoxylin, dehydrated in an ascending series of alcohol reaching from 50 to 98%, and then covered with xylol.

2.4. Staining evaluation

To quantify the specific VDR immunoreactivity in the nuclei and cytoplasm, the semiquantitative immunoreactive scoring system of Remmele and Stegner (IRS) was used (Remmele and Stegner, 1987).

The intensity and distribution pattern of the staining reaction was evaluated by two independent blinded observers. For staining evaluation, a light microscope by Leitz (Immuno-histochemistry Type 307-148.001 512 686, Wetzlar, Germany) and a 3CCD color camera (JVC, Victor Company of Japan, Japan) were used.

The IRS method has been previously described and applied in numerous studies by our group (Zehni et al., 2019, 2020, 2021a,b). In brief, the scoring system ranges from 0 to 12. For that, the staining intensity (score 0 = no staining, score 1 = weak staining, score 2 = moderate staining, score 3 = strong staining) was multiplied by the percentage of positively stained cells (0: no staining, 1: ≤10% of the cells, 2: 11-50% of the cells, 3: 51–80% of the cells and 4: ≥81% of the cells). For each tissue sample, VDR staining in the nucleus and cytoplasm was evaluated in parallel, with the separate determination of nuclear and cytoplasmic IRS. Cut-off scores with an IRS ≥ 1 were defined as positive for either nuclear or cytoplasmic VDR expression.

2.5. Ethical approval

After all diagnostics had been completed, the tissue samples used in this study were retrieved from the archive of Gynecology and Obstetrics, Ludwig Maximilian University in Munich, Germany. All patients gave their consent to participate in the study. Patient data and clinical information from the Munich Cancer Registry were fully anonymized and encoded for statistical analysis. The study was performed according to the standards set in the Declaration of Helsinki in 1975. The current study was approved by the Ethics Committee of Ludwig Maximilian University, Munich, Germany (approval number 048-08, 18th March 2008). The authors were blinded to the clinical information during the experimental analysis.

2.6. Statistical analysis

For the statistical analysis, the IBM Statistical Package for Social Sciences (IBM SPSS Statistic v26.0 Inc., Chicago, IL, USA) was used. In an implied manner, the results were inserted into the SPSS database, building the TC. The TC was tested for significance using the different statistical devices listed below. To be considered significant, a p-value <0.05 was determined in this study. All p-values and the number of patients analyzed in each group are listed for each chart. To assess the distribution of clinical-pathological variables, the chi-squared test was used. Spearman's analysis tested for correlations between immunohistochemical staining findings. By applying the nonparametric Kruskal-Walli's test, differences between cytoplasmic and nuclear VDR expression regarding the set prognostic markers were tested for significance. As prognostic markers in our study, OS and DFS (in years) were set. With

Kaplan–Meier curves, the differences in patients' OS and DFS were illustrated, and the chi-square of the log-rank test tested for significance. In addition, multivariate analyses were conducted by using Cox regression for the set prognostic markers. The following factors were included: pT and pN of the TNM staging system, grading, histology type, and Her2-neu-, estrogen-, and progesterone receptors.

3. Results

3.1. Nuclear and cytoplasmic VDR in BC samples

Figure 1 shows a selection of the immunohistochemically stained VDR in BC tissue. Positively stained cells appeared in a brownish color, and unstained tissue appeared blue (Fig. 1 a-d). To assess the specificity of the immunoreactions, negative and positive controls were performed. Human placenta tissue sections incubated with preimmune IgGs (supersensitive rabbit negative control, BioGenex, Fremont, CA, USA) instead of the primary antibody served as a negative control and are colored blue. As positive controls, we used human placenta and vaginal samples for VDR detection. Here, positively stained cells appeared in a brownish color. Pictures were taken with a digital charged-coupled device camera system (JVC, Tokyo, Japan).

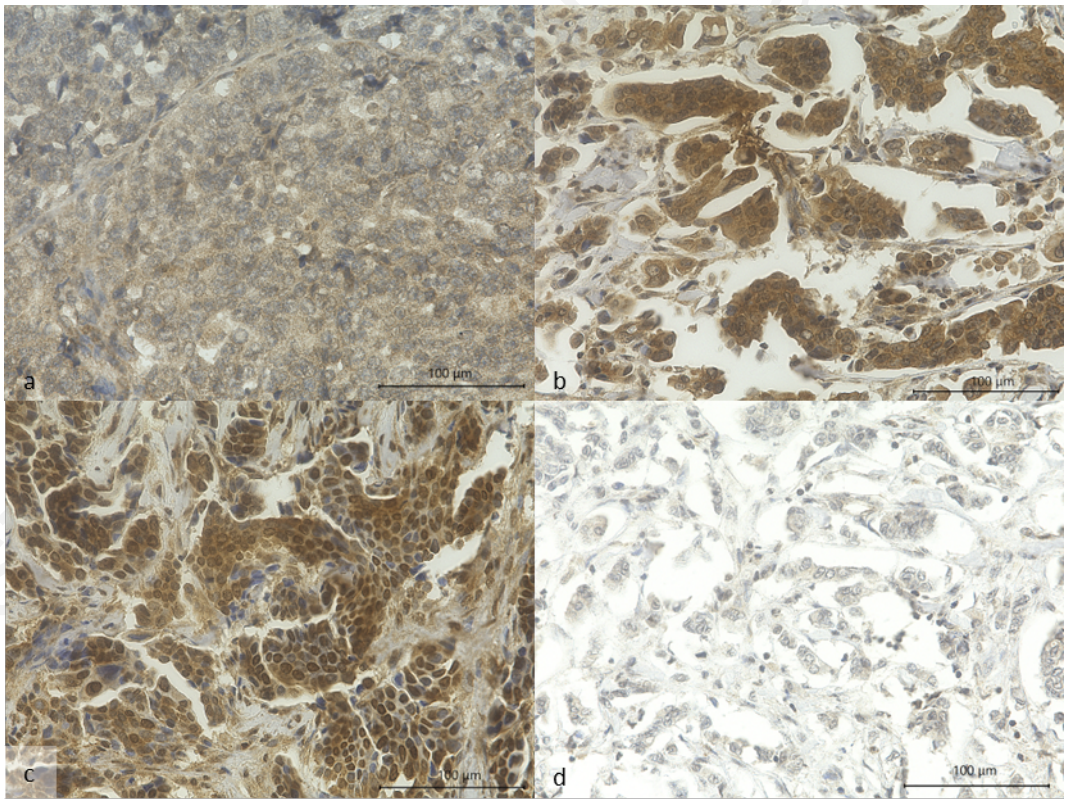
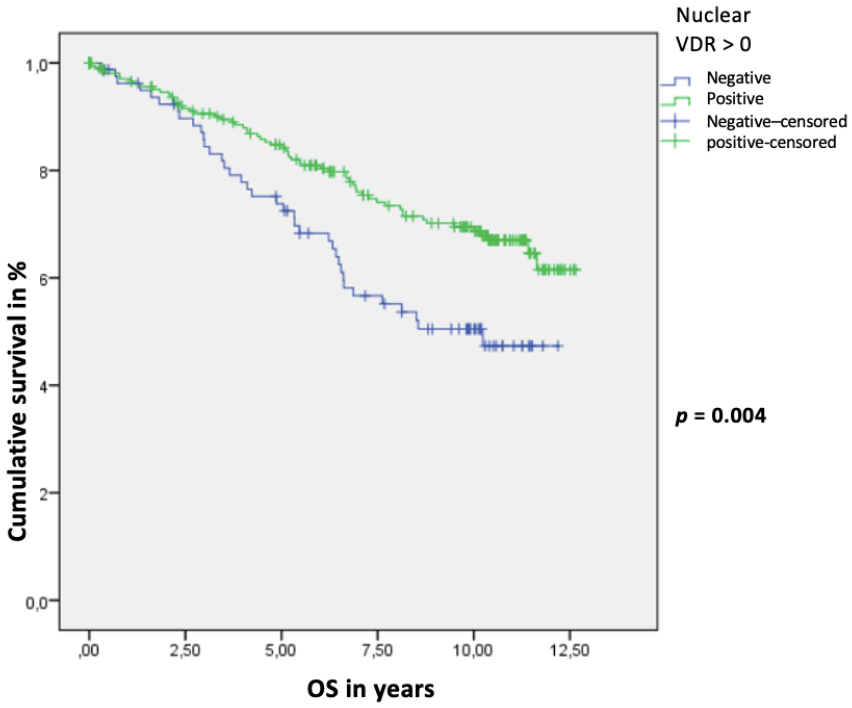


Figure 1. Immunohistochemical staining of VDR in human breast cancer samples is illustrated in (**a-d**). (**a**) Positive cytoplasmic VDR expression only; 25x (scale bar = 100 µm). (**b**) Positive nuclear and cytoplasmic VDR expression with a nucleocytoplasmic IRS ratio of 10:6; 10×, scale bar = 200 µm. (**c**) Positive nuclear VDR expression only, 25x (scale bar = 100 µm). (**d**) Negative nuclear and cytoplasmic VDR expression; 25x (scale bar = 100 µm).

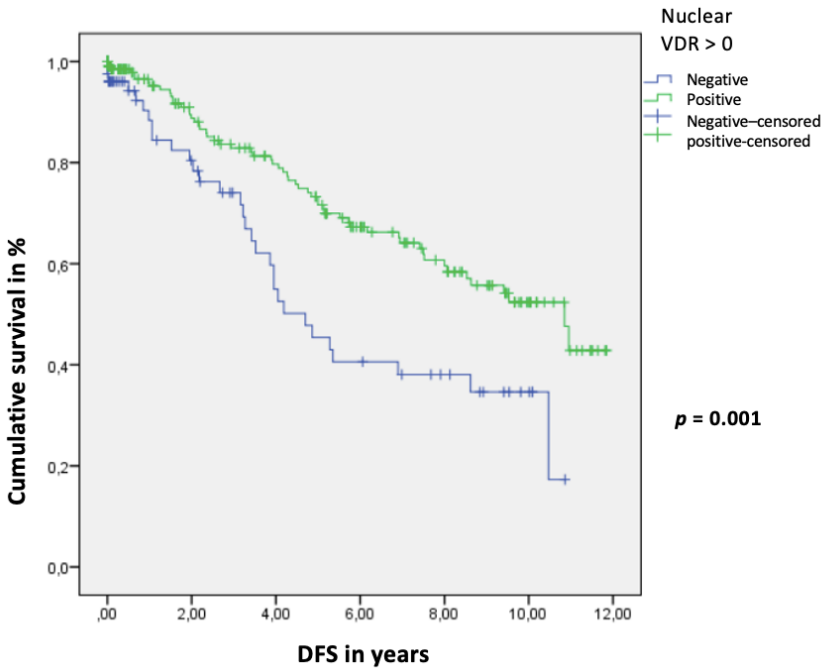
3.2. Nuclear VDR expression correlates significantly with an improved BC prognosis

In this cohort, nuclear VDR expression revealed a significant positive association with BC prognosis. Patients showed a significantly longer OS when their tumors expressed nuclear VDR. This was visualized in the Kaplan-Meier curve shown in Figure 2, and the log-rank test indicated a significant *p-value* of 0.004. Not only OS but also DFS was significantly positively affected by nuclear VDR expression. These results are displayed in Figure 3, where the Kaplan-Meier curve shows that patients with nuclear VDR expression have a significantly longer DFS (p=0.001). Supporting these results, multivariate Cox regression revealed that nuclear VDR expression is an independent marker of DFS (HR 0.574, 95% CI 0.358-0.921, p=0.021) (Table 2).



	Mean OS time (years)			
			95% confidence interval (CI)	
Nuclear VDR	Mean	SEM	lower border	higher border
Negative (n=124)	8.357	0.477	7.421	9.293
Positive (n=168)	10.101	0.286	9.540	10.662
Overall (n=292)	9.662	0.253	9.165	10.159

Figure 2. Kaplan-Meier survival analysis of positive and negative nuclear VDR expression in relation to OS. The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.



Mean DFS time (years)				
			95% confidence interval (CI)	
Nuclear VDR	Mean	SEM	lower border	higher border
Negative (n=82)	5.837	0.575	4.711	6.964
Positive (n=216)	8.310	0.359	7.607	9.014
Overall (n=298)	7.737	0.320	7.109	8.365

Figure 3. Kaplan-Meier survival analysis of positive and negative nuclear VDR expression in relation to DFS. The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.

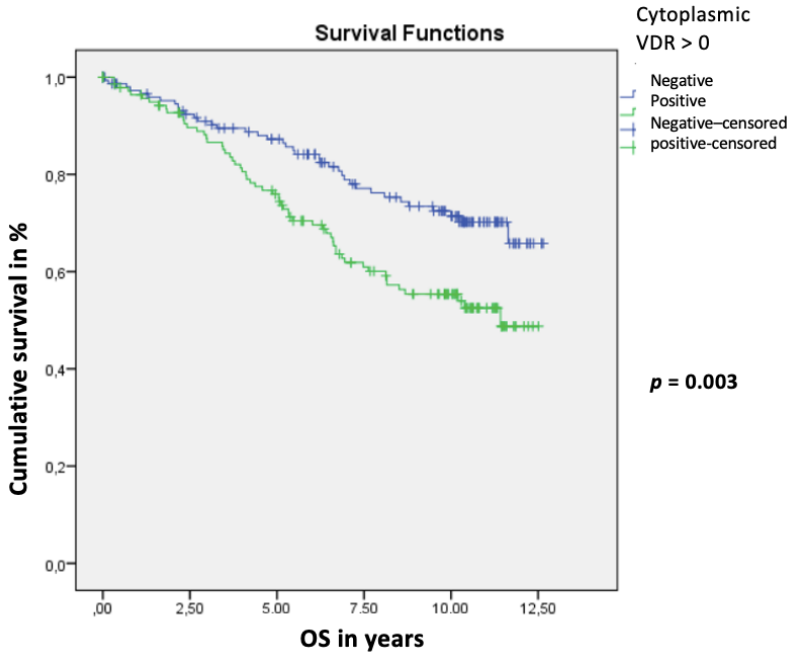
Table 2. Multivariate Cox regression analysis of nuclear VDR expression regarding DFS.

Variable	Coefficient	HR (95%CI)	*p*-value
Nuclear VDR >0	-0.556	0.574 (0.358-0.921)	**0.021**
Age	0.003	1.003 (0.984-1.022)	0.768
Histology	-0.067	0.935 (0.865-1.012)	0.096
Grading	0.001	1.001 (0.996-1.018)	0.812
pT	0.309	1.362 (1.136-1.632)	**0.001**
pN	0.004	1.004 (0.990-1.018)	0.592
Her2neu	-0.006	0.994 (0.988-1.001)	0.098
ER	-0.077	0.926 (0.545-1.574)	0.777
PR	0.081	1.084 (0.639-1.841)	0.764

Significant results are shown in bold (p<0.05); HR: hazard ratio; CI: confidence interval, pT = tumor size, pN = lymph node involvement.

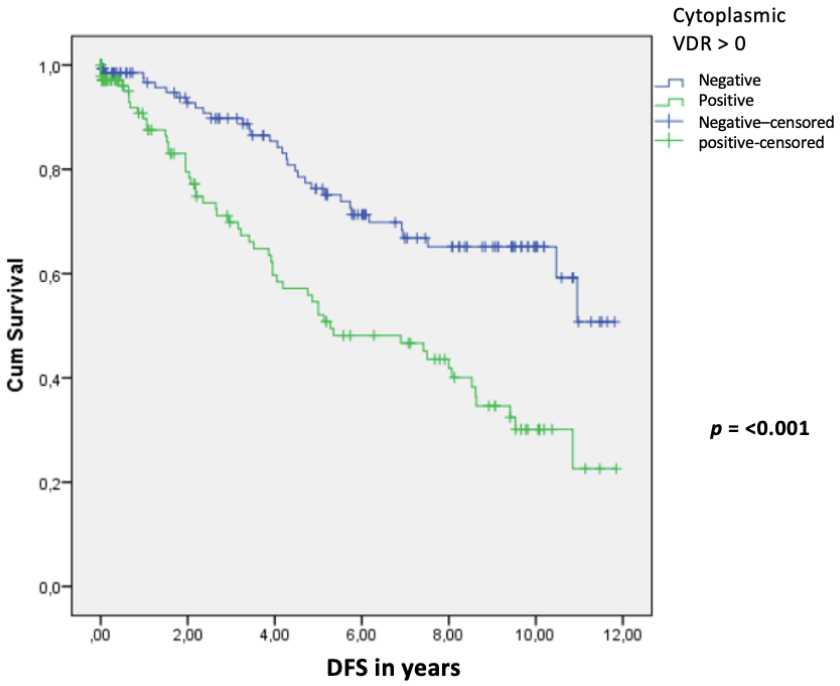
3.3. Cytoplasmic VDR expression significantly correlates with a worse BC prognosis

Cytoplasmic VDR expression was significantly associated with a worse BC prognosis. The Kaplan-Meier curve was visualized, and log-rank tests calculated a significantly worse course regarding OS (p=0.003, Fig. 4) and DFS (p<0.001, Fig. 5) when cytoplasmic VDR was expressed in BC tissue. Similar to the results of nuclear VDR cases, multivariate Cox regression identified cytoplasmic VDR as an independent prognostic factor of DFS (HR 2.288, 95% CI 1.468-3.566, p<0.001) (Table 3).



Mean OS time (years)				
			95% confidence interval (CI)	
Cytoplasmic VDR	Mean	SEM	lower border	higher border
Negative (n=124)	10.387	0.327	9.745	11.028
Positive (n=168)	8.861	0.371	8.133	9.588
Overall (n=292)	9.662	0.253	9.165	10.159

Figure 4. Kaplan-Meier survival analysis of positive and negative nuclear VDR expression in relation to OS. The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.



Mean DFS time (years)				
			95% confidence interval (CI)	
Cytoplasmic VDR	Mean	SEM	lower border	higher border
Negative (n=155)	8.953	0.405	8.160	9.747
Positive (n=143)	6.375	0.464	5.466	7.284
Overall (n=298)	7.737	0.320	7.109	8.365

Figure 5. Kaplan-Meier survival analysis of positive and negative cytoplasmic VDR expression in relation to DFS. The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.

Table 3. Multivariate Cox regression analysis of cytoplasmic VDR expression regarding DFS.

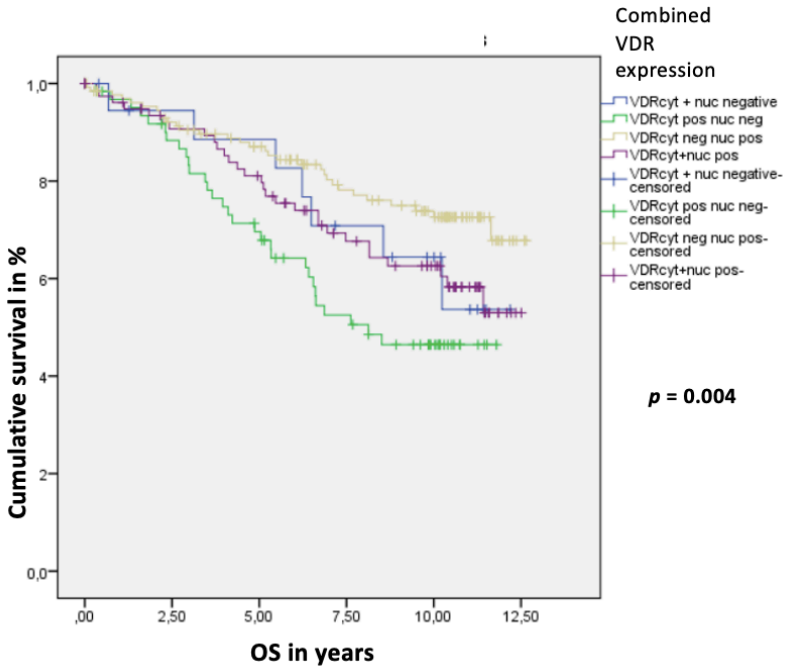
Variable	Coefficient	HR (95%CI)	*p*-value
Cytoplasmic VDR >0	0.828	2.288 (1.468-3.566)	**<0.001**
Age	0.002	1.002 (0.983-1.021)	0.843
Histology	-0.043	0.958 (0.889-1.032)	0.256
Grading	0.001	1.001 (0.996-1.005)	0.080
pT	0.324	1.383 (1.167-1.640)	**<0.001**
pN	0.001	1.001 (0.987-1.015)	0.925
Her2neu	-0.007	0.993 (0.987-1.00)	**0.047**
ER	-0.044	0.957 (0.561-1.632)	0.871
PR	0.048	1.050 (0.616-1.788)	0.859

Significant results are shown in bold (p<0.05); HR: hazard ratio; CI: confidence interval, pT = tumor size, pN = lymph node involvement

3.4 Subcellular localization of VDR and correlation with prognosis

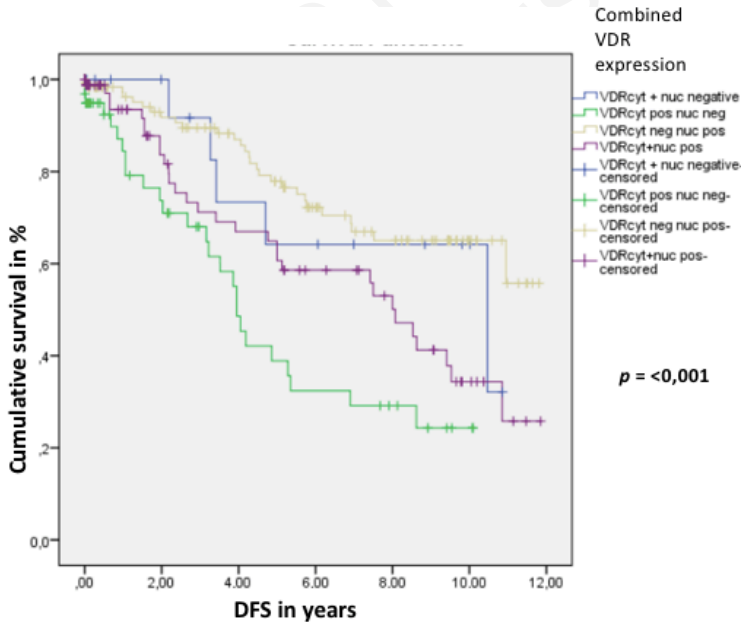
Furthermore, the cohort was divided into four phenotypic combinatorial groups as follows: cytoplasmic and nuclear VDR negative (*n*=19), only cytoplasmic VDR positive (*n*=63), only nuclear VDR positive (*n*=138), and cytoplasmic and nuclear VDR positive (*n*=80). Cut-off scores >0 for the IRS were defined as either cytoplasmic or nuclear VDR positive. In some cases, where VDR expression was observed in the nucleus and the cytoplasm, an IRS-ratio was given.

Supporting the results mentioned in 3.3 and 3.4, the Kaplan-Meier curve visualized a significantly better outcome regarding OS (Fig. 6) and DFS (Fig. 7) for the patient cohort only expressing nuclear VDR and a worse prognosis when only expressing cytoplasmic VDR. In contrast, the results of the combined VDR expression groups were in between those of the other groups. The log-rank test calculated significant results for both: OS *p*-value=0.004 and DFS *p*-value of <0.001.



Mean OS time (years)				
			95% confidence interval (CI)	
Combined VDR expression	Mean	SEM	lower border	higher border
Cytoplasmic and nuclear VDR negative (n=19)	9.488	0.873	7.777	11.199
Cytoplasmic VDR positive (n=63)	7.868	0.530	6.830	8.906
Nuclear VDR positive (n=138)	10.492	0.348	9.810	11.174
Cytoplasmic and nuclear VDR positive (n=80)	9.428	0.475	8.498	10.359
Total (n=300)	9.662	0.253	9.165	10.159

Figure 6. Kaplan-Meier survival analysis of the four combinatorial phenotypic VDR groups and their expression in relation to OS. Statistical significance is shown as the *p*-value from the log-rank test (**p*<0.05). The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.



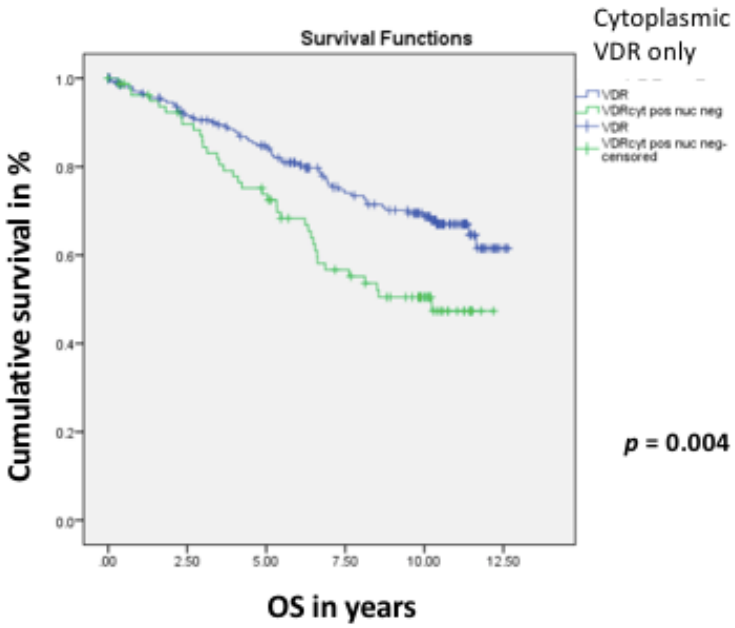
Mean DFS time in years				
			95% confidence interval (CI)	
Combined VDR expression	Mean	SEM	lower border	higher border
Cytoplasmic and nuclear VDR negative (n=19)	8.069	1.055	6.001	10.137
Cytoplasmic VDR positive (n=63)	4.927	0.595	3.762	6.093
nuclear VDR positive	9.049	0.431	8.205	9.893

(n=136)				
Cytoplasmic and nuclear VDR positive (n=80)	7.101	0.595	5.934	8.267
Total (n=298)	7.737	0.320	7.109	8.365

Figure 7. Kaplan-Meier survival analysis of the four combinatorial phenotypic VDR groups and their expression in relation to DFS. Statistical significance is shown as the *p*-value from the log-rank test (*$p<0.05$). The risk table demonstrates the mean survival time, standard error of the mean (SEM), 95% confidence interval (CI) for univariate analyses.

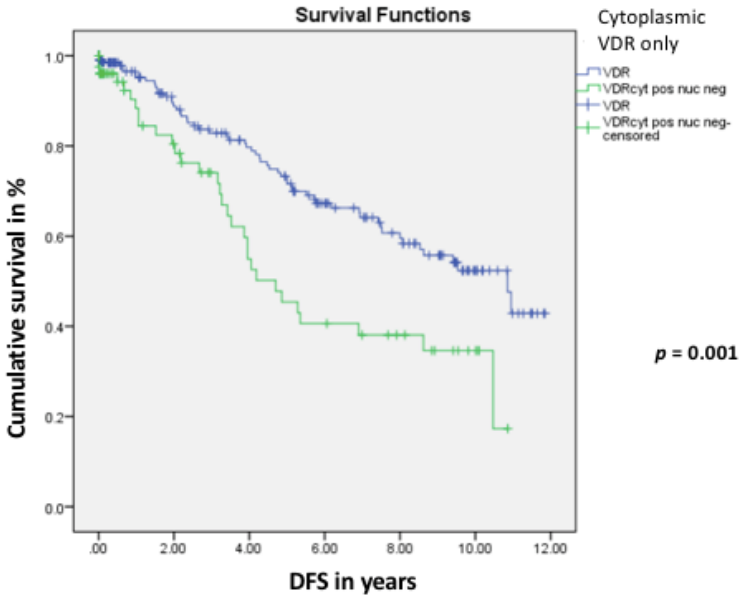
3.5 Cytoplasmic VDR expression is an independent marker of DFS

For further analysis of the prognostic impact of cytoplasmic VDR, the combined VDR group (n= 216) was correlated with cytoplasmic VDR only group (n=82), being cytoplasmic VDR positive and nuclear VDR positive, for prognosis. Matching the results of the subcellular analysis of VDR (Section 3.4), significantly reduced outcomes for OS ($p=0.004$) and DFS ($p<0.001$) were calculated via the log-rank test and visualized with Kaplan-Meier analysis when only expressing cytoplasmic VDR compared with combined VDR expression (Figures 8 and 9). Additionally, multivariate Cox regression identified cytoplasmic VDR expression as an independent prognostic marker of DFS (HR 2.138, 95% CI 1.244-3.674, $p<0.001$) (Table 4).



	Mean OS time (years)			
			95% confidence interval (CI)	
Cytoplasmic VDR only	Mean	SEM	lower border	higher border
Cytoplasmic VDR only (n=82)	8.357	0.477	7.421	9.293
combined VDR (n=216)	10.101	0.286	9.540	10.662
Overall (n=298)	9.662	0.253	9.165	10.159

Figure 8. Kaplan-Meier survival analysis of patients with cytoplasmic VDR positivity only and combined VDR expression in relation to OS. The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.



Mean DFS time (years)				
			95% confidence interval (CI)	
Cytoplasmic VDR only	Mean	SEM	lower border	higher border
Cytoplasmic VDR only (n=82)	5.837	0.575	4.711	6.964
combined VDR (n=216)	8.310	0.359	7.607	9.014
Overall (n=298)	7.737	0.320	7.109	8.365

Figure 9. Kaplan-Meier survival analysis of patients with cytoplasmic VDR positivity only and combined VDR expression in relation to DFS. The risk table demonstrates the mean survival time, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.

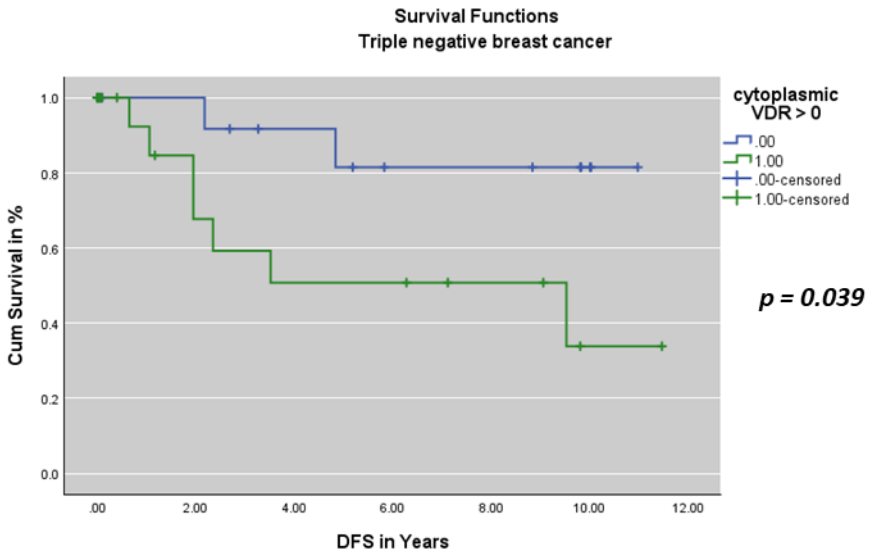
Table 4. Multivariate Cox regression analysis of cytoplasmic VDR expression only regarding DFS.

Variable	Coefficient	HR (95%CI)	*p*-value
Cytoplasmic VDR only	0.760	2.138 (1.244-3.674)	**0.006**
Age	0.008	1.008(0.987-1.029)	0.459
Histology	-0.035	0.965 (0.905-1.029)	0.279
Grading	-0.003	0.997 (0.992-1.002)	0.274
pT	0.371	1.449 (1.202-1.746)	**<0.001**
pN	0.008	1.008 (0.994-1.023)	0.271
Her2neu	-0.004	0.996 (0.989-1.003)	0.254
ER	-0.417	0.659 (0.336-1.290)	0.223
PR	-0.182	0.834 (0.477-1.458)	0.523

Significant results are shown in bold (p<0.05); HR: hazard ratio; CI: confidence interval, pT = tumor size, pN = lymph node involvement

3.6. Cytoplasmic VDR as a prognostic marker in triple-negative BC patients

The correlations between subcellular VDR expression were also investigated specifically in the subcohort of triple-negative BC (TNBC) cases using the same statistical devices for evaluation. In the TNBC patient cohort, cytoplasmic VDR expression revealed a significant negative correlation (*p-value*=0.039) with DFS (Fig. 10).



Mean DFS (years) in triple-negative BC patients

			95% confidence interval (CI)	
Cytoplasmic VDR	**Mean**	**SEM**	**lower border**	**higher border**
Negative (n=15)	9.621	0.876	7.905	11.338
Positive (n=20)	6.453	1.315	3.876	9.031
Overall (n=35)	8.209	0.891	6.463	9.955

Figure 10. Kaplan-Meier survival analysis of cytoplasmic VDR expression in the triple-negative patient cohort in relation to DFS. Statistical significance is shown as the *p*-value from the log-rank test (p = 0.039). The risk table demonstrates the mean survival time in triple-negative patients, standard error of the mean (SEM), and 95% confidence interval (CI) for univariate analyses.

3.7. Ratio of nuclear/cytoplasmic VDR expression as an independent marker of OS and DFS

Finally, we performed an analysis of the prognostic value of the ratio between the nuclear and cytoplasmic expression of VDR (VDRnuc/cyt). To calculate this ratio, negative cytoplasmic expression was set to 0.01 to avoid division by zero. As a result, we obtained three groups: 1. VDRnuc/cyt = 0—in this group, the nuclear expression of VDR is zero; 2. VDRnuc/cyt = balanced—in this group, there is positive staining in both the nucleus and the cytoplasm; and 3. VDRnuc/VDRcyt = infinite—in this group, there is almost no VDR expression in the cytoplasm. Kaplan-Meier analysis showed that the VDRnuc/cyt ratio is a very good prognostic marker of OS and DFS (Figure 11). In addition, multivariate Cox regression identified the VDRnuc/cyt ratio as an independent prognostic marker of OS and DFS (Table 5).

Table 5. Multivariate Cox regression analysis of the VDRnuc/cyt ratio regarding OS and DFS.

Variable (OS)	Coefficient	HR (95%CI)	*p*-value
VDRnuc/cyt ratio	-0.294	0.746 (0.565-0.983)	**0.037**
Age	0.029	1.029 (1.014-1.045)	**<0.001**
Histology	-0.001	0.999 (0.975-1.024)	0.951
Grading	0.004	1.004 (0.999-1.008)	0.082
pT	0.450	1.569 (1.348-1.826)	**<0.001**
pN	0.011	1.011 (1.002-1.020)	0.013
Her2neu	-0.002	0.998 (0.992-1.004)	0.508
ER	0.163	1.177 (0.698-1.983)	0.541
PR	-0.160	0.852 (0.506-1.434)	0.547

Variable (DFS)	Coefficient	HR (95%CI)	*p*-value
VDRnuc/cyt ratio	-0.548	0.578 (0.436-0.767)	**<0.001**
Age	0.004	1.004 (0.985 - 1.023)	0.672
Histology	-0.056	0.946 (0.876 -1.022)	0.158
Grading	0.000	1.000 (0.996 -1.005)	0.830
pT	0.283	1.328 (1.114 -1.582)	**0.002**
pN	0.003	1.003 (0.989 -1.017)	0.680
Her2neu	-0.006	0.994 (0.988 -1.001)	0.104
ER	-0.011	0.989 (0.582-1.681)	0.986
PR	0.015	1.015 (0.598-1.724)	0.955

Significant results are shown in bold (p < 0.05); HR: hazard ratio; CI: confidence interval, pT = tumor size, pN = lymph node involvement

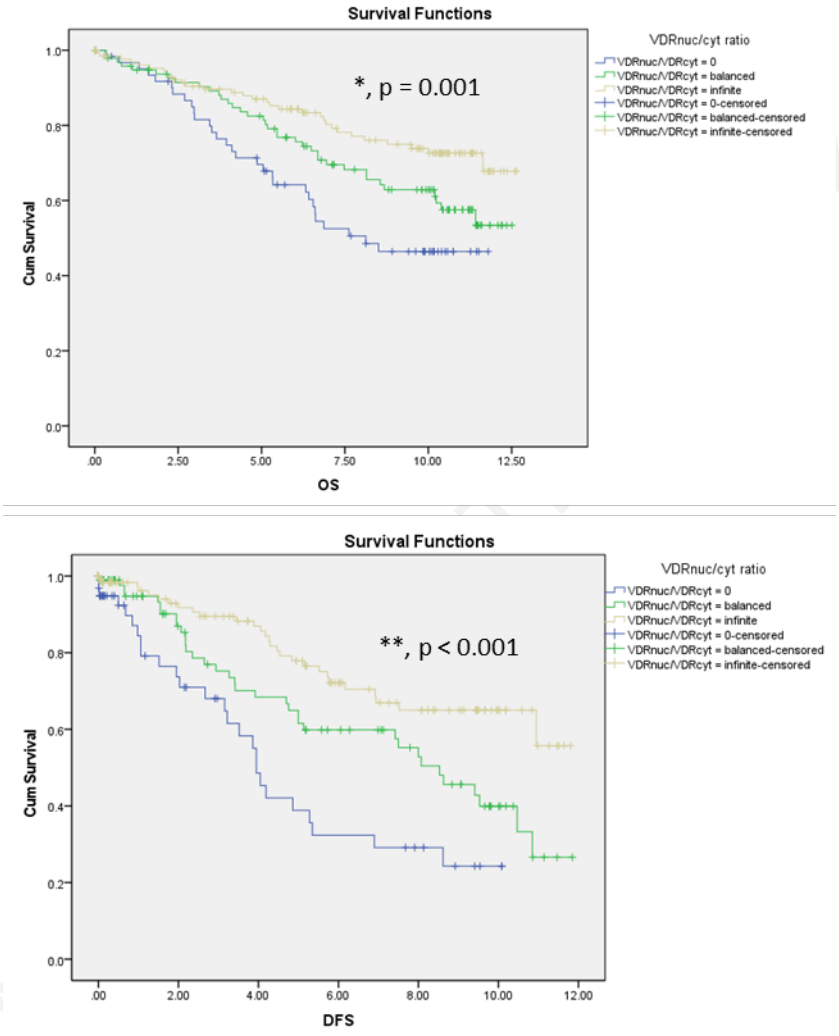


Figure 11. Kaplan-Meier survival analysis of the ratio between the nuclear expression and cytoplasmic expression of VDR (VDRnuc/cyt). Patients with a ratio =0 showed the lowest OS and DFS in comparison with patients with an infinite ratio of VDRnuc/cyt; p=0.001 for OS and <0.001 for DFS.

4. Discussion

Analysis and evaluation of the subcellular expression of VDR in BC and the correlation of these results with clinicopathological criteria was the research focus of this retrospective study. This is the first examination to outline the prognostic impact of cytoplasmic versus nuclear expression of VDR in BC. This study was based on a relatively large patient cohort that did not receive any treatment before surgery and had a long-term follow-up. Our data provide evidence that nuclear VDR expression is a protective factor, whereas the expression of cytoplasmic VDR is a significant negative prognostic marker. These results were strengthened by multivariate analysis, where cytoplasmic and nuclear VDR were found to be independent prognostic markers of DFS, with poorer and better outcomes, respectively.

VDR is involved in cell growth, differentiation, and apoptosis in BC and normal mammary cells. This receptor is expressed in epithelial as well as stromal and immune cells of the mammary gland. In

the epithelial compartment, it is dynamically regulated in special hormonal phases, such as puberty and pregnancy (Zinser et al.,2002; Zinser and Welsh, 2004). 1,25(OH)2D3 induces cell cycle arrest, differentiation, and/or apoptosis through VDR in both estrogen-dependent and estrogen-independent BC cells (Lazzaro et al., 2000; Narvaez and Welsh, 2001) through suppression of estradiol and growth factor signaling pathways and induction of negative growth regulators, such as transforming growth factor (TGF) (Stoica et al., 1999; Lazzaro et al., 2000; Cordero et al., 2002). Additionally, animal models have revealed that a lack of VDR expression is related to alterations in the proliferation and apoptosis of epithelial cells. For example, in VDR-KO mice, post-lactational involution, a process driven by apoptosis of mammary epithelial cells, is delayed (Zinser and Welsh, 2004).

In our study, the patient cohort positive for nuclear VDR expression and negative for cytoplasmic VDR revealed a significant correlation with improved OS and DFS when compared with the other combinatorial VDR expression cohorts. In contrast, the worst prognostic outcomes in both OS and DFS were seen in the patient cohorts with only cytoplasmic VDR expression. We suggest that the nuclear and cytoplasmic forms of VDR may exhibit opposite roles in mammary carcinogenesis. This is possibly due to the activation status of VDR. While the antiproliferative effect has been shown to be mediated predominantly via the nuclear pathway by binding of the activated receptor to VDREs (Omdahl et al., 2002; Carlberg, 2003, 2014), a similar antiproliferative effect through VDR expression in the cytoplasm could not be demonstrated in our analysis. Furthermore, the presence of inactive cytoplasmic VDR could also be due to the absence of a corresponding ligand or decreased receptor response to the ligand. Moreover, the results suggest that the protective effects of nuclear VDR expression may be neutralized by a balanced expression of VDR in the cytoplasm and nucleus. This hypothesis is supported by the significant results from the VDRnuc/cyt ratio analyses. Here, the importance of the subcellular distribution of VDR expression is demonstrated, as well as its excellent suitability as an independent prognostic marker. In our previous survey, nuclear VDR expression was significantly positively associated with smaller tumor size, as well as lower regional lymph node involvement. These findings strengthened the hypothesis of a specific role of the subcellular localization of VDR.

To date, no studies have examined the particular role of VDR in diverse cell compartments in BC tissue. The VDR is mainly localized in the nucleoplasm. In addition, it is localized in the cytosol, while its location in intermediate filaments is still discussed in the literature [65]. VDR, in the absence of its ligand, seems to distribute between the nucleus and the cytoplasm and undergo nuclear translocation upon ligand binding (Hsieh et al., 1998; Prufer et al., 2000). Moreover, the nuclear import of VDR is promoted in the presence of RXR, suggesting that the process involves RXR-VDR heterodimers (Prufer et al., 2000; Prufer and Barsony, 2002). Published research indicates that VDR is imported into the nucleus by distinct pathways by binding importin α. This observation further proves the association of

VDR with its cognate importin α; hence, its nuclear import is increased by the respective ligands, but the magnitude of the ligand response is noticeably different (Yasmin et al., 2005). VDR weakly interacts with importin α in the absence of its ligand, and the association is considerably enhanced in the presence of calcitriol (Yasmin et al., 2005). However, the mechanisms that underlie the nuclear import of RXR-containing heterodimers are not completely understood.

In accordance with the present findings, we demonstrated in an earlier study a correlation with nuclear RXRα expression regarding improved OS, whereas cytoplasmic RXRα expression was significantly correlated with shorter outcomes in terms of both OS and DFS (Zehni et al., 2021a). Furthermore, consistent with our data, a direct link between the nuclear localization of THR and increased OS in epithelial ovarian cancer has been proven (Ditsch et al., 2020). However, THR has also been identified to represent cancer-promoting activities during BC development (Shao et al., 2020.

Interestingly, cytoplasmic VDR expression showed a significant negative correlation with DFS (Fig. 10) in the TNBC patient subcohort. Other known BC receptors demonstrate no statistically significant correlation between prognosis and subcellular VDR expression. Further research on the intracellular localization of the VDR in BC, especially in TNBC, should be of major interest, as this BC subtype is characterized by shorter OS, as well as DFS and increased metastatic potential compared with other BC subtypes. The identification of reliable predictive biomarkers is essential in the process of finding new therapeutic regimens and approaches.

Epidemiological and preclinical evidence advocates the risk-reducing influence of vitamin D in gynecologic carcinomas (Valdivielso and Fernandez, 2006). Besides, it is a widely shared point of view that vitamin D supplementation decreases the risk of developing cancer (lappe et al., 2007; Walentowicz-Sadlecka, 2013). Compelling epidemiologic evidence proposes that inadequate VDR expression is associated with a more aggressive disease, which has led to the present standardized vitamin D supplementation for BC prevention and therapy (Walentowicz-Sadlecka et al., 2013; AWMF, Al-Azhri et al., 2017). In contrast, different studies have postulated increased VDR expression in diverse gynecological cancers, such as multifocal BC and ovarian, cervical, and endometrial cancer (Deuster et al., 2017; Zehni et al., 2019). Therefore, a critical reconsideration of vitamin D as a target subjected to downregulation during BC progression is suggested. Additionally, VDR polymorphisms have been shown to affect the risk of ovarian cancer (Deuster et al., 2017).

Certain aspects limit this study, such as the sample size, which was relatively low and may thus be insufficient to interpret all the heterogeneous entities in BD. Furthermore, it is a retrospective analysis based on a single database. Moreover, specific information on possible toxic environmental aspects or a history of endocrine therapy and other patient characteristics could enrich the investigation of how additional factors interact with VDR. Additionally, the guidelines for surgical, radiation oncology, and

chemotherapy treatment options changed significantly within the observation time of the survey. Consequently, oncological treatment details were not included in the analysis.

Nevertheless, aside from these limitations, the results strongly indicate that the VDR pathway could represent a promising therapeutic target in future BC treatment. These findings might serve to impulse further investigation of the crosstalk between potential NR ligands and the VDR pathway regarding its therapeutic potential in BC.

5. Conclusions

In this paper, the prognostic potential of the nuclear localization of the VDR compared with its cytoplasmic expression in human BC samples was investigated. Beyond that, the correlation between clinicopathological criteria, as well as patient treatment outcomes and the subcellular localization of the VDR, was analyzed. This is the first retrospective cohort study to define the prognostic role of cytoplasmic versus nuclear expression of VDR in sporadic mammary cancer using a large clinical patient cohort and a long-term follow-up.

VDR expression was found to inversely correlate with BC prognosis depending on its intracellular localization: VDR expressed in the cytoplasm of BC tissues was negatively associated with patient OS, while the opposite result was observed for VDR located in the nucleus. Most interestingly, the VDRnuc/cyt ratio was identified as a highly significant and independent prognostic marker in BC. Ultimately, nuclear receptors such as VDR and possible targeted treatments should be the subject of future research. Further studies on the subcellular expression of VDR and other members of the NR family are essentially required. Besides, the interaction between the VDR and other NRs (including estrogen, progesterone, and androgen receptors) should be specifically analyzed. Additional investigations to examine the biomolecular function of VDR in BC would be of major interest. Nevertheless, the evidence provided in this study indicates a key role for the VDR in BC.

Declarations

Ethics approval and consent to participate: The tissue samples used in this study were leftover material after all diagnostics had been completed and were retrieved from the archive of Gynecology and Obstetrics, Ludwig Maximilian University, Munich, Germany. All patients gave their consent to participate in the study. All patient data were fully anonymized; the study was performed according to the standards set out in the Declaration of Helsinki, 1975. The current study was approved by the Ethics Committee of Ludwig Maximilian University, Munich, Germany (approval number 048-08) 05 February 2018. The authors were blinded from the clinical information during the experimental analysis.

Consent for publication: Informed consent was obtained from all subjects involved in the study.

Availability of data and materials: The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to ethical issues.

Conflicts of Interest: Sven Mahner: research support, advisory board, honoraria, and travel expenses from AbbVie, AstraZeneca, Clovis, Eisai, GlaxoSmithKline, Medac, MSD, Novartis, Olympus, PharmaMar, Roche, Sensor Kinesis, Teva, and Tesaro. All other authors declare no conflict of interest.

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Author Contributions: U.J., V.C., and N.D. conceived and designed the project. A.Z.Z. and C.S. wrote the paper. A.Z.Z. carried out the statistical evaluation. A.Z.Z. carried out the method. U.J. provided the concept, substantially contributed to the manuscript, and supervised the research. T.V., F.B., V.C., S.S., S.M. M.K., and T.K. revised the manuscript for critical content and helped with statistical evaluation. Funding acquisition, S.M. All authors have analyzed and interpreted the data and read and agreed to the published version of the manuscript.

Abbreviations

BC	Breast cancer
DFS	Disease-free Survival
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
HR+	Hormone receptor-positive
NRs	Nuclear receptors
OS	Overall-survival
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
IRS	Immunoreactive Score
RXRα	Retinoid X receptor
TC	Total collective
THRs	Thyroid hormone receptors
VDR	Vitamin D receptor
VDRE	Vitamin D-responsive element

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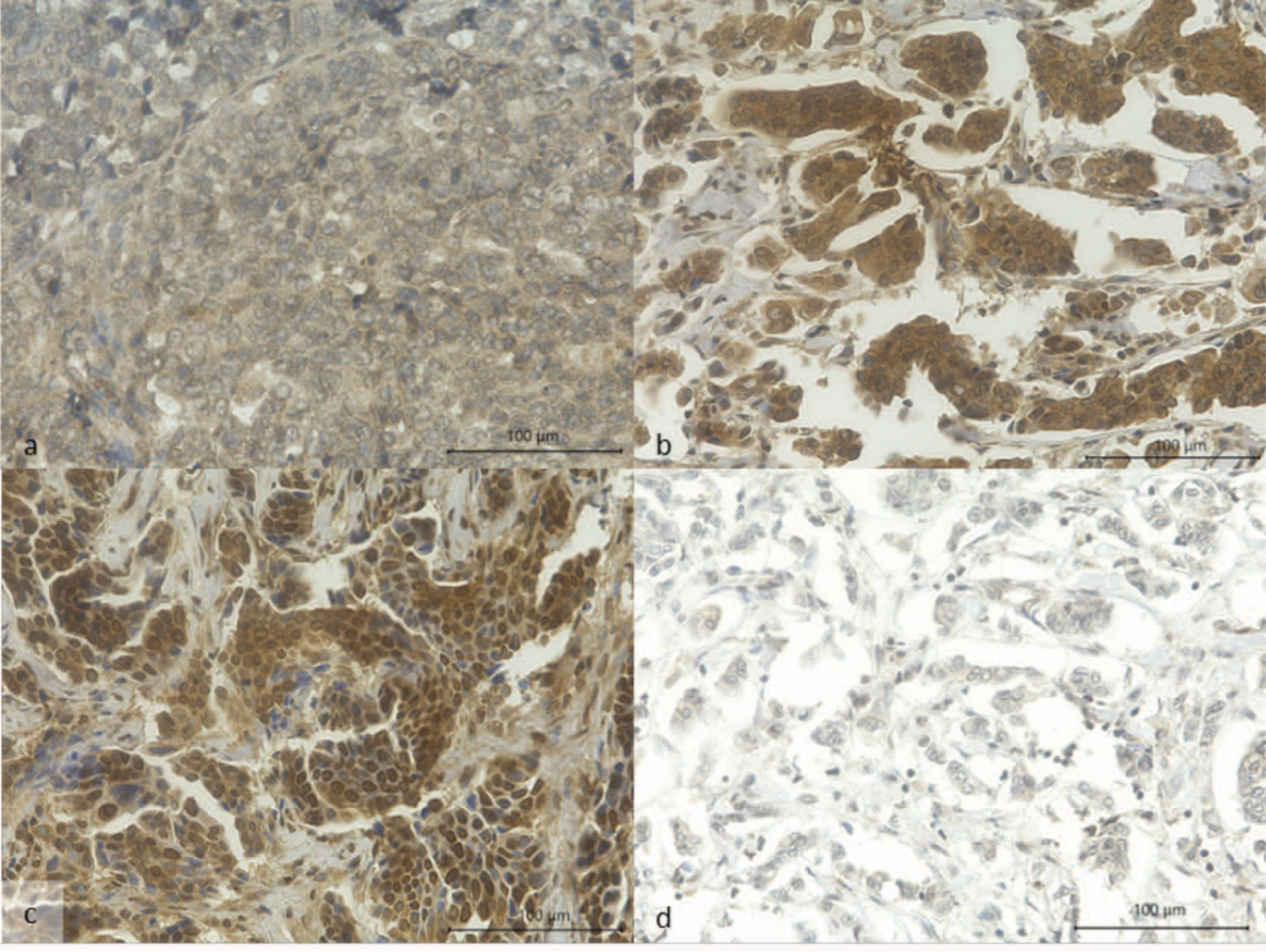
a
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b
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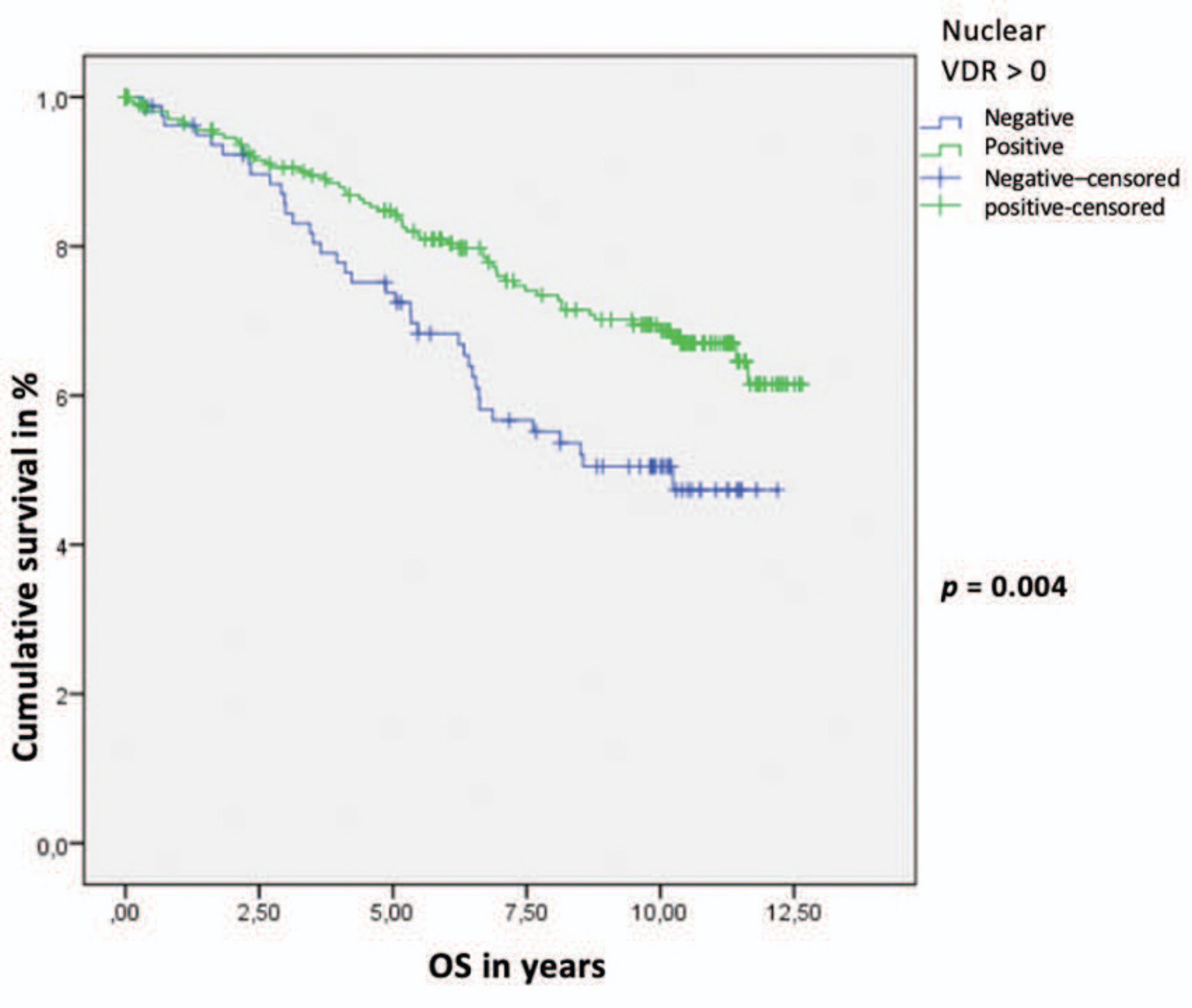
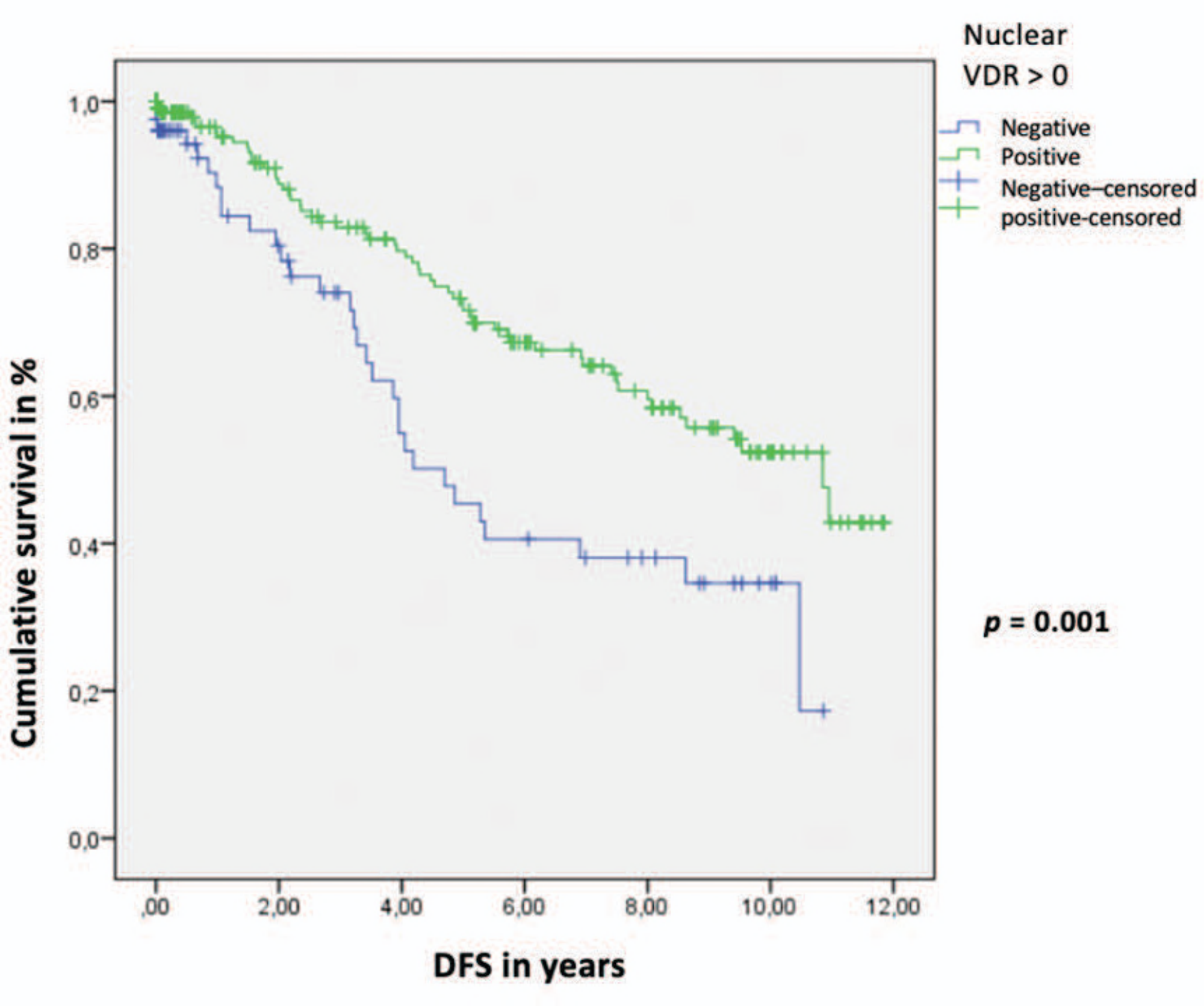
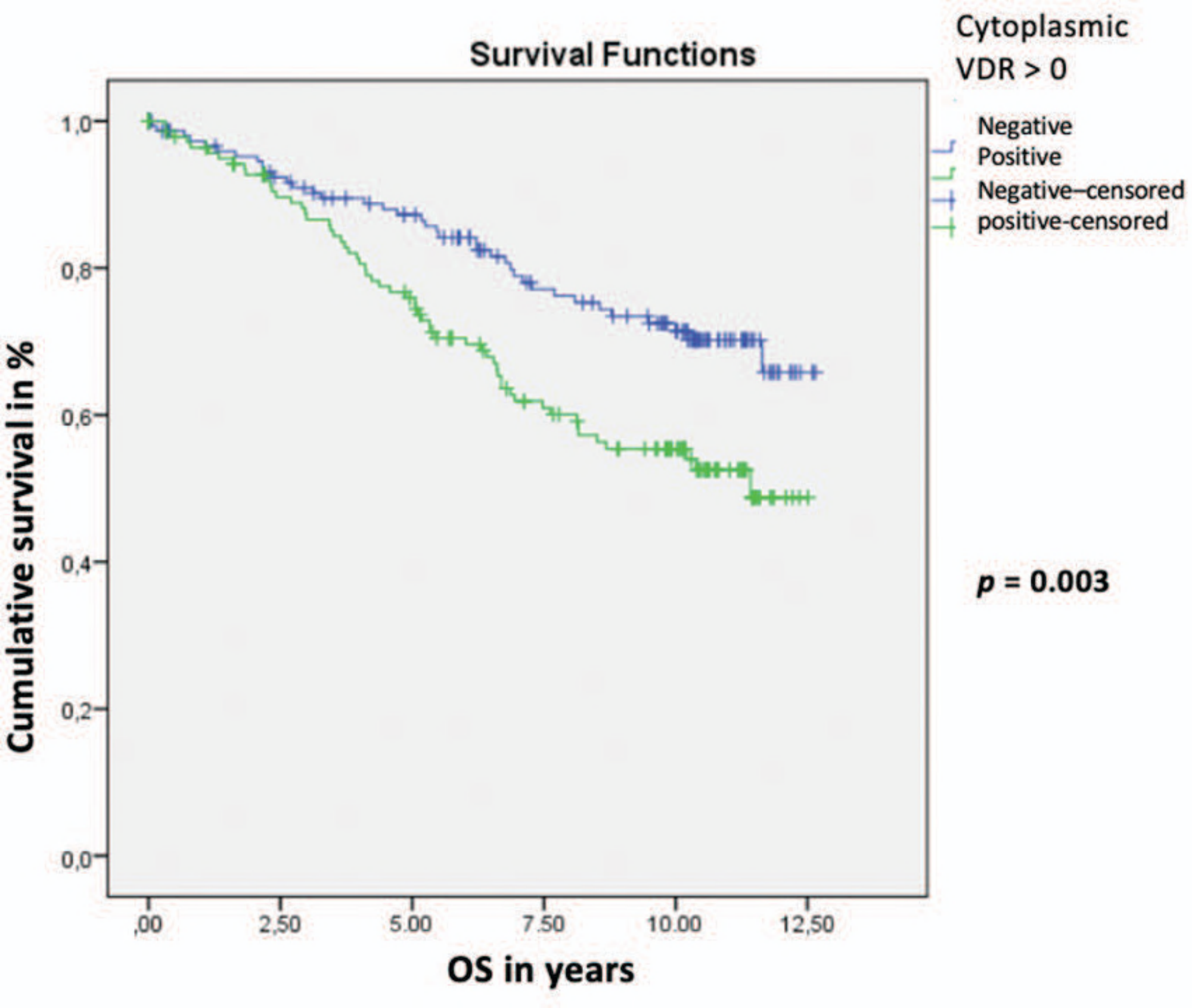
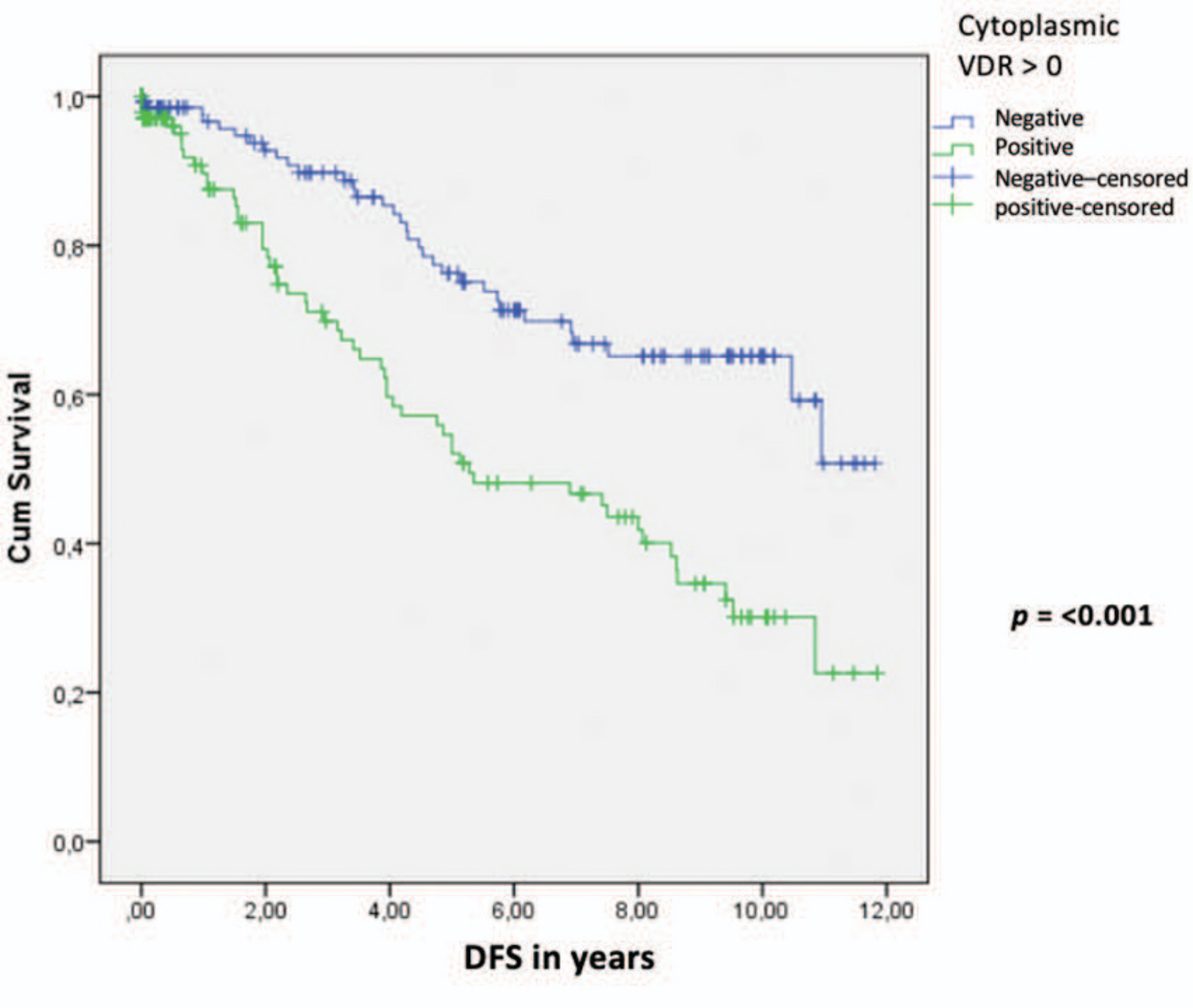
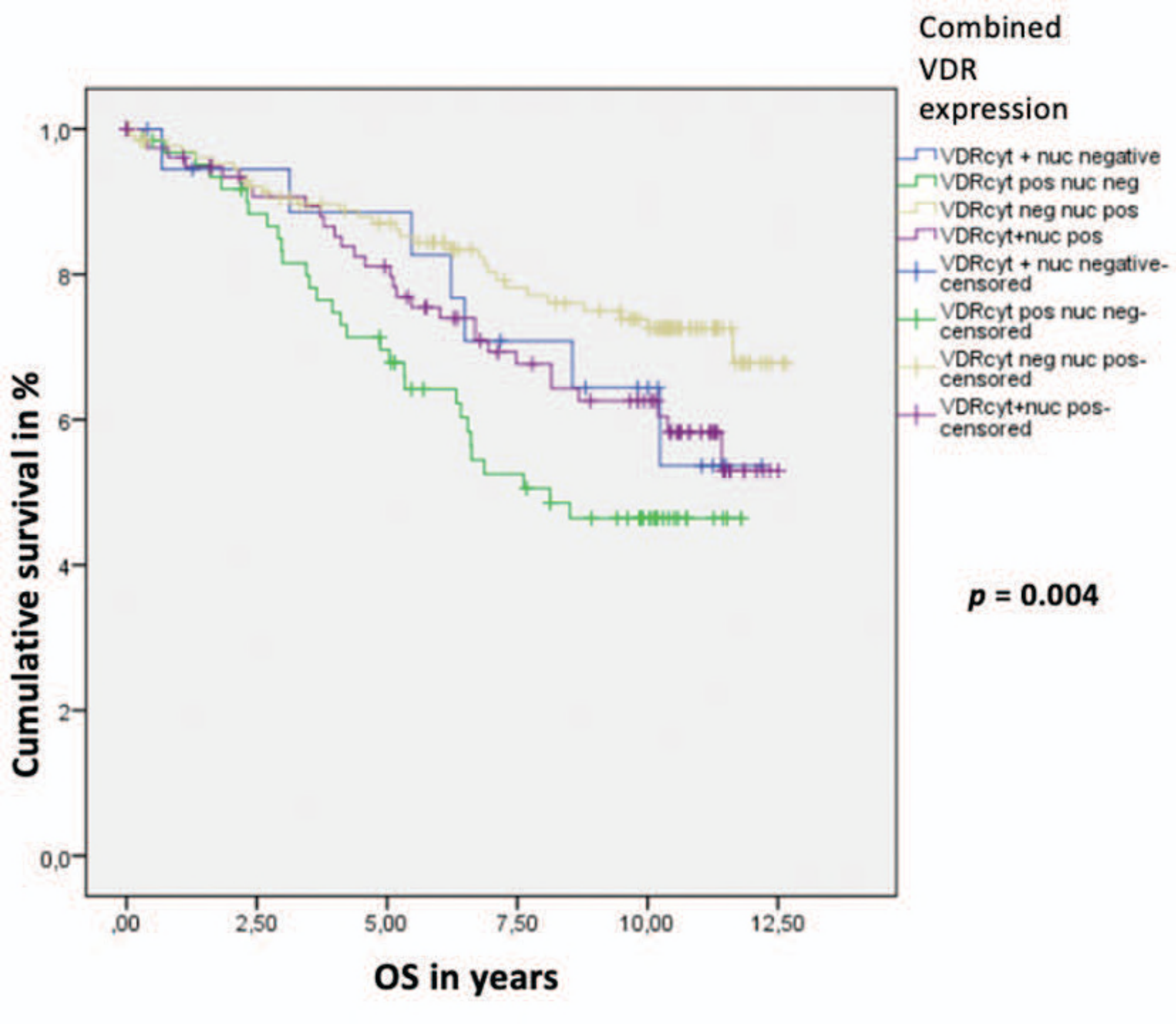
c
100 µm
d
100 µm

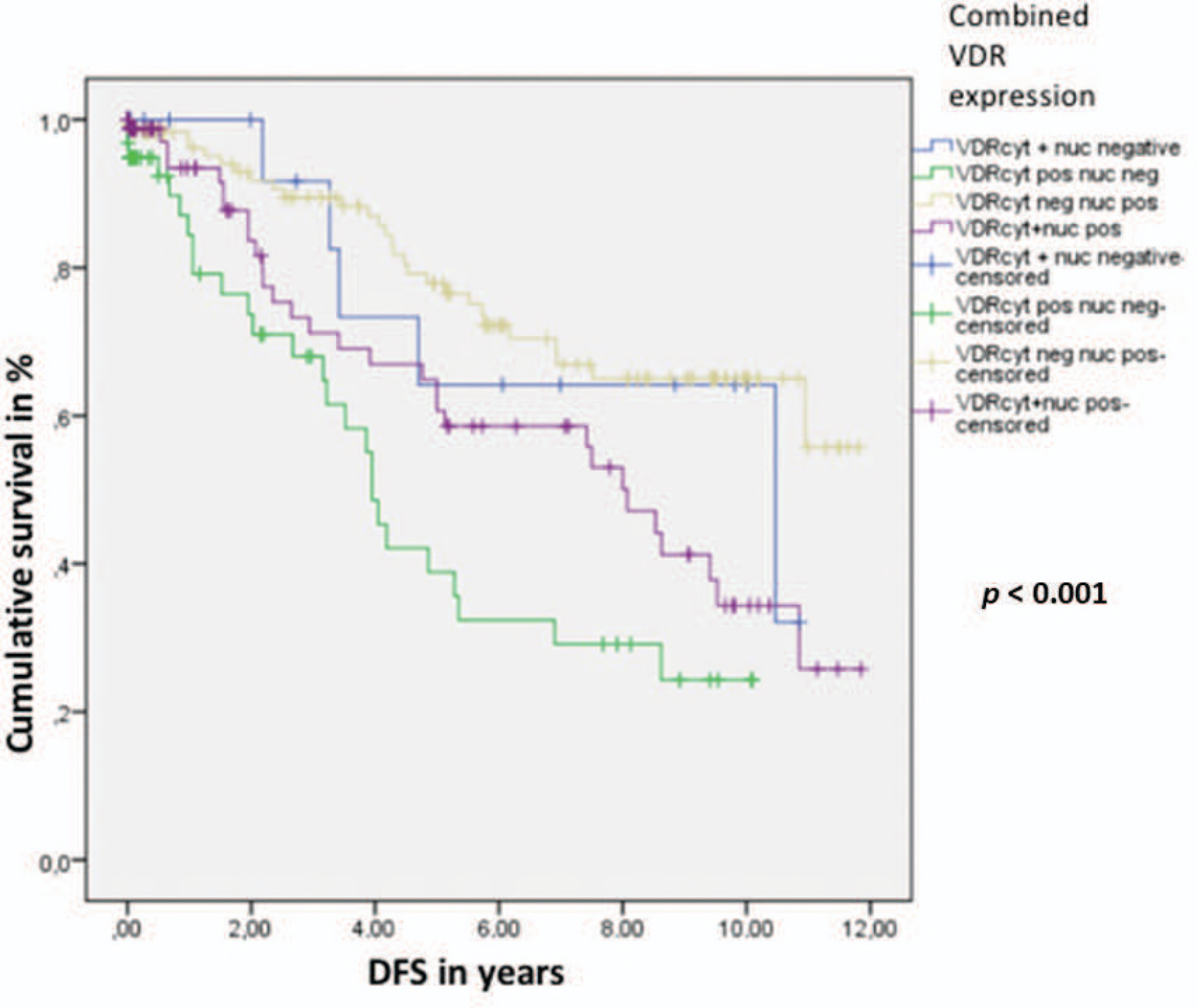

Nuclear
VDR > 0
Negative
Positive
Negative–censored
positive-censored
1,0
8
6
4
2
0,0
Cumulative survival in %
,00
2,50
5,00
7,50
10,00
12,50
OS in years
p = 0.004

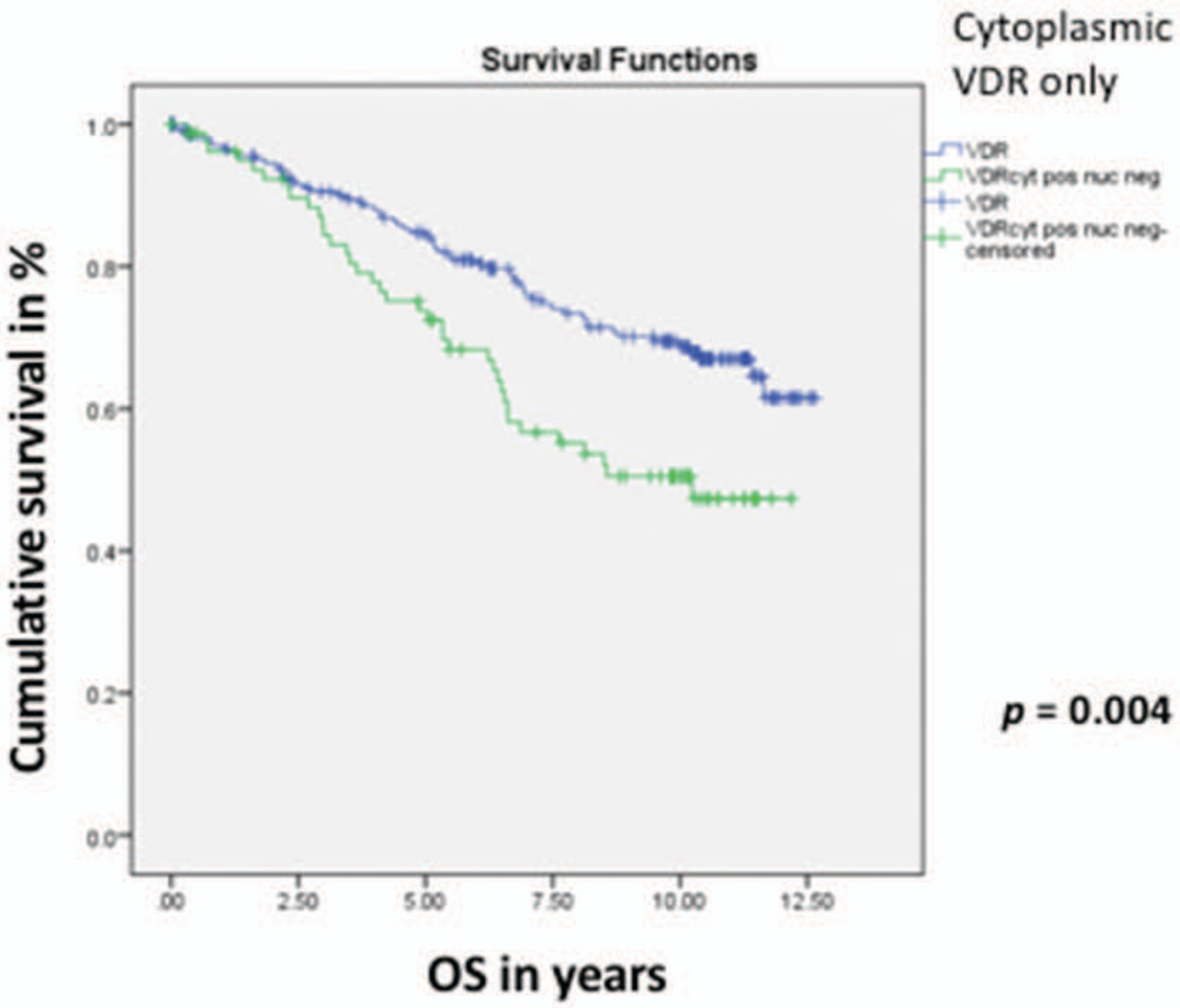

Nuclear
VDR > 0
Negative
Positive
Negative–censored
positive-censored
Cumulative survival in %
1,0
0,8
0,6
0,4
0,2
0,0
,00
2,00
4,00
6,00
8,00
10,00
12,00
DFS in years
p = 0.001

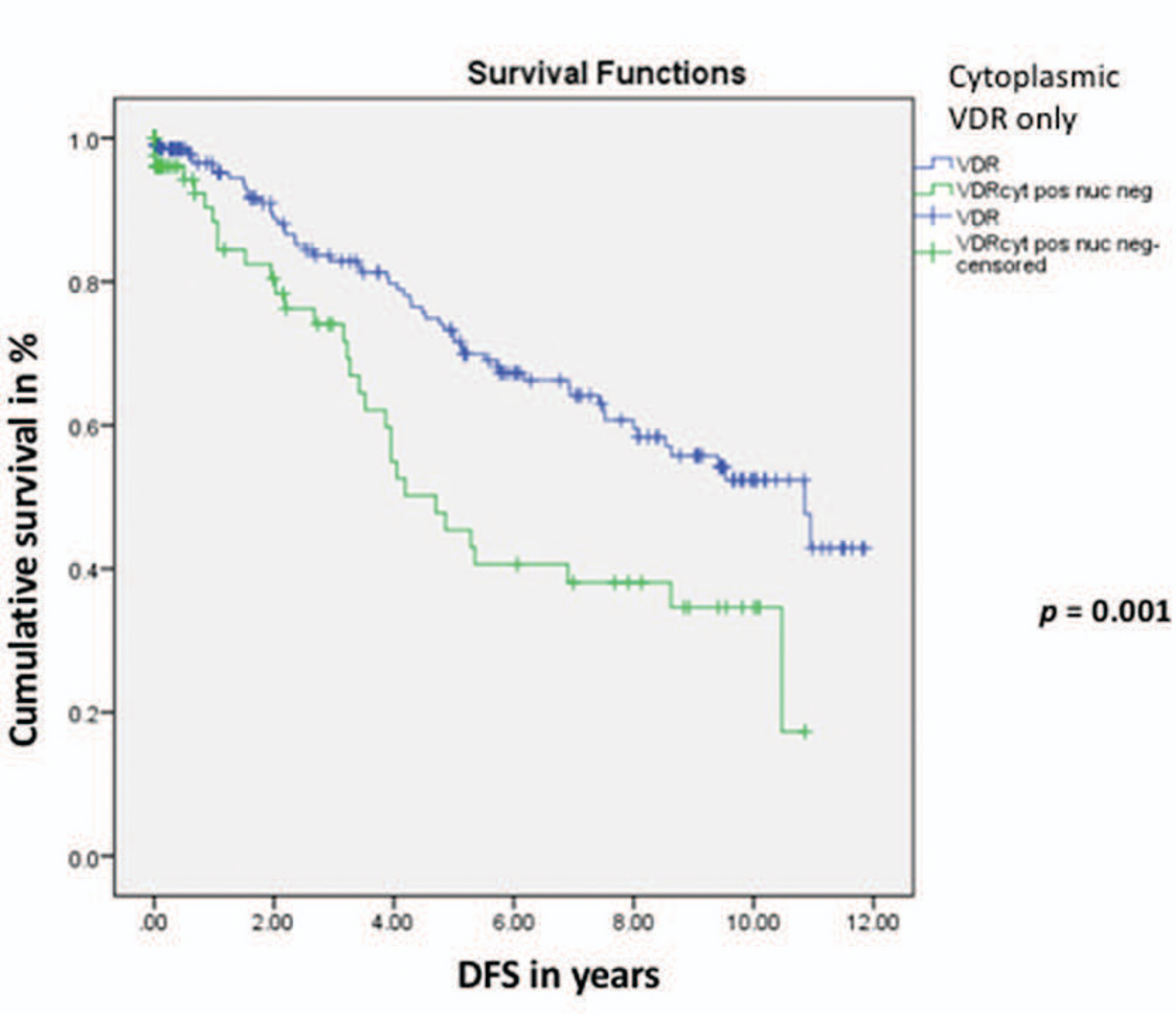

Survival Functions
Cytoplasmic
VDR > 0
Negative
Positive
Negative–censored
positive-censored
1,0
0,8
0,6
0,4
0,2
0,0
Cumulative survival in %
,00
2,50
5.00
7.50
10.00
12,50
OS in years
p = 0.003

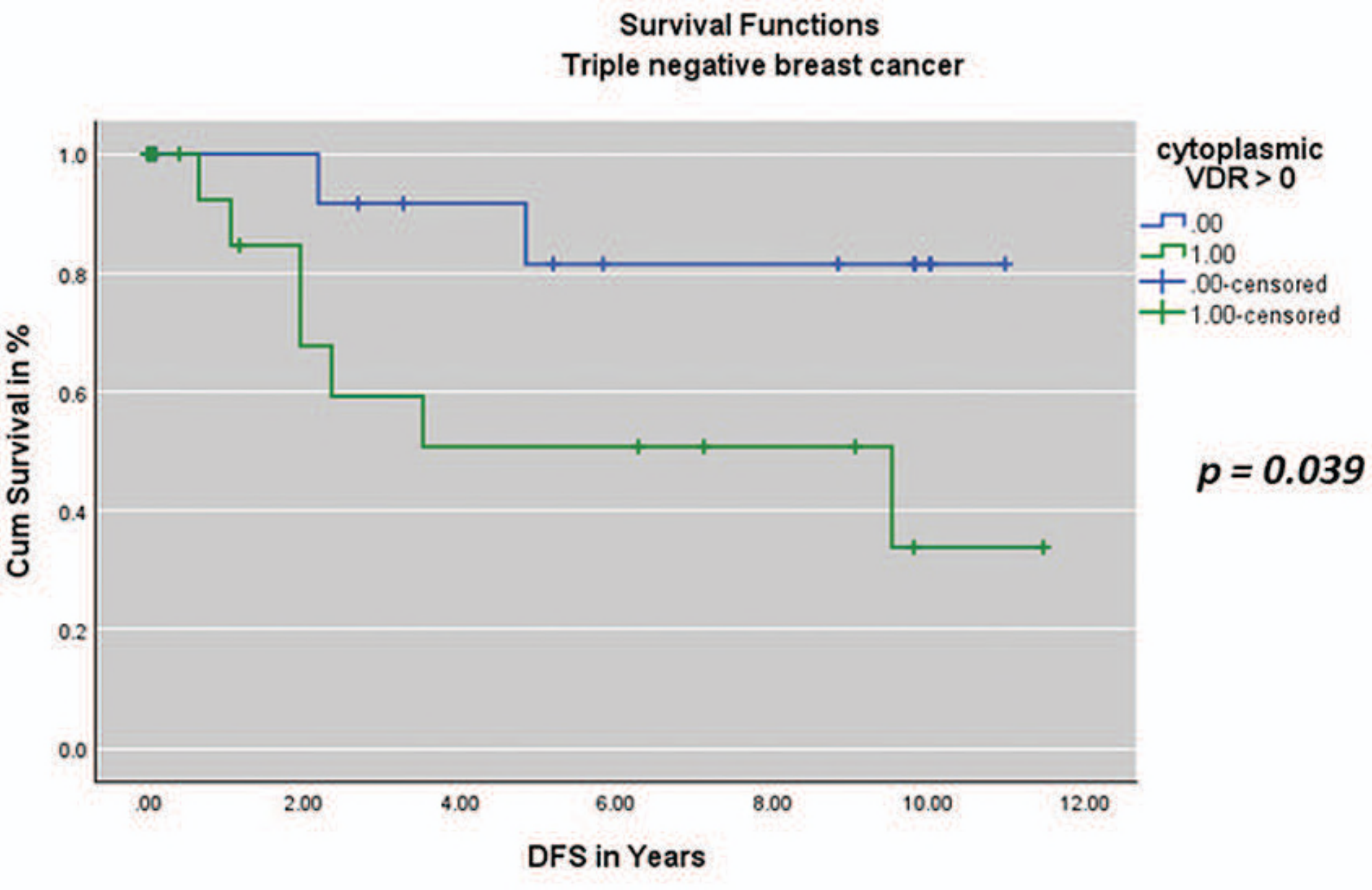

Cytoplasmic
VDR > 0
Negative
Positive
Negative–censored
positive-censored
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
,00
2,00
4,00
6,00
8,00
10,00
12,00
DFS in years
p = <0.001

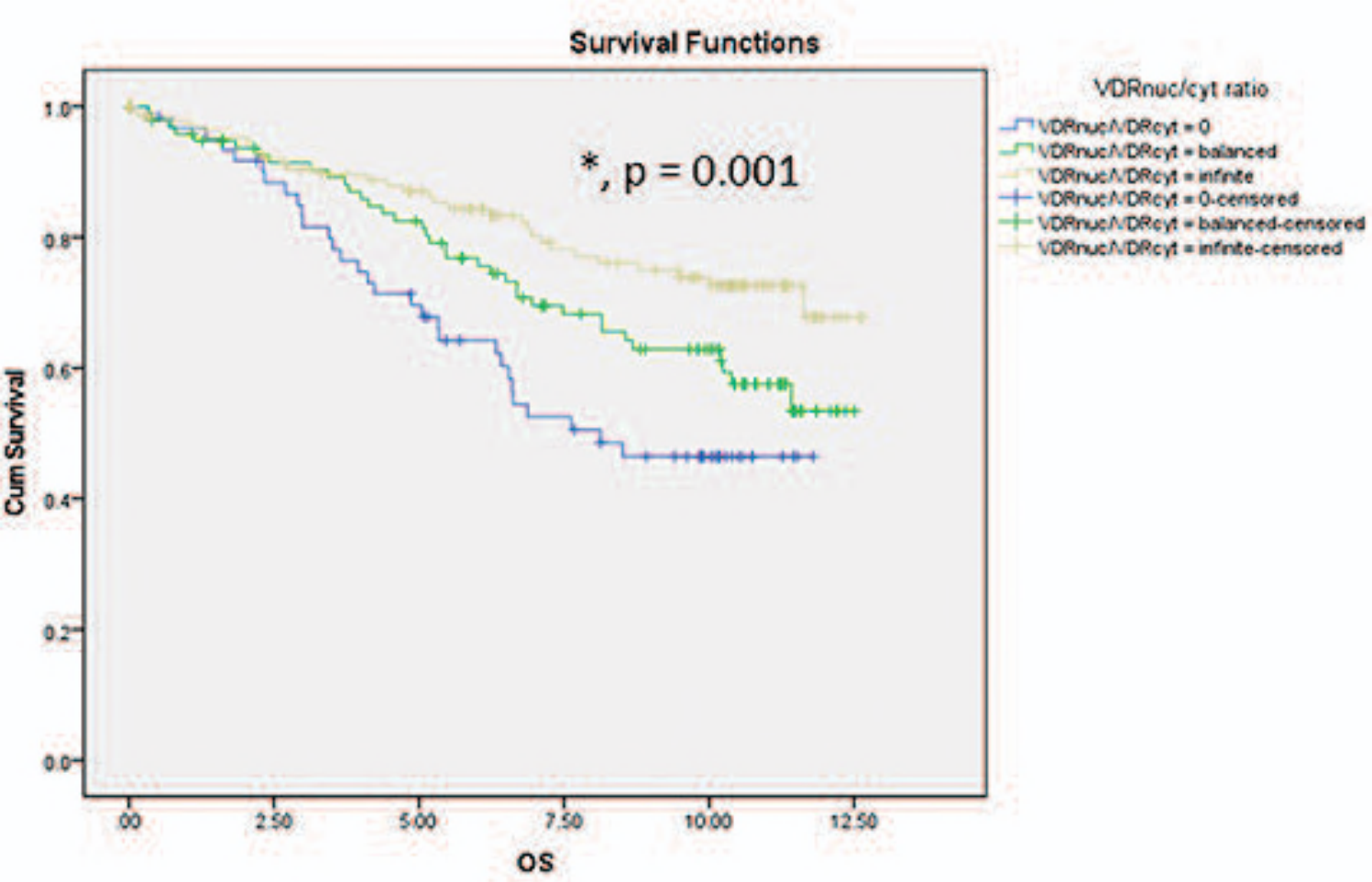

Combined VDR expression
VDRcyt + nuc negative
VDRcyt pos nuc neg
VDRcyt neg nuc pos
VDRcyt+nuc pos
VDRcyt + nuc negative-censored
VDRcyt pos nuc neg-censored
VDRcyt neg nuc pos-censored
VDRcyt+nuc pos-censored
p = 0.004
Cumulative survival in %
1,0
8
6
4
2
0,0
,00
2,50
5,00
7,50
10,00
12,50
OS in years

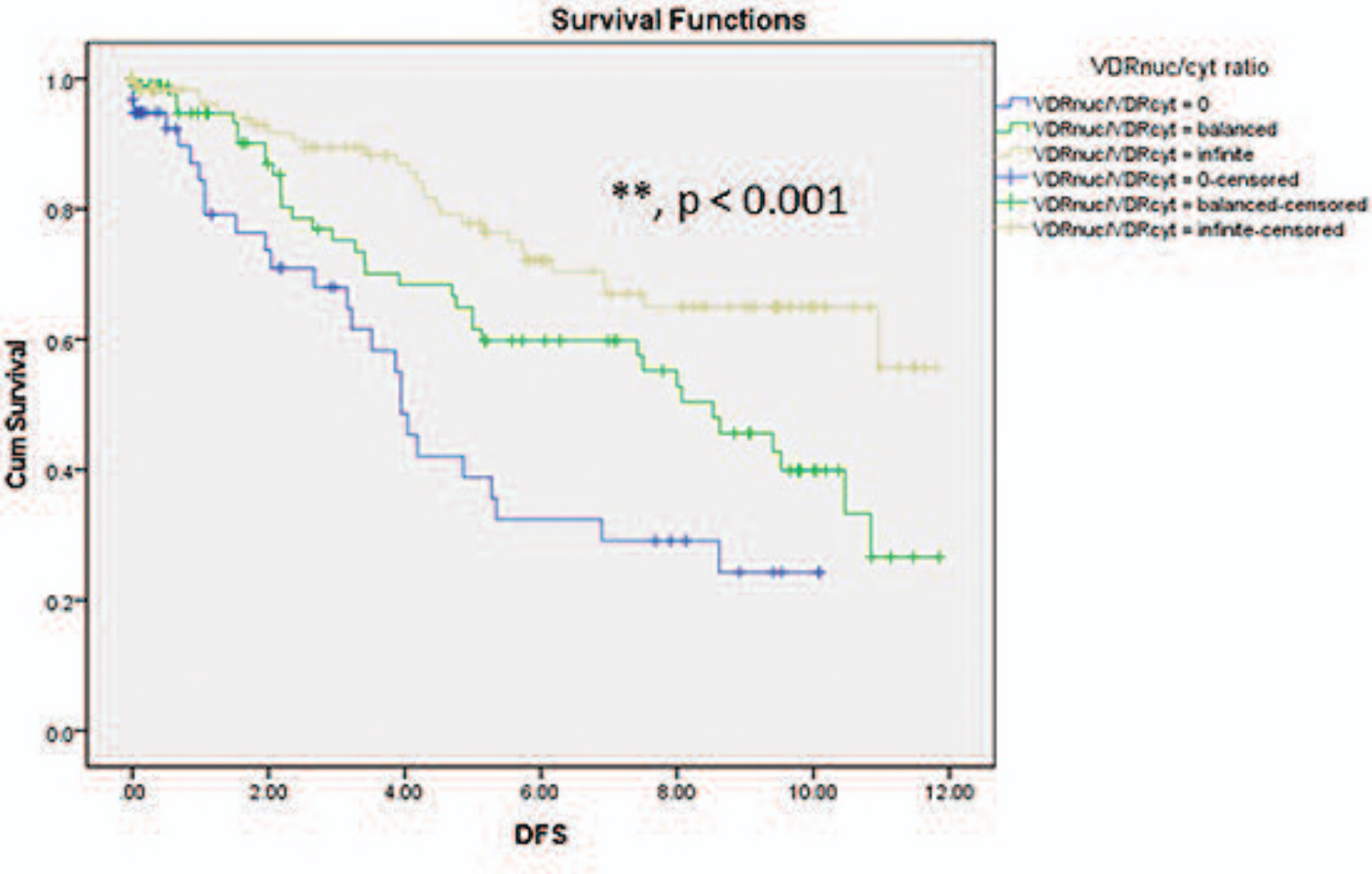

Combined VDR expression
VDRcyt + nuc negative
VDRcyt pos nuc neg
VDRcyt neg nuc pos
VDRcyt+nuc pos
VDRcyt + nuc negative-censored
VDRcyt pos nuc neg-censored
VDRcyt neg nuc pos-censored
VDRcyt+nuc pos-censored
1,0
,8
,6
,4
,2
0,0
Cumulative survival in %
,00
2,00
4,00
6,00
8,00
10,00
12,00
DFS in years
p < 0.001


Cytoplasmic VDR only
Survival Functions
VDR
VDRcyt pos nuc neg
VDR
VDRcyt pos nuc neg-censored
Cumulative survival in %
1.0
0.8
0.6
0.4
0.2
0.0
.00
2.50
5.00
7.50
10.00
12.50
OS in years
p = 0.004


Survival Functions
Cytoplasmic VDR only
VDR
VDRcyt pos nuc neg
VDR
VDRcyt pos nuc neg-censored
Cumulative survival in %
1.0
0.8
0.6
0.4
0.2
0.0
.00
2.00
4.00
6.00
8.00
10.00
12.00
DFS in years
p = 0.001


Survival Functions
Triple negative breast cancer
cytoplasmic VDR > 0
.00
1.00
.00-censored
1.00-censored
p = 0.039
Cum Survival in %
1.0
0.8
0.6
0.4
0.2
0.0
.00
2.00
4.00
6.00
8.00
10.00
12.00
DFS in Years


Survival Functions
*, p = 0.001
VDRnuc/cyt ratio
VDRnuc/VDRcyt = 0
VDRnuc/VDRcyt = balanced
VDRnuc/VDRcyt = infinite
VDRnuc/VDRcyt = 0-censored
VDRnuc/VDRcyt = balanced-censored
VDRnuc/VDRcyt = infinite-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
.00
2.50
5.00
7.50
10.00
12.50
OS


Survival Functions
**, p < 0.001
VDRnuc/cyt ratio
VDRnuc/VDRcyt = 0
VDRnuc/VDRcyt = balanced
VDRnuc/VDRcyt = infinite
VDRnuc/VDRcyt = 0-censored
VDRnuc/VDRcyt = balanced-censored
VDRnuc/VDRcyt = infinite-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
.00
2.00
4.00
6.00
8.00
10.00
12.00
DFS