Prognostic significance of S100A4-expression and subcellular localization in early stage breast cancer

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Abstract

Purpose: Prognostic factors are useful in order to identify early stage breast cancer patients that might benefit from adjuvant treatment. The metastasis-promoting protein S100A4 has previously been associated with poor prognosis in breast cancer patients. The protein is expressed in diverse subcellular compartments, including the cytoplasm, extracellular space and nucleus. Nuclear expression is an independent predictor of poor outcome in several cancer types, but the significance of subcellular expression has not yet been assessed in breast cancer.

Methods: Nuclear and cytoplasmic expression of S100A4 was assessed by immunohistochemistry in prospectively collected tumor samples from early stage breast cancer patients using tissue microarrays.

Results: In patients not receiving adjuvant systemic therapy nuclear or cytoplasmic expression was found in 44/291 tumors (15%). Expression of either nuclear or cytoplasmic S100A4 was associated with histological grade III, triple-negative subtype and Ki-67-expression. Patients with S100A4-positive tumors had inferior metastasis-free and overall survival compared to S100A4-negative. When expression was analyzed separately, nuclear S100A4 was a significant predictor of outcome, while cytoplasmic was not.

In patients who received adjuvant treatment 23/300 tumors (8%) were S100A4-positive, but no tumors displayed nuclear staining alone. S100A4-expression was strongly associated with histological grade III and triple-negative subtype. Although not significant, metastasis-free and overall survival was numerically reduced in patients with S100A4-positive tumors.

Conclusions: S100A4-expression was associated with poor outcome in early stage breast cancer, but the low percentage of positive tumors and the modest survival differences imply that the clinical utility in selection of patients for adjuvant treatment is limited.

Introduction

Breast cancer is the most common cancer among women in the Western world. While increased focus on early detection combined with adjuvant systemic treatment has improved survival in breast cancer patients [1], a more detailed disease characterization is still important to identify patients who are most likely to benefit from adjuvant treatment. Patients are stratified according to the TNM system (primary tumors, regional lymph nodes and distant metastasis) and histological grade, but treatment decisions also take into account molecular characteristics such as presence of growth factor or hormone receptors (human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and estrogen receptor (ER)) [2]. Metastatic disease is the main cause of breast cancer deaths, and elucidation of mechanisms causing progression and treatment resistance is necessary to more efficiently target the disease and discover novel therapies. Classification algorithms guiding treatment decisions are still imprecise, and an increased number of prognostic and predictive markers would be helpful to assign optimal treatment for each patient.

S100A4 is a small Ca^{2+}-binding protein involved in several biological functions enhancing the metastatic capacity of cancer cells [3]. The mechanisms causing S100A4-induced migration [4], invasion [5,6] and angiogenesis [7] are starting to be unraveled, but exact knowledge is still to be determined [8]. We, and others, have convincingly demonstrated that the protein can induce expression of matrix metalloproteinases (MMPs) and thereby enhance the tumor cells' ability to invade surrounding tissue [9-11]. It has also been proposed that S100A4-expression is induced when epithelial cells undergo reprogramming and gain mesenchymal traits during epithelial-to-mesenchymal transition (EMT) [12,13]. This is in agreement with the fact that the protein is expressed in highly migratory and often de-differentiated cells [14,15]. Interestingly both normal and cancer cells may secrete S100A4, and when present in the stroma the protein stimulates secretion of pro-inflammatory factors from cancer cells [14]. Recently it was shown that S100A4 is induced when resident cells take up tumor-derived exosomes in metastatic organs, and that the presence of S100A4 may aid in preparing a growth-promiscuous niche for metastatic tumor cells [16].

Thus, S100A4 is found involved in numerous biological processes and an association between protein expression and poor patient outcome has been reported in various types of cancer [17,18]. S100A4-expression is demonstrated to be a strong predictor of reduced survival in breast cancer [19-21], but conflicting results have also been reported [22]. All these studies are either based on rather small or relatively old patient cohorts. Hence, evaluating the prognostic value in a larger cohort where the patients are given treatment more relevant to the presently used regimes would be of great interest. Furthermore, in colorectal [23,24], liver [25] and ovarian [26] cancer nuclear localization of S100A4 has been associated with poor outcome, but the relevance of nuclear versus cytoplasmic localization has not been assessed in breast cancer. The aim of the present study was therefore to examine prognostic value and associations with clinicopathological parameters of nuclear and cytoplasmic S100A4-expression in a large and clinically relevant cohort. The present study adheres to the REMARK criteria [27].

Material and methods

Patient cohort

The Oslo Micrometastasis Project was conducted in the period 1995 to 1998 and a total of 920 patients with localized operable breast cancer were enrolled in the study. The patients were recruited from five different hospitals in the Oslo region (described in detail previously [28]). Tumor tissue was collected at time of primary surgery and written informed consent was obtained from the patients. Date of death was retrieved from the Norwegian Death Cause Registry. After excluding patients with benign lesions, carcinoma *in situ*, non-epithelial cancers and patients with distant metastasis, 860 patients were available for analysis (Supplementary Fig. S1).

A subgroup of 323 patients did not receive adjuvant systemic therapy and is designated the "no-adjuvant cohort". This included patients with pT1N0- or pT2pN0G1-status, as well as HR-negative patients over 65 years (described in Table 1 from [29]). Median follow-up in this sub-cohort was 7.4 years (range 0.6-10.4) for metastasis-free and 13.8 years (range 12.1-15.7) for overall survival. Six patients had no relapse status available. The majority of the no-adjuvant cohort has been reported previously [30].

Patients with pT2pN0G2-3 or pN+ received systemic therapy according to the Norwegian guidelines between 1995 and 1998 [29]. This "adjuvant cohort" included 421 patients and the median follow-up was 7.4 years (range 0-10.3) for metastasis-free survival and 13.6 years (range 12-15.7) for overall survival. Relapse status was unavailable for eleven patients, while time of death was unavailable for 35 patients.

Tissue microarrays

Tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded archival blocks. Areas of invasive breast cancer were identified by a pathologist after hematoxylin and eosin (H&E) staining and transferred to a recipient block.

The analyses were performed utilizing two distinct TMAs. The no-adjuvant patient cohort (n=323) was initially analyzed with a set of TMAs containing three 0.6 mm tissue cores per tumor, while the remaining analyses were performed utilizing a second set of TMAs with two tissue cores per tumor. The second TMA contained tumor tissue both from patients receiving systemic adjuvant therapy (n=421) and from patients not receiving adjuvant therapy. The latter tumor samples allowed us to evaluate potential differences between the two sets of TMAs.

Immunohistochemistry

Sections of formalin-fixed paraffin embedded tissue were immunostained with an in-house mouse monoclonal anti-S100A4 antibody [31]. Optimization of the antibody concentration was performed (dilutions from 1:250 to 1:2000) and a dilution of 1:500 (from a stock solution of 0.9 mg/ml) was chosen, which is similar to a parallel study in colorectal cancer (CRC) [32]. The staining procedure was done according to the EnVision™ FLEX+ (mouse; Dako, Agilent Technologies, Glostrup, Denmark) protocol (described in [33]). Hematoxylin (in house) was used for visualization of nuclei. Tissue from colorectal adenocarcinoma known to express S100A4 was used as positive control, while equal concentration of mouse-myeloma-protein IgG1 was included as negative control.

Scoring of S100A4-immunoreactivity

Cytoplasmic and nuclear S100A4-staining was scored as separate variables, without assessing immunoreactivity in adjacent normal tissue. The number of S100A4-positive cells was semi-quantitatively estimated and divided into categories from 0 to 4 (percentage of positive carcinoma cells in parenthesis): 0 (0%), 1 (<1%), 2 (1-10%), 3 (11-50%) and 4 (>50%). Intensity was scored from 0 to 3: 0 (negative), 1 (weak), 2 (intermediate) and 3 (strong). If there were discrepant results between cores from the same tumor, the highest S100A4-immunoreactivity was used in the analyses. All scorings were performed by the study pathologist (D. P.).

Statistical analysis

Associations between S100A4 and the clinicopathological parameters were examined using two-tailed Fisher's exact- or Pearson's chi-square-tests as appropriate. Survival curves were made according to the Kaplan-Meier-method, and were analyzed using the log-rank-test. Time to systemic recurrence was measured from time of surgery, until first evidence of recurrence. Metastases to skeleton, liver, lungs or the central nervous system were considered as systemic relapse. Patients without metastases were censored at the last follow-up visit or at the time of death (both cancer and non-cancer related death). Overall survival was measured from date of surgery until death of any cause. Patients still alive were censored at the end of the study follow-up (31.12.2010).

Data analysis was performed using SPSS 21.0 (SPSS Inc., Chicago, MO, USA). *P*-values <0.05 were considered statistically significant.

Results

S100A4-expression and associations with clinicopathological parameters in patients not receiving adjuvant therapy

Of the 323 patients included in the no-adjuvant cohort, tumor tissue from 291 patients was successfully stained for S100A4, while staining was not evaluable in 32 cases due to technical reasons or no tumor tissue present. The intensity and fractions of positive tumor cells are described in Table 1. While the intensity mainly was evaluated to be strong or intermediate in both cytoplasm and nucleus,

some tumors with cytoplasmic immunoreactivity were considered weakly stained (Fig. 1). As few cases had S100A4-immunoreactivity, cases with ≥1% stained tumor cells were regarded positive (Supplementary Fig. S2). Using this cut-off, specific expression was found solely in the nuclei in two samples and exclusively in the cytoplasm in two other samples, giving 44 (15%) S100A4-positive cases altogether.

The clinicopathological parameters of patients included in the no-adjuvant cohort (n=291) are summarized in Table 2. Median age at diagnosis was 60 years (range 31-93). The majority of the patients presented with pT1c tumors (55%), with the remaining being mainly pT1b or pT2. The three patients with pT1a tumors were grouped together with pT1b in the subsequent analyses. The tumors were primarily grade I or II (31% and 46%, respectively), as well as ductal carcinomas (75%), with 18% being registered as lobular carcinomas. While the majority of the tumors did not express HER2 (88%), 77% expressed hormone receptors (either PR or ER). Only 27% had more than 10% Ki-67-positive tumor cells.

For analysis of associations with clinical and histopathological parameters, cytoplasmic and nuclear expression was merged into one variable as presented in Table 2. Expression of S100A4 was strongly associated with histological grade as only 5% and 10% of grade I or II tumors, respectively, were S100A4-positive, compared to almost 40% positivity in grade III tumors. Similarly, only 8% of the HR-positive tumors expressed S100A4, while 41% were S100A4-positive in the HR-negative subgroup. HER2 was not significantly associated with S100A4-expression. S100A4-positivity was strongly associated with the triple-negative subtype (HR-HER2-), and 45% of tumors displayed S100A4-immunoreactivity in this category. Furthermore, S100A4-expression was associated with higher pT-stage, Ki-67-expression and other histological subtypes than ductal and lobular carcinomas. In this group, four of six S100A4-positive cases were mucinous adenocarcinomas. With only five mucinous adenocarcinomas in the no-adjuvant cohort, S100A4-expression was strongly associated with this subtype, also when analyzing tumor subtypes separately (data not shown). No associations were found between S100A4-expression and age or vascular invasion (data not shown).

Associations between S100A4 subcellular expression and clinical outcome

Forty patients experienced systemic relapse during follow-up, of which nine had tumors with cytoplasmic S100A4-staining and ten nuclear S100A4-staining. Nuclear expression of S100A4 was associated with reduced metastasis-free survival (Fig. ~~1a~~2a; P=0.031), however, no statistically significant association was seen for cytoplasmic S100A4 (Fig. ~~1b~~2b; P=0.096). When combining nuclear and cytoplasmic immunoreactivity, a similar difference was observed, with S100A4-positive patients having significantly inferior outcome (Fig. ~~1c~~2c; P=0.05). Patients with S100A4-positive tumors had an estimated 5-year metastasis-free survival of 78% compared to 89% for S100A4-negative patients. Similarly, patients with S100A4-positive tumors had reduced overall survival, (Fig. ~~1d~~2d-f), but only S100A4-expression in the nuclei was a statistically significant predictor of poor overall survival (P=0.037). For nuclear and cytoplasmic S100A4, patients had an estimated 5-year overall survival of 76% and 77%, respectively, compared to 90% for S100A4-negative patients. Prognostic significance of S100A4-expression was primarily confined to patients with histological grade II (Supplementary Fig. ~~S2~~S3), as these patients had both lower metastasis-free (P=0.013) and overall survival (P=0.029).

In multivariate analysis including the variables age, pT-status, HR-positivity and histological grade, S100A4 was not found to be associated with metastasis-free or overall survival.

S100A4-expression and clinical impact in the adjuvant patient cohort

The remaining analyses were done utilizing the second set of TMAs, and to address potential discrepancies between the two TMAs S100A4-expression was first assessed in tumor tissue from the patients not receiving adjuvant therapy. The metastasis-free and overall survival were similar to what observed in the initial analyses with the first TMA set (Supplementary Fig. ~~S3~~S4, Fig. ~~1c~~2c&f), but only 20 out of 229 tumors were S100A4-positive (9%).

Next S100A4-expression was evaluated in patients who received adjuvant systemic therapy to investigate the clinical impact of the protein in this subgroup. Of 421 included patients, tumor cores from 300 patients displayed technically successful S100A4-staining and only 23 (8%) were considered

positive using the same cutoff as in the initial analyses (Table 3). The majority of cases with S100A4-immunoreactivity had <1% positive tumor cells, and compared to the corresponding group in the no-adjuvant cohort this group was three times larger, while the percentage of tumors with no S100A4-immunoreactivity was fairly similar in the two cohorts. In S100A4-positive tumors, both nuclear and cytoplasmic staining was found in most cases, with the exception of five tumors with only cytoplasmic immunoreactivity. As there were few tumors positive for S100A4 along with minimal differences when examining the no-adjuvant cohort based on the protein's subcellular expression, the remaining analyses focus on S100A4-positivity, without considering subcellular localization.

Clinicopathological parameters and associations with S100A4-expression (n=300) are summarized in Table 4. The median age at diagnosis was 55 years (range 27-88). Most patients were diagnosed with ductal carcinomas (80%), and had pT1 (37%) or pT2 (52%) tumors. The majority had presence of tumor cells in one or more axillary lymph node (65%), and was classified as histological grade II (55%), HR-positive (80%) and HER2-negative (86%). Histological grade III, hormone receptor-negativity and triple-negative tumor subtype were strongly associated with S100A4-expression, as 14%, 20% and 26% of these tumors were S100A4-positive, respectively (Table 4). Contradictory to the no-adjuvant series, no associations were found between S100A4 and pT-stage or histological subtype. Moreover, no associations with therapy, lymph node status, age or vascular invasion were observed. Ki-67-scoring was not available for this sub-cohort.

Of 293 patients with available relapse status, 79 experienced systemic relapse but only seven of these had tumors positive for S100A4. Estimated 5-year metastasis-free survival was 65% and overall survival 61% for patients with S100A4-positive tumors, compared to 78% and 77%, for S100A4-negative. However, no statistically significant differences were observed for neither metastasis-free (Fig. ~~2a~~3a; P=0.321) nor overall survival (Fig. ~~2b~~3b; P=0.120). Similar to the no-adjuvant cohort, prognostic significance of S100A4-expression was primarily found in patients with histological grade II, as these had both lower metastasis-free (P=0.066) and overall survival (P=0.020) (data not shown).

Discussion

The metastasis-promoting protein S100A4 has previously been suggested as a strong prognostic marker in early stage breast cancer [19-21,34]. All investigations so far have utilized patient cohorts collected before the current adjuvant systemic treatment was introduced, and it was therefore of interest to examine the prognostic impact of S100A4 in a more recent unselected prospectively collected patient population. Initially, patients not receiving adjuvant therapy were selected to evaluate the potential prognostic value of the biomarker without the influence of various responses to treatments. Subsequently we elaborated our findings in a subgroup consisting of patients who had received adjuvant systemic therapy, where we confirmed that S100A4 is expressed in tumors exhibiting markers of an aggressive phenotype. We observed expression of S100A4 in nuclei and cytoplasm of the tumor cells, and the fraction of positive tumors was 15% in the no-adjuvant cohort. Only four cases showed staining in either subcellular compartment. In the adjuvant cohort, only eight per cent of the tumors were considered S100A4-positive, and none were positive for S100A4 exclusively in the nucleus.

In contrast to the present investigation, previous studies on S100A4 in early stage breast cancer have found expression in most of the examined tumors. The observed differences could have several explanations. First, breast cancer has been detected at earlier stages in recent decades, thereby causing a drift towards a less aggressive biological phenotype in the diagnosed primary tumors. Second, TMAs were used in the present study while Rudland and co-workers analyzed S100A4-expression using tumor tissue sections [19]. S100A4 is heterogeneously expressed in several types of cancer [35], and whole sections might therefore more precisely designate the S100A4-status. In our examinations tumors were considered positive if the protein was expressed in one of the cores examined per tumor. Ten of 24 tumors in the no-adjuvant cohort were positive in only one of three cores, reflecting the heterogeneity and partly explaining the lower frequency of S100A4-positive tumors compared to earlier studies. It might also explain the higher number of positive tumors in the no-adjuvant versus the adjuvant patient cohort, as the latter TMAs only contained two cores per tumor. In concordance with this, of the 27 cases that had tumors positive for S100A4 in the no-adjuvant series also present in the second TMAs, only nine were scored as S100A4-positive in the latter. Furthermore, the percentage

of S100A4-positive tumors was similar for the no-adjuvant and adjuvant subgroups in the TMAs with two tissue cores per tumor (9% and 8%, respectively), compared to 15% in the no-adjuvant cohort analyzed on the TMAs with three cores per tumor. This indicates that the discrepancies in expression between the two cohorts are explained by technical differences rather than variations in S100A4-expression between patient subgroups. Our findings further demonstrate the challenge by evaluating heterogeneous protein expression using TMAs and may suggest that the number of S100A4-positive tumors could be underestimated in the present investigation. Finally, we used a highly specific monoclonal antibody, recognizing one specific epitope. In all previous studies [19,20,22], polyclonal antibodies against either rat or human S100A4 were utilized. While these are more sensitive and through interactions with several epitopes might circumvent problems such as epitope hiding (for instance from multimere formation), they are also more unspecific. This is especially important since the S100-proteins constitute a large family of homologous proteins [8,36] and the use of polyclonal antibodies might result in unspecific binding to other S100-proteins.

Despite few S100A4-positive cases, a clear pattern was observed in the survival analyses. This was evident for both cohorts, however, no statistically significant differences could be found in the adjuvant cohort. These patients received systemic therapy, which normally would influence the patients' survival. Patients with S100A4-positive tumors had lower metastasis-free and overall survival compared to patients with tumors negative for S100A4, however, the differences were not as striking as previously reported by Rudland *et al*. [19]. In this study, only 20% of patients with tumors positive for S100A4 were alive after 19 years, compared to 80% of patients with S100A4-negative tumors. While later studies have reported less pronounced differences [20], S100A4 has consistently been associated with poor outcome. Variations in the number of patients included of each stage could potentially explain some of these differences. The study conducted by Rudland *et al*. contained a higher number of pT-stage 2 and 3 tumors compared to the present study where the majority of patients were either pT1 or pT2, and it is expected that this will affect the patient outcome. Based on our findings, S100A4-expression does not seem to be a useful biomarker for selection of patients for adjuvant treatment. Even though S100A4 is associated with a more aggressive phenotype and poor

prognosis, the fraction of S100A4-positive tumors is small and the impact on outcome is modest, indicating that the clinical utility would be limited.

Our analysis revealed a strong association between S100A4-positivity, histological grade III and HR-negative tumors. In contrast to what has been observed for other types of cancer, there were no substantial differences between cytoplasmic and nuclear localization of S100A4 in survival analyses or in the relationship with clinicopathological variables [23,25,26]. This might suggest that the localization of the protein has no specific effect on disease progression in breast cancer. The biological function of nuclear localized S100A4 is not well described in the literature, ~~W~~but we have previously ~~shown~~ demonstrated that, in addition to be ~~nuclear S100A4 is~~ a robust prognostic marker in CRC [23], ~~and recently we demonstrated~~ nuclear S100A4 ~~to be~~is enriched in the G2/M-phase in CRC cell lines, before localizing to the centrosomes after nuclear membrane breakdown [37]. The latter has not yet been investigated in breast cancer, but the lack of associations between survival and subcellular expression may suggest nuclear S100A4 to have diverse biological roles and clinical impact in different cancer types. We may speculate that nuclear S100A4, and also localization to the centrosomes, imply involvement in processes needed for disease progression in CRC, and that the same processes are less important in breast cancer. Supporting this hypothesis is the fact that improved ability to invade the surrounding tissue has been observed in other cancer subtypes where nuclear localization of S100A4 is associated with poor prognosis [26,38]. Nevertheless, further experiments are needed to elucidate the functional role of S100A4, and in particular how localization to different subcellular compartments influences tumor progression.

S100A4 is believed to support an invasive phenotype [10] and different mechanisms have been proposed for how the protein stimulate such a phenotype. Among others, S100A4 is suggested to be involved in EMT [39]. In line with this hypothesis, knockdown of ER-expression induces an EMT-like process in breast cancer cells [40], and as S100A4 was associated with HR-negative tumors, gain of mesenchymal traits may explain the observed associations with high tumor grade and poor prognosis. Involvement of S100A4 in EMT-like processes has been suggested for the extracellular protein [41].

Similarly, recent reports have shown that microenvironmental S100A4 facilitates metastasis through pre-metastatic niche formation [16,42]. Cancer cells may secrete S100A4 [7,26,43], and intracellular expression, as investigated in the present study, might thus facilitate tumor progression in a similar way as the extracellular protein. It is therefore possible that patients with tumors expressing S100A4 are more prone to develop metastatic disease, either because of the tumor's ability to gain mesenchymal traits or by preparing growth promiscuous niches in metastatic organs. Further characterization of the cellular mechanisms involving S100A4 is, however, needed in order to determine how the protein influences such processes.

Conclusion

We have shown that the metastasis promoting protein S100A4 is associated with an aggressive phenotype in breast cancer, and that S100A4 is a marker of poor metastasis-free and overall survival. However, the clinical utility of S100A4 seems limited because the number of S100A4-positive tumors is rather low.

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Compliance with Ethical Standards

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Conflict of interest

The authors declare that they have no conflict of interests.

Ethics approval

The project was approved by the Regional Ethics Committee of South-East Norway, and all procedures performed were in accordance with their ethical standards and with the 1964 Helsinki declaration and its later amendments.

Informed consent

Written informed consent was obtained from all patients enrolled in the study.

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Titles and legends to figures

Fig. 1: Representative photomicrographs showing immunohistochemical staining of S100A4 (magnification ×100). No immunoreactivity in the cytoplasm or nucleus (a). Tumors with weak (b), moderate (c) or strong (d) cytoplasmic and nuclear staining of S100A4. Corresponding H&E-staining of the four samples (e-h).

Fig. ~~1~~2: Prognostic significance of S100A4 in the no-adjuvant patient cohort. Kaplan-Meier survival plots displaying metastasis-free (a-c) and overall survival (d-e). The distinct plots are based on the presence or absence of nuclear (a, d), cytoplasmic (b, e) or total (c, f) S100A4.

Fig. ~~2~~3: Prognostic significance of S100A4 in the adjuvant patient cohort. Kaplan-Meier plots show metastasis-free (a) and overall (b) survival based on S100A4-positivity.

Table 1: S100A4-immunoreactivity in the no-adjuvant cohort

		Cytoplasmic[1]		Nuclear[1]	
		Number	**Percent**	**Number**	**Percent**
Percentage					
0	0 %	236	81	236	81
1	<1 %	13	5	13	5
2	1-10 %	17	6	19	7
3	11-50 %	6	2	10	3
4	>50 %	19	7	13	5
Intensity					
0	Negative	236	81	236	81
1	Weak	8	3	1	0
2	Medium	12	4	12	4
3	Strong	35	12	42	14

[1] Data from 291 patients

Table 2: Clinicopathological parameters and associations with S100A4 in the no-adjuvant cohort

	All patients*		**S100A4-positive**		
Parameter	Number	Percent	Number	Percent[1]	*P*-value
Patients	291	100	44	15	
Age					0.326[2]
Median (range)	60 (31-93)		64 (31-93)		
Histological subtype					0.043[3]
Invasive ductal carcinoma	219	75	34	16	
Invasive lobular carcinoma	53	18	4	8	
Others	19	7	6	32	
Tumor stage					<0.001
pT1 not possible to classify as	6	2	1		
a-b-c	68	26	7	10	
pT1a-b	158	55	16	10	
pT1c	53	18	20	38	
pT2	6				
pTX					
Histological grade					<0.001
I	89	31	4	5	
II	135	46	14	10	
III	67	23	26	39	
Hormone receptor status[4]					<0.001
Positive	224	77	17	8	
Negative	66	23	27	41	
Missing	1				
HER2 status[4]					0.077
Positive	35	12	9	26	
Negative	256	88	35	14	
Tumor subtypes					<0.001
HER2- HR+	208	72	14	7	
HER2+ HR+/-	35	12	9	26	
HER2- HR-	47	16	21	45	
Missing	1				
Ki-67[4]					0.002
Positive	63	27	15	24	
Negative	175	74	14	8	
Missing	53				

P-values were calculated using Pearson's chi-square test
* All patients with successful S100A4-staining of TMA
[1] Percentage of S100A4-positive patients within the category
[2] Two-sample Student's t-test
[3] Fisher's exact-test
[4] Cut-off at >10% for positive

Table 3: S100A4-immunoreactivity in the adjuvant cohort

		Cytoplasmic[1]		Nuclear[1]	
		Number	**Percent**	**Number**	**Percent**
Percentage					
0	0 %	232	77	235	78
1	<1 %	45	15	47	16
2	1-10 %	12	4	13	4
3	11-50 %	4	1	4	1
4	>50 %	7	2	1	0
Intensity					
0	Negative	232	77	235	78
1	Weak	5	2	0	0
2	Medium	5	2	3	1
3	Strong	58	19	62	21

[1] Data from 300 patients

Table 4: Clinicopathological parameters and associations with S100A4 in the adjuvant cohort

	All patients*		**S100A4-positive**		
Parameter	Number	Percent	Number	Percent[1]	*P*-value
Patients	300	-	23	8	
Age					0.785[2]
Median (range)	55 (27-88)	-	55 (34-88)		
Histological subtype					0.067[3]
Invasive ductal carcinoma	240	80	19	8	
Invasive lobular carcinoma	53	18	2	4	
Others	7	2	2	29	
Tumor stage					0.965[3]
pT1	108	37	8	7	
pT2	152	52	13	9	
pT3	27	9	2	7	
pT4	5	2	0	0	
pTX	8				
Lymph node status					0.238
pN0	102	35	10	10	
pN+	189	65	11	6	
pNX	9		2		
Histological grade					0.015[3]
I	37	13	1	3	
II	162	55	8	5	
III	96	33	14	15	
Missing	5				
Hormone receptor status[4]					<0.001[3]
Positive	240	80	11	5	
Negative	60	20	12	20	
HER2 status					0.755[3]
Positive	43	14	4	9	
Negative	257	86	19	7	
Tumor subtypes					<0.001[3]
HER2- HR+	219	73	9	4	
HER2+ HR+/-	43	14	4	9	
HER2- HR-	38	13	10	26	
Adjuvant systemic therapy					0.487[3]
Tamoxifen	134	50	8	6	
Chemo +/- tamoxifen	134	50	12	9	
Missing	32		3		

P-values were calculated using Pearson's chi-square test
* All patients with successful S100A4-staining of TMA
[1] Percentage of S100A4-positive patients within the category
[2] Two-sample Student's t-test
[3] Fisher's exact-test
[4] Cut-off at >10% for positive

Figure 1

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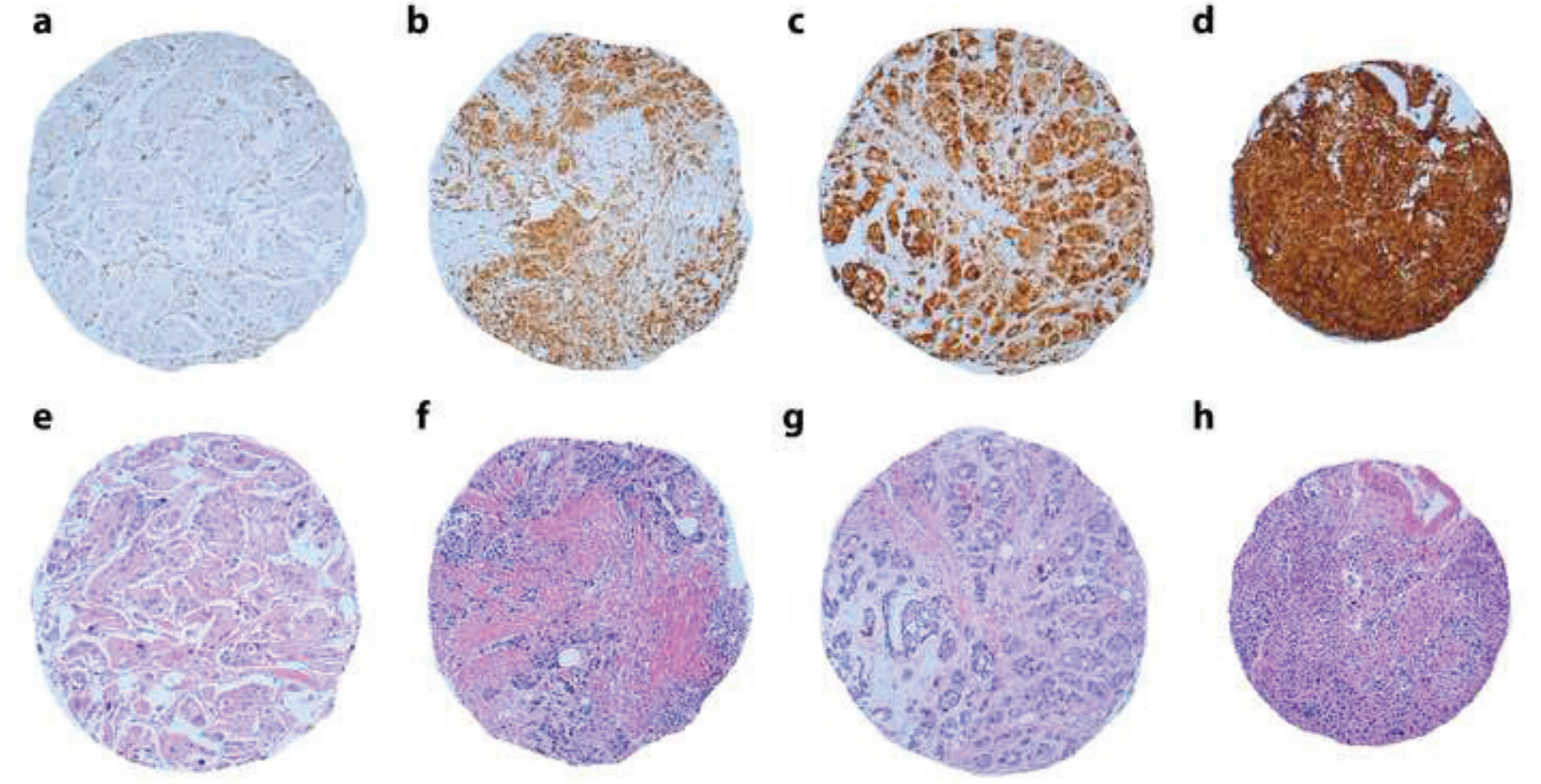

Figure 2

Figure 3

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