New insights into the prognostic value of the tumor-stroma ratio in patients with breast cancer

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The intra-tumoral stroma in patients with breast cancer increases with age

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ABSTRACT

Purpose

The tumor microenvironment in older patients is subject to changes. The tumor-stroma ratio (TSR) was evaluated in order to estimate the amount of intra-tumoral stroma and to evaluate the prognostic value of the TSR in older patients with breast cancer (≥70 years).

Methods

Two retrospective cohorts, the FOCUS study (n = 619) and the Nottingham Breast Cancer series (n = 1793), were used for assessment of the TSR on hematoxylin and eosin stained tissue slides.

Results

The intra-tumoral stroma increases with age in the FOCUS study and the Nottingham Breast Cancer series (B 0.031, 95% CI 0.006-0.057, $p = 0.016$ and B 0.034, 95% CI 0.015-0.054, $p < 0.001$, respectively). Fifty-one percent of the patients from the Nottingham Breast Cancer series <40 years had a stroma-high tumor compared to 73% of the patients of ≥90 years from the FOCUS study. The TSR did not validate as an independent prognostic parameter in patients ≥70 years.

Conclusions

The intra-tumoral stroma increases with age. This might be the result of an activated tumor microenvironment. The TSR did not validate as an independent prognostic parameter in patients ≥70 years in contrast to young women with breast cancer as published previously.

INTRODUCTION

Breast cancer is the leading malignancy in European women (1). A major risk factor for breast cancer development is aging (2).

In the last decade, the tumor microenvironment has gained interest in unravelling cancer development and cancer progression, but also as a source for new therapeutic targets and prognostic parameters. The tumor microenvironment, i.e. tumor stroma, consists of a variety of structures and cells located in the extracellular matrix, such as immune cells, fibroblasts and endothelial cells. Various processes in the tumor microenvironment are involved in tumor progression by influencing the proliferation of cancer cells, the epithelial-mesenchymal transition, tumor metabolism and dissemination capabilities (3). Epidemiological and clinico-pathological characteristics are different in older patients with breast cancer compared to their younger counterparts (4-7). The biology of breast cancer is age-dependent in which alterations in extracellular matrix and products secreted by senescent fibroblasts are thought to promote late-onset breast tumorigenesis, however the extent is still unknown (8). Research into the molecular profile of older patients with triple-negative breast cancer showed a different stromal microenvironment favorable for tumorigenesis, in which senescence-associated secretory profile and autophagy are important aberrant stromal features induced with increasing age (9).

A widely researched prognostic marker based on the tumor-microenvironment is the tumor-stroma ratio (TSR). The TSR reflects the ratio between tumor cells and stromal cells and is visually assessed with conventional light microscopy. Previous studies have shown that the TSR is a valuable prognosticator for breast cancer patients, whereby tumors with a high stromal content are associated with a poor clinical outcome (10-18). This effect was observed and validated in the overall group of breast cancer patients and clinically relevant subgroups (18).

In the current literature, older patients are often defined as patients of 70 years and older (19). In older patients with breast cancer, better risk stratification is desirable. Whilst breast cancer mortality in the total group of patients with breast cancer has decreased over the last decade, this decrease is lower or absent in older patients. This leads to an increased survival gap between older and younger patients with breast cancer (20-23). Invasive breast tumors in the aging women are thought to

have a more favorable biology compared to younger females. Improvement of prognostic tools is needed for more accurate prediction of prognosis in the older breast cancer patient, considering that only very few older patients with breast cancer aged over 70 years receive chemotherapy (24). More accurate stratification of disease aggressiveness could contribute to shared-decision making on the extent of adjuvant therapy. This may minimize the risk of undertreatment which may contribute in the survival gap between younger and older patients with breast cancer. Although extensive research in population-based studies showed that the TSR is an important prognosticator in women with breast cancer, none of these studies have focused on its significance in the older female population.
Therefore, the aims of this study were (1) to investigate the amount of intra-tumoral stroma by the assessment of the TSR in older patients with breast cancer and (2) to evaluate the prognostic value of the TSR in women diagnosed with breast cancer at the age of 70 years or older.

MATERIAL AND METHODS

Study population

This study included two databases with retrospectively collected clinical data from women diagnosed with breast cancer.

The FOCUS study

The FOCUS study consisted of a population-based cohort of women aged 65 years and older, who were diagnosed with breast cancer (n = 3672) between 1997 and 2004 in Comprehensive Cancer Centre Region West (The Netherlands). Women with a history of cancer or in situ tumors, neoadjuvant therapy, distant metastasis at time of diagnosis, age under 70 years or with no available tumor tissue were excluded. In total, 1577 women were suitable for analysis. This cohort was used to answer both study aims, the evaluation of the amount of intra-tumoral stroma and the prognostic value of the TSR in the older women with breast cancer.

The Nottingham Breast Cancer Series

The Nottingham Breast Cancer Series (n = 1809) is a cohort of women ≤70 years of age presenting with primary invasive breast cancer without distant metastasis and primarily treated with surgery in Nottingham City Hospital between 1993 and 2002. Patients were included if hematoxylin and eosin (H&E) stained tissue slides and clinical information (patients and tumor characteristics and survival data) were available. This study was used for the evaluation of the amount of intra-tumoral stroma with the increase of age.

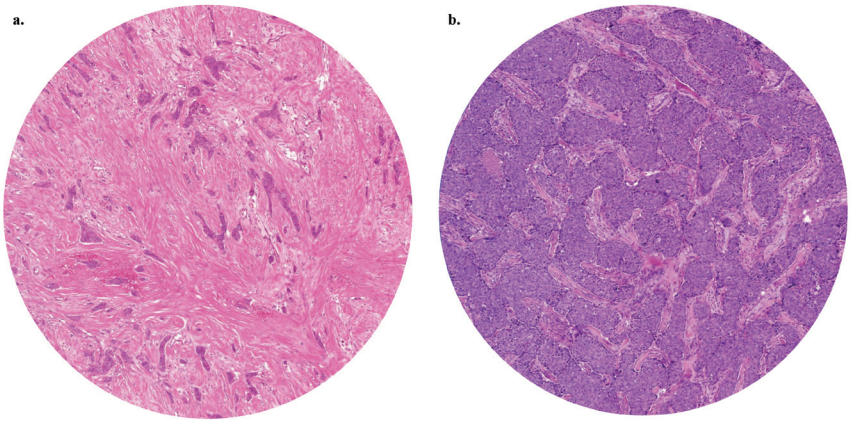
For standard clinical care all resected tumors were assessed by a pathologist, according to the currently applied pathological standards. The clinical data from the Nottingham Breast Cancer series were anonymized and the study was approved by the Nottingham Research Ethics Committee 2 under the title 'Development of a molecular genetic classification of breast cancer'. All samples from the FOCUS study were also anonymized and data were handled according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies").

Tumor-stroma ratio assessment

The tissues slides from the FOCUS study were assessed for the TSR by visual eyeballing with a conventional light microscope on standard H&E stained tissue slides, as previously described by our group (10, 25). The most stroma-rich area on the slide was selected with a 5x objective. A 10x objective was used to select the final most stroma-abundant area. The H&E slides from the Nottingham Breast Cancer series were digitally assessed via CaseViewer 2.2 for Windows (3D HISTECH Ltd.). The original H&E slides were scanned with a 20x magnification using 3D Histech Panoramic 250 Flash II (3DHISTECH Ltd., Budapest, Hungary). The most stroma-abundant area was selected and in the most stroma-rich field a circle with an area of 3.1 mm^2 was annotated. This area corresponded with the magnification used in our previously published research (26). The next steps in the assessment of the TSR on digital images and conventional images were performed in the same manner. The percentage of stromal cells compared to tumor cells in the selected area were scored by increments of 10%. The selected area required tumor

cells at all borders of the image field. Stromal areas with post-biopsy effects were avoided. Finally, the determined percentages were divided into two categories; stroma-low (≤50% stroma) and stroma-high (>50% stroma) (figure 1). The tissues slides were scored double in a blinded fashion. If no consensus could be reached between the two observers a third observer was consulted. Consensus could be reached in all cases.

FIGURE 1. Representative example of tumor-stroma ratio assessment
a. Stroma-high tumor **b.** Stroma-low tumor.



Statistical analyses

For statistical analyses, SPSS statistics version 23.0 (SPSS Inc., IBM Company Chicago, IL, USA) was used. Relative survival analyses were performed with STATA SE software version 12 (StataCorp, College Station, TX). A Cohen's kappa was calculated for the evaluation of inter-observer agreement. A value above 0.6 was considered as a good level of agreement. To evaluate the difference of patient characteristics between women with stroma-low or stroma-high tumors, the χ^2 test was used in case of categorical variables. The distribution of numerical variables was tested with the Shapiro-Wilk test. Non-parametric continuous variables were evaluated using the Mann-Whitney U test. Linear regression analysis was

performed to investigate the association between age (continue) and the intra-tumoral stroma in percentage (increments of 10%). The linear regression analysis was adjusted for tumor size, histology, estrogen receptor (ER) status, progesterone receptor (PR) status, human epidermal growth factor 2 (HER2) status, triple-negative (TN) status and grade, as these parameters might influence the amount of intra-tumoral stroma.

The primary endpoint was recurrence-free period (RFP). The definition for RFP was time from diagnosis to local, regional or distant recurrence or contralateral breast cancer. Censoring was applied at the last date at which patients were known to be recurrence-free and alive. The secondary endpoint was relative survival (RS). This was defined as the observed overall survival (OS) among included patients divided by the expected survival in the general population. Groups were matched by sex, age and calendar year. This analysis was applied according to the Ederer II method with use of the 'strs' command in STATA. A relative survival rate of less than 100% at 10 years after diagnosis means that the survival of patients in the study is lower than expected when compared to survival of the general population. The relative survival data were calculated at 10-year follow-up. The relative excess risk (RER) of death was estimated using a multivariable generalized linear model with a Poisson distribution, based on collapsed relative survival data, using exact survival times. To assess the differences in RFP for our parameter of interest, the Kaplan-Meier curves were compared using the log-rank test. This test was also used for analyzing different TSR cut-off values, other than the normally used 50% (i.e. ≤50% stroma is categorized as stroma-low and >50% stroma is categorized as stroma-high). A *p*-value lower than 0.05 was considered statistically significant for all analyses. Cox regression analyses were used to calculate the prognostic value of the TSR (univariate and multivariate). The TSR was corrected for clinically important confounders. The interaction term was introduced to evaluate the prognostic value of the TSR stratified by confounders. Power analysis showed that at least 618 patients of the FOCUS study must be analyzed to reach a power of 0.80 (1-β) with a type I error rate of 5% (α).

RESULTS

Patients

The FOCUS study

In total, 1577 women included in the FOCUS study were eligible for inclusion. Based on power calculation, 627 patients were selected via computer randomization (minimum of 618 patients). The included (n = 627) and excluded (n = 950) patients were compared for age, tumor grade, histological type, T-stage, N-stage, hormone receptor status, HER2 status, type of operation, radiotherapy, chemotherapy and hormonal therapy. Between these two groups, only hormonal therapy showed to be statistically significant different (p = 0.003). In the included group, more patients were treated with hormonal therapy. However, hormonal therapy has no association with outcome (HR 1.01, 95% CI 0.66-1.54, p = 0.975). The median age of the excluded patients was 78 and the median age of the included women was 79 at time of diagnosis. Eight slides were not suitable for TSR assessment due to poor quality of the staining.

The characteristics of the selected patients are described in table 1. Cohen's kappa inter-observer agreement was 0.77 (33% of slides were scored in a double-blinded fashion).

TABLE 1. Statistically significant difference between stroma-low and stroma-high tumors in the FOCUS study.

		Stroma-low		**Stroma-high**		
	n	n = 204	%	n = 415	%	*p*-value
Age (in years)						
	619	79 (mean)		80 (mean)		0.020
Grade						
I	82	31	22.0	51	17.3	0.126
II	198	69	48.9	129	43.9	
III	155	41	29.1	114	38.8	
Histological type						
Invasive carcinoma of NST	471	148	72.5	323	77.8	0.171
Lobular carcinoma	65	28	13.7	37	8.9	
Other	83	28	13.7	55	13.3	

TABLE 1. Continued.

		Stroma-low		Stroma-high		
	n	n = 204	%	n = 415	%	*p*-value
Tumor size						
pT1	254	96	47.1	158	38.1	0.014
pT2	286	92	45.1	194	46.7	
pT3/4	79	16	7.8	63	15.2	
Tumor involvement in the lymph nodes						
Negative	353	134	66.3	219	54.2	0.004
Positive	253	68	33.7	185	45.8	
ER status						
Negative	95	33	18.9	62	16.9	0.574
Positive	447	142	81.1	305	83.1	
PR status						
Negative	195	64	38.8	131	37.8	0.822
Positive	317	101	61.2	216	62.2	
HER2 status						
Negative	484	151	76.3	333	82.0	0.096
Positive	120	47	23.7	73	18.0	
Type of surgery						
BCS	181	68	33.3	113	27.2	0.117
MST	438	136	66.7	302	72.8	
Radiotherapy						
No	366	121	59.3	245	59.0	0.947
Yes	253	83	40.7	170	41.0	
Chemotherapy						
No	602	199	97.5	403	97.1	0.753
Yes	17	5	2.5	12	2.9	
Hormonal therapy						
No	303	112	54.9	191	46.0	0.038
Yes	316	92	45.1	224	54.0	

Abbreviations: BCS = breast conserving surgery, ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, MST = mastectomy, NST = no special type, PR = progesterone receptor. Missing values were excluded from these analyses.

The Nottingham Breast Cancer Series

An external cohort of primary breast cancer patients diagnosed in Nottingham City Hospital was used for the evaluation of the TSR in order to investigate alterations in the amount of intra-tumoral stroma. Due to bad quality of the tissue, 15 patients were excluded (0.8%), and one patient was excluded because clinical information regarding patients age was unknown. Finally, 1793 patients were used in the analyses. The mean age was 55. An overview of patient characteristics, tumor characteristics and treatment is shown in table 2. All slides were assessed by two observers. If no consensus could be reached a third observer was consulted. Consensus was reached in all cases.

Alterations in stromal amount with the increase of age

For the patients in the FOCUS study (n = 619), the Mann-Whitney U test showed a significant association between age and the TSR ($p = 0.020$). By evaluating the TSR, the results showed a higher amount of intra-tumoral stroma with the increase of age (B 0.025, 95% CI 0.004-0.045, $p = 0.018$). In the group of patients between 70 and <75 years of age, 63% of the tumors were assessed as stroma-high compared to 73% of the tumors in patients aged 90 years or older (figure 2a).

To evaluate this age effect in an independent cohort, the Nottingham Breast Cancer Series (n = 1793), consisting of breast cancer patients of ≤70 years of age, was assessed. The Mann-Whitney U test showed a significant association between age and TSR ($p = 0.003$). In this patient cohort, the evaluation of the TSR showed that the amount of intra-tumoral stroma also increases with age (B 0.033, 95% CI 0.014-0.053, $p = 0.001$). Of the patients under the age of 40, 51% was scored as stroma-high compared to 66% of patients between 65 and 70 years of age (figure 2b).

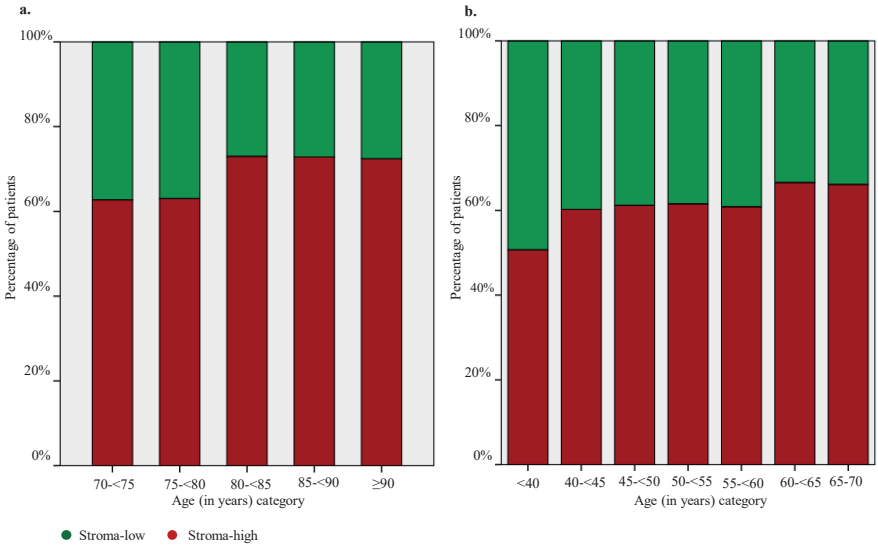
Linear regression was adjusted for tumor size, histology, ER status, PR status, HER2 status, TN status and grade in the FOCUS study and the Nottingham Breast Cancer Series (B 0.031, 95% CI 0.006-0.057, $p = 0.016$ and B 0.034, 95% CI 0.015-0.054, $p < 0.001$, respectively). These results showed that the association between the amount of intra-tumoral stroma and age remained statistically significant after adjustment of pathological tumor-based characteristics.

TABLE 2. Statistically significant difference between stroma-low and stroma-high tumors in the Nottingham Breast Cancer series.

		Stroma-low		Stroma-high		
	n	n = 681	%	n = 1113	%	*p*-value
Age (in years)						
	1793	54 (mean)		55 (mean)		0.003
Grade						
I	279	105	15.4	174	15.6	0.779
II	733	272	40.0	461	41.5	
III	780	303	44.6	477	42.9	
Histological type						
Invasive carcinoma of NST	1128	450	66.1	678	61.0	0.114
Lobular carcinoma	155	53	7.8	102	9.2	
Tubular carcinoma	275	90	13.2	185	16.6	
Others	235	88	12.9	147	13.2	
Tumor size						
T1	1146	505	74.3	641	57.7	<0.001
T2	624	169	24.9	455	41.0	
T3	21	6	0.9	15	1.4	
Tumor involvement in lymph nodes						
Negative	1127	452	66.6	675	60.8	0.013
Positive	663	227	33.4	436	39.2	
ER status						
Negative	331	151	22.2	180	16.2	0.002
Positive	1462	530	77.8	932	83.8	
PR status						
Negative	708	282	42.0	426	38.7	0.168
Positive	1066	390	58.0	676	61.3	
HER2 status						
Negative	1572	594	87.2	978	87.9	0.650
Positive	221	87	12.8	134	12.1	

Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, NST = no special type, PR = progesterone receptor. Missing values were excluded from these analyses.

FIGURE 2. Percentage of patients with stroma-low and stroma-high tumors stratified by age category **a.** The FOCUS study (n = 619), **b.** The Nottingham Breast Cancer Series (n = 1793).



Evaluation of the prognostic value of the TSR in older patients with breast cancer

The FOCUS Study

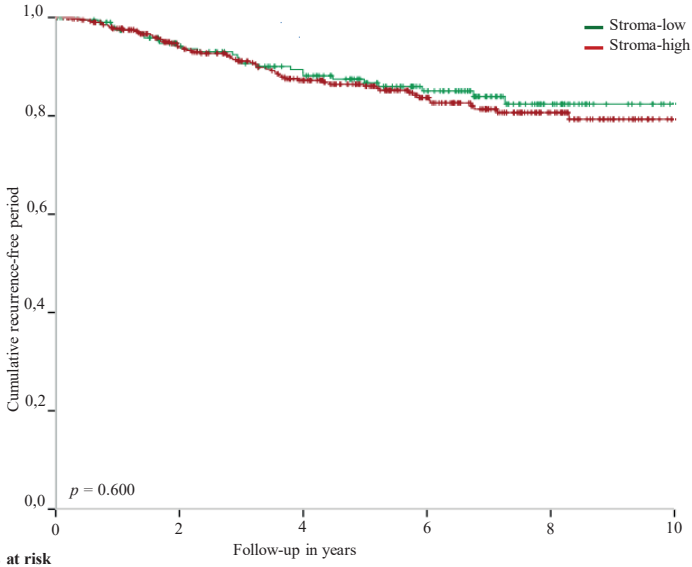
Most of the 619 tumors were categorized as stroma-high (67%). Eighty-five patients developed a tumor recurrence. Among stroma-high tumors, a higher number of patients with positive lymph nodes (p = 0.004), an advanced T-stage (p = 0.014) and hormonal therapy (p = 0.038) was observed. Older age was associated with stroma-high tumors (p = 0.020) (table 1). After a follow-up period of 10 years, no statistically significant differences were observed in recurrence rates between stroma-low and stroma-high tumors, 18% versus 21% respectively (HR 1.13, 95% CI 0.72-1.78, p = 0.602) (figure 3). The results of the multivariate Cox regression analyses were in line with the results of the univariate analysis (HR 1.02, 95% CI 0.59-1.78, p = 0.937) (table 3). After 10-year follow-up, the relative survival rates

of patients with stroma-low compared to stroma-high tumors were 90.2% versus 91.6%, respectively (RER 1.53, 95% CI 0.31-7.47, $p = 0.601$).

The interaction term was added in the Cox regression analysis. These analyses showed no statistically significant value for the TSR if stratified by grade ($p = 0.571$), morphology ($p = 0.449$), ER status ($p = 0.598$), PR status ($p = 0.737$), HER2 status ($p = 0.721$) or tumor size ($p = 0.571$).

In the FOCUS study, survival analyses were performed for the TSR at other cut-off values than the established 50%. The cut-off values ranged from 20% to 70%, but none of the values showed statistically significant differences on clinical outcome (data not shown).

FIGURE 3. Kaplan-Meier analysis for recurrence-free period stratified by the tumor-stroma ratio of patients included in the FOCUS study.



Numbers at risk

Stroma-low	204	130	13
Stroma-high	415	233	31

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Abbreviations: ER = estrogen receptor, HER2 = human epidermal growth factor receptor 2, NST = no special type, PR = progesterone receptor. Missing values were excluded from these analyses.

DISCUSSION

The results in this study showed a significant association between age and intratumoral stroma percentage expressed with the TSR; a higher amount of intratumoral stroma was observed with the increase of age. This may be related to differences in tumor development and tumor microenvironment in older patients with breast cancer compared to their younger counterparts. This could be due to, for instance, age-related pathological alterations which occur in the mamma, such as an increase in fat tissue and collagenous stroma as replacement for glandular tissue (5, 27). The extent of the alterations in the extracellular matrix and products secreted by senescent fibroblasts in the promotion of late-onset breast tumorigenesis is still unknown. A different view on the role of senescent cells is suggested in recent literature. Senescent cells were previously thought to be tumor-protective, but recent research showed that these cells contribute to a tumor-promoting environment (8). A dysregulated response between declining immune function (i.e. immunosenescence) on one hand and a low grade chronic inflammation (i.e. inflammaging) on the other hand may lead to an altered tumor microenvironment. These processes have impact on tumor development and tumor growth in the aging population, probably with the involvement of CD4+ and CD8+ T cells (28). Previous research showed decreased values of these immune cells in mammary tumors in older mice compared to their younger counterparts (29). Brouwers et al. investigated the molecular profile of the microenvironment in older triple-negative breast cancer patients. The authors provided evidence that breast cancer in the older patients is associated with a different stromal microenvironment favorable for tumorigenesis, in which senescence-associated secretory profile and autophagy are important stromal features induced with age. As an illustration, the authors validated in an external publicly available dataset a significant upregulation of fibroblast growth factor 13 (FGF13) in tissues of older breast cancer patients. This gene belongs to the fibroblast growth factor superfamily. Aberrant expression of this superfamily is involved in tumor growth and invasion (9). Another process that occurs with aging are changes in the hormonal status. In postmenopausal women, the production of estradiol takes place in peripheral tissues instead of in the ovaries, like in premenopausal women. This change leads to a consistent but lower level of circulating estrogen (30). Postmenopausal women with relatively

high systemic concentration of estrogen have a higher risk of developing breast cancer (31). The chance of random genetic errors is increased by the proliferative effect of estrogens on breast epithelial cells (32, 33). Whether these processes contribute to the increase of stroma-high patients is not known yet. Also, the contradictory results in this study regarding the prognostic value of the TSR is not fully understood. These results are in strong contrast to the discriminating power of the TSR regarding to clinical outcome presented in the review of Kramer and colleagues. The authors showed that patients with stroma-high tumors have a poor clinical outcome. This was observed in the overall patient population with breast cancer and in clinically relevant subgroups, such as, patients with triple-negative tumors, estrogen positive tumors or lymph node negative tumors (18). Therefore, the understanding and confirming of age-related changes in the microenvironment requires further research.

Regarding the aging patient, the tumors of older patients with breast cancer are, for example more often receptor positive and have a lower grade (34). In contrast to the more favorable biology, Van de Water et al. concluded that the clinical outcome in older patients with breast cancer must not be underestimated, as breast cancer relapse and disease specific mortality is higher in older breast cancer patients compared to their younger counterparts (35). A study performed in Denmark showed results in line with Van de Water and colleagues. The 5-year relative survival decreases with the increase of age; 90% for patients aged between 0-69 years, 80% for patients aged 70-79 and 73% for women aged 80-89 years (22). Also the frequently used online prediction tool PREDICT slightly overestimated the 10-year overall survival of patients aged ≥65 years and must especially be interpreted with caution in patients aged ≥75 years (36, 37). Dutch guidelines contain no explicit recommendations about chemotherapy in older patients, mainly due to the scarce amount of studies specifically focusing on older patients. This results in lack of evidence about the efficiency of chemotherapy in patients over 70 years. In daily clinical practice in the Netherlands, chemotherapy is advised in fit older patients over 70 years. Shared-decision making between oncologists and patients plays a role in this process. A better prediction rule for prognosis combined with research about the definition of ‘fit’ and the effectiveness and side effects of chemotherapy in older patients, might simplify decision making regarding adjuvant therapeutic

options. Based on the result that TSR seems to be an important prognostic marker in patients under the age of 70 in contrast to older patients, we advocate for the importance of validating other prognostic parameters in older patients.
With respect to this study, the chosen endpoint might have an effect on the outcome of the prognostic value of the TSR. With RFP as primary endpoint, it remains possible that metastases or recurrences are not filed if the observation of disease relapse has no clinical consequence, for example if patients are unfit for further treatment. To minimize the effect of competing mortality on survival, the second endpoint was determined as RS instead of OS. A final limitation of this study is that adjuvant treatment options have changed over the years. Advantages of the FOCUS study are the long follow-up period and the amount of patients. In order to give a more definitive conclusion about the prognostic value of the TSR in the older patient with breast cancer, it is necessary to do a large observational population-based cohort study of older breast cancer patients treated following current guidelines assembled in a detailed database with focus on recurrences and disease specific survival.

CONCLUSIONS

The intra-tumoral stroma increases with age. The TSR showed no correlation with survival in patients of 70 years or older in contrast to young women with breast cancer as published previously.

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